Deepwater Horizon Oil Spill Natural Resource Damage Assessment Comprehensive Toxicity Testing Program: Overview, Methods, and Results

Technical Report
Draft

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The *Deepwater Horizon* (DWH) oil spill released millions of barrels of oil in the northern Gulf of Mexico between April 20 and July 15 in 2010. (U.S. District Court, 2015). The combination of water depth, distance to shore, duration, dispersant use, and volume of the DWH oil spill exposed natural resources to oil and dispersants on an unprecedented spatial scale and magnitude. The timing of the spill and the vast geographical area that it contaminated coincided with the height of the reproductive season for multitudes of Gulf of Mexico species; the spill also intersected with critical habitats for a wide range of these species and their lifestages. The oil that remains in benthic habitats and environments associated with the shoreline continues to pose an exposure risk to organisms in these areas. The vast temporal and geographic scale of this oil spill resulted in a complex series of exposure pathways, durations, and contaminant composition profiles. Exposure to oil and dispersant occurred through various pathways, which included direct exposure as well as ingestion of contaminated water and sediments or consumption of contaminated prey.

The Trustees' toxicity testing program included more than 40 species of fish, aquatic invertebrates, phytoplankton, reptiles, and birds (Table 1). The lifestages we included in our bioassays ranged from gametes to adults depending on the species and study objective. Most of the tested species were native to the Gulf of Mexico, while a handful of non-native surrogate species were used as model test species. The species and lifestages we included in our toxicity testing matrix represent a range of habitats, including marsh and sandy areas on the shoreline, pelagic and benthic estuarine areas, and offshore pelagic habitats. The oil samples we used in bioassays were all DWH oil collected from the well or the field (i.e., floating slick oil and oiled sediments) and ranged in weathering state from fresh oil to highly weathered oil [e.g., 85–90% loss of polycyclic aromatic hydrocarbons (PAHs) relative to hopane; Forth et al., 2015; Morris et al., 2015]. We also used dispersant (COREXIT® 9500) in bioassays to study the effects of exposure to dispersant or chemically dispersed oil on organisms.

Our study designs used several different contaminant exposure routes (Figure 1), including waterborne [water accommodated fractions (WAFs); Forth et al., 2015], contaminated sediments (Krasnec et al., 2015), direct contact with oil, and ingestion. In addition, we conducted bioassays to study other stressors that organisms would also have experienced in the field during the oil spill, such as exposure to elevated water temperatures; decreased dissolved oxygen (anoxia); ranges in salinity; and exposure to ultraviolet (UV) light, which results in photo-induced toxicity.

Finally, to determine and quantify the nature and extent of adverse biological effects induced through exposure to oil, we measured a large suite of toxicological endpoints. These endpoints included survival; growth; reproduction; behavior; cardiac defects; histological abnormalities; gene expression; immune function; physical performance, such as swimming; and general physiological parameters (Figure 2). More than 25 Principal Investigators (Table 0.1 in Attachment 1) from collaborating university, government, and private laboratories and consultants conducted these studies. These collaborations produced more than 500 toxicological bioassays and chemical characterizations.

Table 1. Species and exposures included in the Trustees' toxicity testing program

		Exposure				
Species	**Scientific name**	**WAF/ surface slick**	**Sediment/ oiled substrate**	**Dietary**	**Dermal**	**UV**
Fish						
Atlantic croaker	*Micropogonias undulatus*	X				
Bay anchovy	*Anchoa mitchilli*	X				X
Cobia	*Rachycentron canadum*	X				
Gulf killifish	*Fundulus grandis*	X	X			X
Gulf menhaden	*Brevoortia patronus*	X				
Gulf toadfish	*Opsanus beta*	X				
Inland silverside	*Menidia beryllina*	X				
Mahi-mahi	*Coryphaena hippurus*	X				X
Pacific bluefin tuna[a]	*Thunnus orientalis*	X				
Pacific mackerel[a]	*Scomber japonicus*	X				
Red drum	*Sciaenops ocellatus*	X	X			X
Red snapper	*Lutjanus campechanus*	X				X
Sand seatrout	*Cynoscion arenarius*	X				
Sheepshead minnow	*Cyprinodon variegatus*	X				X
Shovelnose sturgeon[a]	*Scaphirhynchus platorynchus*	X				
Southern bluefin tuna[a]	*Thunnus maccoyii*	X				
Southern flounder	*Paralichthys lethostigma*	X	X			
Speckled sea trout[b]	*Cynoscion nebulosus*	X				X
Yellowfin tuna	*Thunnus albacares*	X				
Yellowtail amberjack[a]	*Seriola lalandi*	X				
Zebrafish[a]	*Danio rerio*	X				
Invertebrates						
Amphipod	*Leptocheirus plumulosus*		X			
Blue crab	*Callinectes sapidus*	X	X			X
Brown shrimp	*Farfantepenaeus aztecus*		X			
Copepod	*Acartia tonsa*	X				X
Eastern oyster	*Crassostrea virginica*	X	X	X		X
Fiddler crab	*Uca longisignalis*	X	X			X

Table 1. Species and exposures included in the Trustees' toxicity testing program (cont.)

		Exposure				
Species	**Scientific name**	**WAF/ surface slick**	**Sediment/ oiled substrate**	**Dietary**	**Dermal**	**UV**
Fiddler crab	*Uca minax*		X			
Grass shrimp	*Palaemonetes pugio*	X	X			X
Marsh periwinkle	*Littoraria irrorata*		X			
Mysid shrimp	*Americamysis bahia*	X				X
White shrimp	*Litopenaeus setiferus*	X	X			
Pacific white shrimp[a]	*Litopenaeus vannamei*	X	X			X
Phytoplankton						
Diatom	*Skeletonema costatum*	X				
Reptile						
Common snapping turtle[a]	*Chelydra serpentina*			X		
Red-eared slider[a]	*Trachemys scripta elegans*			X		
Birds						
Double-Crested Cormorant	*Phalacocorax auritus*			X	X	
Homing Pigeon[a]	*Columba livia*			X	X	
Laughing Gull	*Leucophaeus atricilla*			X		
Western Sandpiper	*Calidris mauri*			X	X	

a. Surrogate species.
b. Many common names for this species, including spotted seatrout.

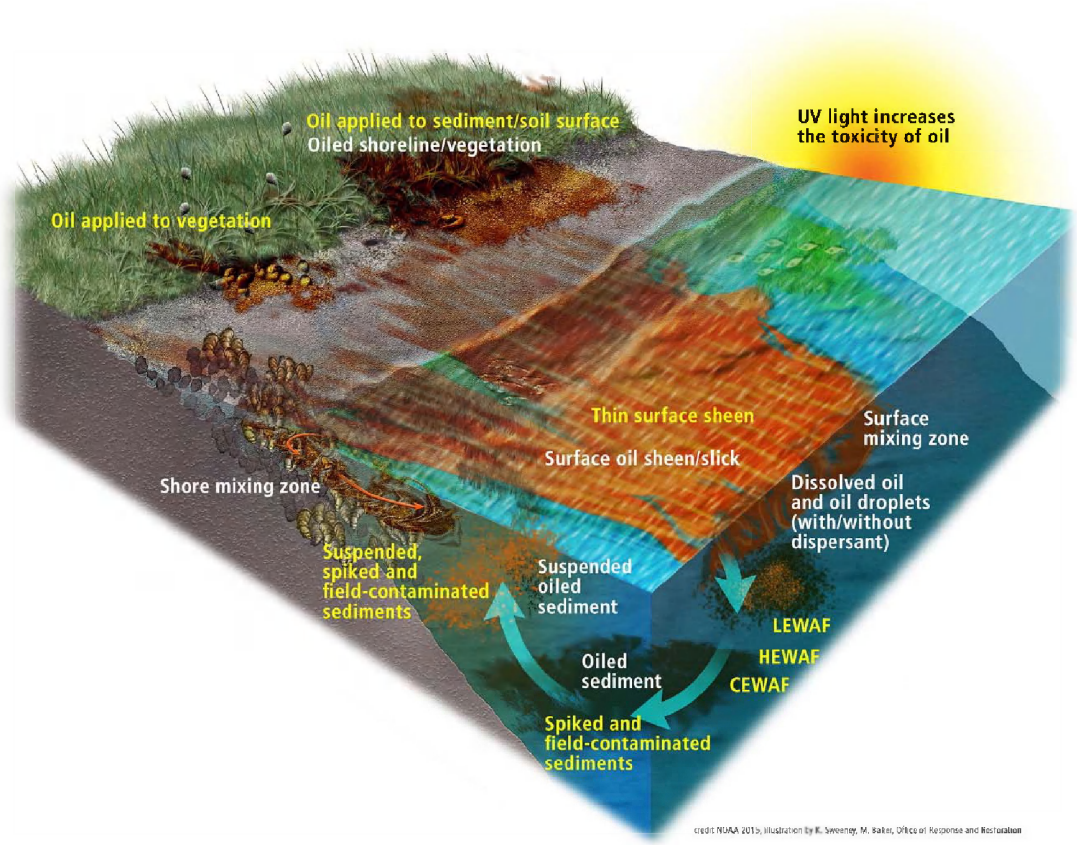

Figure 1. Conceptual diagram of oil present in the northern Gulf of Mexico following the DWH oil spill (white labels) and the toxicity testing preparations we used to represent each type of oil (yellow labels). Although this diagram illustrates slicks and the surface mixing zone near the shoreline, these types of oil exposures would be similar throughout the offshore DWH oil slick and the surface mixing zone. See Forth et al. (2015) and DWH Trustees (2015, Section 4.3, Toxicity) of the Programmatic Damage Assessment and Restoration Plan (PDARP) for definitions of chemically enhanced WAF (CEWAF), high-energy WAF (HEWAF), and low-energy WAF (LEWAF).

Source: K. Sweeney.

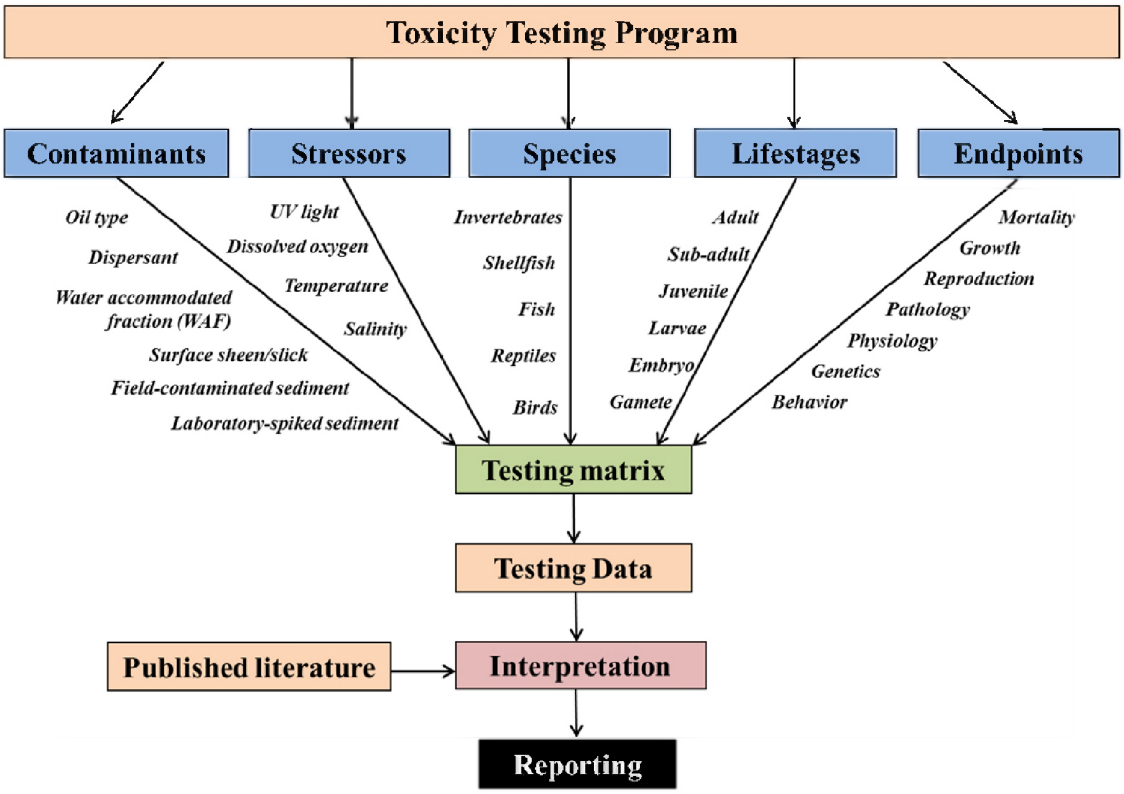

Figure 2. An overview of the Trustees' toxicity testing program approach.

We designed a comprehensive toxicity testing program with many contributors; to ensure consistency across laboratories, we standardized our methods and procedures as much as possible at the outset of testing. These efforts included using the same oil samples for testing, creating exposure solutions using the same methods, thoroughly reviewing testing plans and procedures before conducting each test, and collecting data in a standardized format. We designed a programmatic Quality Assurance Project Plan (QAPP) describing many of these procedures and steps (see Attachment 3 in this report). Finally, we hired a qualified third party to audit each toxicity testing laboratory to ensure that the laboratory was following the pre-approved procedures (see Attachment 4 in this report).

The purpose of this document is to provide detailed protocols for the procedures and bioassays that we conducted on fish, invertebrates, and phytoplankton under the Trustees' DWH toxicity testing program. This report also provides bioassay results and dose-response figures for specific toxicity tests referenced in DWH Trustees (2015, Section 4.3, Toxicity) and in other technical reports supporting this section. For specific details on toxicity tests on birds, see Bursian et al.

(2015a, 2015b), Dorr et al. (2015), Maggini et al. (2015), and Pritsos et al. (2015); for toxicity tests on turtles, see Mitchelmore et al. (2015); and for toxicity tests on sturgeon, see USFWS (2015).

The remainder of this document is organized as follows:

- Attachment 1 provides a detailed list of all definitive toxicity tests completed as of July 23, 2015, and the General Laboratory Procedures and Practices (GLPP) that each laboratory followed to conduct these tests.
- Attachment 2 provides the results of our statistical analyses of bioassay data and test-specific details for tests specifically referenced in the toxicity section of the PDARP (Section 4.3) or in other technical reports that support this section.
- Attachment 2 also includes a description of toxicity test conditions for each test used in the PDARP, a description of methods used to produce calculated values, and a description of analytical methods.
- Attachment 3 is the QAPP, which all laboratories followed when conducting bioassays under the Trustees' toxicity testing program.
- Attachment 4 shows the quality assurance/quality control (QA/QC) procedures that we followed to ensure accuracy throughout data processing and analysis.

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USFWS. 2015. Exposure and Injuries to Threatened Gulf Sturgeon (*Acipenser oxyrinchus desotoi*) as a Result of the Deepwater Horizon Oil Spill.

1. General Laboratory Procedures and Practices: *Deepwater Horizon* Laboratory Toxicity Testing

Contents

0. Overview

This *General Laboratory Procedures and Practices: Deepwater Horizon Laboratory Toxicity Testing* document contains a collection of standard laboratory protocols, procedures, and practices used, under the guidance of Stratus Consulting,[1] by institutions conducting toxicity testing in support of the *Deepwater Horizon* (DWH) Natural Resource Damage Assessment (NRDA). The principal investigators (PIs) from each institution and their corresponding team members are listed in Table 0.1. Each chapter describes the protocols and procedures for a specific institution or institutions. With the exception of Pacific EcoRisk (Chapter 10), the PI from each associated institution wrote the protocols and procedures, in close collaboration with Status Consulting. Stratus Consulting created the Pacific EcoRisk chapter based on Pacific EcoRisk reports.

All of the details provided in this overview and the accompanying documents pertain to definitive tests completed by July 23, 2015. In most cases, testing protocols and test conditions tables (TCTs) were developed before the initiation of any test and were approved by Stratus Consulting. Following the completion of a test, minor modifications may have been made to the testing protocol to accurately reflect actual testing procedures. This document contains the final/modified versions of protocols and test conditions. Any minor in-test modifications to the test conditions (e.g., test duration, replicate numbers) are reflected in the definitive test conditions reported for each test in the toxicity testing database. As additional tests are completed, new information will be added to this document. Additional information regarding each test can be found in the DWH Trustee toxicity database generated in support of the DWH NRDA. Below is a brief description of how each chapter is organized, as well as information regarding supporting documents.

- **Chapter**
  - **General laboratory procedures and practices (GLPP; main chapter text)**

    The main text in each chapter provides institution-specific information regarding GLPP. It also includes general standard operating procedures (SOPs) that were followed in day-to-day testing activity for most tests (e.g., measuring basic water quality parameters).

1. Stratus Consulting merged with Abt Associates in 2015; since much of this work was conducted by Stratus Consulting prior to the merger, both firm names will appear.

- **Testing protocols (chapter appendices)**

  The appendices provide specific protocols for the various types of toxicity tests conducted at each institution.

- **Supporting documents**

  - **Quality Assurance Project Plan: DWH Laboratory Toxicity Testing**

    Stratus Consulting prepared this document to outline the procedures used to ensure that data were collected and analyzed to meet project requirements; the document also includes some project-wide SOPs, such as the preparation of water accommodated fractions (WAFs).

  - **TCTs**

    Each test that was conducted is accompanied by a test-specific TCT that describes the conditions of the test (e.g., species, life stage, contaminant tested, WAF preparation method, nominal exposure concentrations, test duration, temperature). Any modifications to the GLPP or testing protocols were captured in the test-specific TCT. TCTs will be available through the National Oceanic and Atmospheric Administration (NOAA) DIVER data repository (DIVER, 2015). Appendix A contains abbreviated information relating to each definitive test.

Note: When stated, samples were sent to ALS Environmental, formerly Columbia Analytical Services (CAS). Water sampling series or schedules listed in a specific testing protocol may have been modified from test to test because of specific testing conditions or earlier test results.

Each chapter contains institution-specific information regarding toxicity testing and is split into two major sections. The first section of the chapter contains the GLPP from a specific institution. The second section contains appendices that comprise laboratory-specific testing protocols for definitive tests. Stratus Consulting does not endorse or recommend any commercial products cited in this document. The stated use of any commercial products by authors of this report may not be used for advertising or product endorsement purposes.

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
Auburn University	Auburn, AL	James Stoeckel	Adam Kelly Catlin Ames Ginger Stuckey	Ian Palmer Michael Hart
Florida Gulf Coast University and University of North Carolina, Wilmington	Fort Myers, FL	Aswani Volety	Ai Ning Loh Andy Griffith Anne Rolton Ashley Demey Ben Woodall Brooke Denkert Cecile Jauzein Chelsea Miley Christophe Lambert David Segal Emily Nickols Fu-Lin Chu Hunter Cox Ian Campbell Jeff Devine John Roberts Josh Forbes	Julie Neurohr Julien Guyomarch Julien Vignier Kathleen McNatt Kelsey McEachern Kyle Chenevert Leslie Haynes Lindsay Castret Ludovic Donaghy Michael Parsons Molly Rybovich Myrina Boulais Nelly Legoic Nicole Martin Philippe Soudant Rheannon Ketover Thomas Dolan
Louisiana State University, Department of Biological Science	Baton Rouge, LA	Fernando Galvez	Benjamin Dubansky Kali Holder Catherine Simoneaux Brittney Keosayasing Courtney Poulos Eben Smitherman	Gabi Borel Gregory Long Florence Louann Johnson Charles Brown Sydney Hebert Tiffany Simms Lindsey
Louisiana Universities Marine Consortium	Chauvin, LA	Edward Chesney	Evan Kwityn Kathryn O'Shaughnessy Sam Leberg	Sarah Webb Tara Duffy Taylor Alexander William Childress
Marin Biologic Laboratories, Inc.	Novato, CA	Peter Ralph	Erin Accurso	Tania Weiss
Miami University	Oxford, OH	James Oris	Andrew Tucker Dale Coffey Marlo Jeffries Leah Thornton	Alison Willis Graham Hughes Nora Covy Lucas Smith

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members (cont.)

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
Mote Marine Laboratory	Sarasota, FL	Dana Wetzel	Carlos Yanes-Roca Erin Pulster Kevan Main Kylee Bowling Matthew Resley Michael Nystrom Mike Henry Nathan Brennan	Nicole Rhody Patricia Blum Paula Puiggerver-Caldentrey Rebecca Medvecky Richard Pierce Samantha Harlow
Northwest Fisheries Science Center	Seattle, WA	Nathaniel Scholz John Incardona	Allisan Aquilana-Beck Barbara French Bernadita Anulacion Catherine Sloan Cathy Laetz Darlye Boyd David Baldwin Gina Ylitalo Heather Day	James Cameron Jana Labenia Jennie Bolton Mark Tagal Nathaniel Scholz Richard Edmunds Ron Pearce Tanya Brown Tiffany Linbo Tony Gill
Pacific EcoRisk	Fairfield, CA	Scott Ogle	Drew Gantner Krista Prosser	Padrick Anderson
Stanford University	Stanford, CA	Barbara Block	Ben Machado Fabien Brette John Dale	Luke Gardner Robert Schallert
Stratus Consulting	Boulder, CO	Jeffrey M. Morris	Andrew McFadden Anthony Berenguel Chad Mansfield Claire Lay David Cacela Fiona Garvin Heather P. Forth Ian Lipton James V. Holmes Jeffrey Cegan Joshua Lipton Karen Dean	Liza Hernandez Mary Huisenga Michael Carney Michael Duckworth Michel Gielazyn Michelle Krasnec Robert Vega Ronald Hall Ryan Takeshita Shane Bonnot David Abrego

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members (cont.)

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
University of Maryland Center for Environmental Science	Solomons, MD	Carys Mitchelmore	Christopher Rowe Eric Schott Gregory Danvers Hannah Pie Jum Sook Chung	Maureen Strauss Nicole Chigounis Sarah Funck Steve Suttles
University of Miami/Grosell	Miami, FL	Martin Grosell Daniel Benetti	Andre Faul Andrew Esbaugh Charlotte Bodinier Daniel Benetti Danielle McDonald Edward Mager Jennifer Panlilio Jessica Wingar	John Stieglitz Kathleen Munley Kevin Brix Maria Rodgers Ron Hoenig Sasa Miralao Theresa Mackey Zack Daugherty
University of North Texas	Denton, TX	Aaron Roberts	Alexis Wormington Brian Matherne Brianne Soulen Carmen Overturf Celeste Ortega-Rodriguez Charles Mansfield Erin Ussery James Smith Jason Magnuson	Jennifer Gnau Jessica Trevino Kristin Nielsen-Bridges Lauren Sweet Leigh Taylor Matt Alloy Morgan VanAken Thomas (Ty) Curran Thomas Garner
University of Southern Mississippi	Ocean Springs, MS	Robert (Joe) Griffitt	Arthur Karels Beth Jones Binnaz Bailey Bryan Hedgpeth Carly Somerset Danielle Simning Idrissa Boube Jay Grimes Jean Jovonovich	Jenish Kumar Jeremy Johnson Jeremy Lindsey Jessica Holland Kim Griffit Lyndsay Carrigee Nancy Brown-Peterson Natalie Ortell (Cumbaa) Ryan Gordon
U.S. Army Engineer Research and Development Center	Vicksburg, MS	Guilherme Lotufo	James Biedenbach Ashly Harmon Daniel J. Farrar Jacob Stanley	James Lindsay Jamma Williams Jen Chappel Jennifer Laird

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A. Abbreviated Tables of Toxicity Testing Conditions

Table A.1. Description of headings used in Tables A.4 and A.5

Heading	Definition
Test ID	Identification number of a test
Species	Test Species
Life stage	Life stage of test organisms
Test duration	Duration (h) of the test
Contaminant	Contaminant used during the test (Forth et al., 2015; Krasnec et al., 2015)
Exposure type	Exposure mixture or media that was used during a test (Forth et al., 2015; Krasnec et al., 2015)
Endpoints	Endpoints that were measured during a test
Other conditions	Additional test design parameters
Laboratory	Institution that conducted a test (Table A.4 and Table 4.5)
GLPP chapter	Chapter of the GLPP containing testing the procedures and protocols of a test*
Protocol	The number of the test-specific protocol within the GLPP chapter[a]

a. NYA = Not yet available, NA = Not applicable.

Table A.2. Description of testing laboratory abbreviations used in Tables A.4 and A.5

Abbreviation	Testing laboratory
Auburn	Auburn University
FGCU	Florida Gulf Coast University
GCRL	University Southern Mississippi (GCRL)
Hopkins	Stanford University (Hopkins Marine Station)
LSU	Louisiana State University
Mote	Mote Marine Lab
MUO	Miami University (Ohio)
NWFSC	Northwest Fisheries Science Center (NOAA)
PER	Pacific EcoRisk
RSMAS	University Miami (RSMAS)
UMD	University Maryland
UNT	University North Texas
USACE	U.S. Army Engineer Research and Development Center
Stratus	Abt Associates (formerly Stratus Consulting)

Table A.3. Description of contaminant abbreviations used in Tables A.4 and A.5

Contaminant	Description
A	Slick A (Oil)
ANSCO	Alaska North Slope crude oil
B	Slick B (Oil)
COR	Dispersant (COREXIT 9500)
FIELD SED	Field collected sediment
FLU	Fluoranthene
S	Source (Oil)
WS	Weathered Source (Oil)

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
535	Fiddler crab	Adult	456	A	SED CLEAN + surface oiling	Behavior		Auburn	1	3
572	Fiddler crab	Adult	240	A	SED CLEAN + surface oiling	Offspring mortality (test 578)	Maternal exposure	Auburn-UNT	1	1
578	Fiddler crab	Zoea	96	A	Maternal exposure (572)	Mortality	Offspring from 572	Auburn-UNT	1	2
196	Fiddler crab	Adult	336	A	HEWAF	Reproduction		Auburn-UNT	1	4
382	Marsh periwinkle	Adult	72	B	Oiled substrate	Mortality, movement		Auburn-UNT	1	5
598	Marsh periwinkle	Adult	72	B	Oiled substrate	Mortality	Differential exposure duration	Auburn-UNT	1	6
602	Marsh periwinkle	Adult	72	B	Oiled substrate	Mortality	Differential exposure duration	Auburn-UNT	1	7
102	Eastern oyster	Embryo	96	WS	CEWAF	Mortality, growth, development		FGCU	2	1
103	Eastern oyster	Embryo	96	A	CEWAF	Mortality, growth, development		FGCU	2	1
105	Eastern oyster	Veliger	96	WS	CEWAF	Mortality, growth, development		FGCU	2	1
106	Eastern oyster	Veliger	96	A	CEWAF	Mortality, growth, development		FGCU	2	1
107	Eastern oyster	Early spat	336	A	HEWAF	Mortality, growth		FGCU	2	1
109	Eastern oyster	Early spat	168	A	CEWAF	Mortality, growth		FGCU	2	1
110	Eastern oyster	Embryo	96	COR	DISP	Mortality, growth, development		FGCU	2	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
111	Eastern oyster	Veliger	96	COR	DISP	Mortality, growth, development		FGCU	2	1
124	Eastern oyster	Gamete to veliger	96	A	CEWAF	Fertilization, mortality, growth, development		FGCU	2	1
175	Eastern oyster	Veliger	96	A	CEWAF	Mortality, growth		FGCU	2	1
176	Eastern oyster	Veliger	96	A	HEWAF	Mortality, growth, development		FGCU	2	1
178	Eastern oyster	Veliger	96	COR	DISP	Mortality, growth, development		FGCU	2	1
189	Eastern oyster	Gamete to veliger	96	A	LEWAF	Fertilization, mortality, growth, development		FGCU	2	1
190	Eastern oyster	Embryo	96	A	LEWAF	Abnormality, growth, mortality		FGCU	2	1
191	Eastern oyster	Veliger	96	A	LEWAF	Mortality, growth, development		FGCU	2	1
263	Eastern oyster	Embryo	96	A	HEWAF	Mortality, growth, development		FGCU	2	1
264	Eastern oyster	Gamete to veliger	96	A	HEWAF	Fertilization, mortality, growth, development		FGCU	2	1
305	Eastern oyster	Early spat	168	WS	CEWAF	Mortality, growth		FGCU	2	1
306	Eastern oyster	Early spat	168	COR	DISP	Mortality, growth		FGCU	2	1
307	Eastern oyster	Early spat	336	WS	HEWAF	Mortality, growth, development		FGCU	2	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
368	Eastern oyster	Gamete to veliger	96	B	HEWAF	Mortality, abnormality, growth, reproduction		FGCU	2	1
370	Eastern oyster	Embryo	96	B	HEWAF	Mortality, abnormality, growth		FGCU	2	1
371	Eastern oyster	Veliger	96	B	HEWAF	Mortality, abnormality, growth		FGCU	2	1
400	Eastern oyster	Gamete to veliger	96	COR	DISP	Fertilization, mortality, growth, development		FGCU	2	1
506	Eastern oyster	Embryo	96	A	HEWAF	Mortality, growth, development		FGCU	2	1
507	Eastern oyster	Veliger	96	A	HEWAF	Mortality, growth, development		FGCU	2	1
508	Eastern oyster	Gamete to veliger	96	A	HEWAF	Fertilization, mortality, growth, development		FGCU	2	1
519	Eastern oyster	Gamete to veliger	96	WS	CEWAF	Fertilization, mortality, growth, development		FGCU	2	1
521	Eastern oyster	Embryo	96	WS	HEWAF	Mortality, growth, development		FGCU	2	1
522	Eastern oyster	Veliger	96	WS	HEWAF	Mortality, growth, development		FGCU	2	1
525	Eastern oyster	Gamete to veliger	96	WS	HEWAF	Fertilization, mortality, growth, development		FGCU	2	1
188	Eastern oyster	Adult	720	A	Algae + oil	Histology		FGCU	2	2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
302	Eastern oyster	Adult	504	A, COR	Algae + CEWAF	Mortality		FGCU	2	2
318	Eastern oyster	Adult	504	WS	Algae + oil	Mortality		FGCU	2	2
541	Eastern oyster	Adult to offspring	720	A	Algae + oil	Fertilization, mortality, growth, development		FGCU	2	3
542	Eastern oyster	Adult to offspring	672	WS	Algae + oil + dispersant	Fertilization, mortality, growth, development		FGCU	2	3
576	Eastern oyster	Embryo	96	FIELD SED	Sediment elutriate	Mortality, growth, development		FGCU	2	4
609	Eastern oyster	Gamete to veliger	96	FIELD SED	Sediment elutriate	Fertilization, mortality, growth, development		FGCU	2	4
610	Eastern oyster	Veliger	96	FIELD SED	Sediment elutriate	Mortality, growth, development		FGCU	2	4
534	Eastern oyster	Adult	336	FIELD SED	FIELD SED	Mortality		FGCU	2	5
573	Eastern oyster	Adult to offspring	672	A	Algae + oil	Fertilization, mortality, growth, development		FGCU	2	2, 3
128	Eastern oyster	Spat	240	A	HEWAF	Mortality	Salinity / temperature	FGCU	2	7
132	Eastern oyster	Veliger	96	A	CEWAF	Mortality, growth, development	Salinity / temperature	FGCU	2	6, 1
133	Eastern oyster	Veliger	96	A	HEWAF	Mortality, growth, development	Salinity / temperature	FGCU	2	6, 1
582	Eastern oyster	Embryo	96	A	CEWAF	Mortality, growth, development	Salinity / temperature	FGCU	2	6, 1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
583	Eastern oyster	Embryo	96	A	HEWAF	Mortality, growth, development	Salinity / temperature	FGCU	2	6, 1
585	Eastern oyster	Gamete to veliger	96	A	HEWAF	Fertilization, mortality, growth, development	Salinity / temperature	FGCU	2	6, 1
628	Eastern oyster	Gamete to veliger	96	A	CEWAF	Fertilization, mortality, growth, development	Salinity / temperature	FGCU	2	6, 1
156	Eastern oyster	Adult to offspring	336	B	SPIKED SED	Mortality, growth, development		FGCU	2	8
218	Eastern oyster	Adult to offspring	336	B	SPIKED SED	Mortality, reproduction, histology		FGCU	2	8
343	Eastern oyster	Adult	336	B	SPIKED SED	Immune, histology, mortality		FGCU	2	8
558	Eastern oyster	Veliger	336	A	Algae + oil	Growth, mortality		FGCU	2	9
230	Eastern oyster	Pediveliger	72	A	HEWAF	Mortality, settlement		FGCU	2	10
233	Eastern oyster	Pediveliger	72	A	CEWAF	Mortality, settlement		FGCU	2	10
256	Eastern oyster	Pediveliger	72	B	SPIKED SED	Mortality, settlement		FGCU	2	10
125	Blue crab	Zoea	96	A	HEWAF	Mortality		GCRL	3	1
126	Blue crab	Zoea	96	B	HEWAF	Mortality		GCRL	3	1
127	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality		GCRL	3	1
134	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality		GCRL	3	1
135	Sheepshead minnow	Juvenile	96	A	HEWAF	Mortality		GCRL	3	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
136	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		GCRL	3	1
137	Sheepshead minnow	Juvenile	96	A	CEWAF	Mortality		GCRL	3	1
138	Sheepshead minnow	Larvae	96	B	CEWAF	Mortality		GCRL	3	1
139	Sheepshead minnow	Juvenile	96	B	CEWAF	Mortality		GCRL	3	1
141	Speckled sea trout	Juvenile	96	A	HEWAF	Mortality		GCRL	3	1
142	Speckled sea trout	Larvae	96	A	CEWAF	Mortality		GCRL	3	1
143	Speckled sea trout	Juvenile	96	A	CEWAF	Mortality		GCRL	3	1
144	Blue crab	Zoea	96	B	CEWAF	Mortality		GCRL	3	1
148	Blue crab	Zoea	96	A	CEWAF	Mortality		GCRL	3	1
154	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality	Salinity	GCRL	3	1
155	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality	Salinity	GCRL	3	1
167	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		GCRL	3	1
173	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality	Dissolved oxygen	GCRL	3	1
184	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		GCRL	3	1
185	Sheepshead minnow	Embryo	144	A	HEWAF	Mortality	Temperature	GCRL	3	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
192	Sheepshead minnow	Embryo	144	A	HEWAF	Mortality, hatching	Temperature	GCRL	3	1
193	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		GCRL	3	1
194	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality		GCRL	3	1
225	Sheepshead minnow	Larvae	24	A	HEWAF	Mortality	Differential exposure duration	GCRL	3	1
227	Sheepshead minnow	Larvae	24	A	CEWAF	Mortality	Differential exposure duration	GCRL	3	1
236	Sheepshead minnow	Larvae	48	A	HEWAF	Mortality	Differential exposure duration	GCRL	3	1
238	Sheepshead minnow	Larvae	48	A	CEWAF	Mortality	Differential exposure duration	GCRL	3	1
248	Sheepshead minnow	Larvae	24	A	HEWAF	Mortality	Differential exposure duration	GCRL	3	1
253	Sheepshead minnow	Larvae	24	A	CEWAF	Mortality	Differential exposure duration	GCRL	3	1
254	Sheepshead minnow	Larvae	48	A	HEWAF	Mortality	Differential exposure duration	GCRL	3	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
255	Sheepshead minnow	Larvae	48	A	CEWAF	Mortality	Differential exposure duration	GCRL	3	1
261	White shrimp	Juvenile	96	A	HEWAF	Mortality		GCRL	3	1
267	White shrimp	Juvenile	96	COR	DISP	Mortality		GCRL	3	1
268	White shrimp	Juvenile	96	A	CEWAF	Mortality		GCRL	3	1
283	Grass shrimp	Adult	96	A	CEWAF	Mortality		GCRL	3	1
289	Grass shrimp	Adult	96	S	HEWAF	Mortality		GCRL	3	1
293	Grass shrimp	Adult	96	S	CEWAF	Mortality		GCRL	3	1
310	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality	Variable renewal frequency	GCRL	3	1
311	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality	Variable renewal frequency	GCRL	3	1
312	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality	Variable dispersant	GCRL	3	1
325	Blue crab	Zoea	96	A	CEWAF	Mortality		GCRL	3	1
352	Sheepshead minnow	Embryo	96	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1
353	Sheepshead minnow	Embryo	24	A	HEWAF	Mortality, heart rate, hatching	Differential exposure duration	GCRL	3	1
354	Sheepshead minnow	Embryo	6	A	HEWAF	Mortality, heart rate, hatching	Differential exposure duration	GCRL	3	1
364	Sheepshead minnow	Embryo	6	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
365	Sheepshead minnow	Embryo	12	A	HEWAF	Mortality, hatching, heart rate		GCRL	3	1
367	Sheepshead minnow	Embryo	24	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1
523	Grass shrimp	Adult	96	A	HEWAF	Mortality		GCRL	3	1
524	Blue crab	Zoea	96	A	HEWAF	Mortality		GCRL	3	1
536	Sheepshead minnow	Embryo	12	A	HEWAF	Mortality, heart rate, hatching	Temperature	GCRL	3	1
538	Sheepshead minnow	Embryo	12	A	HEWAF	Mortality, heart rate, hatching	Temperature	GCRL	3	1
612	Sheepshead minnow	Embryo	168	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1
613	Sheepshead minnow	Embryo	12	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1
614	Sheepshead minnow	Embryo	12	A	HEWAF	Mortality, heart rate, hatching		GCRL	3	1
632	Southern flounder	Larvae	96	A	HEWAF	Mortality		GCRL	3	1
633	Southern flounder	Larvae	72	A	HEWAF	Mortality		GCRL	3	1
634	Southern flounder	Larvae	72	B	SPIKED SED	Mortality		GCRL	3	1
635	Southern flounder	Larvae	96	A	HEWAF	Mortality		GCRL	3	1
661	Grass shrimp	Adult	96	B	CEWAF	Mortality		GCRL	3	1
662	Grass shrimp	Adult	96	B	HEWAF	Mortality		GCRL	3	1
170	Sheepshead minnow	Larvae	672	A	HEWAF	Mortality		GCRL	3	2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
171	Sheepshead minnow	Larvae	672	A	CEWAF-VARDISP	Mortality		GCRL	3	2
540	Southern flounder	Juvenile	672	FIELD SED	FIELD SED	Mortality, histology		GCRL	3	3
551	Grass shrimp	Adult, embryos, larvae	1104	A	HEWAF	Reproduction		GCRL	3	4
568	Grass shrimp	Adult, embryos, larvae	672	A	CEWAF	Reproduction		GCRL	3	4
627	Grass shrimp	Adult, embryos, larvae	672	A	HEWAF	Mortality, growth		GCRL	3	5
215	Sheepshead minnow	Adult, embryos, larvae	1008	A	CEWAF	Reproduction		GCRL	3	6
571	Sheepshead minnow	Adult, embryos, larvae	840	A	HEWAF	Reproduction		GCRL	3	6
113	Southern flounder	Juvenile	768	B	SPIKED SED	Mortality, growth, qPCR, histology, microbiomics		GCRL	3	7
163	Grass shrimp	Adult	672	B	SPIKED SED	Mortality, reproduction, hatching, growth		GCRL	3	8
129	Sheepshead minnow	Adult	336	A	HEWAF	Mortality, growth		GCRL	3	9
130	Sheepshead minnow	Adult	336	A	CEWAF	Mortality, growth		GCRL	3	9

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
586	Grass shrimp	Adult	672	FIELD SED	FIELD SED	Mortality		GCRL	3	10
909	Red snapper	Juvenile		A	HEWAF	Mortality, growth, qPCR, BKA		GCRL	3	11
916	Atlantic croaker	Juvenile	264	A	HEWAF	Mortality, growth, qPCR, RBC, PCV		GCRL	3	11
921	Red drum	Juvenile	264	A	HEWAF	Mortality, growth, qPCR, RBC, PCV		GCRL	3	11
900	Southern flounder	Juvenile	408	B	SPIKED SED	Mortality, growth, qPCR, transcriptomics, microbiomics, BKA		GCRL	3	12
904	Southern flounder	Juvenile	408	B	SPIKED SED	Mortality, growth, qPCR, transcriptomics, microbiomics, BKA		GCRL	3	12
450	Pacific mackerel	Adult	72	WS	GWAF	Respirometry		Hopkins	4	3
451	Pacific mackerel	Adult	72	WS	GWAF	Respirometry		Hopkins	4	3
452	Pacific mackerel	Adult	72	WS	GWAF	Respirometry		Hopkins	4	3
453	Pacific mackerel	Adult	72	WS	GWAF	Respirometry		Hopkins	4	3
454	Pacific mackerel	Adult	96	WS	GWAF	Respirometry		Hopkins	4	3
455	Pacific mackerel	Adult	96	WS	GWAF	Respirometry		Hopkins	4	3

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
456	Pacific mackerel	Adult	96	WS	GWAF	Respirometry		Hopkins	4	3
457	Pacific mackerel	Adult	72	WS	GWAF	Respirometry		Hopkins	4	3
458	Pacific mackerel	Adult	96	WS	GWAF	Respirometry		Hopkins	4	3
411	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
412	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
413	Pacific bluefin tuna	Cardio-myocyte	6	WS	HEWAF	Electrophysiology		Hopkins	4	1, 2
414	Pacific bluefin tuna	Cardio-myocyte	6	WS	HEWAF	Electrophysiology		Hopkins	4	1, 2
415	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
416	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
417	Pacific bluefin tuna	Cardio-myocyte	6	A	HEWAF	Electrophysiology		Hopkins	4	1, 2
418	Pacific bluefin tuna	Cardio-myocyte	6	A	HEWAF	Electrophysiology		Hopkins	4	1, 2
420	Pacific bluefin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
421	Pacific bluefin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
423	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
424	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
426	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
427	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
428	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
429	Pacific bluefin tuna	Cardio-myocyte	6	A	HEWAF	Electrophysiology		Hopkins	4	1, 2
430	Pacific bluefin tuna	Cardio-myocyte	6	A	HEWAF	Electrophysiology		Hopkins	4	1, 2
438	Pacific bluefin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
440	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
441	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
442	Pacific bluefin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
443	Pacific bluefin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
444	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
445	Pacific mackerel	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
447	Pacific mackerel	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
448	Pacific mackerel	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
449	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
490	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
491	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
492	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
493	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
494	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
498	Pacific bluefin tuna	Cardio-myocyte	6	NA	HEWAF	Electrophysiology		Hopkins	4	1, 2
499	Pacific bluefin tuna	Cardio-myocyte	6	NA	HEWAF	Electrophysiology		Hopkins	4	1, 2
H06	Pacific bluefin tuna	Cardio-myocyte	6	S, WS, A	HEWAF	Electrophysiology		Hopkins	4	1, 2
H07	Pacific bluefin tuna	Cardio-myocyte	6	S, WS, A	HEWAF	Electrophysiology		Hopkins	4	1, 2
H08	Pacific bluefin tuna	Cardio-myocyte	6	S, WS, A	HEWAF	Electrophysiology		Hopkins	4	1, 2
H09	Pacific bluefin tuna	Cardio-myocyte	6	S, WS, A	HEWAF	Electrophysiology		Hopkins	4	1, 2
H11	Pacific bluefin tuna	Cardio-myocyte	6	WS	HEWAF	Electrophysiology		Hopkins	4	1, 2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
419	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
422	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
425	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
431	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
432	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
433	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
434	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
435	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
436	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
437	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
439	Yellowfin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
446	Yellowfin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
495	Yellowfin tuna	Cardio-myocyte	6	S	HEWAF	Electrophysiology		Hopkins	4	1, 2
496	Yellowfin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
497	Pacific bluefin tuna	Cardio-myocyte	6	B	HEWAF	Electrophysiology		Hopkins	4	1, 2
H10	Pacific bluefin tuna	Cardio-myocyte	6	WS	HEWAF	Electrophysiology		Hopkins	4	1, 2
477	Southern bluefin tuna	Embryo	36	WS	HEWAF	Mortality, cardiac effects		Hopkins-NWFSC	13	4
164	Gulf killifish	Larvae	96	A	HEWAF	Mortality, heart rate		LSU	11	1
165	Gulf killifish	Larvae	96	A	CEWAF	Mortality, heart rate		LSU	11	1
195	Gulf killifish	Embryo	480	B	SPIKED SED	Heart rate, hatching, mortality		LSU	11	2
152	Gulf killifish	Embryo	96	A	CEWAF	Mortality, heart rate, hatching		LSU	11	3
157	Gulf killifish	Embryo	480	A	HEWAF	Mortality, heart rate, hatching		LSU	11	4
158	Gulf killifish	Embryo	480	A	CEWAF	Mortality, heart rate, hatching		LSU	11	4
217	Gulf killifish	Embryo	480	B	HEWAF	Mortality, heart rate, hatching		LSU	11	4
377	Gulf killifish	Embryo	480	A	Slick	Heart rate, hatching		LSU	11	5
378	Gulf killifish	Embryo	480	B	Slick	Heart rate, hatching		LSU	11	5
K01	Gulf killifish	Embryo	480	A	Slick	Mortality, heart rate, hatching		LSU	11	5
K02	Gulf killifish	Embryo	480	B	Slick	Mortality, heart rate, hatching		LSU	11	5
914	Sand seatrout	Embryo	96	B	HEWAF	Mortality		LUMCON	16	1
911	Gulf menhaden	Larvae	72	B	HEWAF	Mortality		LUMCON	16	1
912	Gulf menhaden	Embryo/ larvae	48	B	HEWAF	Mortality		LUMCON	16	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
913	Gulf menhaden	Embryo to larvae	96	B	HEWAF	Mortality		LUMCON	16	1
925	Bay anchovy	Embryo	48	B	HEWAF	Mortality		LUMCON	16	2
926	Bay anchovy	Embryo	48	B	HEWAF	Mortality		LUMCON	16	2
927	Bay anchovy	Embryo	24	B	HEWAF	Mortality	Differential exposure duration	LUMCON	16	2
929	Bay anchovy	Embryo	48	A	HEWAF	Mortality		LUMCON	16	2
931	Bay anchovy	Embryo	48	B	LEWAF	Mortality		LUMCON	16	2
936	Bay anchovy	Embryo	48	A	LEWAF	Mortality		LUMCON	16	2
943	Bay anchovy	Embryo	72	A	HEWAF	Mortality		LUMCON	16	2
937	Bay anchovy	Embryo	48	A	Slick	Mortality		LUMCON	16	3
938	Bay anchovy	Embryo	48	B	Slick	Mortality		LUMCON	16	3
944	Bay anchovy	Embryo	48	A	Slick	Mortality		LUMCON	16	3
945	Bay anchovy	Embryo	2, 6, 48	A	Slick	Mortality		LUMCON	16	3
949	Bay anchovy	Embryo	6, 10, 48	A	Slick	Mortality		LUMCON	16	3
950	Bay anchovy	Embryo	6, 10, 48	A	Slick	Mortality		LUMCON	16	3
956	Red snapper	Embryo	48	A	HEWAF	Mortality		LUMCON	16	4
970	Red snapper	Embryo	48	A	HEWAF	Mortality		LUMCON	16	4
B01	Adrenal cell H295R	Na	48	B	HEWAF	Mortality		Marin	15	3
B02	Adrenal cell H295R	Na	48	WS	HEWAF	Adrenal		Marin	15	3
670	Adrenal cell H295R	Na	48	A	HEWAF	Adrenal		Marin	15	4
672	Adrenal cell H295R	Na	48	A	LEWAF	Adrenal		Marin	15	4

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
200	Red drum	Embryo	24	WS	HEWAF	Hatching, development		Mote	6	1
201	Red drum	Embryo	24	A	HEWAF	Hatching, development		Mote	6	1
202	Red drum	Embryo	24	A	CEWAF	Hatching, development		Mote	6	1
204	Red drum	Embryo	24	COR	DISP	Hatching, development		Mote	6	1
205	Red drum	Larvae	96	WS	HEWAF	Mortality		Mote	6	1
206	Red drum	Larvae	96	A	HEWAF	Mortality		Mote	6	1
207	Red drum	Larvae	96	A	CEWAF	Mortality		Mote	6	1
209	Red drum	Larvae	96	COR	DISP	Mortality		Mote	6	1
210	Inland silverside	Larvae	96	A	HEWAF	Mortality		Mote	6	1
211	Inland silverside	Larvae	96	A	CEWAF	Mortality		Mote	6	1
212	Inland silverside	Juvenile	96	A	HEWAF	Mortality		Mote	6	1
213	Inland silverside	Juvenile	96	A	CEWAF	Mortality		Mote	6	1
214	Red drum	Larvae	96	A	CEWAF	Mortality		Mote	6	1
888	Red drum	Juvenile	16	A	CEWAF	Behavior		Mote	6	2
889	Red drum	Juvenile	16	COR	DISP	Behavior		Mote	6	2
890	Red drum	Juvenile	16	A	CEWAF	Behavior		Mote	6	2
891	Red drum	Juvenile	16	COR	CEWAF	Behavior		Mote	6	2
198	Amberjack	Embryo	48	WS	HEWAF	Mortality, heart rate		NWFSC	13	5
274	Amberjack	Embryo	48	WS	HEWAF	Heart rate		NWFSC	13	6

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
360	Zebrafish	Embryo to juvenile	4–6	A, B, WS, ANSCO	HEWAF	Mortality, development, growth		NWFSC	13	1, 2, 3
114	Leptocheirus	Juvenile	240	A	SPIKED SED	Mortality		PER	10	1
115	Leptocheirus	Juvenile	240	B	SPIKED SED	Mortality		PER	10	1
262	Leptocheirus	Juvenile	240	B	SPIKED SED	Mortality		PER	10	1
340	Leptocheirus	Juvenile	240	B	SPIKED SED	Mortality		PER	10	1
342	Leptocheirus	Juvenile	240	A	SPIKED SED	Mortality		PER	10	1
630	Leptocheirus	Juvenile	240	FIELD SED	FIELD SED	Mortality		PER	10	I
631	Leptocheirus	Juvenile	240	FIELD SED	FIELD SED	Mortality		PER	10	1
580	Mysid shrimp	Juvenile	96	A	HEWAF	Mortality		PER	10	2
591	Mysid shrimp	Juvenile	96	A	LEWAF	Mortality		PER	10	2
592	Mysid shrimp	Juvenile	96	B	HEWAF	Mortality		PER	10	2
600	Mysid shrimp	Juvenile	96	B	LEWAF	Mortality		PER	10	2
601	Mysid shrimp	Juvenile	96	B	HEWAF	Mortality		PER	10	2
622	Mysid shrimp	Juvenile	48	A	HEWAF	Mortality		PER	10	2
275	Diatom	Cells	96	A	LEWAF	Growth		PER	10	3
596	Diatom	Cells	96	B	LEWAF	Growth		PER	10	3
597	Diatom	Cells	96	B	HEWAF	Growth		PER	10	3
603	Diatom	Cells	96	B	LEWAF	Growth		PER	10	3
604	Diatom	Cells	96	B	HEWAF	Growth		PER	10	3
623	Diatom	Cells	96	A	HEWAF	Growth		PER	10	3
279	Mahi-mahi	Embryo	96	A	HEWAF	Mortality		RSMAS	7	5
285	Mahi-mahi	Embryo	96	S	CEWAF	Mortality		RSMAS	7	5
291	Mahi-mahi	Embryo	96	WS	CEWAF	Mortality		RSMAS	7	5
298	Mahi-mahi	Embryo	96	WS	HEWAF	Mortality		RSMAS	7	5
299	Mahi-mahi	Embryo	96	S	HEWAF	Mortality		RSMAS	7	5

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
304	Mahi-mahi	Embryo	96	COR	DISP	Mortality		RSMAS	7	5
316	Mahi-mahi	Embryo	96	A	CEWAF	Mortality		RSMAS	7	5
320	Cobia	Embryo	96	S	HEWAF	Mortality		RSMAS	7	5
328	Mahi-mahi	Embryo	96	A	LEWAF	Mortality		RSMAS	7	5
329	Mahi-mahi	Embryo	96	B	LEWAF	Mortality		RSMAS	7	5
330	Mahi-mahi	Embryo	96	A	LEWAF	Mortality		RSMAS	7	5
339	Cobia	Embryo	96	WS	HEWAF	Mortality		RSMAS	7	5
663	Mahi-mahi	Embryo	96	A	LEWAF	Mortality		RSMAS	7	5
314	Mahi-mahi	Embryo	48	A	HEWAF	Swim performance		RSMAS	7	7
550	Mahi-mahi	Embryo	48	A	HEWAF	Swim performance, cardiac effects		RSMAS	7	7
317	Mahi-mahi	Sub-adult	24	A	HEWAF	Swim performance, mortality		RSMAS	7	8
220	Mahi-mahi	Juvenile	24	A	HEWAF	Swim performance, mortality		RSMAS	7	9
270	Mahi-mahi	Juvenile	24	A	HEWAF	Swim performance		RSMAS	7	9
324	Mahi-mahi	Juvenile	24	A	HEWAF	Swim performance, mortality		RSMAS	7	9
552	Mahi-mahi	Juvenile	24	A	HEWAF	Swim performance, mortality		RSMAS	7	9
554	Mahi-mahi	Juvenile	24	A	HEWAF	Swim performance, mortality		RSMAS	7	9
619	Mahi-mahi	Juvenile	3–6	A	HEWAF	Swim performance		RSMAS	7	9
620	Mahi-mahi	Juvenile	12	A	HEWAF	Swim performance		RSMAS	7	9
199	Mahi-mahi	Embryo	96	A	Unsettled HEWAF	Mortality		RSMAS	7	10

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
239	Mahi-mahi	Embryo	96	WS	Unsettled HEWAF	Mortality		RSMAS	7	10
260	Mahi-mahi	Embryo	96	WS	Unsettled HEWAF	Mortality		RSMAS	7	10
359	Mahi-mahi	Embryo	96	A	HEWAF	Mortality		RSMAS	7	10
559	Mahi-mahi	Embryo	96	A	LEWAF	Mortality		RSMAS	7	10
234	Mahi-mahi	Embryo	24	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
235	Mahi-mahi	Embryo	24	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
292	Mahi-mahi	Embryo	6	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
341	Mahi-mahi	Embryo	2	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
362	Mahi-mahi	Larvae	6	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
373	Mahi-mahi	Larvae	6	A	HEWAF	Mortality	Differential exposure duration	RSMAS	7	11
273	Mahi-mahi	Embryo	48	WS	HEWAF	Mortality, gene expression, cardiac effects		RSMAS	7	12

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
667	Mahi-mahi	Embryo	36	A	LEWAF	Mortality	Differential exposure duration	RSMAS	7	13
668	Mahi-mahi	Embryo	36	A	LEWAF	Mortality	Differential exposure duration	RSMAS	7	13
669	Mahi-mahi	Embryo	6	A	LEWAF	Mortality	Differential exposure duration	RSMAS	7	13
332	Mahi-mahi	Embryo	2, 6, 96	A	Slick	Mortality	Differential exposure duration	RSMAS	7	14
334	Mahi-mahi	Embryo	12, 24, 96	A	Slick	Mortality	Differential exposure duration	RSMAS	7	14
337	Mahi-mahi	Embryo	70, 88, 96	A	Slick	Mortality	Differential exposure duration	RSMAS	7	14
338	Mahi-mahi	Embryo	66, 68, 96	A	Slick	Mortality		RSMAS	7	14
527	Mahi-mahi	Embryo	62, 66, 96	A	Slick	Mortality		RSMAS	7	14
664	Mahi-mahi	Embryo	12	A	Slick	Mortality	Differential exposure duration	RSMAS	7	14
326	Mahi-mahi	Embryo	96	A	Slick	Mortality		RSMAS	7	15
327	Mahi-mahi	Embryo	96	B	Slick	Mortality		RSMAS	7	15
R54	Mahi-mahi	Embryo	96	A	Slick	Mortality		RSMAS	7	15
R55	Mahi-mahi	Embryo	96	B	Slick	Mortality		RSMAS	7	15
910	Copepod	Adult	96	A	HEWAF	Mortality		RSMAS	7	16

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
946	Copepod	Adult	48	A	HEWAF	Mortality		RSMAS	7	16
947	Copepod	Adult	48	A	LEWAF	Mortality		RSMAS	7	16
951	Copepod	Adult	48	A	HEWAF	Mortality, reproduction		RSMAS	7	16
953	Copepod	Adult	48	A	LEWAF	Mortality, reproduction		RSMAS	7	16
930	Copepod	Nauplii	48	A	HEWAF	Mortality		RSMAS	7	17
932	Copepod	Nauplii	48	A	HEWAF	Mortality		RSMAS	7	17
934	Copepod	Nauplii	96	A	HEWAF	Mortality		RSMAS	7	17
276	Gulf toadfish	Adult	24	A	HEWAF	Adrenal		RSMAS	7	18
528	Yellowfin tuna	Embryo	96	A	HEWAF	Mortality		RSMAS-NWFSC	7	1
539	Yellowfin tuna	Embryo	72	WS	HEWAF	Mortality		RSMAS-NWFSC	7	1
588	Yellowfin tuna	Embryo	72	A	HEWAF	Mortality		RSMAS-NWFSC	7	2
615	Yellowfin tuna	Embryo	72	Oxytetracycline	NA	Mortality		RSMAS-NWFSC	7	2
616	Yellowfin tuna	Embryo	60	WS	HEWAF	Mortality		RSMAS-NWFSC	7	2
537	Yellowfin tuna	Embryo	48	A	HEWAF	Mortality, cardiac effects		RSMAS-NWFSC	7	3
543	Yellowfin tuna	Embryo	36	A	HEWAF	Mortality, heart rate, transcriptomics		RSMAS-NWFSC	7	3
621	Yellowfin tuna	Embryo	24	WS	HEWAF	Cardiac effects, gene expression		RSMAS-NWFSC	7	4
625	Yellowfin tuna	Embryo	24	WS	HEWAF	Mortality, cardiac		RSMAS-NWFSC	7	4

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
626	Yellowfin tuna	Embryo	24	WS	HEWAF	Mortality, cardiac		RSMAS-NWFSC	7	4
278	Mahi-mahi	Embryo	48	A	HEWAF	Mortality, cardiac effects, gene expression		RSMAS-NWFSC	7	5, 6
288	Mahi-mahi	Embryo	48	A	CEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
290	Mahi-mahi	Embryo	48	WS	HEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
294	Mahi-mahi	Embryo	48	WS	CEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
296	Mahi-mahi	Embryo	48	COR	DISP	Mortality, cardiac effects		RSMAS-NWFSC	7	5, 6
512	Mahi-mahi	Embryo	48	S	HEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
515	Mahi-mahi	Embryo	48	S	CEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
563	Mahi-mahi	Embryo	48	A	HEWAF	Mortality, gene expression, cardiac effects		RSMAS-NWFSC	7	5, 6
380	Red drum	Embryo	72	B	HEWAF	Mortality		Stratus	14	1
381	Red drum	Embryo	72	A	HEWAF	Mortality		Stratus	14	1
384	Red drum	Embryo	72	B	HEWAF	Mortality		Stratus	14	1

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
385	Red drum	Embryo	72	B	HEWAF	Mortality		Stratus	14	1
387	Red drum	Embryo	72	B	HEWAF	Mortality		Stratus	14	1
388	Speckled sea trout	Embryo	72	B	HEWAF	Mortality		Stratus	14	1
394	Speckled sea trout	Embryo	72	A	HEWAF	Mortality		Stratus	14	1
398	Speckled sea trout	Larvae	24	A	HEWAF	Mortality		Stratus	14	1
399	Red drum	Larvae	36	A	HEWAF	Mortality		Stratus	14	1
640	Red drum	Embryo	60	A	LEWAF	Mortality		Stratus	14	1
641	Red drum	Embryo	60	B	LEWAF	Mortality		Stratus	14	1
647	Speckled sea trout	Embryo	72	B	CEWAF	Mortality		Stratus	14	1
653	Red drum	Embryo	60	B	HEWAF	Mortality		Stratus	14	1
X17	Red drum	Embryo	60	B	CEWAF	Mortality		Stratus	14	1
386	Red drum	Embryo	24	B	HEWAF	Mortality	Differential exposure duration	Stratus	14	2
397	Red drum	Embryo	24	A	HEWAF	Mortality	Differential exposure duration	Stratus	14	2
X18	Red drum	Embryo	60	B	Slick	Mortality		Stratus	14	4
X19	Red drum	Embryo	60	A	Slick	Mortality		Stratus	14	4
654	Red drum	Juvenile	336	B	SPIKED SED	Mortality, growth		Stratus	14	5
655	Pacific white shrimp	Post-larvae	168	B	SPIKED SED	Growth, mortality		Stratus	14	6

Table A.4. Metadata describing "definitive" tests performed without UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
636	Red drum	Embryo	36	A	HEWAF	Mortality, cardiac effects		Stratus-NWFSC	14	3
637	Red drum	Embryo	36	B	HEWAF	Mortality, cardiac effects		Stratus-NWFSC	14	3
638	Red drum	Embryo	36	A	LEWAF	Mortality, cardiac effects		Stratus-NWFSC	14	3
639	Red drum	Embryo	36	B	LEWAF	Mortality, cardiac effects		Stratus-NWFSC	14	3
350	Red-eared slider	Juvenile	336	A	OTHER	Mortality, histology, pathology		UMD	NA[b]	NA[b]
351	Common snapping turtle	Juvenile	336	A	OTHER	Mortality, histology, pathology		UMD	NA[b]	NA[b]
569	Blue crab	Juvenile	672	FIELD SED	FIELD SED	Mortality, growth, molting		UMD	9	1
679	Pacific white shrimp	Post-larvae	24	A	HEWAF	Mortality		UNT	8	NYA[a]
683	Pacific white shrimp	Post-larvae	24	A	HEWAF	Mortality		UNT	8	NYA[a]
247	Leptocheirus	Neonates	672	B	SPIKED SED	Mortality, growth, reproduction, reburial		USACE	12	1
272	Leptocheirus	Neonates	672	B	SPIKED SED	Mortality, growth, reproduction, reburial		USACE	12	1

a. Tests conducted after 7/23/2015; protocols not yet available.
b. Protocols found in Mitchelmore and Rowe (2015).

Table A.5. Metadata describing "definitive" tests performed with UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
579	Fiddler crab	Zoea	96	A	Maternal exposure (572)	Mortality	Maternal exposure	Auburn-UNT	8	5
594	Gulf killifish	Embryo	14	A	Slick	Mortality, hatching, heart rate		LSU	11	6
595	Gulf killifish	Embryo	14	B	Slick	Mortality, hatching; heart rate		LSU	11	6
617	Gulf killifish	Embryo	14	A	Slick	Mortality, hatching, heart rate		LSU	11	6
618	Gulf killifish	Embryo	14	B	Slick	Mortality, hatching, heart rate		LSU	11	6
959	Bay anchovy	Embryo	24	A	Slick	Mortality		LUMCON-UNT	8	14
964	Bay anchovy	Embryo	24	A	Slick	Mortality	Differential UV duration	LUMCON-UNT	8	14
969	Bay anchovy	Embryo	24	A	Slick	Mortality	Short slick exposure period	LUMCON-UNT	8	14
962	Red snapper	Embryo	24	A	Slick	Mortality		LUMCON-UNT	8	14
939	Bay anchovy	Embryo	24	B	HEWAF	Mortality		LUMCON-UNT	8	15
940	Bay anchovy	Embryo	24	A	HEWAF	Mortality		LUMCON-UNT	8	15
941	Bay anchovy	Embryo	24	B	HEWAF	Mortality		LUMCON-UNT	8	15
942	Bay anchovy	Embryo	24	B	LEWAF	Mortality		LUMCON-UNT	8	15

Table A.5. Metadata describing "definitive" tests performed with UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
961	Red snapper	Embryo	24	A	HEWAF	Mortality		LUMCON-UNT	8	15
258	Mahi-mahi	Embryo	48	WS	CEWAF	Mortality, hatching		MUO	5	1
555	Mahi-mahi	Embryo	48	WS	HEWAF	Mortality, hatching		MUO	5	1
250	Sheepshead minnow	Embryo	168	A	CEWAF	Mortality		MUO	5	2
251	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality		MUO	5	2
252	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		MUO	5	2
321	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		MUO	5	2
562	Sheepshead minnow	Embryo	168	A	HEWAF	Mortality, hatching		MUO	5	2
161	Mahi-mahi	Embryo	48	A	CEWAF	Hatching		RSMAS-UNT	8	2
160	Mahi-mahi	Embryo	96	A	HEWAF	Mortality		RSMAS-UNT	8	7
611	Mahi-mahi	Juvenile	48	A	HEWAF	Swim performance		RSMAS-UNT	8	8
923	Copepod	Adult	96	A	HEWAF	Mortality, reproduction		RSMAS-UNT	8	16
389	Speckled sea trout	Larvae	24	A	HEWAF	Mortality		Stratus-UNT	8	12
391	Red drum	Larvae	24	A	HEWAF	Mortality		Stratus-UNT	8	12
392	Red drum	Larvae	24	B	HEWAF	Mortality		Stratus-UNT	8	12
396	Red drum	Embryo	24	B	HEWAF	Mortality		Stratus-UNT	8	12
650	Red drum	Larvae	24	B	LEWAF	Mortality		Stratus-UNT	8	12

Table A.5. Metadata describing "definitive" tests performed with UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
393	Speckled sea trout	Larvae	24	A	HEWAF	Mortality		Stratus-UNT	8	12
395	Speckled sea trout	Larvae	24	B	HEWAF	Mortality		Stratus-UNT	8	12
645	Speckled sea trout	Larvae	48	B	HEWAF	Mortality		Stratus-UNT	8	12
646	Speckled sea trout	Larvae	48	B	CEWAF	Mortality		Stratus-UNT	8	12
648	Speckled sea trout	Larvae	24	B	HEWAF	Mortality		Stratus-UNT	8	12
649	Speckled sea trout	Larvae	24	B	CEWAF	Mortality		Stratus-UNT	8	12
643	Speckled sea trout	Embryo	24	A	Slick	Mortality		Stratus-UNT	8	13
644	Speckled sea trout	Embryo	24	B	Slick	Mortality		Stratus-UNT	8	13
544	Sheepshead minnow	Embryo	168	A	HEWAF	Mortality, hatching		UNT	8	1
545	Sheepshead minnow	Larvae	96	A	HEWAF	Mortality		UNT	8	1
547	Sheepshead minnow	Larvae	96	A	CEWAF	Mortality		UNT	8	1
229	Mahi-mahi	Embryo	48	A	HEWAF	Hatching		UNT	8	2
231	Mahi-mahi	Embryo	48	WS	CEWAF	Hatching		UNT	8	2
295	Mahi-mahi	Embryo	48	WS	HEWAF	Hatching		UNT	8	2
587	Mahi-mahi	Embryo	48	A	HEWAF	Hatching		UNT	8	2
117	Blue crab	Zoea	48	B	HEWAF	Mortality		UNT	8	3
118	Blue crab	Zoea	48	A	HEWAF	Mortality		UNT	8	3

Table A.5. Metadata describing "definitive" tests performed with UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
237	Blue crab	Zoea	96	A	HEWAF	Mortality		UNT	8	3
322	Blue crab	Zoea	48	A	HEWAF	Mortality		UNT	8	3
323	Blue crab	Zoea	48	A	CEWAF	Mortality		UNT	8	3
116	Fiddler crab	Zoea	48	A	HEWAF	Mortality		UNT	8	3
131	Fiddler crab	Zoea	48	B	HEWAF	Mortality		UNT	8	3
177	Eastern oyster	Veliger	24	A	HEWAF	Mortality, growth, development		UNT	8	4
181	Eastern oyster	Embryo	24	A	HEWAF	Mortality, growth, development		UNT	8	4
245	Eastern oyster	Embryo	96	A	HEWAF	Mortality		UNT	8	4
246	Eastern oyster	Veliger	48	A	HEWAF	Mortality		UNT	8	4
564	Eastern oyster	Gamete	1	A	HEWAF	Fertilization		UNT	8	4
565	Eastern oyster	Gamete	1	A	HEWAF	Fertilization		UNT	8	4
566	Eastern oyster	Veliger	48	FLU, A, COR	HEWAF	Abnormality		UNT	8	4
567	Eastern oyster	Gamete	1	A	HEWAF	Fertilization		UNT	8	4
197	Fiddler crab	Zoea	F1	A	HEWAF	Mortality		UNT	8	5
149	Fiddler crab	Zoea	24	A	HEWAF	Mortality		UNT	8	6
153	Fiddler crab	Zoea	48	A	HEWAF	Mortality		UNT	8	6
379	Grass shrimp	Larvae	48	B	HEWAF	Mortality		UNT	8	9
590	Grass shrimp	Larvae	48	B	SPIKED SED		Maternal exposure	UNT	8	10
548	Grass shrimp	Adult	168	B	SPIKED SED	Mortality		UNT	8	11
517	Mysid shrimp	Juvenile	96	A	HEWAF	Mortality		UNT	8	17
529	Mysid shrimp	Juvenile	96	A	CEWAF	Mortality		UNT	8	17
530	Mysid shrimp	Juvenile	96	A	CEWAF	Mortality		UNT	8	17
665	Mysid shrimp	Juvenile	96	COR	DISP	Mortality		UNT	8	17

Table A.5. Metadata describing "definitive" tests performed with UV. Organized by testing laboratory, GLPP chapter, and protocol number.

Test ID	Species	Life stage	Test duration (h)	Contaminant	Exposure type	Endpoints	Other conditions	Laboratory	GLPP chapter	Protocol
666	Mysid shrimp	Juvenile	96	A	Slick	Mortality		UNT	8	18
680	Pacific white shrimp	Post-larvae	24	A	HEWAF	Mortality		UNT	8	NYA[a]
681	Pacific white shrimp	Post-larvae	24	A	Slick	Mortality		UNT	8	NYA[a]

a. Tests conducted after 7/23/2015; protocols not yet available.

1. Auburn University General Laboratory Procedures and Practices

1.1 Test Organism Collections and Husbandry

1.1.1 Artificial seawater

Artificial seawater (ASW) was made by bringing reverse osmosis, deionized (RO/DI) water up to the appropriate salinity with Instant Ocean synthetic sea salt. RO/DI water was obtained by running city water through an AquaFX water treatment system purchased from Aquatic Ecosystems and housed in a secure laboratory setting.

1.1.2 Fiddler crab: *Uca minax*

U. minax were obtained from Weeks Bay, Alabama. Male and female crabs were transported back to Auburn University (Auburn) in two 40-gal coolers that each contained 5 gal of seawater from the collection site, as well as bioballs to decrease water sloshing and provide substrate to minimize antagonistic interactions between crabs. In the laboratory, males and females were held in separate coolers in the dark, with aeration, at room temperature (~ 24°C). Bioballs were left in the coolers to serve as biofilters and to minimize aggressive interactions between crabs. The water was changed twice weekly with 12 ppt ASW, which matched the salinity of the water at the crab collection site. Crabs were fed commercial shrimp pellets *ad libitum* three times weekly.

1.1.3 Fiddler crab: *Uca longisignalis*

U. longisignalis were collected at Point Aux Pines, Alabama [Alabama Department of Conservation and Natural Resources (ADCNR) Wildlife Management Area]. General culture procedures were the same as for *U. minax,* except that crabs were held outdoors in a secure area and exposed to ambient light and temperature regimes. Also, in addition to shrimp pellets, clumps of sediment from the collection site were added to the holding tanks to serve as an additional source of food. Water was changed three times per week with ~ 15 ppt ASW, which matched the salinity of the water at the crab collection site.

1.1.4 Marsh periwinkle: *Littoraria irrorata*

Marsh periwinkle snails (*L. irrorata*, ~ 560 individuals) were collected from the Alabama Coastline on Dauphin Island, Alabama, near Mobile Bay (~ 30°25'16.91"N, 88°08'27.46"W).

Simultaneously, *Spartina alterniflora* shoots were collected from the Alabama coastline southwest of Bayou La Batre near Sandy Bay (~ 30°22'48.93"N, 88°18'45.65"W) for use in snail toxicity studies. Snails and *Spartina* were transported from the collection site to the laboratory in a 5-gal cooler containing a small amount of seawater included for maintaining humidity levels. Upon arriving at the laboratory, and before the start of the experiment, the snails were placed in a 5-gal plastic bucket containing shoots of *Spartina* and ~ 15 ppt ASW with aeration. The *Spartina* shoots served as habitat and a food source for the snails

1.2 Toxicity Test Exposure Media Preparations

Protocols for test solution preparations are found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The types of toxicity testing solutions follow.

1.2.1 Oil emulsions

Oil emulsions were prepared according to the procedure for preparing high-energy water accommodated fractions (HEWAFs), except that after blending, the resultant emulsion was used in its entirety rather than transferred to a separatory funnel for a 1-hr separation of layers. Oil emulsions were prepared using Slick A (CTC02404-02) oil obtained from Stratus Consulting.[1]

1.2.2 Sediments

Sediments were collected and oiled at appropriate levels according to more detailed procedures described in individual test protocols.

1.2.3 Seawater

All tests used ASW prepared with Instant Ocean and RO/DI water as described in the Fiddler Crab Culturing section.

1. Stratus Consulting merged with Abt Associates in 2015; since much of this work was conducted by Stratus Consulting prior to the merger, both firm names will appear.

1.2.4 Water accommodated fraction exposure media

Water exposures were conducted using HEWAFs prepared as outlined in *Protocols for Preparing Water Accommodated Fractions* found in the QAPP. Water accommodated fractions (WAFs) were prepared with ASW and Slick A (CTC02404-02) oil.

1.3 General Testing Standard Operating Procedures

1.3.1 Standard operating procedure: Water quality monitoring

Water quality measurements [temperature, pH, dissolved oxygen (DO), salinity, ammonia, alkalinity, and hardness] were taken from water sources and specific frequencies as described in specific test protocols.

Standard operating procedures

Meters were bench calibrated or their accuracy verified against standards or alternate methodologies prior to use. Bench calibration (i.e., verification) records specific to the National Oceanic and Atmospheric Administration (NOAA) *Deepwater Horizon* projects were maintained in a laboratory file specific to instrument type and traceable to individual units by serial number. Calibrations and calibration verifications were performed prior to sampling; calibration or verification protocols were based on the manufacturer's specifications and/or standard methods. For measurements using probes in test chambers or sumps, measurements began in the lowest concentration, and then proceeded to the next higher concentration until the highest concentration chamber was measured. Probes were rinsed with RO/DI water between measurements to reduce the risk of cross-contamination.

pH: HANNA HI 9813-6 meter

Record meter and probe serial numbers.

1. Turn the meter on.

2. Calibrate the meter prior to each sampling event.

3. Immerse the probe in the calibration solution. Use pH 7.01 calibration solution if you expect the sample to be near neutral, pH 4.01 if you are going to measure acidic samples, or pH 10.01 if you expect the samples to be alkaline (basic).

4. Press °C and record the calibration solution temperature.

5. Adjust the pH knob to display the solution value at the measured temperature (refer to table "pH vs. temperature" on the solution packet).

6. Take sample measurements.

7. Submerge the probe in the sample to be tested.

8. Push the pH button.

9. Record the pH measurement once it stabilizes.

DO: YSI ProODO

1. Record meter and probe serial numbers.

2. Calibrate the meter at the beginning of each day of use. Calibration will hold for the remainder of the day, but adjust salinity compensation if needed.

3. Moisten (do not saturate) the sponge in the calibration/transport sleeve with a small amount of clean water.

4. Turn the meter on and wait 5 minutes for the calibration/transport sleeve to become fully saturated.

5. Press the Calibration button, highlight DO, and press Enter.

6. Once DO and temperature readings are stable (wait at least 30 seconds), highlight Accept Calibration, and press Enter. The screen will indicate if the calibration has been accepted.

7. If taking measurements in saltwater, enter the salinity value of the water to be tested in the Probe menu. This will allow for the proper salinity compensation of the mg/L DO values.

8. Take sample measurements.

9. Turn the meter on.

10. Insert the probe into the sample. Give an initial "shake" of the probe to release any air bubbles. Note that continuous movement/stirring is not required.

11. Wait 25–30 seconds for the readings to stabilize.

12. Record the DO value.

ProOBOD probes when used with a ProODO meter have built-in temperature sensors. Temperature calibration is neither required nor available. Record temperature directly from the meter display as you read the DO measurement.

Ammonia: Tetra Easy Strips and YSI 9300 photometer

1. Calibration is not applicable for the Tetra Easy Strips. The YSI 9300 photometer has its own internal calibration curve that is not adjustable by the user.

2. Remove one test strip from the bottle and replace and tighten the cap.

3. Hold the strip at the end and dip it into the sampled water collected from the appropriate chamber, tank, or system, swishing the water back and forth for 10 seconds.

4. Remove and shake the excess water from the strip.

5. Compare the strip immediately to the color chart for saltwater.

Calcium hardness and alkalinity: YSI 9300 photometer

The YSI 9300 photometer has its own internal calibration curve that is not adjustable by the user.

General instructions

1. Do not pour out the samples or prepare the tests directly over the instrument.

2. Cap the test tubes after preparing the blank and test samples.

3. Wipe the test tubes with a clean Kimwipe® and RO/DI water to remove dirt and residue, and dry with a clean Kimwipe to remove excess water, drips, and/or condensation immediately before placing in the photometer.

4. Remove tubes from the photometer immediately after each test.

5. Immediately wipe up any drips or spills on the instrument or in the test chamber with a clean tissue.

6. Clean the test chamber regularly using a Kimwipe or cotton ball moistened with RO/DI water.

7. Ensure that the carrying case is dry before the case is closed and the instrument is stored.

Calcium and alkalinity

1. See the manual for specific test instructions.

2. Follow the specific instructions for saltwater – reagents and protocols may vary between freshwater and saltwater.

3. Record the appropriate units.

Salinity: Pinpoint salinity meter or YSI Y30 SCT meter

Pinpoint salinity meter

1. Record the salinity meter serial number.

2. Calibrate the salinity meter before each sampling event.

3. Empty 53.0 mS standard calibration fluid into a small, clean cup and swirl the probe to dislodge air bubbles.

4. Wait 1–2 minutes for the proper temperature compensation.

5. Use a screwdriver to turn screw #3 (inside the battery cover) until the display reads 53.0 mS ± 0.5.

6. Do not turn screw #4 (also inside the battery cover).

Take sample measurement

1. Calibrate pinpoint salinity meter as described above or calibrate the YSI Y30 SCT meter according to manufactures directions.

2. Place the probe into the sample and swirl to dislodge air bubbles.

3. Record the salinity in mS and ppt units of measure, taking care to use the appropriate units (you will need to use the chart to convert salinity units of measure from mS to ppt).

4. Do not get the salinity meter wet as it is not waterproof.

1.3.2 Standard operating procedure: Analytical chemistry

The final confirmatory analytical chemistry was conducted offsite at ALS Environmental, as described in the QAPP. Offsite analytical testing was used to characterize oil emulsions, exposure sediments, and natural sediments. Samples were shipped to the offsite laboratory under strict chain-of-custody procedures in laboratory-provided sample containers and shipping coolers, as described in the QAPP.

A. Testing Protocol 1: Assessing the Effects of Maternal Oiled Sediment Exposure on Fiddler Crab Larval Production and Survival

A.1 Crab Collection and Maintenance

Fiddler crabs (*Uca longisignalis*) were collected from the Alabama coastline near Sandy Bay (~ 30°22'46.11"N, 88°18'23.27"W) and transported to Auburn, Alabama. Male and female crabs were housed outdoors in separate 30-gal coolers containing aerated ASW (~ 15 ppt; same salinity as the collection site) and bioballs prior to initiation of the experiments. Bioballs served as a biofilter, as well as a structure to reduce antagonistic interactions between crabs.

A.2 Sediment Collection

Sediments were collected from the same location as the fiddler crabs on the Alabama coastline near Sandy Bay (~ 30°22'46.11"N, 88°18'23.27"W).

A.3 Maternal Exposure Experimental Design

The maternal exposure consisted of 20 individual chambers (4 treatments plus a control, each with 4 replicates). Each chamber contained 5 male and 5 female crabs. The individual chambers were 18 in. in diameter and contained approximately 8 gal of non-oiled sediments obtained from the crab collection site. The sediment was sloped from 5 to 8 in. deep. Each chamber was housed inside a larger container that was connected to its own sump pump and water supply. ASW (~ 15 ppt to match the crab collection site) pumped between the sump and the outer container simulated low and high tides that were set to mimic ambient cycles near the collection site. Small holes drilled into the sides of the chamber allowed the water to move in and out of the sediments during tidal flow (see Figure A.1 for schematics). The chambers were kept outdoors so that the crabs were exposed to natural sun and lunar cycles.

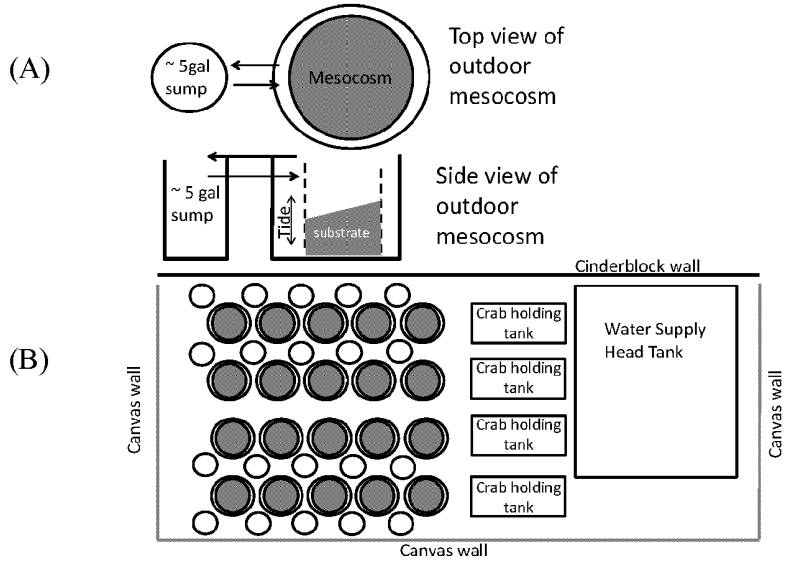

Figure A.1. Experimental design schematics. (A) Schematic of individual exposure chambers showing tidal flow between the chamber and a 5-gal sump. (B) Schematic of the layout of the replicate chambers.

A.4 Maternal Exposure Experimental Methods

At the initiation of the experiment (8/5/2012), 5 males and 5 females were placed in each of the 20 outdoor chambers (see Figure A.1). Each female crab was checked carefully for eggs before being placed in the chambers. If eggs were observed, that female was not used in the study.

After crabs were allowed to acclimate (~ 24 hours) to the chambers (8/6/2012), the chambers were dosed with oil. The general oiling procedures were as follows: the tidal water of the chambers was brought up to a level at least 2 cm above the surface of the sediments. An oil:ASW emulsion was then added to the top of each chamber and allowed to spread across the surface of the tidal water. For controls, the same volume of clean ASW was added to each chamber. After the addition of oil, the tidal water was slowly drained to at least 2 cm below the surface of the sediments, and sediments were allowed to drain for 30 minutes. A modified tidal flux (a 15-minute flooding of all sediments was followed by a 15-minute drain, until it reached 2 cm below the lowest sediment surface) was then repeated 3 times to allow oil to saturate the sediments. Note that the oil emulsion was only added during the initial tidal cycle. After the fourth tidal flux of the oiling process, the pumps were set so that normal tidal fluxes were resumed.

The oil:ASW emulsion that was added to the chambers was made using the procedure described in the Auburn Toxicity Testing General Laboratory Procedures and Practices (GLPP). Once prepared, the emulsion was immediately separated into beakers, each containing an appropriate volume to dose an individual chamber with the desired amount of oil. Once the emulsion had been added to each beaker, clean ASW was added to bring the total volume to 1,000 mL. These solutions were well mixed before being poured into their respective chambers using stir bars and a stir plate. To minimize residual oil in the beaker after the emulsion had been added to the chambers, the beakers were rinsed once with ~ 100 mL of clean ASW, and that rinse water was added to its respective chamber.

A.5 Chamber Observations

Once daily for 9 consecutive days, observations of mortality, number of females at the sediment surface, number of open burrows, and number of sealed burrows (i.e., a male burrow into which a female had entered and subsequently sealed) were recorded for each chamber. To help track the location and status of the burrows within a chamber, burrow locations were marked on a standardized diagram each day. Courtship behavior was recorded with 24-hour surveillance cameras above each chamber. If dead crabs were discovered, they were removed, given a unique identification (ID) number, wrapped in aluminum foil, and frozen at -20°C according to the archive protocols in the QAPP.

Crabs remained in oiled chambers for 9 days (~ 1.5 weeks), starting from the date of oiling, and removed on 8/16/2013. Any gravid females in the chamber were placed into individual Aquatic Habitat (AHAB) tanks (Figure A.2) containing ASW at ~ 20 ppt (salinity was increased to better match the higher salinities experienced by early life stage fiddler crabs in the environment). The chamber from which each female was taken was marked on the AHAB tanks. The females were kept in these tanks until their eggs hatched, or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Any larvae used in subsequent tests were removed before the zoeae were anesthetized in ethanol. The number of remaining zoeae was estimated for each female. To facilitate counting, the zoeae were sacrificed and preserved in ethanol. Preserved zoeae were diluted to 200 mL in tap water, and then mixed with a plunger to ensure an even distribution. Then 5-mL subsamples were removed and the zoeae were counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined.

Following these counts, the samples were retained in ethanol at 4°C for archiving. Females were put on ice to anesthetize, wrapped in aluminum foil, given a unique ID, and stored at -20°C.

Figure A.2. Recirculating AHAB tank system used to hold gravid females and collect and enumerate fiddler crab larvae. Water first circulates through each tank, and then through biological and mechanical filters to maintain water quality. Each tank drains through a spigot under which collection filter cups can be placed. Gravid females were placed in individual tanks, and filter cups were checked daily for crab larvae.

Source: AHABs (photographic example from catalog; all tanks used during testing were the same size).

A.6 Larval Production

Gravid female crabs from the maternal exposure study were placed in individual AHAB tanks 7 days following their first observable eggs. The AHAB tanks contained ~ 20 ppt ASW. The aquarium from which each female came was marked on the appropriate AHAB tank. Gravid females were held until their eggs hatched or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Zoeae used in survivorship tests were removed immediately and transferred to appropriate experimental vessels. Remaining zoeae were sacrificed by chilling to -20°C. Frozen zoeae were thawed and then clean 20 ppt ASW added until the total liquid volume reached 200 mL. The sample was then mixed with a plunger to ensure an even distribution, 5-mL subsamples were removed, and zoeae were counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined. The number of zoeae per female was estimated as:

$$N = S/ss \times n + x$$

where:

N = # zoeae per female
S = sample volume (mL)

ss = cumulative subsample volume (mL)
n = cumulative count of larvae in subsamples
x = # live larvae removed before preservation.

Adult females were anesthetized on ice, wrapped in aluminum foil, given a unique ID, and stored at -20°C for archival purposes. After enumeration, larvae were re-frozen and shipped to the Roberts Lab at the University of North Texas (UNT) for storage and potential polycyclic aromatic hydrocarbon (PAH) analysis.

A.7 Water Quality

Three times weekly, pH, DO, salinity, and ammonia were monitored in the outer container tidal water. Temperature was measured hourly from each chamber with a data logger buried ~ 4 in. below the surface of the sediment in the center of the chamber. Temperature was also measured three times weekly in the water of the outer container during routine water-quality measurements. Water that evaporated from the system during the course of the experiment was replaced with RO/DI water to maintain consistent tidal cycles without affecting salinity levels.

A.8 Sediment, Water, and Tissue Sampling

Two 8-oz jar samples of the bulk sediment used to load the chambers were collected and analyzed for PAH, total extractable hydrocarbon (TEH), total organic carbon (TOC), metals, pesticides/polychlorinated biphenyls (PCBs), and grain size. The high concentration oil:ASW emulsion that was used to oil the chambers was also sampled for PAH analysis.

Water samples were collected from the sumps of each chamber every 3 to 4 days during the test. Replicate water samples within each treatment were composited and sent to ALS Environmental for PAH analysis.

A.9 Collection of Crabs, Sediment Samples, and Temperature Loggers

The collection of crabs, sediment samples, and temperature loggers was conducted according to the following protocol:

1. Move cameras off the pad.

2. Move sumps off the pad.

3. Arrange tubs by treatment type, with a workup space by each group of tubs.

4. Remove surface males from each chamber and freeze in tinfoil and Ziploc baggies. Males from the same chamber can be frozen together. Label bags with a unique ID number for that sampling event.

5. Remove surface females from each chamber.

    a. Freeze non-gravid females and archive in tinfoil and Ziploc baggies. Non-gravid females from the same chamber can be frozen together. Label the bags with a unique ID number for that sampling event.
    b. Place gravid surface females in a cup labeled with the appropriate chamber number (1 female per cup). Immediately bring the female(s) to the crab laboratory and place in the appropriate and labeled AHAB.

6. Score the sediment surface to a depth of 2 cm (using a spatula) in parallel lines across the supra-tidal zone (top one-third). Lines should be ~ 5 cm apart.

    a. Remove sediment to a depth of 2 cm and place on appropriate clean glass plate (each treatment/tidal zone gets its own plate), and mix with a spatula.
    b. Place 20 oz (one-quarter of the sample bottle) of sediment into the appropriate surface, supratidal, composite sample jar.
    c. Place 80 oz of sediment (the full sample bottle) into the appropriate surface, supratidal, individual chamber sample jar.
    d. Repeat Steps 6a–c for the remaining 3 chambers in the treatment. Rinse and wipe the spatula clean after each chamber workup.
    e. Repeat Steps 6a–d for the subtidal surface samples, scraping the lower half of the chamber surface to 2 cm.
    f. Place the excess sediment into 5-gal buckets with lids, labeled for waste pickup.
    g. Ensure that all surface sediment has been removed from the chambers.

7. Collect gravid females and data loggers.

    a. Carefully dig through the sediment to find burrowed males and females.
        i. Freeze burrowed males as in previous steps.
        ii. Freeze non-gravid females as in previous steps.
        iii. Clean each gravid female with an ASW squirt bottle. Quickly transport gravid females to the crab laboratory in labeled cups and place into the appropriate AHAB tank (as in previous steps).
    b. Collect data logger from each chamber. Ensure that each logger is correctly labeled as to the chamber it was in.

8. Collect subsurface sediments.

    a. Mix sediments in each chamber.
    b. Place 20 oz (one-quarter of the sample jar) from each chamber into the appropriate subsurface composite jar (one jar per treatment).
    c. Place 80 oz (full sample jar) of sediment into the appropriate subsurface chamber jar (one jar per chamber).

All collected samples were labeled, stored, and shipped according to the QAPP.

B. Testing Protocol 2: Larval Survival Studies Experimental Design

For this study, the survival of larvae hatched from all gravid females (from Auburn GLPP Testing Protocol 1) was assessed. The larval survival studies were done indoors as well as outdoors, with and without ultraviolet (UV) exposure. For the indoor study, four replicate dishes with 20 larvae each were assessed for each gravid female. For the outdoor study, three replicate dishes with UV exposure and three replicate dishes without UV exposure, each with 20 larvae, were assessed for every gravid female (see UNT GLPP Testing Protocol 5).

B.1 Testing Apparatus Design

Both the indoor and outdoor testing apparatuses consisted of a water table containing a small aquarium heater and/or chiller and a small recirculation pump to maintain an even temperature throughout the water bath. The water table acted simply as a warming/cooling bath, and organisms were never in contact with this water. Water was fed into the water table, and the rate of flow varied to maintain a constant temperature during the test. Test dishes were then placed in foam blueboard insulation floats (blueboard is available at Home Depot, Lowes, etc.) and floated in the water table.

For the outdoor tests, the water table was covered in either UV transparent or UV opaque clear plastic sheeting (Aclar and Cortgard are recommended). Plastic sheeting was attached to the system to allow some airflow over the table for cooling. The water table was placed in a location that was unshaded between 8:00 a.m. and 6:00 p.m. UV measurements were made either continuously [BioSpherical profiling ultraviolet (PUV)] or regularly at approximately 20-minute intervals (OceanOptics JAZ). Mortality was assessed twice daily (morning and evening) for up to 96 hours for the outdoor tests and up to 2 weeks for the indoor tests.

AHAB tanks were checked at least twice daily, in the morning and again in the evening, to determine if gravid females had hatched their eggs. For the outdoor tests, if larvae were found in the morning, they were collected from the filter cups and a subsample was immediately transferred to test dishes to be placed outdoors for the day (see steps 1–4 in Section B.2). However, if larvae were found in the evening, test dishes were prepared and kept in the dark until the following morning. For the indoor tests, newly hatched larvae were transferred to test dishes and held overnight on the table top (same conditions as for the outdoor tests) and tests were started the following day. Details are provided below.

B.2 Indoor Experimental Methods

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with the test treatment and tank # (replicate number).

2. Fill each dish with 200-mL ASW using a glass cylinder.

3. Place 20 organisms in each test dish and feed with 20 rotifers/mL every day. On days 2 and 3, supplement with a 100-µL shellfish diet (Reed Mariculture) per dish and then reduce to a 25-µL shellfish diet per dish for each subsequent day.

4. Complete test forms as described in the QAPP.

5. Turn the water table heaters on and heat to the desired temperature.

6. Once the water table has acclimated, randomly place test dishes in foam blueboard floats in the water bath.

7. Once daily, renew test dish water and assess organisms for mortality (see steps 8–10 below).

8. Place dish under a microscope and pipette all live larvae into a clean dish filled with new ASW.

9. Do not transfer or replace dead larvae. Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Place "new" test dishes back into the water bath.

11. Repeat steps 7–10 until the last organism in the lowest WAF concentration has died or for a maximum of 2 weeks.

C. Testing Protocol 3: Assessing Burrowing Activity by Fiddler Crabs Following a Simulated Oil Slick

The indoor testing apparatus (see Figure C.1) consisted of a 26"L × 9"H × 0.75"W artificial burrowing chamber (ABC) modified from the design of Stoeckel et al. (2011), with the two large sides made from glass. Perforated polyvinyl chloride (PVC) formed the inner chamber that was filled with sediment, and a sump was located underneath the ABC. A timer was hooked up to a sump pump that pumped water into the ABC every 24 hours to simulate high tide. An adjustable standpipe allowed water to rise ~ 2 in. above the sediment surface before overflowing back into the sump. Low tide was simulated 12 hours later by manually lowering the standpipe to drain water ~ 5 in. below the sediment surface, back into the sump. High tide came in very rapidly, with water levels rising above the sediment surface and flooding burrows within 0.5 hour. Low tide occurred slowly as water gradually seeped out of the sediments and drained back into the sump over a period of several hours. The sump was filled with ~ 12 ppt salinity ASW, matching the salinity of the crab collection site for *U. minax*, the fiddler crab species used in this study. Sediment was obtained from the fiddler crab collection site at Weeks Bay, Alabama, where sediment primarily comprises organic material rather than sand.

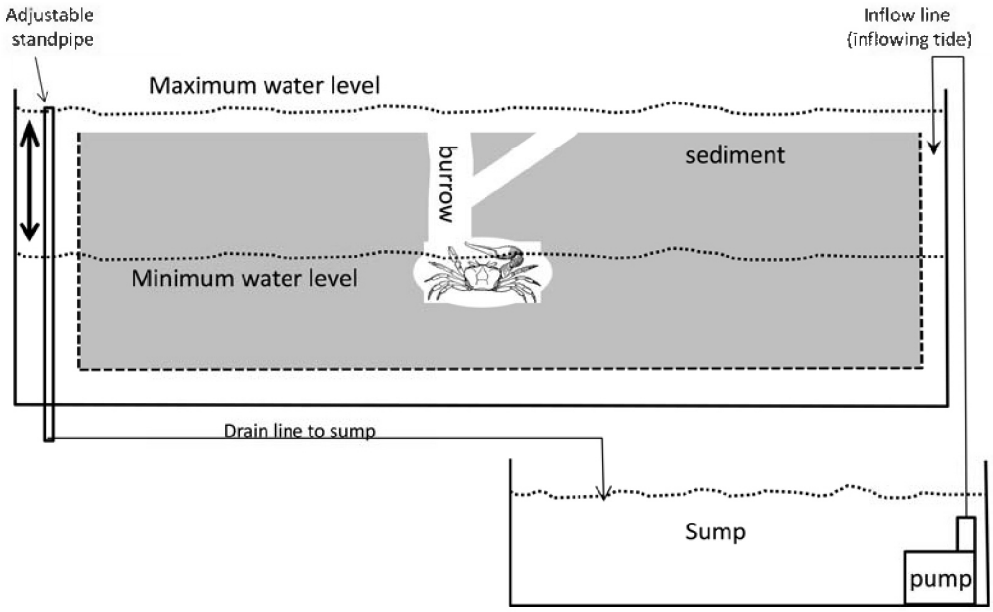

Figure C.1. Schematic of crab ABCs.

A total of 20 chambers were used for this experiment. Ten of these chambers (five randomly chosen chambers from each treatment) each had an infrared (IR)-capable, high-resolution surveillance camera mounted above it to monitor surface-burrowing activity, as well as another camera mounted near the side to monitor subsurface-burrowing activity (i.e., 2 cameras for each of the 10 chambers). In addition to the surveillance cameras, 2 high-definition IR camcorders were periodically used to record high-quality images of surface and subsurface activities in representative chambers for illustrative purposes (e.g., close-up shots of burrow excavation).

C.1 Test Procedure

C.1.1 Sample organisms

All crabs (*U. minax*) in this study were collected from Weeks Bay, Alabama. Male crabs were used in this experiment.

C.1.2 Acclimation

On day 0, one male fiddler crab was placed into each ABC and allowed to acclimate and burrow until all crabs had constructed a burrow and inhabited it for > 2 days. Tides were simulated during acclimation as previously described. Burrow construction and activity in 10 of the 20 chambers were filmed with surveillance cameras. Burrow outlines were traced immediately after initial burrow construction, as well as on the day before the experiment began.

C.1.3 Treatments

Upon initiation of the experiment, Auburn personnel subjected 10 of the 20 ABCs to a simulated oil spill, while the other 10 ABCs remained oil-free. A 2-L oil emulsion was created using ~ 20 g of Slick A oil blended into the ASW (see the Auburn GLPP). After initiation of the lowering of the water level to simulate low tide, approximately 200 mL of the oil emulsion was poured into the 10 "oil spill" ABCs when water levels were even with the surface. A sufficient volume of oil emulsion (~ 200 mL/chamber) was added such that there was a 2-cm deep layer of oil emulsion over the sediment. A bead of room-temperature, weathered oil was then applied on top of the oil emulsion using a syringe. The volume of oil emulsion and volume of oil applied to each chamber were recorded for each chamber. The oil emulsion and oil bead were allowed to coat sediment surfaces and the insides of burrows as water slowly receded during the low-tide drawdown. Any excess oil emulsion that drained into the sump was recirculated above the sediments during subsequent high tides. Five of the oiled and five of the non-oiled chambers were equipped with surveillance cameras as described previously.

C.1.4 Sample collection and endpoints

The experiment was run for 19 days post-oiling. Burrows were traced daily for the first 16 days in each chamber followed by a final tracing on the 19th day. Tracings were scanned and image analysis software was used to determine (1) total burrow area, (2) total burrow depth, (3) burrow area and depth below the minimum water line, (4) number of entrances/burrows, and (5) number of separate burrows.

Surface camera videos were utilized to determine (1) the proportion of time crabs spent on the surface during diurnal hours, and (2) the proportion of time crabs spent on the surface during nocturnal hours for 5 of the 10 crabs in each treatment. Side camera videos were analyzed to determine (1) the proportion of time crabs spent in the water while in their burrows, and (2) the proportion of time crabs spent actively burrowing.

On the final day of the experiment, 100 mL of "high tide" water was collected from each chamber and shipped to ALS Environmental for PAH analysis. Water was then drained from each chamber. Each chamber was then placed flat on its side and the side panel was removed. Crabs were then removed and held in individual cups for use in a subsequent escape-response study. Sediment was divided into three equal 2-in. layers (i.e., top, middle, and bottom), and each layer was homogenized with a stainless steel spatula. Individual ~ 8-oz samples were collected from each layer in each oiled chamber and placed into a glass 8-oz jar (3 layers × 10 oiled chambers = 30 samples). Soil from each layer was composited between control chambers (3 layers × 1 composite sample/layer = 3 samples) and ~ 8 oz of sediment from each composite sample placed into a glass 8-oz jar. Sample jars were stored at 4°C in darkness and shipped to ALS Environmental.

C.1.5 Escape-response experiment

The escape-response experiment followed the protocol of Culbertson et al. (2007) and Krebs and Valiela (1974). A single fiddler crab was placed underneath a plastic cup in the middle of a 44-cm diameter circle. At a prearranged signal, one person lifted the cup away from the crab and a second person immediately released a 5 × 5 cm weighted black square that swung on a pendulum directly above the crab. This process was repeated for each crab from all 20 burrowing chambers and the time to exit the circle was recorded for each crab.

Immediately following this experiment, each crab was individually wrapped in tinfoil, and frozen in individually labeled Ziploc bags.

D. Testing Protocol 4: Assessing the Effects of WAF Maternal Exposure on Fiddler Crab Larval Production

D.1 Crab Collection and Maintenance

Fiddler crabs (*U. longisignalis*) were collected from the Alabama coastline near Sandy Bay (~ 30°22'46.11"N 88°18'23.27"W) and transported to Auburn. Male and female crabs were housed outdoors in separate 30-gal coolers containing aerated ASW (Instant Ocean ~ 15 ppt; same salinity as collection site), and PVC ribbons prior to the initiation of experiments. PVC ribbons served as a biofilter while simultaneously serving as a structure to reduce antagonistic interactions between crabs. Auburn previously had good success housing crabs in this system for several months.

D.2 WAF Preparation

Exposures were conducted using Slick A HEWAF preparations (see the QAPP).

D.3 Maternal Exposure

The maternal exposure study occurred in 38-L glass aquaria containing 6 L of ASW (15 ppt) spiked with the appropriate amount of stock WAF. A PVC ribbon was added to each aquarium to serve as a structure. Previous pilot studies showed that fiddler crabs will develop broods in this setup, in the absence of sediment. At the initiation of this experiment, five males and five females were placed into each aquarium. Crabs were fed unlimited commercial shrimp feed. Each female crab was checked carefully for eggs before being placed into an aquarium. If eggs were observed, that female was not used in the study. Once added, crabs were allowed to acclimate for 12 hours before the aquaria were dosed with WAF.

Water in the aquaria was renewed every 48 hours. At each renewal approximately 80% of the water from each tank was siphoned out, and newly prepared WAF was diluted to the appropriate concentration before added to the aquaria.

Females were checked every 48 hours for the presence of newly fertilized eggs. If a female was found with eggs, that female was marked with a unique tag before being placed back into its respective aquarium. Each gravid female was held in an aquarium for an additional 7 days after the first observation of eggs. After 7 days, the gravid females were transferred to an individual

AHAB tank containing clean ~ 20 ppt ASW where they were kept until eggs hatched or for no more than 2 weeks. The larvae from each gravid female were collected to determine larval production (Section D.4) and to follow larval survival (described in the UNT GLPP Testing Protocol 5). The unique tags on females allowed them to be tracked individually during this process.

During the course of the test, additional females and males were added to the tanks to replace crabs that were removed. However, there were never more than five females and five males in a tank at one time.

D.4 Larval Production

Gravid female crabs from the WAF maternal exposure study were placed into individual AHAB tanks 7 days following their first observable eggs. The AHAB tanks contained ~ 20 ppt ASW. The aquarium from which each female came was marked on the appropriate AHAB tank. Gravid females were held until their eggs hatched or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Zoeae used in survivorship tests were removed immediately and transferred to appropriate experimental vessels. The remaining zoeae were sacrificed by chilling to -20°C. Frozen zoeae were thawed and clean 20-ppt ASW was added to bring the total volume to 200 mL. The sample was then mixed with a plunger to ensure an even distribution, 5-mL subsamples were removed, and zoeae counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined. The number of zoeae per female was estimated as:

$$N = S/ss \times n + x$$

where:

N	= # zoeae per female
S	= sample volume (mL)
ss	= cumulative subsample volume (mL)
n	= cumulative count of larvae in subsamples
x	= # live larvae removed before preservation.

Adult females were anesthetized on ice, wrapped in aluminum foil, given a unique ID, and stored at -20°C for archival purposes. After enumeration, larvae were re-frozen and shipped to the Roberts Lab (UNT) for storage and potential PAH analysis.

D.5 Water Quality

Three times weekly, pH, temperature, DO, salinity, and ammonia measurements were taken for each tank. Water was renewed every 2 days. If ammonia levels rose above 20 mg total ammonia nitrogen (TAN)/L (~ 10% of the 24-hour LC50 for *Uca* sp. as reported by Azpeitia et al., 2013), additional water modifications would have been made; however, these modifications were not needed during this study. In addition, tanks were not initially aerated in order to minimize potential PAH loss; however, DO levels were monitored closely, and it was determined 2 days after initiation of the test that aeration was needed to maintain DO levels. Thus, all tanks were aerated using an airstone for the remainder of the test.

D.6 Water Sampling

Water samples were collected and analyzed for PAH levels. All collected samples were labeled, stored, and shipped according to the QAPP.

E. Testing Protocol 5: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Movement and Survival

These tests were conducted in collaboration with the University of North Texas.

E.1 Direct Oil Exposure

For the direct oil exposures, 24-L (55.3 cm x 33.3 cm x 15.6 cm) plastic Sterilite® storage containers were used as experimental chambers, with 5 replicates each of one oil treatment and one control treatment, for a total of 10 chambers. Containers were filled with roughly 5 gal of sand so that a baking pan could lay flush with the edges of the test chambers. Each baking pan was filled with 200 g of trimmed *Spartina alterniflora* shoots and leaves. Baking pans were then filled with 15 ppt ASW so that the *Spartina* could hydrate overnight. Before the snails were exposed to the oil or to the controls, baking pans were drained of water and placed inside experimental chambers to mimic "fallen" *Spartina* beds. In addition, 2 rows consisting of 8 shoots each of *Spartina* were inserted vertically into the sand at the rear of the exposure chamber to mimic unaffected *Spartina* beds (Figures E.1 and E.2).

In the oiled exposure chambers, the vertical grass shoots served as an unoiled habitat where the snails could go to escape the oil. Trimmed blades of *Spartina* were placed at the rear of the baking pans to bridge the horizontal and vertical vegetation. Once the vegetation was set up in each chamber, 1 L of Slick B oil was added to the horizontal vegetation in each of the 5 oiled exposure chambers and allowed to settle. Once the oil settled, the layer of oil across the exposure chambers was approximately 1-cm thick.

Twenty snails were counted for each exposure chamber, painted with Liquid Paper, and labeled according to treatment. This procedure improved the ability of laboratory personnel to see the snails, enabled the camera to take better images, and enabled personnel to identify any snails that escaped the exposure chambers. Once painted and labeled, snails were placed in the center of the baking pan, roughly 23 cm from the vertical vegetation. Exposure chambers were placed outdoors, on a tarp on top of gravel and under a tent at ambient temperature. See Figure E.2 for a depiction of the individual chamber design and the layout of the exposure chambers under the tent.

Snail movement was assessed and photographed at the following time points: 0, 0.8, 0.25, 0.42, 0.58, 0.75, 0.98, 1.17, 2.5, 4.25, 19.5, 24.25, 28, 44, 48, 52, 68, and 72 h. At each assessment point, movement was documented by categorizing each individual snail into one of three groups:

on the horizontal vegetation, on the vertical vegetation, or exiting the exposure chamber. In addition, one control and one oiled replicate were video recorded for a cumulative total of 4 hours and 9 minutes in approximately 30-minute segments. The video camera only allowed for recording videos of this length, which provided approximately 9 segments before the battery ran out of power.

If snails exited the horizontal vegetation from a direction other than toward the vertical vegetation, or if they successfully traversed the horizontal vegetation into the vertical vegetation, laboratory personnel recorded this information and removed those snails from the exposure chambers and movement assay. Once the snails were removed from the exposure chamber, they were placed into individual scintillation vials using large forceps for additional monitoring (see Section E.2, Post-exposure Monitoring). At the end of 72 hours, snails that remained in the horizontal vegetation were also transferred into individual scintillation vials for post-exposure monitoring. All bench sheets were completed as described in the QAPP.

Figure E.1. Control chamber, with horizontal and vertical stalks and no oil.

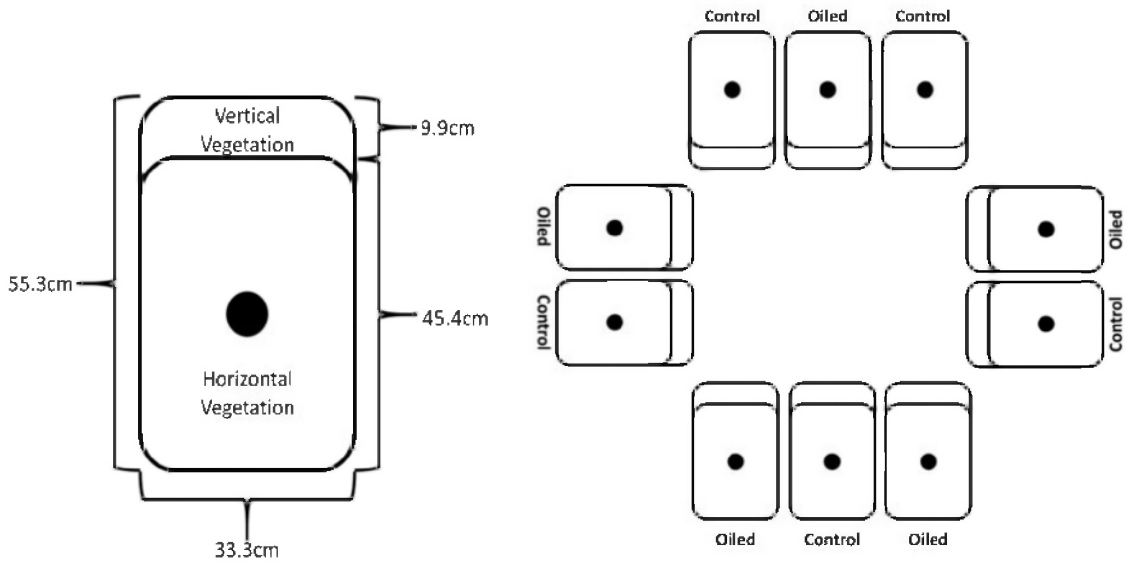

Figure E.2. Exposure Chamber Design and Layout.

E.2 Post-exposure Monitoring

After direct oil exposure, snails were monitored for 7 days to assess post-exposure mortality. All live snails were collected from the exposure chambers according to the procedures described above (Section E.1) and placed into individually labeled scintillation vials. Each vial contained a small volume of 15-ppt ASW to provide moisture, and a 2 in. stalk of *Spartina* to allow the snail to crawl up and out of the water. In addition, a small hole was drilled into the lid of the scintillation vial to allow for air movement. At the start of the 7-day monitoring period and every 24 h afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive, with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. If snails did not respond to the prodding, they were placed into a dish with 15-ppt ASW for 5–10 minutes; those that did not emerge out of their shells were noted as dead and archived according to the procedures described in the QAPP. Snails that did emerge were returned to their respective scintillation vial. At the end of 7 days, all living and dead snails were recorded, and then live snails were sacrificed and all dead snails archived according to the procedures described in the QAPP.

F. Testing Protocol 6: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Survival at Cool Temperatures

These tests were conducted in collaboration with the University of North Texas.

F.1 Testing Apparatus Design

The direct exposure outdoor testing area consisted of a concrete foundation over which a shade tent was placed. The water bath (Figure F.1) consisted of two shallow fiberglass tanks placed on top of a metal frame with a sump below the tanks to allow for placement of heaters and a pump to circulate the heated water between the sump and tanks. Water never came into direct contact with the organisms. The exposure chambers were set into foam blueboard floats which maintained an even spacing between chambers and minimized heat escape from the water bath. The foam board kept chambers from resting on the bottom of the tanks, thus allowing the water to flow underneath and around each chamber for even heat distribution. Small pumps maintained an even flow of water within the tanks, and the water level was controlled through the use of standpipes emptying into the sump below.

The exposure chambers were moved indoors immediately following the 32-hour exposure time point because of a storm that blew the shade tent over and caused outdoor temperatures to drop. Thus, exposure periods for the 1- to 32-hour time points were done outdoors and exposure periods for the 32- to 72-hour time points were done indoors.

At a predetermined time point, individual snails were moved into glass jars for a 7-day mortality assessment [see test-specific test conditions tables (TCTs)]. During the time points that the outdoor water bath was running (1–32 hours), glass jars used in the seven-day, post-exposure, mortality assessment were held in the same water bath as the experimental chambers. The spaces in the foam board were labeled by treatment and replicate number to ensure that jars containing the surviving snails were placed back into the same space from which the matching exposure chambers had been removed. After the 32-hour time point, all glass jars were placed indoors on a tabletop for the remainder of the trial.

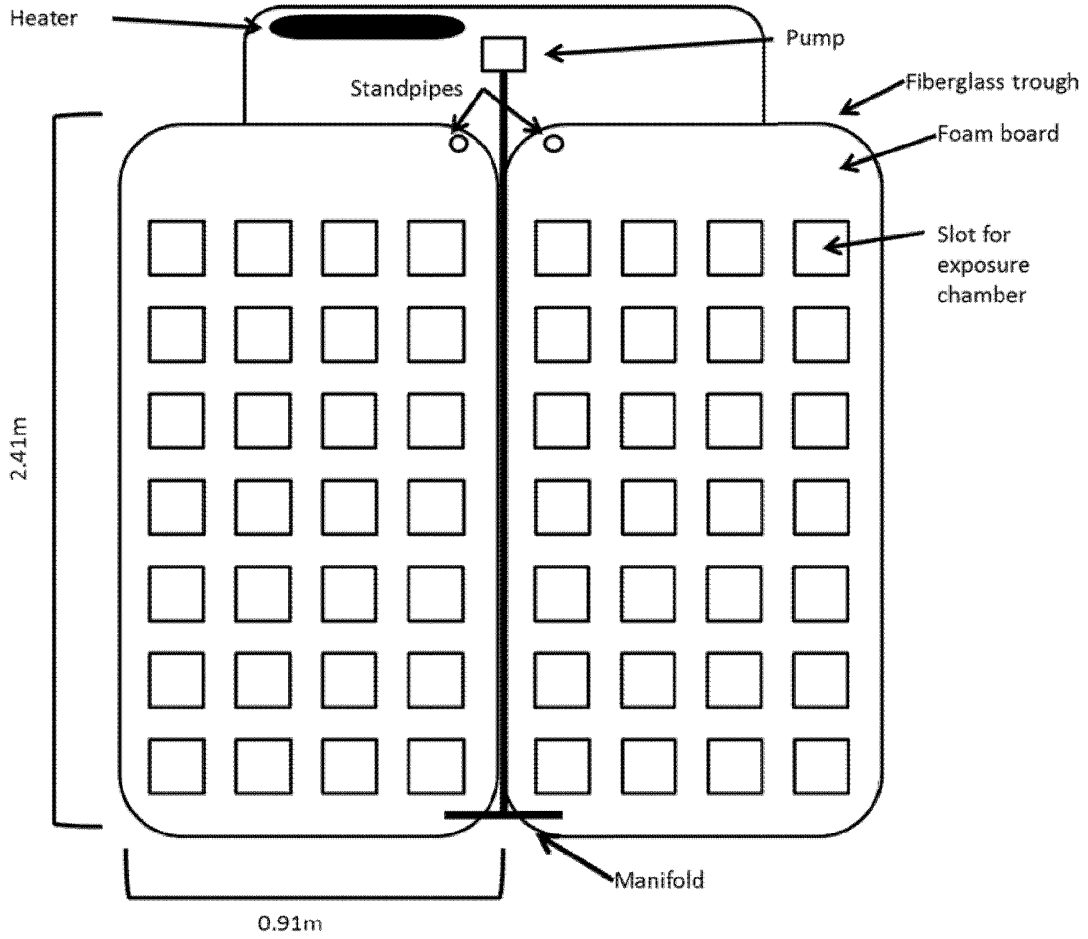

Figure F.1. Water bath design and layout.

F.2 Direct Oil Exposure

The direct exposure portion of this study used 0.9-L (0.15 m x 0.15 m x 0.045 m) plastic storage containers as experimental chambers (see Figure F.2). Each chamber was fitted with a stainless steel, wire-bristle brush around the container lip to prevent the snails from exiting the chamber. Each chamber contained a single layer of trimmed *Spartina alterniflora* and enough 15-ppt ASW to wet the *Spartina*. The *Spartina* soaked in the ASW overnight, and any excess water was removed the following morning. After the overnight soak, a 1-cm layer of Slick B oil (~ 200 g) was added to each oiled chamber. All chambers were placed in a shallow water bath. At the

32-hour time point, all chambers were brought indoors as described in Section F.1. The temperatures of the air, water bath, and chambers were monitored using daily minimum-maximum thermometers. At the start of the test, 10 snails were randomly assigned to each chamber. The treatments for these tests included 1, 2, 4, 8, 16, 32, and 72 hours of exposure in the oiled and control chambers (7 oil treatments plus 7 control treatments x 4 replicates each = 56 total chambers). At the end of each exposure period, snails from the appropriate control and oiled chambers were removed using large forceps, checked for mortality (see Section F.3), and placed into labeled quart jars corresponding to their treatment and replicate number for post-exposure monitoring.

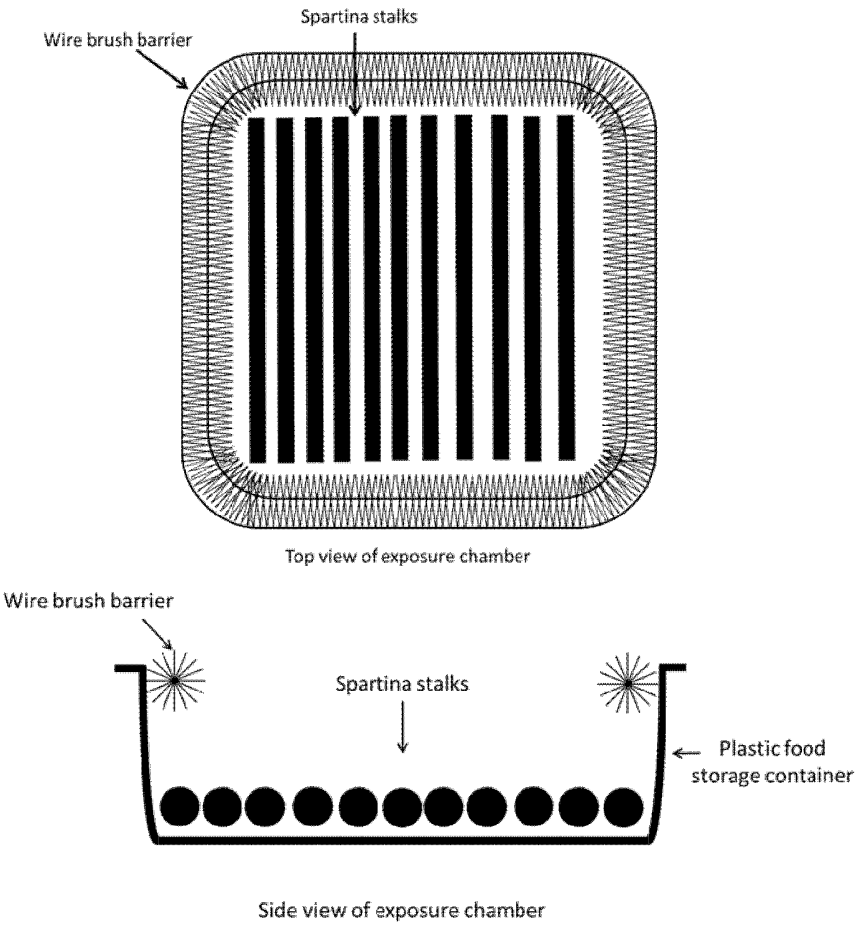

Figure F2. Design of exposure chamber.

F.3 Post-Exposure Monitoring

After the direct oil exposure, snails were monitored for 7 days to assess post-exposure mortality. All live snails were collected from the exposure chambers and transferred to corresponding 1-qt jars (see Section F.2). Each jar contained a small volume of 15-ppt ASW to provide moisture and a small stalk of *Spartina* to allow the snails to crawl up and out of the water. In addition, the jar lid was screwed down over a piece of window screen to allow for air movement. At the start of the 7-day monitoring period and every 24 hours afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. Snails that did not respond to the prodding were placed into a dish with 15-ppt ASW for 5–10 minutes; snails that did not emerge out of their shells were noted as dead and archived according to the procedures described in the QAPP. If alive, they were returned to their respective jar. At the end of 7 days, all live and dead snails were recorded, and then archived according to the procedures described in the QAPP.

G. Testing Protocol 7: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Survival at Warm Temperatures

These tests were conducted in collaboration with the University of North Texas.

G.1 Testing Apparatus Design

The direct exposure testing apparatus consisted of an indoor water bath in a room with temperature and photoperiod controls. The water bath (see Figure F.1 in the Auburn GLPP Testing Protocol 5) consisted of two shallow fiberglass tanks placed on top of a metal frame with a sump below the tanks to allow for placement of heaters and a pump to circulate the heated water between the sump and tanks. The water never came into direct contact with the organisms. The exposure chambers were set into foam blueboard floats that maintained an even spacing between chambers and minimized heat escape from the water bath. The foam board kept chambers from resting on the bottom of the tanks, thus allowing the water to flow underneath and around each chamber as well as even heat distribution. Small pumps maintained an even flow of water within the tanks, and water levels were controlled through the use of standpipes emptying into the sump below.

Glass jars used in the 7-day, post-exposure, mortality assessment were held in the same water bath as the experimental chambers. The spaces in the foam board were labeled by treatment and replicate number to ensure that jars containing the surviving snails were placed back into the same space from which the matching exposure chambers had been removed.

G.2 Direct Oil Exposure

The direct exposure portion of this study used 0.9-L (0.15 m x 0.15 m x 0.045 m) plastic storage containers as experimental chambers (see Figure F.2 in the Auburn GLPP Testing Protocol 5). Each chamber was fitted with a stainless steel, wire-bristle brush situated around the container lip to prevent experimental animals from exiting the chamber. Each chamber contained a single layer of trimmed *Spartina alterniflora* and enough 15-ppt ASW to wet the *Spartina*. The *Spartina* soaked in ASW overnight, and any excess water was removed the following morning. After the overnight soak, a 1-cm layer of Slick B oil (~ 200 g) was added to each oiled chamber. To maintain the appropriate temperature for the duration of the exposure, all chambers were placed into a shallow water bath. The temperatures of the air, water bath, and chambers were monitored using daily minimum-maximum thermometers. At the start of the test, 10 snails were

randomly assigned to each chamber. The treatments for these tests included 1, 2, 4, 8, 16, 32, and 72 hours of exposure in the oiled and control chambers (7 oil treatments plus 7 control treatments x 4 replicates each = 56 total chambers). At the end of each exposure period, snails from the appropriate control and oiled chambers were removed using large forceps, checked for mortality (see Section G.3), and placed into labeled 1-qt jars corresponding to their treatment and replicate number for post-exposure monitoring.

G.3 Post-exposure Monitoring

After the direct oil exposure described above, snails were monitored for 7 days to assess post-exposure mortality. Live snails were collected from the exposure chambers and transferred to corresponding 1-qt jars (see Section G.2). Each jar contained a small volume of 15-ppt ASW to provide moisture and a small stalk of *Spartina* to allow the snails to crawl up and out of the water. In addition, the jar lid was screwed down over a piece of window screen to allow for air movement. At the start of the 7-day monitoring period and every 24 hours afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. Snails that did not respond to the prodding were placed into a dish with 15-ppt ASW for 5–10 minutes; snails that did not emerge from their shells were noted as dead and archived according to the procedures described in the QAPP. If a snail did emerge from its shell, it was considered alive and returned to its respective jar. At the end of 7 days, all live and dead snails were noted and archived according to the procedures described in the QAPP.

Appendix References

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2. Florida Gulf Coast University General Laboratory Procedures and Practices

2.1 Methods

These tests examined the response of eastern oysters to experimental exposure to oil constituents, dispersants, and contaminated sediments with regard to survival, reproduction, settlement, immune responses, and disease susceptibility. The oysters were exposed to different concentrations of polycyclic aromatic hydrocarbons (PAHs) and/or dispersants under laboratory conditions. Laboratory exposures involved both early lifestage (embryo, veliger, and early spat) and adult oysters, as well as direct exposure of gametes.

Upon exposure, the larval development, survival, and metamorphosis were used as end points for larval stages, while fecundity, gamete quality, immune system, and disease susceptibility to the oyster pathogen *Perkinsus marinus* were examined in adult oysters (Volety et al., 2009). Gonadal condition and histopathological alterations were examined using histological techniques (Volety, 2008; Volety et al., 2009). Total petroleum hydrocarbons (TPHs) were extracted using a modified Bligh-Dyer extraction (Kimbrough et al., 2006; Loh et al., 2008), followed by subsequent analysis using a gas chromatograph (GC) equipped with DB-5 fused silica capillary column interfaced with a flame-ionization detector (FID). Additional analytical analyses of hydrocarbon constituents were performed by ALS Environmental, and comparisons were made between exposed and control treatments. Methods and experimental protocols are described in detail below.

All organisms and tissues from organisms collected for and/or used during testing were archived according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

2.1.1 Source water

The natural seawater used in these tests was pumped from the channel adjacent to the Vester Marine Field Station in Estero Bay, Florida. All of the water was filtered. Particles greater than 25 µm were removed by sand-and-bag filtration. The seawater was then purified by fiber cartridge and bag filters, ultraviolet (UV) sterilized, and then passed through a 0.1-µm filter before use in experiments. This seawater was adjusted to the desired test salinity via the addition of deionized water. At times when the salinity of the natural seawater in the bay was too low (< 20 ppt), artificial seawater was created by mixing Instant Ocean artificial salts with deionized water.

2.1.2 Experimental oyster sources and husbandry

Healthy oysters were obtained from Estero Bay, Florida, which is Florida's first Aquatic Buffer Preserve and has extensive, healthy oyster reefs. Oysters were acclimated in 250 L tanks for up to 2 weeks at ambient salinity of collection site (~ 20–25 ppt) prior to using them in any experiments. During acclimation, water quality was maintained using flow-through conditions, and seawater from an onsite salt-water well at the field station was used for oysters and preparation of aqueous matrix of the toxicants. Oysters and larvae were fed a mixture of shellfish diet or freshly cultured algae (e.g., a mixture of *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., and *Thallasiosira weissflogii*). For some spat and juvenile oyster exposure experiments, oysters were obtained from the Auburn University Shellfish Hatchery (AUSH). Larvae and spat were shipped overnight from AUSH to the Florida Gulf Coast University (FGCU) Vester Marine Field Station where they were placed in ambient seawater for acclimation and further experimentation.

Conditioning of broodstocks

Adult oysters were maintained at 20–24°C in 0.1-µm filtered seawater (FSW) and fed a daily ration of algae equal to approximately 3% of the oyster dry body weight. Oysters were artificially conditioned to the reproductively active stage (broodstock) by manipulating the culture temperature and feeding them a nutritionally enhanced diet (i.e., algae). Sub-samples of oysters were periodically shucked open and sampled to examine the maturity of gametes under a microscope. Spawning was induced in the laboratory and hatchery by thermal stimulation.

Spawning and larval culture

Oyster gametes, embryos, and larvae were collected from the laboratory-conditioned broodstock. Experimental oysters were thermally induced to spawn at 17–18°C and 30°C in spawning flumes containing seawater. Once spawning occurred, spontaneously spawning oysters were separated and maintained in individual 1-L containers. Sperm and eggs were collected, and the number of eggs was counted using a hemacytometer or a Sedgwick-Rafter counter under a microscope. Sizes of eggs were measured at the same time. Eggs from individual oysters were fertilized with sperm pooled from two to three males of same treatment group. Fertilized eggs were rinsed with seawater to remove unfertilized sperm, then placed in 80-L larval tanks at a concentration of 25–35 fertilized eggs/mL. They developed to straight-hinge larvae (normally 18–24 hours after fertilization at 27–29°C). Straight hinge larvae were cultured to eyed larvae at a density of 5 larvae/mL in larval tanks. Larvae were fed daily with algae and water was changed every other day. Straight hinge (day 1), umbo (day 5), and eyed (pediveliger, day 9) larvae were used in the experiments. In cases where natural spawning did not occur after thermal stimulation, additional oysters were stripped to obtain gametes.

2.1.3 Exposure water and sediment preparations

- Three different water accommodated fraction (WAF) preparations: high energy (HEWAF), low energy (LEWAF), and chemically enhanced (CEWAF) WAFs; use of two oil types: Slick A (CTC02404-02) and weathered source oil (072610-W-A)
- Corexit only exposures – as a definitive test (dose-response)
- Control water – prepared similarly as WAFs but without the addition of oil
- Sediments – a range of contaminated and uncontaminated field-collected sediment brought back to the laboratory and/or uncontaminated field-collected sediment spiked with oil in the laboratory.

WAF preparation methods followed guidelines detailed in the QAPP. However, in some instances Slick A oil was added using a gas tight syringe.

Sediment-derived WAF

Sediment slurry method

Bioassays were conducted using decanted sediment and sediment elutriates according to Geffard et al. (2003). Decanted sediment WAFs were made by adding clean seawater (20–25 ppt) to contaminated sediment in a glass beaker, stirring for 10 seconds, and then allowing sediment to settle for 2 hours. Supernatant solution of seawater was used in the test chambers. Exposure concentration of the sediment was determined in consultation with Stratus Consulting. Sediment from a known uncontaminated control location in Estero Bay, FL (or reference sediment from Louisiana provided by Stratus Consulting) was used for controls.

For the preparation of sediment elutriates, contaminated sediment was mixed in artificial seawater at a ratio of 1:10 (sediment:water) and was mechanically shaken at 300 RPM for 6 hours and allowed to settle for another 12 hours. Supernatant was siphoned off and mixed with FSW.

2.1.4 Testing methods

Acute and chronic aqueous exposure toxicity tests

Methodology of the gamete, embryo, larval, and spat exposures are detailed in Testing Protocol 1 of the FGCU General Laboratory Procedures and Practices (GLPP).

C. virginica embryos, veligers or early spat, and adults were exposed to various concentrations of LEWAF, HEWAF, CEWAF, and dispersant in an aqueous matrix. Embryo and larval exposures generally followed the U.S. Environmental Protection Agency's Office of Prevention, Pesticides and Toxic Substances (OPPTS) protocol 850.1055 (U.S. EPA, 1996) for bivalve acute toxicity tests. Exposure and toxicity evaluation methods are described below.

Embryo/veliger exposures

The starting life stage of these exposures were embryos that, after approximately 24 hours, became veligers. Embryos that were at the 2 to 4 cell stage were exposed to HEWAF, LEWAF, CEWAF, and dispersant-only treatments for 96 hours. Control treatments were run concurrently for each toxicant. Control oysters were exposed to the same FSW used in exposure treatment, but without toxicants. Exposures were conducted in 400-mL glass jars, with 4 replicate jars for each treatment. Each replicate contained ~ 4,000 oyster embryos. Exposures were conducted under static conditions. Embryo survival and growth were assessed after 24 and 96 hours. Embryo development was assessed in morbid and surviving oysters at the end of the exposure (96 hours). These embryos were fixed with buffered 0.1% formalin and evaluated for developmental abnormalities using a microscope.

The developmental success of veligers was determined by their progress to the pediveliger stage, while the effect of various treatments on early spat and adult oysters was determined by examining the survival and growth (see above). In addition, histological sections of adult oysters were made (Volety, 2008; Volety et al., 2009) and any alterations in the somatic and/or gonadal tissues observed under a microscope were compared with that of control oysters (see above). Concentrations of PAH in water and oyster tissues were analyzed.

Pediveliger exposure

The developmental success of pediveligers was determined by monitoring their progression to spat (settlement success). For these tests, pediveligers were exposed for 3 days to HEWAF, CEWAF, or sediment that had been spiked with oil. Each test chamber consisted of approximately 1,000 individuals. Survival and settlement success were assessed at the end of the exposure.

Early spat exposures

Early spat (5–15-mm length, ~ 30 days post-hatch) were exposed to either HEWAF, CEWAF, or dispersant for up to 14 days. Each test chamber consisted of 15–25 individuals. Exposures were conducted under static-renewal conditions. Exposure media were renewed every other day and water quality was monitored throughout the exposure period. Survival was assessed at each water change.

Adult exposures

Adult oysters were exposed to oil using algae as a carrier. Oil was sorbed onto the algae (shellfish diet) with or without dispersant and fed to adult oysters daily for 4 weeks, with gentle aeration to stimulate feeding. Individuals in control treatments were fed uncontaminated algae. Exposures were conducted in 40-L glass tanks with 20–25 individuals per tank and three replicates per treatment. For the duration of the experiment, water was changed thrice a week and oysters were fed a shellfish diet (5% wet weight) for maximal growth. Dissolved oxygen (DO), temperature, and salinity were measured just before water changes. Individuals were examined daily for obvious mortality, although mortality was at times difficult to ascertain from outside of the tank. Individuals were closely inspected for mortality during every water change. Mortality of test organisms was measured in the exposure chambers by evidence of lack of valve closure upon stimulation.

Fertilization success tests

Fertilizations between gametes were performed by mixing 10 mL of sperm with 200 mL of eggs in a 400 mL beaker. Aliquots of fertilized egg samples were fixed at 60-minute post-insemination to assess fertilization success. Remaining embryos were followed for developmental abnormalities for 24 and 96 hours.

Samples were fixed in 3.2% paraformaldehyde in seawater. To label DNA, fixed samples were stained with 1 μg/mL Hoechst 33342 for 15 minutes, and then washed twice with seawater.

Fertilization success was determined by examining the formation of polar bodies and the cleavage of fertilized eggs into embryos. In addition, embryo growth and metamorphosis of embryos into veliger were followed over a span of 96 hours.

Acute effects on sperm activity

Sperm activity after exposure to CEWAF, HEWAF, or dispersant was assessed by examining the viability, motility, mitochondrial membrane potential (MMP) and oxidative activity. Detailed procedures are described in Testing Protocol 1 of the FGCU GLPP under *Sperm Activity Assays*.

Adult dietary exposures, progeny effects assessments

HEWAF, CEWAF, and/or dispersant were added to algal diet where the PAHs adsorb to the algal particles. Oysters were exposed to contaminant-spiked algae on a daily basis, during feeding, filtration, and ingestion of algal particles.

Acute suspended sediment exposure toxicity tests

Effects of sediment-associated contaminants on various life stages of oysters (Larvae-Adult) were assessed using decanted sediment suspension according to the protocol described above for HEWAF and CEWAF.

Adult immunological response effects assessments

Adult oysters collected from the field (e.g., Estero Bay, FL) were exposed to similar concentrations of oil and/or dispersant for up to 30 days. Adult oysters were exposed to Slick A and/or weathered source oil using algae as a carrier. Oil was sorbed onto the algae (shellfish diet) with or without dispersant and fed to adult oysters daily for 4 weeks, with gentle aeration to stimulate feeding. Control treatments were fed algae only without oil and/or dispersant. Oysters from all treatments were sampled at the end of 2 weeks and 4 weeks, and analyzed for susceptibility to *P. marinus* (prevalence and intensity), inflammatory responses, and hemocyte immune responses (viability, phagocytosis, respiratory burst, and MMP). For more information, see Testing Protocol 1 of the FGCU GLPP under the section *Hemocyte Populations, Concentration, and Viability.*

Acute aqueous exposure, salinity effects assessments

Oyster gametes, embryos, and larvae were exposed to multiple concentrations of oil and/or dispersant in an aqueous matrix. The aqueous matrix included two different salinities (5 ppt and 25 ppt) and two different ambient temperatures (25°C and 30°C). Tests were conducted in 400-mL exposure chambers for 4 days, with no water renewal (static exposure). Three replicates were used for each treatment; each replicate contained approximately 4,000 eggs and fertilized embryos, or 3,000 larvae. Survival, growth, and normal development were used as end points for larvae. Larvae were fed with cultured microalgae on day 1 and day 3 (for more details, see Testing Protocol 1 of FGCU GLPP).

Early spat of oysters were exposed to various concentrations of oil and/or dispersant in an aqueous matrix. The aqueous matrix included two different salinities (5 ppt and 25 ppt) and two different ambient temperatures (25°C and 30°C). Tests were conducted in 600-mL exposure chambers for 8–10 days. A minimum of 3–5 replicates were used for each treatment; each replicate contained 15 spat. Survival was used as an end point for early spat (10–15 mm). Clearance rates (i.e., the rate at which the oysters cleared the algae) were also measured. Spat were fed with shellfish diet daily and water was changed every other day.

2.1.5 Water quality monitoring

See the QAPP and test conditions tables (TCTs) for required monitoring.

2.1.6 Histology

Gametogenic stage was identified under a microscope according to Fisher et al. (1996) and Goldberg (1980). This approach has been modified and successfully used by the principal investigator's (PI's) group (Volety et al., 2003, 2009; Volety, 2008).

For histologic sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad (Figure 2.1). Dissected tissue was fixed for one week in Davidson's fixative, and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin. Gonadal portions of the sections were observed by light microscopy to determine gender and gonadal condition (see Table 2.1).

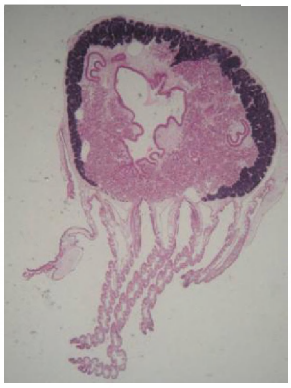

Figure 2.1. A cross-section of oyster tissue (histological section) showing mature gonadal tissue (stained dark). Histological sections are used to estimate the gonadal condition of oysters.

Table 2.1. Microscopic evaluation of gonadal condition in the eastern oyster

Value	Observations
0	Neuter or resting stage with no visible signs of gametes
1	Gametogenesis has begun with no mature gametes
2	First appearance of mature gametes to approximately one-third mature gametes in follicles
3	Follicles have approximately equal proportions of mature and developing gametes
4	Gametogenesis progressing, but follicles dominated by mature gametes
5	Follicles distended and filled with ripe gametes; limited gametogenesis; ova compacted into polygonal configurations, and sperm have visible tails
6	Active emission (spawning) occurring; general reduction in sperm density or morphological rounding of ova
7	Follicles one-half depleted of mature gametes
8	Gonadal area is reduced, follicles two-thirds depleted of mature gametes
9	Only residual gametes remain, some cytolysis evident
10	Gonads completely devoid of gametes, and cytolysis is ongoing

Hemolymph collection

Hemolymph was withdrawn from each oyster through a notch ground on the shell (Volety et al., 1999). Collected hemolymph was then immediately transferred into micro-tubes maintained on ice to minimize the cell clumping. All individual samples were microscopically checked for purity and then filtered through an 80 µm nylon mesh. Subsequent analyses were performed on individual samples (Donaghy et al., 2009a, 2009b, 2010; Haberkorn et al., 2010a).

Flow cytometry analyses

These were performed as previously developed by co-PIs P. Soudant and L. Donaghy (Donaghy et al., 2009b, 2010; Haberkorn et al., 2010a, 2010b). See the *Hemocyte Populations, Concentration, and Viability* section in Testing Protocol 2 of the FGCU GLPP.

2.1.7 Archived water and tissue samples

Archiving water and tissue samples (including any unused material) followed protocols outlined in the QAPP. During the acute toxicity testing, subsamples (~ 10 mL) of the solution containing gametes, embryos, or larvae were fixed using formalin for the analyses of fertilization success, gametogenic and larval development, developmental abnormalities, and survival. Any unused material from the formalin fixed samples was retained and stored at room temperature for further analyses. Any unused tissue from the experiments that was not designated for any specific analyses was pooled from the same replicate tank/exposure chamber and frozen at 20°C.

2.1.8 Analytical chemistry sampling

Analytical chemistry of tissue or CEWAF, HEWAF, and LEWAF was conducted onsite at FGCU; Centre of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE); France; and offsite at ALS Environmental. Oyster tissue sample analyses were conducted onsite and offsite at ALS Environmental, in addition to the water sample analyses. Generally, onsite analytical testing was conducted when quick turnaround results were needed. Offsite analytical testing was used to characterize the water chemistry of exposure treatments when conducting definitive testing. Samples were shipped to the offsite laboratory under strict chain-of-custody (COC) procedures in laboratory-provided sample containers and shipping coolers.

Concentrations of PAHs in oyster tissues and gametes were determined using gas chromatography-mass spectrometry (GC-MS) according to Munschy et al. (2005). When applicable, PAH concentrations in the water were determined according to Roy et al. (2005).

2.2 General Testing Standard Operating Procedures

2.2.1 Solutions preparation

Three or four treatments plus one control (below) were used in these studies. Dilutions were made from the stock solution to obtain appropriate concentrations for each treatment.

Final numbers and types of experimental treatments were provided in TCTs provided to Stratus Consulting during the test approval and test identification (ID) assignment process.

Oil and dispersant preparation

Preparation of LEWAFs, CEWAFs, and HEWAFs were carried out according to procedures outlined in the QAPP. Seawater at a salinity of ≈ 20 ppt was used as a control.

2.2.2 Collection and maintenance of the broodstock

Test oysters were collected from Estero Bay, FL, and acclimated to test conditions (e.g., 25°C and ≈ 20 ppt) at the Vester Marine Station. They were thoroughly cleaned by hand using a brush to remove biofouling organisms and weighed; 50 adult oysters were stocked in a 250 L tank inside the hatchery. Oysters were maintained at 23°C ± 1°C in coarsely filtered (20 µm) UV-treated seawater at ambient salinity (≈ 20 ppt). They were fed a mixture of freshly cultured microalgae (T-*Isochrysis*, *Tetraselmis* sp. and *Chaetoceros* sp.) at a daily ration of 3% of dry body weight for a period of 2 to 5 weeks to develop mature gametes at 23–25°C. Ten oysters were periodically sampled and examined for ripeness under a microscope.

2.2.3 Spawning method and gamete recovery

Mature oyster broodstocks were induced to spawn by thermal stimulation, alternating immersion in seawater at 17–18°C and 30°C every 30 minutes. Spawning females were isolated and placed in 1-L beakers filled with FSW for collection of oocytes, while spawning males were placed in about 200 mL of FSW to obtain a dense sperm solution. Oocytes and sperm were examined under a microscope for motility and viability and the best broodstocks were selected. After filtration through a 35-µm mesh to remove debris, sperm from several males were pooled in a 1-L sterile beaker. Eggs from several females, after successive sieving through 100-µm and 55-µm mesh to remove tissue and debris, were transferred into a 2-L sterile beaker filled with FSW. Fifty µL of eggs were taken from the 2-L stock and fixed and stained using Lugol for egg counts after continuous and gentle mixing (by means of a rod with attached perforated disc), using a Sedgwick-Rafter cell and a dissecting microscope.

2.2.4 Water quality analyses

Salinity, temperature, DO, and pH: Monitored daily in one replicate, and ammonia was tested from one replicate before each water change.

Temperature: Temperatures in the test chambers and stock solutions were obtained by a thermometer or alternatively using a YSI Pro ODO meter with an oxygen and temperature probe. When this probe was used, it was calibrated prior to every test.

Salinity: Salinity was obtained using a refractometer. This instrument is commonly used in the laboratory and field. Accuracy of the refractometer was checked once monthly using salinity standards.

DO: A YSI Pro series sonde with an optical DO probe was used to measure DO in all the stock solutions and test chambers. The probe was calibrated according to manufacturer's specifications prior to every experiment.

pH: pH was measured in a subset of exposures and litmus paper was used to determine any pH changes in the oiled treatments.

Ammonia: Ammonia in the stock solutions and test chambers followed the Seal Analytical Ammonia analyses in water and seawater procedure [Method No. G 171-96 Rev. 14 (Multitest MT19)]. This method uses the Berthelot reaction, in which a blue-green colored complex is formed and measured at 660 nm. A complexing agent was used to prevent the precipitation of calcium and magnesium hydroxides. Sodium nitroprusside was used to enhance the sensitivity.

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A. Testing Protocol 1: Acute Toxicity Testing – Gametes, Embryos, Veliger Larvae, and Spat WAF Exposures

For all of the toxicity testing experiments described below, the placement of treatment chambers was randomized.

Final testing conditions were specified in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

A.1 Gamete Exposures

Sperm and eggs were exposed to various concentrations of CEWAF, LEWAF, HEWAF, or dispersant for 30 minutes before being combined for assessment of fertilization rates and subsequent embryonic and larval development. Fertilization rates were determined by the presence of the polar body and/or division of the egg into an embryo.

Eggs were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and stocked in the beakers at a density of 20 eggs/mL. Each replicate jar (n = 4; 400-mL beaker) contained 200 mL of CEWAF, LEWAF, HEWAF, or dispersant solution (~ 4,000 eggs/replicate/treatment). In a separate container, 10 mL of sperm solution was added to 40 mL of CEWAF, LEWAF, HEWAF, dispersant solution, or seawater. Gametes were exposed to these solutions for 30 minutes before the addition of sperm (10 mL) solution to eggs for fertilization. Fertilization of eggs by sperm began immediately, first resulting in the formation of a polar body (30 minutes), and rapidly progressing to cell division and embryo formation (45 minutes).

Fertilization success was estimated by taking a subsample of 5–10 mL from the test chamber. At least 50–100 eggs/embryos were visually examined under a microscope to assess fertilization rate (% fertilization). Embryos remaining in the test chamber were allowed to divide and progress to the D stage for 24 hours. At the end of the 24-hour period, a subsample of 5–10 mL was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated under a microscope for developmental abnormalities, and examined for developmental success, abnormalities, and viability of the embryos. Any abnormal development, as embryos developed into veliger larvae, was noted.

The live and dead veliger larvae in the 24-hour subsample were counted and recorded, although it should be noted that undeveloped/dead veliger larvae may have disintegrated and thus may not have been counted. At least 50 larvae were counted for each replicate/treatment. Remaining larvae in the test chambers were allowed to progress to veliger stage. After 96 hours, larval survival was assessed under a microscope. Prior to September 2012, veliger larvae remaining after 96 hours were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, veliger larvae remaining after 96 hours were collected on a sieve, rinsed, resuspended with 30 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted. Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia was tested in representative chambers at the start and the end of the experiment.

Photoenhanced toxicity exposures were carried out similarly, except that they were done using outdoor UV exposure systems as detailed in other University of North Texas (UNT) GLPP protocols.

A.2 Embryo Exposures

Two to four cell-staged embryos were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and stocked in the beakers at a density of 20 mL. They were exposed to various concentrations (5 test treatments + control) of seawater, CEWAF, LEWAF, dispersant, or HEWAF in 400-mL beakers containing 200 mL of seawater. Stocking density of embryos was 20 embryos/mL (4,000 embryos/replicate/treatment). There were 4 replicates/treatment. At the end of 24 hours and 96 hours, a subsample of 5–10 mL was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated for developmental abnormalities and were examined for developmental success, abnormalities, growth, and viability of the embryos and veliger larvae. Any abnormality in the development or morphology was noted. For example, failure to develop into a D-shaped veliger larvae, a lack of shell, or a deformed shell were noted as abnormalities. After 96 hours, larval survival was assessed: prior to September 2012, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final

survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia was tested in representative chambers at the start and the end of the experiment.

A.3 Veliger Larvae Exposures

Veliger larvae that were 24-hours old and D-shaped were collected on a 35-µm sieve and used in toxicity testing. Veliger larvae were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and loaded in each exposure chamber at a density of 15/mL. There were 5 treatments with 4 replicates per treatment, which included HEWAF, LEWAF, CEWAF, and dispersant only exposures. All tests were conducted at approximately 25°C and 20 ppt with gentle aeration. Algae were provided as food when larvae were 1 and 3 days old at a ratio of 1×10^5 cells/mL of *Isochrysis* sp. At test initiation and after 48 hours of the exposure, a subsample of either 5 mL (prior to September 2012) or 10 mL (after September 2012) was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated under a microscope for developmental abnormalities, and were examined for developmental success, abnormalities, growth, and viability of the veliger larvae. Any abnormalities in development or morphology, such as failure to progress toward an umbo stage or having a deformed shell, were noted. After 96 hours, larval survival was assessed: prior to September 2012, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted. Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia in representative chambers at the start and the end of the experiment.

A.4 Spat Exposures

Early spat (5–15-mm length, ~ 30 days post-hatch) were exposed to either HEWAF, CEWAF, dispersant treatments or control treatments consisting of FSW for up to 14 days (see test-specific TCTs). For oyster spat, each test chamber consisted of 15–25 individuals (see test-specific TCTs). Exposures were conducted under static-renewal conditions. Exposure media were

renewed every other day and water quality was monitored throughout the exposure period. Survival was assessed at each water change.

A.5 Sperm Activity Assays

The percentage of motile spermatozoa was assessed using a two-step dilution in a salt sperm activating solution – "Moti-gigas" – (Brizard et al., 2004) and observed through a dark-field microscope. Flow cytometry analyses were performed to assess gamete health as previously developed by Dr. Soudant's laboratory (co-PI; Haberkorn et al., 2010). An aliquot of 100 µL of spermatozoa suspension from each selected male oyster was transferred into a tube containing 900-µL FSW. Spermatozoa DNA was stained with two fluorescent DNA specific dyes, SYBR-14 and propidium iodide, in the dark at 18°C for 10 minutes before flow-cytometric analysis. Propidium iodide permeates only spermatozoa that lose membrane integrity and are considered to be dead (necrotic), whereas SYBR-14 permeates both dead and live cells. Fluorescence of SYBR-14 and propidium iodide was measured at 500–530 nm (green) and at 590–600 nm (orange), respectively, by flow cytometry. This method distinguishes viable, "dying" (propidium iodide partially incorporated), and dead cells. By counting the cells stained by SYBR-14 green and cells stained by propidium iodide, it was possible to estimate the percentage of dead cells in each sample.

The MMP of oyster spermatozoa was measured using the potential dependent JC-10 assay kit. This probe enters selectively into mitochondria and exists as two forms – monomeric or aggregate – depending upon membrane potential (Reers et al., 1991). The JC-10 monomer form predominates in cells with low MMP and emits in the green wavelength (500–530 nm). The JC-10 aggregate form accumulates in mitochondria with high membrane potential and emits in the orange wavelength (590 nm). JC-10 forms can change reversibly.

A sample of the spermatozoa suspension was diluted in FSW containing JC-10. Samples were incubated for 10 minutes at room temperature. Fluorescence intensities of JC-10 monomers and aggregates were quantified, respectively, by FL1 (green) and FL2 (orange) detectors of the flow cytometer. The JC-10 aggregate/monomer ratio is assumed to be proportional to MMP (Reers et al., 1991; Cossarizza et al., 1996).

Determination of oxidative activity was performed at room temperature (20–22°C) using 2'7'-dichlorofluorescein diacetate (DCFH-DA), a membrane-permeable, non-fluorescent probe. Inside cells, the -DA radical is first hydrolyzed by esterase enzymes. Intracellular hydrogen peroxide (H_2O_2) and superoxide ion (O_2-•) then oxidize DCFH to the fluorescent 2',7'-dichlorfluorescein (DCF) molecule. Oxidation of DCFH can also be mediated by nitrite radicals (NO_2 or N_2O_3) and various oxidase and peroxidase enzymes. DCF green fluorescence, detected on the FL1 detector of the flow cytometer, is proportional to the total oxidative activity

of cells, including reactive oxygen species (ROS). Briefly, the spermatozoa suspension was incubated with DCFH-DA (10 μM final concentration) for 120 minutes in the dark and then transferred on ice before flow cytometry analysis. The oxidative activity was expressed as fluorescence arbitrary units (AUs).

B. Testing Protocol 2: Chronic Toxicity Testing – Adult Oysters

Adult oysters collected from the field (e.g., Estero Bay) were exposed to similar concentrations of oil and/or dispersant for an extended period (e.g., 30 days). Adult oysters were exposed to a slurry of Deepwater Horizon (DWH) oil and algae. Oil was mixed with the algae (shellfish diet) with and without dispersant and fed to adult oysters daily, with gentle aeration to stimulate feeding. Control treatments were fed algae only, without oil and/or dispersant. Oysters from all treatments were sampled periodically and analyzed for *P. marinus* prevalence, inflammatory responses, hemocyte immune responses, etc. Representative samples of oysters from each treatment were analyzed for tissue bioaccumulation, histology, and other endpoints, at predetermined points (e.g., day 14) and at the end of the experiment.

Final testing conditions were specified in TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

B.1 Preparation of Contaminated Algae

Shellfish Diet (Reed Mariculture) was used to feed oysters for the duration of in vivo exposures. Adult oysters were fed 0.5 mL of algae per oyster each day. Deepwater Horizon oil (concentrations varied by test and were specified in the TCTs) and/or dispersant was added to the vortex of spinning algae in a beaker and stirred using a stir bar for 30 minutes to allow the oil to adsorb to the algae. Control oysters received only algae (0.5 mL per oyster each day). The amount of algae added to each test chamber was adjusted based on the number of oysters in each test tank. Any mortality in oysters and the amount of algae added to each tank were logged.

B.2 *P. marinus* Prevalence and Intensity

Differences in disease expression (i.e., prevalence/percentage of infected oysters) and intensity of disease in various treatments were examined. Disease prevalence, intensity of *P. marinus*, growth, and survival among adult oysters were used as end points. The prevalence and intensity of *P. marinus* in adult oysters were analyzed using Ray's fluid thioglycollate medium technique (Ray, 1954; Volety et al., 2000, 2003, 2009). Samples of gill and digestive diverticulum were incubated in the medium for 5–7 days. Prevalence of infection was calculated as a percentage of infected oysters. The intensity of infection was recorded using a modified Mackin scale (Mackin, 1962), in which 0 = no infection, 1 = light, 2 = light-moderate, 3 = moderate, 4 = moderate-heavy, and 5 = heavy (Volety et al., 2009).

B.3 Inflammation

Inflammatory responses were histologically determined by observation of potential hemocyte infiltrations in tissues using histological techniques. Oysters were opened and underwent gross examination for abnormalities, and a section of soft tissues that included all organs was excised and fixed in Davidson's solution prior to sectioning. For histologic sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad. Dissected tissue was fixed for at least 1 week in Davidson's fixative and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin prior to observation (Fisher et al., 2000; Oliver et al., 2001).

B.4 Hemocyte Populations, Concentration, and Viability

In oysters, three main hemocyte populations can be observed: blast-like cells, hyalinocytes, and granulocytes. The concentration and percentage of each hemocyte subpopulation were determined in collected hemolymph using flow cytometry (Donaghy et al., 2009).

Cellular parameters (viability, oxidative activity, MMP, phagocytosis, and lysosomal content) were analyzed on circulating cells of both exposed and non-exposed oysters. Mitochondrial respiration was assessed through the evaluation of both the oxidative activity and the membrane potential (Donaghy et al., 2009; Haberkorn et al., 2010).

Populations, concentration, and viability of hemocytes were evaluated using a double-staining procedure that included SYBR Green I and propidium iodide. Hemolymph was diluted in FSW containing SYBR Green I and propidium iodide and incubated for 120 minutes in the dark at 10°C prior to flow cytometric data acquisition.

SYBR Green I is a membrane-permeable fluorescent dye that binds to double-stranded DNA and then emits green fluorescence (520 nm). Detection of this fluorescence allows distinction between single cells and aggregates, as well as debris. Hemocyte morphology is based upon relative flow-cytometric parameters, Forward Scatter (FSC), and Side Scatter (SSC). FSC and SSC commonly measure particle size and internal complexity, respectively. Morphological parameters are expressed in flow-cytometric AUs, and total hemocyte count (THC) is reported as the number of cells per milliliter of hemolymph. Membranes of viable cells do not allow propidium iodide to penetrate, whereas altered membranes are permeable by propidium iodide. Dead cells are characterized by loss of membrane integrity and are therefore double-stained by SYBR Green I and propidium iodide. Hemocyte mortality is expressed as the percentage of double positive cells.

B.5 Lysosome Quantification

The presence of lysosomes in hemocytes was determined using LysoTracker Red, a membrane-permeable, fluorescent red probe (emission maximum at 590 nm) that accumulates within lysosomal compartments. Hemolymph was diluted in FSW containing LysoTracker Red. Mixed solutions were incubated for 60 minutes in the dark, at room temperature. Tubes were then transferred and held on ice until flow cytometric analysis. Relative intracellular lysosomal quantity was expressed as the level of red fluorescence (Donaghy et al., 2009; Haberkorn et al., 2010).

B.6 Phagocytosis Capacities

An oyster hemolymph sample was mixed with an equal volume of FSW containing fluorescent latex beads (2.0 μm in diameter). After 120 minutes of incubation at room temperature in the dark, the reaction was stopped by transferring tubes to ice until flow cytometry analysis. The percentage of phagocytic cells, defined as the percentage of cells that have internalized three or more of the fluorescent beads (Donaghy et al., 2009; Haberkorn et al., 2010), was then determined by flow cytometry.

B.7 Intracellular Oxidative Activity

The intracellular oxidative activity, including the production of ROS, was determined using DCFH-DA. A solution of DCFH-DA was added to hemolymph diluted in FSW. The mixtures were then incubated at room temperature for 120 minutes prior to flow cytometry analysis. The oxidative activity was expressed as fluorescence AU (Donaghy et al., 2009; Haberkorn et al., 2010).

B.8 Mitochondrial Membrane Potential

Estimation of MMP was performed using JC-10. Hemolymph was diluted in FSW containing JC-10 and incubated for 30 minutes in the dark at 20°C prior to flow cytometric analysis. Relative MMP intensity was expressed as the ratio between the levels of green fluorescence (FL1 detector of the flow cytometer) and MMP orange fluorescence (FL2 detector) (Donaghy et al., 2009; Haberkorn et al., 2010).

B.9 Water Sampling for Chemical Analysis

Water samples were collected throughout exposure and analyzed for PAHs at ALS Environmental.

The water samples were taken directly from the tank in 250-mL amber bottles. Sample volume, container type, and labeling and handling methods followed the guidelines provided in the QAPP.

Spot check water samples were sometimes collected when the tank water in treatments was renewed. Samples were made by filling the required type and number of sample bottles with water from one tank before the renewal process. Spot check samples were collected from the water column under the water surface, and tank detritus was avoided. Spot check water samples were not filtered. Sample volume, container type, and labeling and handling methods followed the guidelines provided in the QAPP, and samples were sent to ALS Environmental for PAH analysis.

Oyster tissue samples were collected at 14 days and at the end of the exposure. Oyster samples were held at 4°C and sent to ALS Environmental, where the oysters were shucked and PAH and total lipid analyses on the composite samples were conducted.

B.10 Archiving Water and Tissue Samples

Archiving water and tissue samples (including any unused material) followed the protocols outlined in the QAPP. During the acute toxicity testing, subsamples (~ 10 mL) of the solution containing gametes, embryos, or veliger larvae were fixed using formalin for the analyses of fertilization success, gametogenic and larval development, developmental abnormalities, and survival. Any unused material from the formalin fixed samples was retained and stored at room temperature for further analyses. Any unused tissue during or at the end of the experiments that was not designated for any specific analyses was pooled from the same replicate tank/exposure chamber and frozen at -20°C.

B.11 Renewal Procedures

Tank water was renewed every Monday, Wednesday, and Friday and renewals occurred after water sampling. See test-specific TCTs and bench sheets for information regarding the exact days samples were collected. 100% of the water in each tank was removed and replaced with new, clean water. After filling the tank, algae/oil mixtures were added.

C. Testing Protocol 3: Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation

Effects of Deepwater Horizon oil and/or dispersant on the F-2 generation were examined using adult oysters. Reproductively active broodstock of oysters were exposed to Deepwater Horizon oil and/or dispersant using algae as carrier particles for 2–4 weeks in 40-L aquaria. A concentration of oil (see test-specific TCT) was added to the algal diet where the PAHs adsorb to the algal particles, as described in the previous section. Oysters ingest these PAHs during filtration and ingestion of algal particles. This method was successfully used by Chu and Hale (1994) and Choy et al. (2007) to deliver PAHs to *C. virginica* and *C. gigas* oysters.

Oysters were fed a shellfish diet daily and water was changed every Monday, Wednesday, and Friday during the exposure period. After the exposure period, oysters were induced to spawn, and reproductive and developmental success (i.e., fertilization success, embryonic development, metamorphosis to veliger larvae/umbo larvae) were determined at 1, 24, 48, 72, and 96 hours. In addition, histological analyses were used to examine the gonadal state of a subset of oysters from different treatments. The gametogenic stage was identified under a microscope according to Fisher et al. (1996) and the National Academy of Sciences (1980). This approach has been modified and successfully used by the PI's group (Volety et al., 2003, 2009; Volety, 2008). For histological sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad. Dissected tissue was fixed for approximately 1 week in Davidson's fixative and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin. Gonadal portions of the sections were observed by light microscopy to determine gender and gonadal condition.

All analytical chemistry sampling was conducted as described in Section B.9, "Water Sampling for Chemical Analysis," with the exception that tissue samples were collected at both 2-week and 4-week assessment time points. Water renewals followed the methods described above for adult chronic exposures.

D. Testing Protocol 4: Acute Toxicity Testing – Gametes, Embryos, and Veliger Larvae – Sediment Elutriate Exposures

D.1 Preparation of Sediment Elutriates

Bioassays were conducted using sediment elutriates according to modified protocol from Chu and Hale (1994) and Geffard et al. (2003). Seawater (25 ppt) was added to contaminated (LAAR38-B0123-SX401) or reference (LAAR42-C0208-SX403) sediment in a ratio of 10:1 and mechanically stirred (300 RPM) for 6 hours. Sediment was allowed to settle for 12 hours. For the preparation of sediment elutriates, supernatant (100% stock) was siphoned off and mixed with FSW in a dilution series to nominal concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 (control) percent elutriate.

Gametes, embryos, and veliger larvae of *C. virginica* were exposed to varying dilutions of the sediment elutriate described above. Protocols for exposure were similar to those described in FGCU GLPP Appendix A: *Acute Toxicity Testing – Gametes, Embryos, Veliger Larvae, and Spat WAF Exposures*. After 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

D.2 Water Sampling Schedule

Water samples were collected for chemical analyses of PAHs and fluorescence. Specifically, water chemistry samples were collected from the highest nominal exposure treatment concentration (50% supernatant stock) after diluting and mixing, just before test initiation. Water chemistry samples were not filtered. PAH water chemistry samples were collected, handled, and analyzed as discussed in the QAPP and sent to ALS Environmental. Observations of oil sheen and turbidity in water chemistry samples or exposure waters were noted in data reporting tables for each test.

Water samples were collected for fluorescence analysis of dilution splits. Specifically, a standard curve was created using a fluorescence sample from the highest exposure treatment concentration after diluting and mixing, just before test initiation. Additional fluorescence analysis samples were collected from each treatment and analyzed using the standard curve to verify nominal dilution concentrations. Fluorescence sampling and analyses were conducted

in-house as discussed in the QAPP. If fluorescence samples were not taken and analyzed, additional water chemistry samples were taken from each dilution series. These samples were analyzed for PAHs or sent to the analytical laboratory for extraction and archival.

E. Testing Protocol 5: Whole Sediment Exposures – Adult Oyster Toxicity Testing

Adult oysters collected from the field (e.g., Estero Bay) were exposed to field-collected or prepared sediments potentially impacted with oil for a total of 14 days. Reference sediments were used as a negative control. Adult oysters were exposed to a sediment:water slurry added to the aquaria, with gentle aeration using a mini-pump to help maintain suspension of particles within the aquaria. The slurry was added every 3 days, just after the water renewal, at a rate of 3-g sediment per oyster (e.g., 60 g/aquaria, given 20 oysters/replicate).

All adult oysters were maintained in the exposure aquaria throughout the exposure period. Both reference and exposure treatments were similarly maintained. Water quality was maintained via static renewal conditions, where approximately 95% of the water and sediment particulates was removed and renewed every 3 days. Water suspended particulates were removed by siphoning. Care was taken to remove as much of the sediment as possible at each renewal. New sediment:water slurry was added to the aquaria just after renewal of water.

During exposures, the light cycle was held at 12L:12D and oysters were fed daily with a commercially prepared shellfish micro-algae diet at a rate of 0.5 mL of algae diet per oyster. On the days that sediment:water slurry was added, the algae was added just after the slurry. The amount of algae and slurry added to each test chamber was adjusted based on the number of oysters in each test tank.

Adult oysters were inspected for mortalities and general health each day. Oysters from all treatments were sampled periodically and analyzed for *P. marinus* prevalence, inflammatory responses, and hemocyte immune responses as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters* above. After the exposure period, oysters were induced to spawn, and reproductive success (fertilization success, embryonic development, metamorphosis to veliger/pediveliger larvae) was determined as described in FGCU GLPP Testing Protocol 3, *Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation*. In addition, histological analysis was used to examine the gonadal state of a subset of oysters from different treatments. Representative samples of oysters from each treatment were also analyzed for tissue bioaccumulation, histology, etc., at the end of the experiment. Specific endpoints assessed for each test were listed in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process. Dead oysters were removed from exposure tanks and archived according to the QAPP. Numbers of individual oysters used for endpoint assessments depended on the total number of oysters that remained after sediment exposures.

E.1 Sediment Preparation

Test sediments, provided by NOAA in buckets or bags, were used as collected or blended before being used for toxicity testing. Table E.1 provides a list of sediment types that were tested. When not used, sediments were stored frozen, under strict COC. Care was taken to not defrost the entire sediment lot when aliquoting volumes needed for tests at hand. Excess aliquoted sediment was saved and archived.

Table E.1. Sediment types that may be used in adult sediment exposures

Loomis II 2012 (reference) (LAAR42-C0208-SX403)
Blend: 12.5% Black Hole 2011, 87.5% Loomis II 2012
Black Hole 2012 (LAAQ43-C0409-S61317-A)
Black Hole 2011 (LAAR38-B0123-SX401)

When required, test sediment blending was done prior to aliquoting sediments. Blending ratios were made on a weight-to-weight basis before adding water.

E.2 Test Preparation

Sediments were prepared for testing 48 hours before test initiation. For tests performed in triplicate, 1.3 kg of sediment was removed from the freezer (the blend was 12.5% Black Hole 2011 and 87.5% Loomis II 2012) and placed into a large glass jar or stainless steel mixing bowl. An equal volume (1.3 L) of 20 ppt artificial salt water was added to the frozen sediment. The jar was covered, wrapped in aluminum foil, and allowed to thaw and equilibrate for 24 hours at room temperature. After equilibration, the sediment:water slurry was thoroughly homogenized using stainless steel spoons. For each treatment, homogenized sediment was placed into 3 separate clean jars for individual tank dosing. In-between filling each replicate jar for tank dosing, an aliquot of slurry was added to a sample jar that was then sent to ALS Environmental for analyses. Slurry doses were stored in the refrigerator until test initiation. At the initiation of the test and every 3 days after test initiation, one of the jars of sediment:water slurry was emptied into the aquaria. Each replicate jar contained 3 g of sediment slurry per oyster. To ensure complete transfer of the slurry, the jar was rinsed several times by dunking the jar into tank water.

During the aliquot process, an additional 1,000 g of sediment were placed into two 8-oz jars (495 g each) for chemical analysis. The two sample jars were filled simultaneously by adding an aliquot to each sample jar after every two scoops of sediment were added to the dosing jars. The jars of slurry were stored at 4°C until used to dose tanks.

E.3 Analytical Chemistry Sampling

Both sediment and water samples were collected during this experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP, with the following specifications:

E.3.1 Initial sediment sampling

Sediment samples were collected from each treatment group (sediment type) when preparing tanks for sediment exposures. When preparing sediments for dosing the tanks, sediment samples from each treatment group and control were also collected for analytical chemistry (two 8-oz jars). These initial sediment samples were analyzed for PAHs, total organic carbon (TOC), total extractable hydrocarbons plus alkanes, and particle grain size at ALS Environmental.

E.3.2 Initial and spot check water sampling

An initial composite water sample was collected from each treatment group 30 minutes after addition of the first slurry. Sufficient water was collected for a filtered and unfiltered composite water sample. A second set of spot check composite water samples was collected approximately 1.5 days after initial slurry addition. A third set of composite water samples was collected just prior to the water renewal and new slurry addition on the third day. This sampling scheme was repeated once more at the end of the test. Samples were sent to ALS Environmental and extracted and archived for the possibility of future analyses.

At each water-sampling time point, a subsample from each replicate tank within each treatment (sediment type) was collected and composited into a clean decontaminated glass mixing container. Subsamples were collected by dipping a clean, decontaminated glass beaker into each tank. The same transfer beaker was used for all tanks within a single treatment. A separate clean, decontaminated beaker was used when collecting subsamples from each additional treatment. After mixing subsamples, the composite was used to fill the required analytical chemistry containers for requested chemical analyses (see the QAPP). This process was repeated for each treatment group and control. Water samples were sent to ALS Environmental.

F. Testing Protocol 6: Multiple Stressors – Gametes, Embryos, and Veliger Larvae Toxicity Testing

Gametes, embryos, and veliger larvae of oysters were exposed to various concentrations of oil prepared following CEWAF and HEWAF methods, under two temperatures and two salinities. Gametes, embryos, and veliger larvae were exposed in an aqueous matrix with salinities of 5 ppt and 25 ppt at 25°C and 30°C (i.e., 5 ppt at 25°C and 30°C and 25 ppt at 25°C and 30°C) in 400-mL exposure chambers for 4 days, with no water renewal (static exposure). Three replicates were used for each treatment; each replicate contained 4,000 eggs and fertilized embryos or 3,000 veliger larvae. Survival, growth, and normal development were used as end points for veliger larvae. Veliger larvae were fed with cultured microalgae on day 0 and day 2 of the exposure (for more details, see FGCU GLPP Testing Protocol 1). Prior to September 2012, after 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. After September 2012, after 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking three subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

G. Testing Protocol 7: Multiple Stressors – Spat Toxicity Testing

Oyster spat from the experimental hatchery of Auburn University, AL, were exposed to DWH oil prepared following CEWAF and HEWAF methods, under two temperatures and three salinities. Early spat (10–15 mm, ~ 1 g individual wet weight) were exposed in 500 mL of solution in 600-mL beakers, at a stocking density of 15 spat per beaker. A range of 5 concentrations of HEWAF were tested (100%, 25%, 5%, 2.5%, 0.5%) with a seawater control, with 3 replicates per concentration and 5 replicates per control. Three different salinities (prepared by the addition of distilled water into FSW), 5‰, 10‰, and 25‰, and two temperatures, 25°C (room temperature) and 30°C (incubator), were tested for each concentration of oil and control. The exposure lasted 7 (CEWAF) to 10 days (HEWAF), and was conducted under static-renewal conditions (exposure media changed every 2 days). Effects of various treatments on early spat were determined by examining the survival at each water change, using a 2-mm sieve. Beakers were cleaned and wiped using Kimwipes, and refilled with new exposure media.

Water samples (analytical chemistry) as well as ammonia and fluorescence were taken at each water change from each dilution, and water quality (temperature, salinity, pH, and DO) was monitored daily. Each test chamber was aerated to maintained oxygen levels above 3 mg/L and the light photoperiod was maintained at 12-hours light/12-hours dark (LD 12h:12h) using fluorescent lamps. Oyster spat in each test chamber were fed a ration of 2 mL of Shellfish Diet/TW1200 daily.

In order to measure the clearance rate, a single species algal paste, called Instant Ocean Thalasiossira (TW1200), was used. Unlike Shellfish Diet, which is a mix of 4 species of microalgae, TW1200 cells have a square shape and are large (20–40 μm), which makes them easy to count and to discriminate from oil droplets. On day 0, day 2, and day 6, oyster spat in each beaker were fed a ration of 2 mL of TW1200 and 1.5-mL subsamples were taken at T0, T4, T8, T12, and T24 after algae was added. Aliquots were fixed by adding a drop of Glutaraldehyde and cell counts were later determined under a microscope using a counting hemocytometer.

H. Testing Protocol 8: Whole Sediment Intertidal Exposure – Adult Oyster – Spiked Sediment

Adult oysters collected from the field (e.g., Estero Bay) were exposed to sediments collected at Estero Bay and spiked with oil for a total of 14 days. Sediment that had not been spiked was used as a negative control along with a seawater-only control. Adult oysters were exposed to a 1–2 cm thick layer of spiked sediment at the bottom of the aquaria; gentle aeration and mixing by hand before feedings helped maintain some suspension of particles within the aquaria.

All adult oysters were maintained in the exposure aquaria throughout the exposure period. Both controls and exposure treatments were similarly maintained. Water quality was maintained via static renewal conditions, where approximately 95% of the water was removed and renewed every 3 days. Water was removed by siphon, and care was taken to disturb the sediment as little as possible. During exposures, the light cycle was held at LD 12h:12h and oysters were fed a commercially prepared shellfish micro-algae diet at a rate of 0.5 mL of algae diet/oyster/day. Immediately prior to every feeding (except directly after water changes where the sediment is suspended due to filling), the water above the sediment was mixed for 10 seconds by hand with a stainless steel long-handled spoon starting from the top of the aquaria to just above the oysters, and then back to the top of the aquaria.

Final testing conditions, including numbers and size of replicates, were specified in TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

Adult oysters were inspected for mortalities and general health during water changes. If *P. marinus* prevalence was being assessed (see test-specific TCTs), oysters from all treatments were sampled on day 14 and were analyzed for *P. marinus* prevalence, as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters*. If immune response was being assessed (see test-specific TCTs), oysters from all treatments were sampled and immune response was assessed, as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters*. If reproductive success was being assessed (see test-specific TCTs), after the exposure period, oysters were induced to spawn and reproductive success (fertilization success, embryonic development, metamorphosis to veliger/pediveliger larvae) were determined as described in FGCU GLPP Testing Protocol 3, *Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation*. Representative samples of oysters from each treatment could be collected for histology and/or tissue bioaccumulation at the end of the experiment. Specific endpoints assessed for each test were listed in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process. Dead oysters were removed from the exposure tanks and archived according to the QAPP. Numbers of individual oysters used for endpoints assessments depended on the total number of oysters that remained after sediment exposures.

Preparation of sediments

Oil was mixed into uncontaminated Estero Bay sediments using a Cuisinart stand mixer. For each treatment, 7 kg of sediment was weighed and thawed overnight. Oil was added to each sediment treatment following treatments listed in the appropriate TCT. Oil was mixed into the sediment for 30 minutes at a moderate speed (5 on mixer), scraping the sides of the bowl with a metal spatula every 2–4 minutes as needed. The oil-sediment mixture was weighed and placed into 10-gal aquaria (L = 50.5 cm, W = 25.5 cm, H = 32 cm) for each treatment (2,200 g sediment/aquarium). When preparing tanks, sediment samples for the analytical laboratory from each treatment group were prepared by aliquoting approximately 100 g of sediment into the analytical chemistry sediment jar, between filling each replicate tank with sediment, so that each 8-oz sample jar was at least three-quarters full. Sediment samples were stored at 4°C until shipment to ALS Environmental for analysis. The sediment in the tanks was allowed to settle in the tanks overnight under static conditions prior to the addition of the oysters.

Sediments were prepared for testing prior to test initiation and were stored at 4°C until used to dose the tanks. After the sediment had been added to each tank, the tanks were placed into the exposure system, covered with foil, and allowed to settle for another 24 hours. After the settling period, oysters were randomly assigned to each tank and aeration began. Sediments were not removed or replaced throughout the exposure period. Effort was made to not disturb sediments when conducting water changes and water sampling.

Analytical chemistry sampling

Both sediment and water samples were collected during this experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP, with the following specifications:

1. ***Initial sediment sampling:*** Sediment samples were collected from each treatment group (including the control) in one full 8-oz jar per treatment. The sample jars were filled by adding an aliquot to each the jar after filling each tank for dosing. These initial sediment samples were analyzed for PAHs and TOC at ALS Environmental.

2. ***Water sampling:*** An initial composite water sample was collected from each treatment group just prior to the first water renewal.

   Water samples were taken throughout the exposure; at each water sampling time point, a composite water sample consisting of a subsample from each replicate tank was collected and placed into a clean decontaminated glass mixing container for each treatment. Subsamples were collected by dipping a clean, decontaminated glass beaker into each tank. The same transfer beaker could be used for all tanks within a single treatment. A

clean, decontaminated beaker was used when collecting subsamples from each additional treatment. After mixing subsamples, the composite was used to fill the required analytical chemistry containers for requested chemical analyses (see the QAPP). This process was repeated for each treatment group and control. Water samples were analyzed for PAHs at ALS Environmental.

3. ***Final sediment sampling:*** Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a stainless steel scoop or similar utensil until uniform in color and consistency. Once the sediment was thoroughly mixed, a scoop was used to fill the analytical chemistry sediment jar (8-oz glass). Sediments were sent to ALS Environmental for PAH analysis.

Any changes to the analytical sampling plan described in this section were documented in the laboratory notebooks and/or test results reporting tables.

The standard COC forms provided in the QAPP were used to request chemical analyses and relinquish samples to ALS Environmental.

I. Testing Protocol 9: Exposure of Veliger Larvae Using Dietary Pathways (Oil + T-Iso)

Veliger larvae that were 5 days old were used in chronic toxicity testing. Veliger larvae that were larger than 90 µm (retained on a 55-µm sieve) with a D-shape were used in the assays. Larval oysters were exposed to a slurry of DWH oil and algae (T-Iso) for 14 days, in 1,500-mL glass jars. Oil was prepared using the HEWAF method, mixed with the algae for 6 hours (see below for more details), and fed to larval oysters every other day after water renewal with gentle aeration to stimulate feeding. Control treatments were fed algae only without oil. There were 5 treatments with 4 replicates per treatment (see TCTs). All tests were conducted at 25°C, with salinities of 25 to 30 ppt, with gentle aeration. Just before each renewal, a subsample of 10 mL was taken from all test chambers, put into 15-mL centrifuge tubes, and fixed with buffered 0.1% formalin. These samples were evaluated using a microscope to examine abnormality, growth, and viability of the veliger larvae. Any abnormality in the development or morphology was noted. For example, failure to progress toward a pediveliger stage or a deformed shell was noted as an abnormality. Shell length was measured every 2 days (minimum of 200 individuals per concentration) to assess growth. In addition, gut fullness or percentages of veliger larvae with food in the stomach was assessed for each sample.

At the end of the exposure (day 14), larval survival was assessed. Remaining veliger larvae were collected on a sieve, rinsed, and resuspended with seawater in 50-mL centrifuge tubes. Final survival was assessed by taking 3 subsamples of 150 or 300 µL (see bench sheets) after homogenization from each 50-mL centrifuge tube and counted. Lastly, remaining veliger larvae were centrifuged and stored in plastic centrifuge tubes and stored at -80°C for later biochemical analysis (PAH content in larval tissue).

See TCTs for nominal treatment concentrations, replicates, and other test details.

Preparation of contaminated algae

T-Isochrysis (CCMP 1324) was used to feed oyster veliger larvae for the duration of exposures. DWH oil prepared as HEWAF was added to algae in a beaker (300 mL), gently stirred (250 RPM) using a stir bar for 6 hours to allow the oil to adsorb to the algae in the dark, and covered with parafilm. Control oysters received only algae. Algae + oil solutions were added to each exposure vessel at test initiation and at each water change. See TCTs for feeding regime and algae concentrations.

Flow cytometry

Each exposure solution (oil + algae) was analyzed using flow cytometry: SSC, FSC, and fluorescence were used to determine complexity and size of the particles.

Renewal procedures

Exposure vessel water was renewed on Monday, Wednesday, and Friday by pouring the entire contents (water, veliger larvae, algae, and oil) through a Nitex screen filter (55 µm). The screen separated the veliger larvae from the exposure media. Retained veliger larvae were gently washed into a cleaned exposure vessel containing fresh seawater filtered at 0.1 µm. After filling exposure vessels, algae/oil mixtures were added at a feeding regime of 150,000 cells/mL of culture. During every water change and feeding day, new solutions of contaminated or control algae (HEWAF + T-Iso) were prepared and added to the exposure vessels. Control treatments received non-contaminated algae (no HEWAF), prepared the same way as the contaminated algae solutions (6 hours spinning, covered, in the dark).

Water for chemical analysis

Water samples were collected throughout the 2-week exposure and sent to ALS Environmental for PAH analysis. Two distinct sets of water samples were collected. The first set was referred to as sham samples and the second as effluent water samples.

A single sham sample was taken from one pseudo replicate from each treatment group, including controls at test initiation (day 0), for a total of five sham water samples for each test. The sham exposure vessel, volume, and oil preparation were the same as respective treatment exposure vessels, except that the sham vessels did not contain larval oysters. Each sham was prepared when all other test replicates were prepared. Sham water samples were taken from under the water surface in a well-mixed exposure vessel and were not filtered or decanted. If a surface slick was present in the sham vessel when the sample was taken, it was noted on the test Analytical Sample Inventory Bench Sheet.

Effluent water samples were taken during two time points during the 2-week exposure. The first set of effluent samples was taken during the first water renewal at day 3 (72 hours after feeding). The second set of effluent samples was taken on day 5 (48 hours after feeding). A total of 8 effluent water samples for each test; no samples were collected from control vessels.

In each exposure treatment, all replicate exposure vessel effluent (approximately 6 L) was composited into a single mixing vessel (with veliger larvae filtered out). The effluent water sample was taken from under the water surface in the well-mixed composite and was not filtered or decanted. If a surface slick was present in the composited effluent when the sample was taken, it was noted on the test Analytical Sample Inventory Bench Sheet.

J. Testing Protocol 10: Pediveligers WAF and Spiked Sediment Settlement Assessments

Pediveligers were exposed to HEWAFs, CEWAFs, or spiked sediment and settlement success was assessed.

J.1 Pediveliger Exposure to WAF

Pediveligers (retained on 200-µm Nitex screen sieve; about 12–15 days post fertilization) were exposed to 450 mL of HEWAF or CEWAF for 3 days (see test-specific TCTs). WAFs were prepared following the protocols in the QAPP.

Each test consisted of five treatments and a seawater control, with four replicates of each treatment (see test-specific TCTs). Prior to test initiation, two settlement plates, consisting of HardieBacker Cement Board tiles (120 mm × 58 mm), were placed vertically into each testing beaker. To help condition the tiles, the cement tiles were soaked in seawater a minimum of 2 weeks prior to test initiation. At test initiation, pediveligers were stocked into the exposure beakers at a density of approximately 1,000 per beaker. To accurately estimate stocking densities, a "dummy beaker" was loaded at the same stocking density, for each treatment. These dummy beakers were sieved immediately after the test initiation and the total number of pediveligers that were stocked into the beaker was recorded on the appropriate bench sheets.

During the exposure, aeration (gentle bubbling) was supplied for 30 minutes every 2 hours using a timer-controlled air pump. Pediveligers were fed microalgae at approximately 100,000 cells T-Iso/mL on days 0 and 2.

J.2 Endpoint Assessment of WAF Exposure

The developmental success of pediveligers was determined by their progression to spat (i.e., settlement success), and mortality was assessed at the end of the experiment. To assess settlement rates, settlement plates and container walls were observed under magnification using a dissecting microscope, and newly settled oysters were counted. Any pediveligers remaining in the water column were collected on a sieve (150 µm), rinsed, and resuspended in 29.1 mL of seawater in a 50-mL centrifuge tube. Then, 0.9 mL of 10% formalin was added to the 50-mL centrifuge tube for later estimation of survival. The total volume of solution in the 50-mL centrifuge tube was 30 mL. Final survival was assessed by taking 3 subsamples of 1,000 µL

from each 50-mL centrifuge tube after homogenization to estimate the total number of oyster remaining.

J.3 Pediveliger Exposure to Spiked Sediment

Pediveligers (retained on a 200-µm Nitex screen sieve; about 12–15 days post-fertilization) were exposed to sediment spiked oil in 600-mL beakers (350 mL of overlying water) for 3 days. Spiked sediment was prepared following the guidelines in Section I.4.

Each test consisted of five spiked-sediment treatments, an unspiked sediment control, and a seawater control, with four replicates of each treatment (see test-specific TCTs). Prior to test initiation, each container was loaded with 150 g of sediment (≈ 1.5 cm). Following the loading of the exposure chambers with sediment, two settlement plates, consisting of HardieBacker Cement Board tiles (120 mm × 58 mm), were placed vertically into each testing beaker. The tiles were in contact with the sediment approximately 0.5-cm deep. The tiles were soaked in seawater a minimum of 2 weeks prior to test initiation. At test initiation, pediveligers were stocked at a density of approximately 1,000 per beaker. To accurately estimate stocking densities, a dummy beaker was loaded at the same stocking density for each treatment. The dummy beakers were sieved immediately after the test initiation and the total numbers of pediveligers that were stocked into the beakers were recorded on the appropriate bench sheets.

During the exposure, aeration (i.e., gentle bubbling) was supplied for 30 minutes every 2 hours using a timer-controlled air pump. Oysters were fed microalgae at approximately 100,000 cells T-Iso/mL on days 0 and 2.

J.4 Sediment Preparation

J.4.1 General guidelines

The control sediments were prepared using the same technique used for spiked sediments, with the exception of adding oil. Each sediment-oil concentration was prepared separately. Equipment was cleaned and prepared, using the *Decontaminating Glassware* SOP in the QAPP. Unused sediments were stored in a zip-lock bag in the dark at 4°C (short-term) or in the freezer (-20°C; long term).

J.4.2 Preparation of sediment

- After sediment was thawed, all the debris (grass, shells, etc.) was removed from the sediment and placed into a mixer bowl
- Using a Cuisinart SM-70 7-quart stand mixer, the sediment was homogenized by mixing for 2 minutes at low speed (1).

J.4.3 Mixing oil into sediments

The appropriate amount of oil was weighed out as outlined below.

- A weigh boat and two or three Kimwipes were tared on the top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was placed onto the weigh boat. The oil was transferred onto the sediment in the mixing bowl and placed in several areas around the bowl using a spatula. Any oil remaining on the spatula was wiped with the tared Kimwipes. The weigh boat was reweighed with the Kimwipes to calculate and record the actual mass of oil transferred.
- The oil was mixed into the sediment at medium speed (5) with the Cuisinart mixer. Every 2 to 4 minutes, the mixer was briefly stopped and the sides of the mixing bowl were scraped with a putty knife.
- Once the mixing was complete, the mixer paddle was scraped down with a putty knife to remove all excess oiled sediment. The oiled sediment was either transferred from the mixing bowl into the test containers using a stainless steel spoon or stored in the dark at 4°C until test initiation.

J.4.4 Sediment and overlying water sampling

Sediment samples for the analytical laboratory were collected from each treatment group by adding approximately 100 g of sediment into the analytical chemistry sediment jar between filling each replicate tank with sediment. Each 8-oz sample jar was filled at least three-quarters full. Sediment samples were stored at 4°C until they were shipped to ALS Environmental for analysis. After the addition of sediment, the exposure beakers were allowed to settle overnight under static conditions prior to the addition of the oysters.

After the test was completed, overlying water from each replicate was carefully siphoned out of the beaker, composited by treatment, and sent to ALS Environmental for analysis.

J.5 Endpoint Assessment of Sediment Exposure

The developmental success of pediveligers was determined by their progression to spat (i.e., settlement success), and mortality was assessed at the end of the experiment. To assess settlement rates, settlement plates and container walls were observed under magnification using a dissecting microscope, and newly settled oysters were counted. Larvae remaining in the water column and/or settled on the sediment were collected on two sieves, 150 µm (to retain live and dead pediveligers) and 250-µm (to retain newly settled spat). Both were rinsed and the remaining material was re-suspended in 48.5 mL of seawater in a 50-mL centrifuge tube. Then, 1.5 mL of 10% formalin was added to the 50-mL centrifuge tube for later estimation of survival. Separate 50-mL centrifuge tubes were prepared according to sieve size. Final survival was assessed by taking 3 subsamples of 1,000 µL from each 50-mL centrifuge tube after homogenization.

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3. University of Southern Mississippi General Laboratory Procedures and Practices

3.1 Introduction

The University of Southern Mississippi Gulf Coast Research Laboratory (GCRL) conducted a series of toxicity tests to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil spill on resident fish and invertebrate species in the Gulf of Mexico. This chapter describes General Laboratory Procedures and Practices (GLPP) used at GCRL.

3.2 Methods

3.2.1 Water

Artificial seawater (ASW) used at GCRL was prepared using a formulation from Fritz Aquatics (Mesquite, Texas). To make the ASW, approximately 900 gal of unbuffered well water was mixed with 1 bucket of Fritz Pro Aquatics Super Salt Concentrate and 80 pounds of Tru-Soft Water Softener Salt Pellets (United Salt Corporation, Houston Texas). The mixed ASW was recirculated through a 10–25-µm filter before transfer into another large reservoir within the toxicology building where the water was filtered and temperature-adjusted. This water was used for husbandry, to prepare exposures, and also as dilution water in flow-through exposures.

3.2.2 Test organism sources and husbandry

This section describes the sources and husbandry for test organisms used for toxicity tests. Tests were conducted with sheepshead minnow (*Cyprinodon variegatus*), speckled seatrout (*Cynoscion nebulosus*), blue crab (*Callinectes sapidus*), southern flounder (*Paralichthys lethostigma*), grass shrimp (*Palaemonetes pugio*), fiddler crab (*Uca longisignalis*), white shrimp (*Litopenaeus setiferus*), red snapper (*Lutjanus campechanus*), Atlantic croaker (*Micropogonias undulatus*), and red drum (*Sciaenops ocellatus*).

Sheepshead minnow

Wild-type sheepshead minnow were in stock in the Shoemaker Toxicology Laboratory (STL) at the University of Southern Mississippi, housed in 80-gal (200-L) raceways under recirculating conditions with a 10% water change each week. Fish were fed pelleted feed at a rate of 2% body weight daily and were available on demand.

Speckled seatrout

Speckled seatrout were in culture at the Cedar Point Aquaculture Facility at GCRL. They were transported to the STL at least 4 days prior to use and acclimated to 15-ppt salinity prior to exposure initiation. Seatrout e.g.gs were obtained from tank spawns of captive adults maintained under controlled environmental conditions similar to previously described methods (Arnold et al., 1978). Eggs were incubated at a density of 1/mL in 120-L conical-bottomed tanks on a recirculating system at 25-ppt salinity and 27°C. At 33 hours post-hatch, larvae were stocked at a density of 15/L into 1,500-L conical-bottomed black tanks (diameter = 1.5 m) filled with approximately 25-ppt salinity ASW, *Artemia* nauplii, and 0.05 × 106 cells/mL T-iso. Gentle aeration was applied from the bottom center of the tanks. Light intensity at the water surface was approximately 400 lux from standard fluorescent bulbs with constant illumination. Dissolved oxygen (DO) was maintained above 4.5 mg/L, water temperature was maintained at approximately 28.0°C, and salinity was maintained at approximately 25 ppt. Total ammonia (NH_3-N), nitrite (NO_2-N), and alkalinity were measured daily using Hach (Hach Co., Loveland, Colorado) test strips. Alkalinity was maintained at > 150 mg/L by addition of sodium bicarbonate. The pH was measured daily using a YSI 556.

Blue crab

Ovigerous female blue crabs were collected from waters of the Mississippi Sound in an area south of the mouth of Biloxi Bay. Crabs were collected using industry standard crab traps with a soak time of less than 48 hours (preferred). Laboratory personnel accompanied a local fisherman to select healthy females with early bright orange sponges. The females were placed in individual coolers containing aerated site water for transport to GCRL. They were held in quarantine tanks for 24 hours, where they were acclimated to culture conditions (25°C and 28-ppt salinity) and checked for disease (white spot syndrome virus and *Hematodinium* sp.) before being placed in hatching tanks. After approximately 1 week, the zoeae were collected, counted, and stocked (approximately 100,000 per tank) in 1,400-L recirculating culture systems equipped with biological and mechanical filtration. Depending on their stage, the zoeae were fed a diet of rotifers (*Brachionus rotundiformis*) and Instant Algae (*Nannochloropsis*, *Isochrysis*, and Shellfish Diet), newly hatched and enriched *Artemia*, and frozen copepods (Cyclop-eeze). The zoeae molted through 7 zoeal stages to megalopae. When the culture contained ~ 85% megalopae, the systems were drained and the megalopae were collected.

Grass shrimp

Grass shrimp were collected by dip netting in marshes adjacent to the GCRL shore, and stored in 800–4,000-L tanks (15-ppt ASW, 26°C, 16:8 L:D cycle) in the STL quarantine facility. Grass shrimp were held a minimum of 7 days prior to testing, and fed commercial flake food twice daily. Ovigerous females were separated daily from the general population and put into a separate 1,600-L aquarium under the same conditions and feeding regime. Water quality

parameters (temperature, salinity, DO, ammonia) were measured twice weekly in the shrimp-holding aquaria.

Southern flounder

Southern flounder juveniles were obtained from the University of Texas Marine Science Institute (UTMSI) and transported to GCRL in 32-ppt salinity ASW at 20.5°C. They were put into two Living Streams systems with high-flow recirculating water. Juveniles were acclimated to 15-ppt salinity prior to experimentation, and held at 15-ppt salinity for a minimum of 1 week prior to experimentation. They were fed twice daily with commercial flake food and *Artemia* nauplii.

Southern flounder larvae were obtained from the Texas Parks and Wildlife Department and were held in 200-L aquaria at 30-ppt salinity until they were used in experiments. They were fed algae (*Nannochloropsis* spp.), rotifers, and *Artemia* nauplii depending on size.

Fiddler crab

Fiddler crab zoeae were obtained from Auburn University and were used immediately in toxicity testing. See the Auburn University GLPP for fiddler crab collection and culture procedures.

White shrimp

White shrimp were obtained from Florida Organic Aquaculture, Vero Beach, Florida, and transported to STL in 30-ppt salinity ASW at 20°C. They were put into two Living Streams systems with high-flow recirculating water. Shrimp were acclimated to 24°C and 30-ppt salinity water prior to experimentation and held at these conditions a minimum of 4 days prior to experimentation. They were fed twice daily with commercial shrimp pellet food and frozen mysids.

Red snapper

Red snapper were in culture at the Cedar Point aquaculture facility at GCRL, and 8- to 12-week-old fingerlings were transported to STL at GCRL at least 4 days before use and acclimated to 30-ppt salinity before exposure initiation. Captive cultures were started from adult males collected from the field. Broodstocks were reared at Cedar Point and were induced for gamete maturation using previously described methods (Arnold et al., 1978). Eggs were collected by strip spawning, then incubated at a density of 1/mL in 120-L conical-bottomed tanks on a recirculating system at 30 ppt and 26°C. At 14-h post-fertilization, larvae were stocked at a density of 50/L into 1,000-L conical-bottomed black tanks (diameter = 1 m) filled with 30-ppt salinity seawater. Gentle aeration was applied from the bottom-center of the tanks. Light intensity at the water surface was approximately 1,000 lux from standard fluorescent bulbs with constant illumination under a 16 L:8 D photoperiod. DO was maintained above 4.5 mg/L, water temperature was maintained

between 26°C and 28.0°C, and salinity was maintained at approximately 30 ppt. Alkalinity was maintained at > 150 mg/L with the addition of sodium bicarbonate.

Atlantic croaker

Atlantic croaker were in culture at the Cedar Point Aquaculture Facility at GCRL. They were transported to the STL at least one month before use and housed in a 500-gallon circular tank. Fish were originally housed at 26 ppt salinity; the salinity was dropped 1–1.5 ppt every other day until a salinity of 18 ppt was reached. Fish were housed at approximately 28°C and DO was maintained at approximately 6.0 mg/L. Fish were fed Silver Grow Out Feed by Zeigler daily.

Red drum

Juvenile red drum were obtained from Texas Parks and Wildlife aquaculture facility in Lake Jackson, Texas. Upon arrival at GCRL, fish were transferred into living streams. Fish were held for approximately 2 weeks before testing. Salinity was dropped from 29 ppt to 15 ppt in the two-week span in the Living Stream quarantine system. Salinity was dropped 1 to 1.5 ppt per day to reach the experimental design salinity of 15 ppt. Fish were fed Otohime Fish Diet by Reed Mariculture daily.

Pathogenic bacteria

Vibrio anguillarum was used in bacterial exposures. *V. anguillarum* was procured from ATCC (Strain 19264), which was initially isolated from ulcerous lesions in cod. Isolates obtained from ATCC (Manassas, VA) were cultured in T1N3 bacterial media and bacteria were then frozen in glycerol for later use. Media was thawed to ambient temperature before use in toxicity tests.

3.2.3 Exposure media preparations

Toxicity testing at GCRL used multiple oils and preparation methods. There were two different water accommodated fraction (WAF) preparations, high energy (HEWAF) and chemically enhanced (CEWAF) WAFs, using four oil types: Slick A, Slick B, artificially weathered source oil, and source oil. Some tests included Corexit 9500-only as dispersant controls during CEWAF exposures where applicable [see test-specific test conditions tables (TCTs)]. Other tests included exposure to contaminated sediments. In all exposures, appropriate controls were performed. Control waters were managed similarly to WAF preparation methods, except that no oil was added. WAF exposure preparation methods were performed according to protocols described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Sediment exposure preparation methods are included in the individual Testing Protocols listed in the appendix of the GCRL GLPP.

3.2.4 Testing methods

Embryonic, larval, and/or juvenile life stages of sheepshead minnow, speckled seatrout, grass shrimp, southern flounder, fiddler crab, blue crab, red snapper, Atlantic croaker, and red drum were exposed to HEWAF or CEWAF using aqueous exposures. Post-larval, juvenile and adult life stages of southern flounder, brown shrimp, and white shrimp were exposed to spiked sediment. Spiked sediment preparations were prepared as outlined in the GCRL Testing Procedures SOP: *Protocol for Preparation of Oil-Spiked Sediments*.

Acute aqueous exposure toxicity tests

Test solutions for acute toxicity exposures were made according to the QAPP. Acute exposures were performed according to GCRL GLPP Testing Protocol 1. Selected life stages of each species were exposed to multiple concentrations of WAF as acute static or static/renewal tests (see test-specific TCTs for exposure duration). Exposures consisted of different toxicant doses and appropriate controls, and each concentration was performed with 5–20 individuals per replicate, with the exception of blue crab megalopae, which were exposed individually in 20-mL borosilicate scintillation vials containing 20-mL test solution.

At the beginning of the exposure, HEWAF, CEWAF, and control stock solutions were sampled for hydrocarbon analysis and shipped overnight to ALS Environmental according to the QAPP. Dilution series archive and fluorescence samples were collected and handled according to the QAPP.

Test organisms that survived and were used during the tests, but not used for subsequent analyses, were sampled and archived. Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP. Samples requiring long-term storage were shipped to ALS Environmental for storage.

Chronic aqueous exposures

Chronic exposures were performed according to the protocols in *Definitive Chronic Exposures* (GCRL GLPP Testing Protocol 2), *Exposure Systems: General Methods and Materials* SOP, *Calibration of flow-through exposure systems SOP*, *Operation and maintenance of the Hamilton PSD/2 SOP*, and the *ASTM International chamber and flow-through loading calculations SOP*. This was done by performing simultaneous exposures that subjected organisms to HEWAF or CEWAF.

At the beginning of the exposure, the HEWAF, CEWAF, and control stock solutions were sampled for hydrocarbon analysis and shipped overnight to ALS Environmental as described in the QAPP. Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP.

Dilution series archive and fluorescence samples were taken and handled according to the QAPP. When flow-through systems were used during chronic exposures, dilution water was periodically sampled for archival extractions and fluorescence analysis.

Test organisms that survived and were used during the tests, but not used for subsequent analyses, were sampled and archived.

Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP.

Aqueous exposure fertilization tests

Testing staff examined the effects of HEWAF and CEWAF on fertilization and reproductive success in sheepshead minnow and grass shrimp.

Assessments of effects of aqueous exposure on progeny

To assess reproductive effects, adult male and female individuals were exposed to sublethal concentrations of WAF for 4–7 weeks in a flow-through system. Individuals of each sex were sampled from each replicate aquarium and histologically evaluated for treatment-related effects on gonadal development.

Sediment exposures

Oil-contaminated sediments were prepared by adding oil to uncontaminated sediments using a Cuisinart® stand mixer as outlined in the GCRL Testing Procedures SOP: *Protocol for Preparation of Oil-Spiked Sediments.*

3.2.5 Water quality monitoring

Water quality was monitored throughout each exposure according to the *Monitoring water quality parameters during static and flow-through exposures SOP*. See the QAPP and test-specific TCTs for required monitoring.

3.2.6 Analytical chemistry sampling

Analytical sampling and analysis of water and sediments were carried out following the procedures outlined in the QAPP.

3.3 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the QAPP.

3.4 General Testing Standard Operating Procedures

3.4.1 Exposure systems: General methods and materials

Purpose

The purpose of this standard operating procedure (SOP) is to provide guidelines on the material, methods, and construction of exposure systems used in our various studies. Generally, flow-through exposures were performed if the test duration was longer than 7 days; for exposure durations of less than 7 days, static or static/renewal was appropriate.

Procedures

Static and static renewal exposures

A. Static exposures were generally conducted:

1. When the amount of test substance was limited and the requirements for a flow-through exposure exceeded available test material

2. When the test substance was highly hazardous, and it was necessary to keep the waste produced to a minimum

3. When a test organism could not tolerate the stresses of water changes.

B. Static exposures were typically limited to 96 hours or less. In cases where exposure time exceeded 96 hours, scheduled renewals were conducted. For sensitive test organisms that could not tolerate the stress of water changes, tests were run as static exposures.

C. Static exposures were generally conducted in glass vessels with a capacity appropriate for the test volume required to ensure that organism loading and water quality parameters were not compromised during the test. Static exposures were conducted in a temperature-controlled water bath, an incubator, a closed system such as a glove box, or under a laboratory hood. The specific lighting conditions for each test can be found in the test-specific TCTs. Light regimen depended on WAF type and duration of exposure.

Lighting, when present, was generally fluorescent. However, depending upon specifications in the test-specific TCTs, other types of lighting were used.

Flow-through exposures

A. Dosing apparatus:

1. The dosing apparatus was used for metering dilution water and toxicant stock in the proper proportions to each of the exposure aquaria

2. The dosing apparatus in the flow-through system incorporated a water delivery device, which provided either continuous or intermittent flow of test solution or dilution water.

B. The intermittent delivery flow-through system used in toxicology at GCRL was a water partitioning type. The stock delivery system generally used for this type of exposure system was the Hamilton PSD/2, which provided a calibrated volume of toxicant stock on impulse demand. The dosing apparatus was generally located above the exposure aquaria, and gravity aided in the dispensing of test media. Generally, glass tubing was used to transfer test media from the dosing apparatus into mixing chambers or mixing tubes prior to introduction into the exposure aquaria.

Dilution water

A. During flow-through exposures, diluent water was most often pumped continuously through polyvinyl chloride (PVC) pipes from a fiberglass holding reservoir to a wood or fiberglass headbox located above the dosing apparatus system. Excess dilution water was returned to the reservoir through an overflow standpipe or side drain in the headbox.

Toxicant stock

A. During flow-through exposures, the toxicant reservoirs were graduated cylinders, flasks, carboys, and/or other containers that met the individual needs of the stock and exposure. The injector devices transferred toxicant stock from the dispensing carboy to mixing containers through strands of mini-bore tubing.

Mixing chambers, splitter boxes, and exposure aquaria

A. Glass mixing chambers or mixing tubes were used to blend the dilution water and toxicant stock prior to introduction into exposure aquaria or splitter boxes. A splitter box was sometimes used to divide the exposure solution among two or more exposure vessels via glass tubing emerging from bored stoppers that were plugging holes drilled in the bottom or the sides of the boxes. Mixing chambers and splitter boxes were usually

constructed by toxicology personnel by cementing glass sections together with clear silicone adhesive. When volatile compounds were tested, the mini-bore tubing emerging from the injectors was threaded the length of the diluent delivery tubes to the mixing chambers. Because the mini-bore tubing terminates below the standing water level in the mixing chamber, the toxicant stock was delivered under the surface of the diluent water, thereby minimizing volatilization.

B. Exposure aquaria were generally constructed of glass, Plexiglas, or polycarbonate sections. The aquaria dimensions varied depending upon loading density, the number of treatment concentrations, and the number of replications at each treatment. Aquaria pieces were cemented together with either clear silicone cement or plastic solvent (for Plexiglas or polycarbonate) purchased locally. Bored stoppers plugged holes that were drilled on the sides or bottoms of exposure tanks to accommodate overflow tubes, which allowed the aquaria to drain into an effluent trough. The stoppers used for mixing containers, splitter boxes, and exposure aquaria were of a non-toxic material, generally silicone or neoprene.

Exposure chamber

A. An exposure chamber generally housed the test aquaria to minimize environmental disturbances to the test system, and to provide constant temperature and light conditions.

B. The exposure system contained the exposure aquarium in a temperature-controlled water bath inside an exposure chamber. The tanks in the water bath were on supports so that water could circulate under them to control temperature. Outside of the exposure chamber, water pumps that communicate with the water bath operated to circulate and distribute the temperature-controlled bath water. The photoperiod was maintained by fluorescent lights.

3.4.2 Calibration of flow-through exposure systems SOP

Purpose

The purpose of this SOP is to provide guidelines on the calibration of flow-through exposure systems.

General

Any exposure system that was used in the performance of a flow-through toxicity test was calibrated prior to the initiation of the test.

Procedures

A. A water partitioning system provided diluent water to all test concentrations and control(s) by the use of a single series of "water cells." The selected concentrations were achieved by adding various volumes of stock(s) by a series of injectors (usually PSD/2). Instructions for use of the PSD/2 are provided in *Operation and maintenance of the Hamilton PSD/2 SOP*. The volume of stock provided was achieved by altering the syringe size (50 µL to 25 mL), the plunger stroke length, and the number of injections per cycle (details regarding the injection volumes can be found in test-specific TCTs). The electrical pulses for injections were monitored by impulse counters, which tallied the number of injection commands received by the pump as a check of proper system function. Splitter boxes were used to divide test solutions among the replicated test chambers.

B. Calibration of the partitioning system involved a common volume for each treatment and control(s). Volume was checked and adjusted based on the total desired volume minus any addition resulting from the injected stock. Volume of each treatment concentration was checked and recorded 3 times following final adjustments, and the mean of the 3 measurements met protocol specifications or were ± 5% of the target volume, if not explicitly stated.

C. When replicate test chambers were used in a flow-through test system, splitter boxes were employed. The function of the splitter box was to provide its contents equally to each of the replicate test chambers for a specific treatment. If splitter boxes were necessary for a test, the splitter boxes had to be checked for delivery. The volume delivery difference between the replicates met protocol specifications or was within 10%, if not explicitly stated, based on the average of three measurements.

3.4.3 Operation and maintenance of the Hamilton PSD/2 SOP

Purpose

The purpose of this SOP is to direct a user in the proper operation and maintenance of the Hamilton PSD/2.

General

The compact PSD/2 performs accurate liquid dispensing operations at speeds ranging from 1 to 60 seconds per syringe stroke. Operating on a single 24-volt DC power supply with an optional DC/DC converter, each module is capable of functioning as a standalone unit via an onboard EEPROM (Electronically Erasable Programmable Read-Only Memory) with remote activation.

Programs downloaded from an external computer to the EEPROM are retained until erased or replaced, and instruction codes are executed each time remote activation occurs. All operations followed the PSD/2 Installation Guide and/or the Hamilton PSD/2 Operator's Service Manual.

Procedures

A. Power requirements and connection

A power supply that provides 24-V DC (+ 15%, -5%) @ 1A, 25 W was required for operation. Each module requires a maximum of 1 amp. Connection of the power supply to the PSD/2 module was made at the J4 port on the interface board on the back of the module. Connection to the power supply was accomplished with a 4-pin molexR connector.

B. External computer communications requirements and connection

The default settings for both the PSD/2 module and any external computer were used. In some cases, if the default setting was not used, that information was recorded in laboratory notebooks. A set of dual-inline package (DIP) switches are located on the interface board of the PSD/2 module. Except for switch 7, the switches come preset in the off position. The connection of the external computer to the PSD/2 module was made at the J8 (COM IN) port on the interface board on the back of the module. The connection was accomplished with a 4-pin molexR connector at one end of the cable for connection to the PSD/2 at the J8 port; the other end of the cable must be a DB 9 female plug connected to the computer.

C. Remote activation

Remote activation by closed contact was achieved through the TTL IN (J7) port on the interface board on the back of the PSD/2 module. This is an 8-pin molexR connection with the Pin 2 position designated as a trigger. A wire was connected to this position and a separate wire was connected to the Pin 7 position by means of an 8-pin molexR connection. The other ends of these wires were attached to a float switch, timer, or any other normally open contacts. When the contacts were closed, a set of stored instructions was remotely activated.

Downloading stored instructions to the EEPROM

1. Connect the DB 9 end of the communications cable to the external computer at the appropriate port (mouse port or COM 1). Plug the molexR end of this cable into the J8 port on the interface board on the back of the PSD/2 module.

2. Turn on the external computer and access the PSD2DEMO program from either the hard drive or a floppy disk.

3. Supply power to the PSD/2 module. Verify power to the module by the initialization (turning) of the valve motor. It is necessary to periodically interrupt this power later in this procedure. If there is a connection made at the TTL IN (J7) port at this time, ensure that there is no contact closure between the leads. If there has been contact closure, open it, make certain it remains open, and briefly interrupt the power before continuing.

4. Select the first option on the menu of the PSD2DEMO program: Manual ASCII Commands.

5. Type aXR <enter> to verify communications setup. The screen should echo the command and acknowledge (ACK) it. The module should initialize the valve motor and syringe drive. If these things do not happen and/or a screen error appears, check all connections, hit <esc>, interrupt the power to the module, and start again at Step 2.

6. If this is the first time computer communication has been accomplished with the PSD/2 module at hand, or if the user is uncertain of this, type aYSM1R <enter>. This command sets the syringe drive at full resolution (0–2,100 steps) and enables accurate volume adjustments.

7. Based on the amount of WAF that needs to be injected into the splitter box (see test-specific TCTs), choose the correct-size Hamilton syringe (dependent on injection amount) with a threaded hole in the thumb-plate of its plunger. Attach the syringe to the correct valve for the syringe size (part no. 39221 for syringes of 2.5 mL or higher, unless it is determined that the injection stroke speed is slow enough so as not to create excessive backpressure, or part no. 39298 for smaller syringes). The syringe twists clockwise to engage the Luer Lock fitting. The valve and syringe can then be put in place by confirming that the valve-locking handle is in the counter-clockwise position as far as possible; the valve with the syringe is pushed in position until it sits against the valve adapter; the valve-locking handle is rotated clockwise until it locks in a vertical position; and the thumbscrew on the syringe drive arm is secured into the end of the syringe plunger (this may require positioning of the plunger).

8. Type aXS12R <enter> to initialize again for syringe position. The inclusion of S12 in this command is to slow the syringe drive speed in case the dry syringe presents too much resistance for the syringe drive to operate at the default speed of S4. With large syringes of 10–25 mL capacity, the S or Speed Command may need to be increased beyond S12. This to be determined at the time of installation.

9. Type IP(xxxx)S12N5R <enter> with (xxxx) being some step number between 0 and 2,100. This step number is an estimate of what will draw the syringe to the proper volume. As in Step 5, the command should be echoed, acknowledged <ACK>, and executed. Determine the volume called for by the step number chosen and readjust as necessary. Repeat Steps 8 and 9 until the correct injection volume is achieved.

10. Type the following instruction codes in order with <enter> following each line. Fill the (xxxx) with the step number determined in Step 9. While these commands are being entered, the module will echo and acknowledge <ACK> on the computer screen, but it will not execute the functions.

    - a#SP9: Clears the EEPROM memory.
    - a#SP8: Starts the download mode, selects method 8.
    - aXS12R: Initializes the module on the first trigger after any power interrupt. The S or Speed may need to be increased and should be reflected in all S values past this command.
    - aIP(xxxx)S12N5: Sets the valve to the input port, draws syringe the number of steps chosen, and returns 5 steps at speed 12 (12 seconds for a full syringe stroke).
    - aOD(xxxx)S12R: Sets the valve to the output port, and moves the syringe drive backup (dispense) the same number of steps drawn at speed 12. The execute command placed here ensures that the draw and dispense strokes are executed as a unit on each trigger experienced after the first.
    - a#SP0: Ends the download mode.

11. Type <esc> to return to the menu and end communications.

12. Interrupt and re-establish power to the PSD/2 module.

13. Close the contact on the leads from the TTL IN port (J7). The module should initialize and stop. Close the contact again, and the module should execute a complete injection cycle as programmed. Once in place, with nothing connected to the COM IN (J8) port, only power (J4), and TTL IN (J7), the module should execute the downloaded set of functions as described each time remote activation by contact closure occurs. Be aware that the first contact closure after power interruption will trigger initialization only. Computer communication can be achieved only if Steps 1–4 above are followed carefully, and if no untimely contact closure by the remote activation system has occurred.

14. Any changes to downloaded instructions will have to be made by following all steps listed above, except Step 6. No editing options exist. The memory must be cleared and another download executed.

3.4.4 ASTM International chamber and flow-through loading calculations SOP

Purpose

The purpose of this SOP is to provide guidelines for calculating loading in chambers for static, renewal, and flow-through conditions. The number of organisms that were loaded per tank can be found in the test-specific TCTs.

General

1. Loading is the ratio of biomass (wet weight, blotted dry, in grams of whole body tissue) to the volume of solution in a chamber, or passing through it, in a 24-hour period. The loading in culture, testing, and growout was not so high as to affect the health of the animals by an accumulation of excretory waste material, a diminution in DO, or physical contact of organisms to each other. Therefore, loading was maintained below a maximum established level.

2. The loading of organisms in test chambers is generally based on values proposed in ASTM International (ASTM) E729-88a (*Standard Guide for Conducting Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians*). The guideline requires that in static or renewal tests, the loading in each test chamber shall not exceed 0.8 g/L at or below 17°C, and shall not exceed 0.5 g/L at higher temperatures. In flow-through tests at or below 17°C, the loading shall not exceed 10 g/L in the chamber at any time, and shall not exceed 1 g/L of solution passing through the chamber in 24 hours. At temperatures greater than 17°C, the loading in flow-through tests shall not exceed 5 g/L at any time or 0.5 g/L passing through the chamber in 24 hours. This information is summarized in Table 3.1.

Table 3.1. Maximum loading density guidelines

	Maximum loading density	
Holding conditions	**g/L at any time**	**g/L in 24 hours**
Static or renewal, ≤ 17°C	0.8	–
Static or renewal, > 17°C	0.5	–
Flow-through, ≤ 17°C	10	1
Flow-through, > 17°C	5	0.5

Procedures

A. Static or renewal conditions

For organisms maintained under static or renewal conditions, it was necessary to ensure that the maximum chamber loading at any time was not exceeded.

B. Determination of the acceptable number of organisms for a given vessel

1. To determine the acceptable loading (number of organisms) for a given chamber under static or renewal conditions, the volume of solution and the average organism wet weight were determined. The volume of solution was determined for an aquarium by length × width × solution depth. In a vessel that did not have right-angle dimensions, the solution volume was determined by volumetric addition of a liquid medium. The average organism wet weight was determined from a grab or otherwise random sample of organisms comparable to those to be maintained in the chamber. The organisms were preferably individually weighed, but sometimes a pooled weight was taken.

2. To determine the number of organisms that could be placed in the chamber, the solution volume was first multiplied by the appropriate maximum loading density determined from the table. The result was divided by the average wet weight of the organism. The resulting value was the approximate number of organisms that could be added to the chamber to not exceed loading.

C. Determination of the volume of solution necessary to hold a given number of organisms

To calculate the volume required to house a group of organisms in a static or renewal chamber, the total organism wet weight was divided by the maximum loading density. The final result was the minimum liters of solution needed to meet the ASTM guidelines.

D. Flow-through conditions

For organisms that were maintained under flow-through conditions, it was necessary to ensure both that the maximum chamber loading at any time was not exceeded and that the flow of water through the tank in 24 hours was sufficient. Chamber loading calculations were performed in the manner described previously for static or renewal conditions.

E. Determination of the acceptable number of organisms for a given flow rate (cycles/hour)

1. To determine the acceptable loading (number of organisms) for a given flow rate under flow-through conditions, the volume of solution entering a chamber each

day and the average organism wet weight must be known. In intermittent flow-through systems, the daily volume of solution was determined by multiplying the given cycles/hour by the volume of solution entering a chamber each cycle, by the 24 hours in a day. In a continuous flow-through system, the volume of solution per day was calculated by multiplying the flow rate in L/minute by 60 minutes in an hour, by 24 hours in a day.

2. To determine the number of organisms that could be placed in the chamber, the daily solution volume was first multiplied by the appropriate maximum loading density determined from Table 3.1. The result was divided by the average wet weight of the organism. The resulting value was the approximate number of organisms that could be added to the chamber to not exceed loading.

F. Determination of the flow rate (cycles/hour) necessary for a given number of organisms

To calculate the flow rate necessary for a given number of organisms in a flow-through chamber, the total organism wet weight was divided by the maximum loading density. The final result was the minimum volume of solution that must pass through the chamber each day to meet the ASTM guidelines. Intermittent flow rate in cycles/hour was then calculated by dividing this volume by the product of the volume of solution entering a chamber each cycle and the 24 hours in a day. Continuous flow rate in L/minute was calculated by dividing this volume by the 1,440 minutes in a day (60 minutes/hour times 24 hours/day).

3.4.5 Monitoring water quality parameters during static and flow-through exposures SOP

Purpose

The purpose of this SOP is to provide guidelines for monitoring water quality parameters during toxicity tests.

General

Chemical and physical data on test solutions are routinely generated during toxicity tests at times prescribed by the test protocol (see test-specific TCTs). Generally, the temperature, salinity, DO concentration, and pH were measured during the tests.

Equipment and materials

1. DO – YSI ProODO and DO probe

2. pH – pH probe (Hach PHW77-SS)

3. Salinity – Atago handheld refractometer (ATC-S/Mill-E)

4. Temperature – calibrated mercury-filled thermometer, a digital thermometer, or a temperature recorder

5. Total unionized ammonia – measured with Koch Ammonia Strips or Tetra Easy Strips.

Procedures

A. Monitoring methods

1. DO concentration – measured daily in one randomly selected replicate per concentration.

2. pH – measured daily in all exposure containers.

3. Salinity – measured daily in all exposure containers.

4. Temperature – for exposures performed in small volumes in environmental chambers, daily temperature readings were taken from a temperature-equilibrated beaker placed inside the environmental chamber. For exposures performed in larger tanks, daily temperature recordings were taken from each tank.

5. Total unionized ammonia – measured daily in one randomly selected replicate per concentration.

B. Recordkeeping

All chemical and physical measurements taken during tests were recorded on the appropriate bench sheets. Data collected by the probes were digitally stored and printed for a hard-copy reference in the data logbook. Measurements not within protocol/procedural specifications were reported to the study manager immediately.

Measuring devices or methods of determination were recorded for each occasion that the measurements were performed.

Decontamination procedures

DO meters and handheld refractometers were decontaminated after each use by gentle washing with warm soapy water, rinsing with deionized water, and careful rubbing with Kimwipes. When making multiple measurements across multiple treatments, care was taken to ensure that measurements were always made in order from lowest concentration to highest, starting with the controls.

Calibration procedures

DO meters and handheld refractometers were calibrated monthly following manufacturer protocols.

3.4.6 Protocol for preparing oil-spiked sediments SOP

Purpose

The purpose of this SOP is to provide instructions for the preparation of oil-spiked sediments for exposures.

General

- Controls were prepared using the same technique that was used for spiking sediment, with the exception of adding oil.
- Each sediment-oil concentration was prepared separately. For instructions on cleaning and preparing the equipment, refer to the Decontaminating Glassware SOP in the QAPP.
- Unused, prepared sediments were placed into a zip-lock bag and stored in the dark at 4°C (short-term) or in the freezer at -20°C (long-term).

Preparation of sediments

1. Sediments were allowed to thaw overnight at room temperature
2. All debris (sticks, etc.) was removed from thawed sediment and sediment was placed in a mixing bowl
3. Sediment was homogenized by mixing for 2 minutes at low speed (1).

Mixing oil into sediments

1. The appropriate amount of oil was weighed out as outlined below:

   Slick oil was weighed in a pre-cleaned aluminum weigh boat. A weigh boat was tared with 2 to 3 Kimwipes on the top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was added onto the weigh boat. Using the spatula, oil was transferred onto the sediment in the mixing bowl and placed in several areas around the bowl (step 2). Any remaining oil was wiped off of the spatula with the tared

Kimwipes. The weigh boat was re-weighed with the Kimwipes to calculate and record the actual mass of oil transferred.

2. The mixer paddle was lowered into the bowl.

3. Oil was mixed into the sediment with a KitchenAid® KM45 4.5 quart stand mixer at medium speed (4.5) for 30 minutes or with a Cuisinart® SM-70 7-quart stand mixer, also at medium speed (5). The mixer was stopped briefly every 2 to 4 minutes to scrape the sides of the mixing bowl with a putty knife.

4. Once mixing was complete, the mixer paddle was scraped with a putty knife to remove all excess oiled sediment. The sediment was transferred from the mixing bowl to the test containers using a stainless steel spoon, or stored in the dark at 4°C until test initiation.

5. A sample of oil-spiked sediments was collected from each treatment. Samples were stored at 4°C before being shipped

3.4.7 RNA extraction and qPCR for gene expression analyses SOP

Purpose

This protocol describes in detail the experimental procedure for real-time qPCR using SYBR Green I.

General

Real-time qPCR determines relative amounts of genes of interest using a fluorescent reporter, SYBR Green.

Procedures

1. Best laboratory practices were followed to prevent contamination of samples. This included wearing gloves at all times and cleaning the work area and pipettes before prepping the samples or preparing the master mix (step 3).

2. All reagents (step 3) and samples were thawed on ice. Once they had thawed, all components were thoroughly mixed.

3. Enough of the following reaction master mixture was prepared to run each sample in triplicate.

   - Volume per single 20-µL reaction:

     - Two aliquots of 10-µL SYBR Green Mix
     - 1.8 µL of each forward and reverse primer (0.9 µm each)
     - µl H_2O (nuclease-free)
     - µl cDNA template

4. 1.0 µL of template, plus 19 µL of master mix were added to each of the 96 wells in a plate, and the plate was sealed with optically clear film. Samples were run in triplicate along with a no-template control to test for contamination.

	Temp (°C)	Time (s)
Initial denaturation	95	30
40 cycles of:		
Step 1	95	5
Step 2	58	15
Step 3	72	10

5. All qPCR reactions were amplified using the ABI 7500 Fast machine or similar.

6. An endogenous control, such as 18S, was run simultaneously with each gene of interest for all samples.

3.4.8 Method to extract DNA for microbial analyses SOP – conducted at Texas Tech University

DNA was extracted from tissues using a PowerSoil DNA Isolation Kit with minor adaptations (MoBio Laboratories). A thoroughly homogenized aliquot of each tissue sample was added to the PowerSoil bead tube. The extraction proceeded per the directions in the kit, resulting in 100 µL of DNA in the elution buffer (10 mM Tris). Concentrations of DNA in each sample were measured and recorded using a NanoDrop (Thermo) to account for total DNA used in qPCR.

3.4.9 Microbial diversity analysis SOP – conducted at Texas Tech University

The relationship between microbial communities in intestine and gill tissues of oil- versus non-oil-exposed fish was determined by 16S rRNA gene amplification and sequencing as described

by Dowd et al. (2008), targeting the V1–V3 regions. Sequences that failed to return at least half the expected amplicon length (or 250 bp, whichever was shortest) were removed from the data pool. All sequences were then denoised using an algorithm based on USEARCH and checked for chimeras using UCHIME (Edgar, 2010). After denoising and chimera checking, sequence data were separated into operational taxonomic units (OTUs) and annotated using the RDP classifier (Edgar et al., 2011) with GreenGenes v. 12.10 (MacDonald et al., 2012) used as a reference. Finally, relative abundances of taxa at each hierarchical taxonomic level were calculated using the summarize taxa.py QIIME script.

3.4.10 Gill histology image analysis methods SOP

Scope

The scope of this SOP was to provide protocols for gill histology sample preparation and analysis.

General

The purpose of histological analysis was to determine the effect exposure had on the cellular structure of a given tissue.

Histology sample preparation

The entire heads and body cavities of juvenile southern flounder were preserved in 10% neutral buffered formalin (NBF) for at least one week. Tissues were decalcified in Cal-Ex decalcifier (Fisher Scientific) overnight, rinsed for 18 hours in running tap water, and dehydrated in a series of graded ethanols for histological processing. Tissues were embedded in Paraplast and sectioned at 4 µm at two levels: the middle of the eye and completely through the eye. One section from each level was mounted on slides and stained with hemotoxylin and eosin. All areas of gill tissue from each fish on slides were photographed at 100x magnification using a Nikon Eclipse microscope and ACTcamera software. This process resulted in 4 to 12 photographs of gill tissue per fish. Each photograph was numbered with the test number, fish number, tissue type, and photograph number for that tissue type (e.g., ST133 1A6G1).

Photographs were analyzed to quantify the percentage of gill lamellae that presented with various histopathological conditions. Histopathological conditions recorded included telangiectasis in secondary lamellae, epithelial proliferation, swollen/fused lamellae, and the rank of telangiectasis. Ranks were defined based on the percentage of filaments presenting telangiectasis that were observed for each lamella: 0 = no telangiectasis; 1 = < 25% filaments presenting telangectasis; 2 = 25–50% filaments presenting telangectasis; 3 = 51–75% filaments presenting telangiectasis; and 4 = > 75% filaments presenting telangiectasis. Three photographs

were analyzed for each fish – the photographs to be analyzed were selected randomly using a random number table. All lamellae occurring in each photograph were inspected for histopathological conditions. To determine the percentage occurrence of each histopathological condition within the gill, the total number of lamellae present was divided by the total number of lamellae containing the histopathological condition. The mean rank of telangiectasis was determined by adding the rank scores for all lamellae and dividing that number by the total number of lamellae present.

3.4.11 Liver histology image analysis methods SOP

Scope

The scope of this SOP is to provide protocols for liver histology sample preparation and analysis.

General

The purpose of histological analysis was to determine the effect exposure had on the cellular structure of a given tissue.

Histology sample preparation

The entire head and body cavity of juvenile southern flounder were preserved in 10% NBF for at least one week. Tissues were decalcified in Cal-Ex decalcifier (Fisher Scientific) overnight, rinsed for 18 hours in running tap water, and dehydrated in a series of graded ethanols for histological processing. Tissues were embedded in Paraplast and sectioned at 4 µm at two levels: the middle of the tissue and completely through the tissue. One section from each level was mounted on slides and stained with hemotoxylin and eosin. All areas of liver tissue from each fish on a slide were photographed at 100x magnification using a Nikon Eclipse microscope and ACT camera software. This process resulted in 4 to 16 photographs of liver tissue per fish; there was no overlap of liver tissue in any photograph. Each photograph was numbered with the test number, fish number, tissue type, and photograph number for that tissue type (e.g., ST133 1A6L1).

Photographs were analyzed using Image J software to allow quantification of the percentage of liver tissue that presented with various histopathological conditions. Histopathological conditions recorded included macro/micro vesicular vacuolation, congestion, edema, eosinophilic inclusions in exocrine pancreas, and the amount of exocrine pancreas present. Three photographs were analyzed for each fish – the photographs to be analyzed were selected randomly using a random number table. A grid of 80 points (accessed through Plugins-Analyze-Grid) was superimposed on the selected photographs, and histopathological conditions occurring at each point were recorded. Any grid points intersecting non-liver tissue area were recorded as "total excluded

grids." The total number of excluded grid points was subtracted from the total number of grid points (240), and the resulting number of grid points containing liver tissue was used for calculations. To determine the percentage occurrence of each histopathological condition within the liver, the total number of grid points recorded for each condition from all three slides was divided by the total number of grid points containing liver tissue. To determine the percentage of eosinophilic inclusions in exocrine pancreas, the number of grid points containing this condition was divided by the number of grid points containing exocrine pancreatic tissue.

3.5 General Immunotoxicity Testing Standard Operating Procedures

3.5.1 Media preparation and QA/QC SOP

Purpose

The scope of this SOP was to provide protocols for the QC of all microbiological media, including equipment and protocols used for media preparation.

General

All media used in this project came pre-mixed, except for Phosphate Buffered Saline (PBS), which had multiple components. The manufacturer's instructions on preparation were printed on the bottle, were available from the manufacturer, and were printed on the specific QC forms for each media preparation.

Procedures

The following procedures were followed for equipment monitoring:

1. On a monthly basis, the autoclave was checked with a calibrated thermometer on a gravity 20 cycle and the temperatures were recorded.

2. On a monthly basis, the sterility of the autoclave was checked using duo-spore® biological indicator strips. Strips were aseptically inoculated into Tryptic Soy Broth (TSB) tubes and incubated at 55°C (+ /-3°C) for seven days, per manufacturer instructions.

3. The sample incubator temperature was checked twice daily using a digital thermometer. The current temperature readings, along with the minimum and maximum temperatures stored in the thermometer memory, were recorded. The pH meter was calibrated before

each use, using purchased pH 4, pH 7, and pH 10 standards. The before-correction and post-correction readings were recorded for each standard.

4. Before each use of the meter and after calibration, the pH meter was checked using the pH 7 standard. If a reading was off by more than 0.2 pH units, the meter was recalibrated.

The following procedures were followed for the preparation and QA of all media:

1. Ready-to-use mixtures were prepared following the manufacturer's instructions.

2. PBS was prepared by adding 0.58 g NaH_2PO_4-$2H_2O$, 2.5g Na_2HPO_4-$2H_2O$, and 8.5 g NaCl to 1-L deionized water. The resulting solution was then autoclaved at 121°C for 15 minutes.

3. Once autoclaved, the PBS was allowed to cool to room temperature. An aliquot was removed to measure final pH. If the measured pH was not 7.4 +/- 0.2 pH units, the pH meter was checked using the pH 7 standard, and recalibrated if necessary. A second aliquot of PBS was collected and measured; if the pH was again not with the appropriate range of 7.4 +/- 0.2 pH units, the media was discarded and a new batch of PBS was prepared.

4. All plates were prepared in 100-mm disposable petri dishes at a 20-mL volume.

5. All agar plates were tested for sterility using an un-inoculated plate placed in the 33°C incubator for a minimum of 48 hours.

6. TSB was tested by placing un-inoculated broth into a sterile test tube and incubating it at 33°C for a minimum of 48 hours. PBS was tested by placing 1 mL into a sterile test tube that contained previously tested TSB; the tube was then incubated at 33°C for a minimum of 48 hours.

7. A positive control was prepared for each batch of agar by streaking challenge organisms onto a plate. Controls were checked for growth at after one and two days; if no observable growth was present after two days, the agar was discarded and re-prepared.

8. Positive controls on TSB consisted of inoculated tubes containing each of the challenge organisms that were incubated at 33°C for one to two days. Broth that did not produce turbid growth in two days' time was discarded and re-prepared. A 1-µL loop of challenge bacteria was inoculated into 100 µL of PBS and allowed to sit at room temperature for one hour. The PBS was then plated onto an MA plate, which was incubated 33°C for one to two days. Any PBS that did not produce growth was rechecked with a second aliquot. If the second aliquot failed to produce growth, the PBS was discarded.

9. All media was stored in the dark at room temperature.

10. Marine Agar plates were checked for surface moisture and dried if there was condensation present on the agar surface. Plates were also checked for contaminants and discarded if any were present.

3.5.2 Bacterial inoculation

Bacterial inoculation occurred after fish were exposed to oil-contaminated water or sediment. Bacteria (e.g., *Vibrio anguillarum*) were grown in overnight culture to a density of approximately 1 x 10^9 cfu/ml, as assessed by comparison to McFarland Standards. The bacteria were then introduced into the bacterial exposure tanks at the desired concentration by direct addition with a pipette. Bacteria and water were allowed to mix for approximately 30 minutes before fish were exposed. All samples destined for bacterial enumeration were documented using the appropriate bench sheet.

Final bacteria concentration and fish exposure duration were described in test-specific TCTs before beginning definitive tests. The concentration of bacteria for inoculation was determined for each species and, when appropriate, each cohort of fish using test performance information from preliminary dose-response exposures similar to those proposed in this GLPP. For example, we assessed survival rates and bactericidal capacity [using the Bacterial Killing Assay (BKA)] after exposing two flounders, per concentration, to four concentrations of *V. anguillarum*. The exposures lasted for 24-hrs and enabled us to determine the highest concentration (1 x 10^6 cfu/ml) in which all fish survive but with the greatest immune suppression.

3.5.3 Bacteriological Analysis of Sediment and Water SOP

Scope

The scope of this SOP was to provide protocols for bacterial analysis of the water and sediment for pre- and post-pathogen challenge.

General

To obtain a quantitative measurement of bacterial loads before and after exposure, we plated water and sediment directly onto non-selective media. Non-selective media (Marine Agar) provided an understanding of the total bacterial population in the challenge tanks, while allowing phenotypic characterization to aid in further molecular analysis. In addition, water and sediment samples, if applicable, were removed and stored for later microbiomic analyses.

Procedures

Sediment analysis

a. For each sediment type (oiled sediment and non-oiled sediment), five Marine Agar (MA) plates were labeled, brought to room temperature, and checked for contaminants before use.

2. For each sediment type, six 50-mL conical tubes with PBS were prepared; one was labeled 1:2 and the remainder of the tubes were labeled for 10-fold dilutions, from 1:10 to 1:100,000.

3. Twenty millimeters of PBS were added to the 1:10 dilution, and 18 mL of PBS were added to each successive dilution (1:100–1:100,000).

4. Before adding sediment to the tanks, a 25-mL aliquot of sediment was aseptically collected from each pooled sediment batch (oiled sediment and non-oiled sediment) and placed into the appropriate 50-mL conical tube.

5. The conical tube was then filled to the top with PBS, capped, and shaken vigorously.

6. Five millimeters were then removed and placed into the tube labeled 1:10 dilution, which contained 20 mL of PBS.

7. After shaking vigorously, 2 mL were removed and placed into the tube labeled 1:100.

8. Steps 6 and 7 were repeated for the remaining dilutions (1:100–1:100,000).

9. Plates were labeled with the date and dilution.

10. For each dilution, 0.2 g of the well-mixed sediment slurry were plated onto each of the agar plates and carefully spread with a sterile spreader.

11. Slurry liquid was allowed to absorb before inverting the plates and placing them in the incubator.

12. The plates were incubated for 16–18 h at ~ 25°C.

13. After 18 h, colonies on the lowest dilution were counted.

14. Additionally, approximately 2 mL of sediment were collected aseptically into a 2-mL microcentrifuge tube and stored at -80°C for later microbiomic analysis.

Water analysis

1. 1 For each tank, two MA plates were labeled, brought to room temperature, and checked for contaminants before use.

2. For each tank, two 50-mL conical tubes were labeled and 20 mL of PBS were added to one.

3. Approximately 50 mL of water from each tank were collected in one conical by dipping the conical down toward the center of the tank without disturbing the sediment.

4. The cone was lifted and the water in the cone was vigorously mixed and 20 mL of suspended sediment and water were quickly transferred to the second conical, which contained 20 mL of PBS.

5. The PBS/water dilution was vigorously mixed and 500 µL were plated onto an MA plate.

6. The MA plates were incubated for 16–18 h at ~ 25°C.

7. After 18 h, colonies on the lowest dilution were counted.

8. Additionally, 100 mL of water were pooled from the replicate tanks of each treatment type (oiled sediment and non-oiled sediment), aseptically collected, filtered through a 0.20-um mesh filter, and stored at -80°C for later microbiomic analysis.

3.5.4 Fish dissection and sampling SOP

Scope

The scope of this SOP was to provide protocols for dissection and sample collection from fish that were challenged with pathogens.

General

Proper attire was worn at all times while handling the fish. This included, at a minimum, gloves, eye protection, proper clothing, and shoe protection. At no point during biological sample collection was the interior of the fish touched by anything other than autoclaved or alcohol-flamed scissors and forceps. During dissection, proper attire included, at a minimum, gloves and disposable or autoclavable clothing protection. All biological samples were weighed in sterile petri dishes. When possible, samples of each organ were taken and preserved for histological analysis.

Procedures

Fish measurement

Before euthanizing a fish, its weight and standard length (SL) were taken and recorded on the appropriate bench sheet.

Euthanization, blood collection, and visual inspection

1. Individually, fish were removed from their aquaria and visually inspected for lesions before being placed in the anesthetic bath. If any gross pathologies, including lesions, were present, their descriptions were recorded and photographed; lesions were swabbed for bacterial analysis before immersion in the anesthetic bath.

2. To euthanize a fish, it was placed in 50 mg/L solution of MS-222.

3. As soon as a fish stopped pushing water through its gills, blood collection was performed for the purpose of BKA, according to SOP "Bacteriological Analysis of Sediment and Water."

Dissection and sample collection

1. The exterior of the fish was cleaned with iodine and blood was collected from the caudal vein by cutting the tail and collecting the blood in a capillary tube.

2. Using alcohol-flamed scissors, the fish was cut open along the ventral side, from the proximal end to the anterior end and up the left side, removing the skin to expose the organs.

3. Using a newly flamed set of scissors and forceps, the liver, spleen, kidney, gills (upper and lower), and gut were removed and placed individually in 500 µL of RNALater for subsequent examination of gene expression via transcriptomic analysis, bacterial abundance via microbiome analysis, and qPCR quantification.

4. If necessary, tissue samples to be analyzed for bacterial abundance were diluted in PBS and plated on MA plates as in the "Bacterial Analysis of Sediment and Water SOP."

5. The remaining fish carcass was wrapped in aluminum foil, labeled, and stored at -20°C for archive according to the QAPP.

Histological preservation

1. When possible, organ samples for histology were taken after the bacteriological and toxicological samples.

2. To collect histological samples, a small cut of each organ was taken and then weighed.

3. The sample was placed into a histology cassette, which was then placed in 10% buffered formalin. Samples were labeled, stored, and shipped as described in the QAPP.

3.5.5 Bacterial analysis of tissue SOP

Scope

The scope of this SOP was to provide protocols for the biological analysis of samples that were collected from pathogen-challenged fish to provide quantitation of pathogenic infection of fish via lesions and bacterial load in selected organs.

General

Organs were plated directly onto selective and non-selective media to obtain a quantitative measurement of bacterial loads in the organs. Non-selective media (Marine Agar) provided an understanding of the total bacterial population of the organs, while selective media (TCBS) grew only specific bacteria with distinct phenotypic characteristics. This allowed for quick identification (ID) of the pathogens used in the study. The enrichment broth was used to increase the bacterial levels in the organs, which otherwise could have been too low to detect by direct plating.

Procedures

Dissection sample plating

1. For each fish, 10 TCBS and 10 Marine Agar plates were labeled with the appropriate sample ID as described in the QAPP. All TCBS plates were dried for 30 minutes at 33°C. Marine agar plates were checked for surface moisture and dried if necessary.

2. An extra 10 plates of each agar were prepared for any samples of opportunity (e.g., lesions).

3. Fourteen sterile 1.5-mL microfuge tubes were prepared for each fish: four with 1 mL of TB, three with 100 µL of PBS, six with 990 µL of PBS, and one with 35 µL of PBS.

4. All plates and tubes were labeled as described in the QAPP.

5. Blood (10 µL) and organs were removed and prepared at necroscopy, as described in the Fish Dissection and Sampling SOP.

6. Blood and organ samples for bacteriological analysis were split in half; one portion was placed into thioglycollate broth and the other into 95 µL (blood) or 100 µL (organs) of sterile PBS.

7. Both sets of organ samples were macerated by mashing with micropipette tips.

8. The macerated organs and blood in PBS were diluted, respectively, to 1:10 and 1:100 with PBS.

9. 100 µL of each sample were plated directly onto TCBS and Marine Agar plates using appropriate sterile techniques.

10. The thioglycollate broth tubes and plates were transported to the Marine Microbiology Laboratory and incubated at 33°C for 16–18 hours.

Colony analysis

1. Plates and tubes were removed from the incubator after 16–18 hours; the exact time was recorded on sample data sheets.

2. Plates were examined for growth; the lowest dilution containing countable colonies was examined and colonies were counted based on colony morphology for presumptive Pd and Vh on TCBS. Colony counts were back-calculated to CFU/g tissue.

    a. *Vibrio harveyi* produced yellow colonies on TCBS and *Photobacterium damselae* produced dark-green colonies.

3. A representative subsample of each morphology class was analyzed by genetic sequencing with universal 16S primers to verify presumptive identity.

4. If samples did not exhibit growth from direct plating, then 100 µL of broth from enrichment tubes exhibiting visible turbid growth were plated onto TCBS plates, incubated for 16–18 hours, and analyzed for colonies.

3.5.6 BKA SOP

Scope

The scope of this SOP was to provide protocols for the immunological analysis of samples collected from pathogen-challenged fish to provide quantitation of innate and adaptive immunity.

General

The purpose of this assay was to determine the effect the exposure had on both acquired and innate immunity.

Procedures

Bacteria culture preparation

1. T1N3 broth (T1N3) was prepared according to the Media Preparation & QA/QC SOP.
2. Approximately 20 mL of T1N3 was inoculated with *Vibrio anguillarum* and incubated overnight with shaking.

Blood collection

1. Fish were anesthetized with MS222.
2. Using a sterile gauze pad, the outside of the fish was wiped down using ethanol.
3. Using sterilized scissors, the tail was cut off, slicing the caudal vein in the process; blood was collected using a heparinized capillary tube.
4. Blood was displaced from the capillary tube into a 0.2 mL microcentrifuge tube that had been washed with sodium citrate or EDTA (for anti-coagulant properties). After 1.5 µL of blood was removed for each replicate (see below), the remaining blood was reapplied to a capillary tube, then centrifuged to remove plasma. Plasma was stored at -20°C.

BKA assay

1. Using a McFarland Standard, overnight bacterial culture was diluted to 1 x 10^5 CFU/mL with BHIB.
2. 1.5 µL of whole blood was placed into the prepared 35 µL PBS tube.

3. As a positive control, 12.5 µL bacteria to 36 µL PBS were prepared; as a negative control, 48 µL of PBS were prepared.

4. To each of the sample tubes, 12.5 µL bacteria were added.

5. The samples and controls were incubated at 30°C for 30 min.

6. The samples were removed from incubator and vortexed; 250 µL of sterile T1N3 broth was added to each sample and control. The mixture was vortexed and incubated at 30°C for 12 hours.

7. The absorbance at 450 nm was measured for all samples and controls.

8. The anti-microbial activity was determined by calculating:
   1 – (Absorbance of sample/Absorbance of control).

3.5.7 Packed cell volume (PCV) SOP

Scope

The purpose of this SOP was to provide protocols for the indirect evaluation of red blood cell (RBC) volume using PCV, which estimated the proportion of hematocrit to total blood volume.

General

Blood was collected from sacrificed fish using heparinized capillary tubes and stored in EDTA-washed tubes. Centrifugation and PCV measurements were taken soon after blood collection (< 30 minutes).

Procedures

1. Blood collection

1.1 Fish were sacrificed as described in the Fish Dissection and Sampling SOP.

1.2 Once a fish was effectively euthanized, it was sliced just ahead of its tail with a razor blade. The blood was immediately collected with a capillary tube, tipping the fish so that it was above the capillary tube. The blood was collected in two heparinated capillary tubes so that the PCV tube was approximately 3/4 full. The blood was mixed well by an up-and-down motion.

1.3 Both ends of the PCV capillary tube were sealed using Crit-o-Seal.

2. Centrifugation of PCV capillary tubes

2.1 Making sure that the PCV capillary tubes were firmly secured to the rubberized edge of the single-speed capillary microcentrifuge, they were spun for 1 minute.

2.2 Figure 3.1 shows a capillary tube post-centrifugation. Centrifugation separated the total blood sample into RBCs (red layer) and plasma (clear layer). To estimate PCV, we measured the length of the hematocrit layer (red layer) and the length of total blood. PCV was hematocrit length/total blood length. The results for both capillary tubes needed to agree within four percentage points. If they did not, the hematocrit procedure was repeated. If they did agree, the two measurements were averaged.

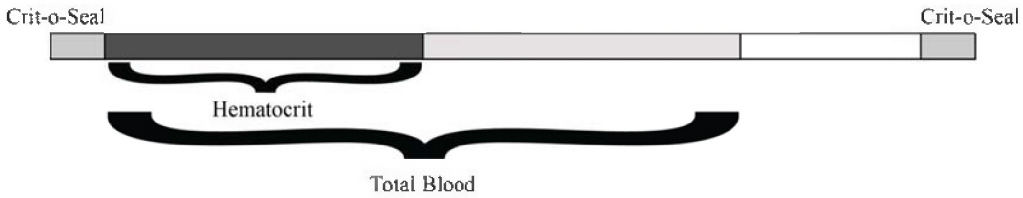

Figure 3.1. Capillary tube post-centrifugation.

2.3 Alternatively, a micro-capillary reader was used to quickly assess the proportion of hematocrit length to total blood length.

2.4 All capillary tubes were properly disposed of in biohazard sharps containers.

3.5.8 RBC count SOP

Scope

The purpose of this SOP was to provide protocols for the direct enumeration of RCBs using a hemocytometer.

General

Blood was collected from sacrificed fish using heparinized capillary tubes at the same time that samples were taken for PCV; blood was stored in EDTA-washed tubes.

Procedures

1. Blood was collected on two heparinated capillary tubes to prevent coagulation. The capillary tube was filled 2/3 full and mixed well by inverting it.

2. A 1:200 dilution was prepared with saline and mixed.

3. The dilution was plated on each side of the chamber to allow cells to settle for 3 minutes.

4. Four outer squares were counted, as well as the center square of the center square mm (sqmm), on each side of the hemocytometer using 40x objective and low light. Cells that were touching the upper and left limits were counted; cells touching the lower and right limits were not counted. If there were fewer than 100 cells in a sqmm, two or more 1-mm square areas were counted and the results were averaged. The center square was used for platelets and red cells.

   Total area counted = 0.4 mm^2
   Center square was divided into 25 squares; each square in the center square was 1/25 mm^2
   5/25 mm^2 counted on each side = 10/25 mm^2 OR 1/5 mm^2 + 1/5 mm^2 = 2/5 mm^2 = 0.4 mm^2

5. RBCs/mL were counted.

   $$\text{RBC} = \frac{\text{\#cells (both sides) x 200}}{\text{0.1 mm x 0.4 sqmm}} \quad \text{or} \quad \frac{\text{total \#cells x 200 x 10 mm}}{\text{0.4 sqmm}}$$

   Example:

   Side 1 305
   Side 2 315

   $$\frac{620 \times 200 \times 10}{0.4} = 3.10 \text{ million/cmm or } 3.10 \times 10^6/\mu L$$

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A. Testing Protocol 1: Definitive Acute Exposures

A.1 Equipment and Materials

CEWAFs, HEWAFs, and Corexit-only exposure solutions were prepared as described in the QAPP; spiked sediment was prepared according to the protocol listed in Section A.3.

A.1.1 Organisms for exposure

1. Sheepshead minnow (*Cyprinodon variegatus*), obtained from GCRL Toxicology laboratory culture
2. Speckled seatrout (*Cynoscion nebulosus*), obtained from GCRL Cedar Point Aquaculture facility
3. Blue crab (*Callinectes sapidus*), obtained from GCRL blue crab program and the University of Maryland
4. Grass shrimp (*Palaemonetes pugio*), obtained from marsh areas around GCRL
5. Southern flounder (*Paralichthys lethostigma*), obtained from Texas Parks and Wildlife
6. Fiddler crab (*Uca longisignalis*), obtained from Auburn University
7. White shrimp (*Litopenaeus setiferus*), obtained from Florida Organic Aquaculture.

A.2 Procedures

The following guidelines were followed when definitive toxicity tests were being planned:

1. Each definitive toxicity test consisted of different concentrations of HEWAF, CEWAF, Corexit, or spiked sediment plus a control, with 3 or 4 replicates per each treatment. The specific concentrations and number of replicates for each test can be found in the test-specific TCTs.
2. Generally 10–20 organisms per replicate were used for exposures, but fewer animals were sufficient if space or animal numbers were limited. The specific number of organisms used per replicate can be found in the test-specific TCTs.

3. The necessary volume of exposure water and corresponding container size from the loading were calculated based on the weight of the test organism. Refer to GCRL GLPP when making these calculations. Water quality (temperature, salinity, pH, DO, ammonia) parameters were recorded daily in at least 1 test container/treatment.

4. The location of test and all other pertinent data were recorded on the appropriate bench sheets.

5. Exposure duration details can be found in the test-specific TCTs. Vessel size/volume was in agreement with approximate body mass of test organisms. In general, fish embryo, larval fish, and crustacean zoeae acute exposures occurred in 15 × 75 Pyrex glass cups (100 mL/cup), and juvenile fish and crustacean exposures were performed in large Carolina bowls (500 mL/bowl). Megalopae exposures occurred in 20-mL borosilicate scintillation vials (1 individual per vial in 10-mL water). Juvenile seatrout were exposed in 10-gal aquaria (20-L water/aquarium), white shrimp juveniles were exposed in 2-gal aquaria (4-L water/aquarium), and adult grass shrimp were exposed in 1-gal jars (3.5-L water/jar) or in 30-gal aquaria (25 L/aquarium). Information regarding the feeding regime, water renewal schedule, and aeration schedule of a particular test can be found in the test-specific TCTs. Aeration was provided with compressed oxygen at a rate of no more than 100 bubbles per minute per aquarium.

6. During static renewal tests (see test-specific TCTs) the following methods were used to transfer organisms to fresh WAF or fresh control solutions during the water renewals:

    a. For the sheepshead minnow embryo and larvae tests, the test container was stirred and the water was rapidly poured through a square of 500-µm nitex mesh. The organisms on the mesh were counted, and the mesh was dipped into the new test container with fresh WAF or control solution to release all of the organisms.

    b. For the white shrimp tests, the shrimp were netted and the old WAF or control solution was discarded from the exposure chambers. Fresh WAF or control solution was added to the exposure chamber and the shrimp were returned to their exposure chamber.

    c. For the speckled seatrout and blue crab tests, 90% of the WAF or control solution was carefully pipetted out of each exposure chamber and then fresh WAF or control solution was added to the exposure chamber.

7. During certain tests that were performed on sheepshead minnow embryos and larvae, after a predetermined exposure duration, organisms were transferred to clean ASW for the remainder of the test (see test-specific TCTs). To transfer organisms, the WAF in the

test container was stirred and the water was rapidly poured through a square of 500-µm nitex mesh. The organisms on the mesh were counted, and the mesh was dipped into the new test container with clean ASW to release all of the organisms. The mesh was rinsed in hot water between replicates, and a new piece of mesh was used for each treatment.

8. For the sheepshead minnow embryo (< 48 hpf) tests, the exposure chambers were maintained on a rotary oscillator set at 70 rpm. Additionally, where heart rate was a listed endpoint, heartbeats of all viable embryos were counted and recorded during the daily observations. The heartbeats of each embryo were counted for 10 seconds using a dissecting microscope; the results were recorded on an appropriate bench sheet.

9. Organisms obtained from Cedar Point or the Blue Crab Hatchery were moved to STL at least 24 hours prior to test initiation for acclimation. All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed and frozen; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed and frozen, except in cases where tissues were collected for further analysis (see test-specific TCT for details on test endpoints).

A.3 SOP: Protocol for Preparation of Spiked Sediment

A.3.1 General guidelines

- Prepare the controls using the same technique used for spiked sediment, with the exception of adding oil.
- Prepare each sediment-oil concentration separately. For instructions on cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.
- Enter all of the appropriate information into the bench sheets provided by Stratus Consulting.
- Place any unused prepared sediment into a zip-lock bag and store in the dark at 4°C (short term) or in a freezer at -20°C (long term). Store large quantities (> 5 kg) of prepared sediment in large metal pans, covered with aluminum foil, and stored in the dark at 4°C.

A.3.2 Glassware preparation

Prepare all of the equipment in accordance with the *Decontamination SOP* in the QAPP.

A.3.3 Preparation of sediments

- Allow the sediment to thaw.
- Remove all debris (grass, shells, etc.) from the thawed sediment, weigh, and place in a mixer bowl.
- Using a Cuisinart SM-70 7-quart stand mixer or a KitchenAid 5-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

A.3.4 Mixing oil into sediment

- Weigh the appropriate amount of oil as outlined below:

  Slick oil should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Transfer the oil into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

- Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.
- Mix the oil into the sediment at medium speed with a Cuisinart SM-70 7-quart stand mixer or a KitchenAid 5-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with the putty knife.
- Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the oiled sediment from the mixing bowl into bags or metal pans for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for test initiation.
- Weigh oiled sediment for each replicate when transferring into test containers.

B. Testing Protocol 2: Definitive Chronic Exposures

B.1 Equipment and Materials

Refer to QAPP *Appendix A: Protocols and Standard Operating Procedures, A.1 Protocols for Preparing Water Accommodated Fractions* for making HEWAF and CEWAF stock solutions.

B.1.1 Organisms for exposure

Sheepshead minnow, obtained from GCRL Toxicology laboratory culture.

B.2 Procedures

Each definitive toxicity test consisted of exposures with different HEWAF and CEWAF concentrations plus a control (see test-specific TCTs for details). All chronic tests were flow-through to maximize water-quality parameters. Generally, 10 organisms per replicate were used for exposure, but fewer animals were sufficient if space or animal numbers were limiting (see test-specific TCTs for details). The necessary volume of exposure water and corresponding container size from the loading was calculated based on the weight of the test organism. Refer to *ASTM International chamber and flow-through loading calculations SOP* in the GCRL GLPP when making these calculations.

Water quality (temperature, salinity, pH, DO, ammonia) was recorded daily in each aquarium on the bench sheet.

Details regarding the light-cycle parameters and feeding schedules, during the exposures, were included in the test-specific TCTs. During the exposure, if the DO fell below 4 mg/L, aquaria had gentle aeration provided from compressed oxygen delivered through Pasteur pipettes. The background data for the organisms were acquired from the culture logs or chain-of-custody transfer sheets prior to or during the exposure and were maintained with the study data.

B.3 Exposure

Treatments were generated by injection of individual stocks of HEWAF, CEWAF, or dispersant into the dilution water, following protocols outlined in Manning et al. (1999). Appropriate volumes of the stock solutions were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after the injection of stock, 2 L of diluent water from a water partitioner were delivered to each splitter box/mixing chamber to

produce the desired concentrations. Each treatment splitter box/mixing chamber then delivered the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that the reduced flow into the aquaria did not result in a drop in DO, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

Stock solutions were replaced every 48 hours with newly prepared stocks. Stock solutions were placed in foil-covered glass containers in a dark cabinet at 20°C. A sample of each stock solution was retained for analysis as specified in the QAPP.

The injector system was allowed to run a minimum of 3 days prior to initiation of the test to ensure that aquaria had the correct concentration of compound, to regulate flow rates, etc.

Test organisms were added to test aquaria in a random order that was assigned prior to test initiation. The day of addition was considered day 0 of the test.

All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed, frozen, and archived according to the QAPP; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed, frozen, and archived according to the QAPP.

C. Testing Protocol 3: Flounder Chronic Sediment Exposures

C.1 Organism for Exposure

Southern flounder were obtained from UTMSI.

C.2 Procedures

C.2.1 Experimental design

1. During the toxicity testing of field-collected sediments, southern flounder were exposed to sediments collected from different field sites. The number of sediments that were tested and the number of replicates per each sediment treatment were recorded in the test-specific TCTs. All sediment tests were performed in a flow-through system.

2. Ten to 15 organisms per replicate were used for exposure, but fewer animals were sufficient if space or animal numbers were limited (see test-specific TCTs for details).

3. The necessary volume of water for each exposure chamber and corresponding container size was calculated based on the weight of the test organism. See the GCRL GLPP *ASTM International chamber and flow-through loading calculations SOP* when making these calculations.

4. Water quality (temperature, salinity, pH, DO, and ammonia) parameters were recorded daily in each test container/treatment. If the DO fell below 4 mg/L, aquaria had gentle aeration from oxygen delivered through Pasteur pipettes.

5. The length of the exposures, the light-cycle parameters, and the feeding schedule can be found in the test-specific TCTs.

6. The data pertaining to the acquisition and maintenance of the southern flounder were maintained in the culture logs or chain-of-custody transfer sheets prior to or during the exposure and were maintained with the study data.

C.2.2 Preparation of sediment/exposure

Sediments were prepared for testing 48 hours prior to test initiation. For tests performed in quadruplicate, 1.3 kg of sediment was removed from the freezer and placed into a large glass jar. An equal volume (1.3 L) of 15-ppt salinity ASW was added to the frozen sediment. The jar was covered, wrapped in aluminum foil, and allowed to equilibrate for 24 hours. After equilibration, the sediment:water slurry was homogenized by hand, and 500 g of the slurry was added to each of the 4 replicate tanks in 100-g aliquots. After the slurry was added to each tank, the tanks were placed into the exposure system, covered, and allowed to settle for an additional 24 hours. After the settling period, individual fish were randomly assigned to each tank. Uncontaminated ASW was dripped into the system on a regular basis for the duration of the test. Sediment samples were collected before and after the experiment and overlying water samples were collected throughout the experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP.

All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed, frozen, and archived according to the QAPP; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed, frozen, and archived according to the QAPP.

C.2.3 Water sampling

Analyses of polycyclic aromatic hydrocarbons (PAHs) in the water of each treatment were performed at ALS Environmental. Water samples were collected from each replicate aquarium and combined into 1 sample/treatment at each collection time point. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles.

C.2.4 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were mixed using a stainless steel scoop or similar utensil until they reached a uniform color and consistency. Once the sediment was thoroughly mixed, the jars for analytical chemistry sediment samples were filled. Sediment samples were sent to ALS Environmental for PAH analysis.

C.2.5 Test termination

At the end of the exposure period, fish were removed from the aquaria and sacrificed with MS-222. Fish were weighed and measured. Liver tissue and gill tissue from a subset of the total number of fish were placed in Eppendorf tubes with 1 mL of RNAlater for potential subsequent RNA extractions. Tissues were stored at -80°C until processing. The remaining whole fish were collected and fixed in 10% NBF for potential histological analyses.

D. Testing Protocol 4: Effects of Chronic Exposure to HEWAF and CEWAF from Oil Slick A on Growth, Reproduction, and Gene Expression of Grass Shrimp

D.1 Test Organisms

Grass shrimp that were wild-caught in Ocean Springs, Mississippi, marshes.

D.2 Acclimation/Holding

Fully mature grass shrimp were used in this study and were in GCRL laboratory culture for a minimum of 1 week prior to experimentation. Shrimp were held in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study. Adult shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

D.3 Procedure

D.3.1 Preparation of stock solutions

1. HEWAF – the HEWAF solution was prepared following standard protocols described in the QAPP. The HEWAF stock was stored in glass Erlenmeyer flasks in the dark and used as the source for injection into test aquaria. New HEWAF stock was prepared every 48 hours.

   Analysis of PAHs in each HEWAF stock was done by ALS Environmental. Additionally, water samples from each dilutor/splitter box (one/treatment concentration – see below) and 1 tank per treatment were sampled during the study period and sent to ALS Environmental.

   Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface by dunking a sample bottle or transfer beaker into the tanks, or using a siphon to directly fill the sample bottles.

2. CEWAF – the CEWAF solution was prepared following standard protocols described in the QAPP. The CEWAF stock was stored in glass Erlenmeyer flasks in the dark and used as the source for injection into test aquaria. The new CEWAF stock was prepared every 48 hours.

   Water samples from each dilutor/splitter box (one/treatment concentration – see below) and 1 tank per treatment were sampled during the study period. Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface by dunking a sample bottle or transfer beaker into the tanks, or using a siphon to directly fill the sample bottles. Analysis of PAHs and dioctyl sodium sulfosuccinate (DOSS) were done by ALS Environmental.

D.3.2 Experimental design

Adult female and male grass shrimp were held in individual Nitex cages in 48.3 cm (L) × 37.5 cm (W) × 12.9 cm (D) aquaria with an overflow drain of 8 cm, providing a maximum water volume of 24.4 L during the exposure and subsequent egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Details regarding dosing, number of individuals per replicate, and number of replicates can be found in test-specific TCTs.

Shrimp were isolated individually in the test aquaria into retention chambers constructed from 10-cm glass petri dish bottoms with a collar of 500-µm nylon mesh with a 10-cm diameter disposable petri lid. The exchange of water within the chamber was ensured by fluctuating the water level within the aquaria 4 to 6 cm periodically with a self-starting siphon. Retention chambers were maintained in a minimum depth of approximately 7 ± 1 cm dilution water.

A flow-through test system was used for this evaluation. Treatments were generated by injection of individual stocks of HEWAF and CEWAF into the dilution water. New HEWAF and CEWAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber delivered 500 mL of the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 3 days

prior to the addition of shrimp to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

1. *Adult exposure*. The exposure was initiated with 15 female and 9 male grass shrimp in individual chambers introduced into each study aquarium. After 4 and 14 days exposure, 3 female shrimp from each aquarium were removed and preserved. The temperature was at 27 ± 1°C and the salinity was maintained at a nominal 15 ppt.

2. *Reproduction*. Following 14 days of HEWAF and CEWAF exposure to male and female adult grass shrimp, spawning pairs were established to monitor fecundity of control and treatment shrimp. Spawning pairs consisted of 1 female and 1 male shrimp placed into the same individual chamber (n = 9 pairs/replicate aquarium). Shrimp pairs were checked daily beginning on day 15 of the experiment for evidence of molts and ovigerous females. Ovigerous females were allowed to remain in the chamber for 7–8 days prior to sacrifice. Seven-eight days after first observation of a clutch of eggs, the female was removed and sacrificed in ice water, and all eggs were removed and counted. The female was weighted and measured, and the hepatopancreas tissue was removed as described above. Twenty embryos were isolated into each of 2 retention chambers (10-cm petri dish with a collar of 300-µm nylon mesh). One chamber was returned to the original aquarium, and 1 chamber was placed into a control aquarium. Embryos were observed once daily until hatch; the percent of successful hatching was determined. Exposure to HEWAF and CEWAF continued throughout this portion of the experiment. The carcasses of each spawning pair were wrapped in foil, given a unique ID number, and stored at -20°C. Hatched larvae were placed into Eppendorf tubes with 500 µL of RNAlater for potential subsequent RNA extractions.

D.3.3 Temperature and lighting

Temperature was regulated at 28°C, and for each single test the temperature remained constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

D.3.4 Test initiation

Test shrimp were distributed to the 12 test aquaria in groups of 24 individual chambers (15 females, 9 males in each treatment replicate). The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

D.3.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 28°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; thus each aquarium was monitored every 5 days.

D.3.6 Biological data

Daily observations of the presence of molts or occurrence of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. If dead organisms were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

E. Testing Protocol 5: Effects of HEWAF from Oil Slick A on Growth, Reproduction, and Gene Expression of Grass Shrimp

E.1 Test Organisms

Grass shrimp, wild-caught in Ocean Springs, Mississippi, marshes.

E.2 Acclimation/Holding

Fully mature grass shrimp that were used in this experiment were maintained in GCRL laboratory culture for a minimum of 1 week prior to experimentation. Shrimp were held in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study. Adult shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding period.

E.3 Procedure

E.3.1 Preparation of stock solutions

The HEWAF solution was prepared following the standard protocols described in the QAPP. The HEWAF stock was stored in a 10-L glass jar in the dark and used as the source for injection into test aquaria. New HEWAF stock was prepared every 48 hours.

Analysis of PAHs from each batch of stock HEWAF was done at ALS Environmental. Additional composite water samples were taken from exposure tanks within each treatment and the control splitter box during the study.

Water samples were collected by pulling water directly from splitter boxes and tanks; samples were not collected from the outflows. Water samples were collected from under the water surface by dunking a sample bottle or transfer beaker into the tanks or by using a siphon to directly fill the sample bottles.

E.3.2 Experimental design

Adult female (n = 5) and male (n = 2) grass shrimp were held in 10-L glass aquaria with an overflow drain of 8 cm providing a maximum water volume of 8 L during the exposure and egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each replicate contained 7 shrimp. Shrimp (adults and embryos) were exposed to treatments for the duration of the experiment.

To facilitate mating, shrimp were allowed to freely interact in the 10-L aquaria. A thin layer of calcium carbonate (crushed coral) sediment was placed on the bottom of each aquarium. When gravid females were observed, they were removed and isolated from the mating aquaria within 48 hours of appearance of an egg mass. Isolated individuals were placed into retention chambers (constructed from 10-cm glass petri dish bottoms with a collar of 300-µm nylon mesh). The retention chambers were placed into a larger glass "brooding" aquarium [48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D), with an overflow drain of 19 cm providing a maximum water volume of 34.4 L] of the same HEWAF treatment level. The water level within the aquaria fluctuated 4 to 6 cm periodically with a self-starting siphon to ensure the exchange of water in the chamber. Retention chambers were maintained in a minimum depth of approximately 7 ± 1 cm dilution water.

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of HEWAF into the dilution water. New HEWAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks (see test-specific TCTs for treatment concentrations) were injected into splitter/mixing boxes by precision syringe pumps. Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber delivered 500 mL of the appropriately diluted study concentration to each replicate 10-L mating aquarium through glass delivery lines. The system cycling rate was maintained at approximately 1 volume addition every 4 hours, which provided approximately 95% replacement every day. To ensure that test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 3 days prior to the addition of shrimp to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

1. *Adult exposure.* The exposure was initiated with 5 female and 2 male grass shrimp introduced into each of the study mating aquaria. Females with an egg mass 24–48 hours old were isolated into individual retention chambers (10-cm petri dish with a collar of 300-µm nylon mesh) and placed in brooding aquaria (1 aquarium/treatment).

Temperature was maintained at 27 ± 1°C and salinity was maintained at a nominal 15 ppt.

2. *Reproduction:* Seven-eight days after isolation in the brooding aquaria, the female was removed and sacrificed in ice water, and all eggs were removed and counted. The female was weighed and measured, and hepatopancreas tissue was placed in Eppendorf tubes with 500 µL of RNAlater for subsequent RNA extractions. Tissues were stored at -80°C until processing. Twenty embryos were isolated into the retention chamber and returned to the female brooding aquaria. The remaining embryos were homogenized in Stat-60 and stored at -80°C. Isolated embryos were observed once daily until hatch; the percentage of successful hatching was determined. Exposure to HEWAF continued throughout this portion of the experiment. The carcasses of each female were wrapped in foil, given a unique ID, and stored at -20°C.

E.3.3 Temperature and lighting

Temperature was maintained at approximately 27°C. For each single test, the temperature remained constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

E.3.4 Test initiation

Test shrimp were distributed to the mating test aquaria in groups of 7 individuals per aquarium (5 females, 2 males in each treatment replicate). The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

E.3.5 Diet

During the exposure, shrimp were fed dry pellet food (B2) twice daily.

E.3.6 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27°C ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all mating and brooding aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

E.3.7 Biological data

Observations of the presence of molts or occurrence of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at least once daily during the exposure. If dead organisms were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

The length and weight of female shrimp at the initiation of the experiment (day 0) were determined from 20 female shrimp randomly chosen from the same culture aquarium as the experimental shrimp. These shrimp were sacrificed in ice water, weighed, measured, wrapped in aluminum foil, and frozen at -20°C.

F. Testing Protocol 6: Effects of WAF on Growth, Reproduction, and Gene Expression of Sheepshead Minnow

F.1 Test Organisms

Sheepshead minnows from the GCRL laboratory culture.

F.2 Acclimation/Holding

Fully mature fish used in this study were in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs for details). Adult fish were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test period.

F.3 Preparation of Stock Solutions

The WAF solutions were prepared following standard protocols described in the QAPP. New stock solutions were prepared every 48 hours. Analysis of PAHs was done by ALS Environmental.

F.4 Experimental Design

Adult female and male sheepshead minnow were held in 48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D) aquaria with an overflow drain of 19 cm providing a maximum water volume of 34.4 L during the exposure and subsequent egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each treatment contained a minimum of 9 male and 11 female fish.

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of WAF into the dilution water. New WAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after the injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chambers to produce the desired concentrations. Each treatment splitter box/mixing

chamber then delivered the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes. The flow-through system was initiated 4 days prior to the addition of fish to the aquaria to ensure the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

F.4.1 Adult exposure

The exposure was initiated with at least 11 female and 9 male sheepshead minnow introduced into each study aquaria. After 4 days exposure, 1 male and 1 female fish from each aquarium was removed and sacrificed and placed in cryo vials and flash-frozen with liquid nitrogen or placed in RNAlater for possible RNA extractions. After 14 days of exposure, 4 female and 4 male fish from each aquarium were removed and sacrificed for tissues for possible histology and gene expression as described above. Temperature was maintained at 27 ± 1°C and salinity maintained at a nominal 15 ppt.

F.4.2 Reproduction

Following 14 days of exposure to male and female adult sheepshead minnow, spawning groups were selected to monitor fecundity of control and treatment fish. Spawning groups consisted of 3 females and 2 males placed in a 20 × 40 × 20 cm chamber with a 0.5-cm polyvinyl mesh bottom to allow passage of eggs out of the chamber. Fish selected for spawning from each replicate treatment were the most robust females and males in all treatments to ensure maximum production. The remaining males and females were placed in the aquaria outside the spawning chambers. Seven days following the placement of fish into the spawning chambers, a 300-µm mesh tray was placed under each chamber to collect spawned eggs. This delay in the collection tray placement allowed for establishment of territories before assessing egg production. During this time, any fish within the chamber that died were replaced with females or males not selected earlier that have been housed in the aquarium outside the chamber. Eggs were harvested daily following placement of the egg collection tray for 11 consecutive days, rinsed with dilution water, counted, and microscopically assessed for viability. Exposure to WAF continued throughout this portion of the experiment. At the end of the egg collection period, female and male spawners were removed from their respective chambers, measured for SL, wet weighed (0.001 g, total weight and gonad weight), and sacrificed. Gonadal tissue (whole testis, half ovary) was placed into individually labeled cassettes and preserved in 10% NBF for histological

analysis. Liver tissue and the other half of the ovarian tissue were placed in cryo vials and flash-frozen with liquid nitrogen or placed in RNAlater for possible RNA extractions. Tissues were stored at -80°C until processing.

F.4.3 F1 generation

Progeny were reared from eggs harvested from each of the spawning groups. Retention chambers containing 20 embryos isolated from each of the spawning groups were used to monitor embryo survival, larval survival, and growth of hatch larvae. One group of 20 embryos was isolated from each replicate. Isolation of progeny began on the first day of embryo collections. Each group of 20 embryos came from a single day's collection to ensure embryos of the same age and maintained at the same density were isolated into retention chambers. Embryos for progeny evaluations were isolated into retention chambers (100-mm petri dish bottoms with a 15-cm tall 40-µm mesh nylon collar) and placed in aquaria from which they came. Embryos were removed from the aquaria daily and counted through hatch. Dead embryos were discarded and live embryos returned to the retention chamber. This procedure was repeated until all living embryos hatched. After hatching, juvenile fish were fed *Artemia* nauplii twice daily. Survival was monitored daily for 10 days post-90% hatch (15 days post-isolation). At the end of the monitoring period, half of the fry were sacrificed and measured (SL, weight) for growth and flash-frozen or placed in RNAlater for possible RNA extraction. The other half were sacrificed and preserved in 10% NBF for histological analysis.

F.4.4 Temperature and lighting

Temperature was regulated at approximately 27°C and for each single test the temperature was constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

F.4.5 Test initiation

Test fish were distributed to the test aquaria in groups of 11 females and 9 males in each treatment replicate. Addition of fish was done in an impartial manner until the required numbers were distributed to each test chamber. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

F.4.6 Diet

During the exposure fish were fed *Artemia* nauplii and commercial flake food twice daily.

F.4.7 Water quality

Ammonia, DO, temperature, pH, and salinity were monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

F.4.8 Biological data

Observations of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead fish were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

G. Testing Protocol 7: Flounder Chronic Spiked Sediment Exposures

G.1 Test Organisms

Southern flounder from UTMSI.

G.2 Acclimation/Holding

Juvenile fish used in this study were in 100% dilution/culture water for 4 days prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Fish were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

G.3 Procedure

G.3.1 Preparation of sediments

Oil was mixed into uncontaminated sediments (ALAJ46-C1127-SB701B) using a KitchenAid stand mixer. For each treatment, 2 kg of sediments was weighed and thawed overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs. Oil was mixed into the sediment for 30 minutes at moderate speed (4.5 on mixer), scraping the sides of the bowl with a metal spatula every 2–4 minutes as needed. The oil-sediment mixture was weighed and placed into mesh cages (10-cm petri dish with a 20-cm column of 2-mm nylon mesh) for each treatment (75-g sediment/cage). When preparing tanks, sediment samples for chemical analysis from each treatment group were prepared by aliquoting 50 g of sediment into the analytical chemistry sediment jar between filling each replicate tank. Samples were stored at 4°C until shipment. Cages were placed in treatment aquaria and sediment was slowly added to each aquarium to a depth of 15 cm. The sediment was allowed to settle in the cages overnight under static conditions prior to beginning flow-through water and the addition of the fish.

G.3.2 Analysis of water and sediment samples

Analyses of PAHs in the water of each treatment were done by ALS Environmental. Filtered water samples were collected from each replicate aquarium and combined into 1 sample/treatment at each collection time point; the same was done for unfiltered samples.

Methods for filtration can be found in the QAPP Appendix A. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis; 3.5 mL of water was added to 3.5 mL of 100% ethanol (ETOH) and sample vials were stored at 4°C until analysis within 4 days.

G.3.3 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were mixed using a stainless steel scoop or similar utensil until they were a uniform color and consistency. Once sediment was thoroughly mixed, the jars for analytical chemistry samples were filled. Sediment samples were sent to ALS Environmental for PAH analysis.

G.4 Experimental Design

Juvenile southern flounder were held in 10-gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 20 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Details regarding the number of treatments and the number of replicates per treatment can be found in the test-specific TCTs. Each treatment contained juvenile flounder in individually numbered mesh cages containing 75 g of prepared sediment.

Each fish was weighed (0.001 g) and measured (SL) and assigned to a mesh cage; flounder were randomly selected for all treatments. Test day 0 began with the initial exposure of the test organisms to the test substance. At the end of the exposure period, fish were removed from the aquarium and sacrificed with MS-222. Fish were weighed and measured, and liver tissue and gill tissue from 4 fish/replicate were placed in Eppendorf tubes with 1 mL of RNAlater for potential subsequent RNA extractions. Tissues were stored at -80°C until processing. Tissues were also removed from the remaining 4 fish/replicate and placed into individually labeled cassettes and fixed in 10% NBF for histological analysis.

The flounder in each mesh cage was video recorded twice each week to provide images from which growth measurements were collected. A 7-cm etched glass rod or a 2.5-cm stir rod was placed into each cage during recording to provide a reference for making measurements from the

video. For each recording session, prior to recording each cage, a label with the name of each treatment was filmed to indicate which treatment was being filmed. The label at the top of each cage contained a small piece of tape with the cage ID (example B-2-7 = treatment B, replicate 2, cage 7). For each treatment, recording always began with replicate 1 followed by replicates 2, 3, and 4, respectively. Any fish that died during the experiment was removed from its mesh cage, although the cage remained in the tank for the duration of the experiment. A piece of marking tape was placed at the end of each mesh cage where a fish had been removed so that when the video was taken of that cage, the viewer could easily identify that there was no longer a fish in that cage. The sequence of recording always followed the recording scheme described above regardless of the presence of fish (i.e., there may be occasions when a video was taken of an empty mesh cage). The video for each week was stored on separate secure digital (SD) cards that were backed up at GCRL prior to shipment of the SD card to Stratus Consulting for growth measurements.

G.5 Test Design

A flow-through test system was used in this evaluation. Clean ASW (15-ppt salinity) flowed from a head box partitioner into 6 splitter boxes and then into the aquaria. To ensure test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

G.5.1 Fish exposure

The exposure was initiated with 8 southern flounder introduced into each study aquarium. Each flounder was in an individual mesh cage containing 75 g of sediment. After 60 days of exposure, all fish from each aquarium were removed and sacrificed. Tissues were removed for possible gene expression, microbiome, or histology analyses as described above.

G.5.2 Temperature and lighting

Temperature during the test was maintained at 22 ± 1°C, and salinity maintained at a nominal 15 ppt. A 12-hour light and 12-hour dark photoperiod was maintained.

G.5.3 Test initiation

Test fish were distributed to the test aquaria in groups of 8 juveniles in each treatment replicate (1 fish/cage). Fish were added to each cage in a random order that was assigned prior to test initiation. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

G.5.4 Diet

During exposure, fish were fed *Artemia* nauplii and/or commercial pellet food twice daily.

G.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 22 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

G.5.6 Biological data

All fish were weighed and measured at test initiation and at test termination. Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead fish were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. Measurements of gene expression and histopathology followed the same methods described in Testing Protocol 12. Although the fish were removed, the cage of the dead fish remained in the tank for the duration of the test. All fish were video recorded twice weekly to provide images that were analyzed for growth measurements.

H. Testing Protocol 8: Effects of Spiked Oiled Sediment on Growth, Reproduction, and Gene Expression of Grass Shrimp

H.1 Test Organisms

Grass shrimp from Ocean Springs, Mississippi, marshes.

H.2 Acclimation/Holding

Grass shrimp used in this study were in 100% dilution/culture water for 14 days prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

H.3 Procedure

H.3.1 Preparation of sediments

Oil was mixed into uncontaminated sediments (ALAJ46-D0515-SB701B) using a Cuisinart stand mixer. For each treatment, 4 kg of sediments was weighed and thawed overnight. Oil was added to each sediment treatment following the test-specific TCTs. Oil was mixed into the sediment for 30 minutes at a moderate speed (4.5 on mixer), scraping the sides of the bowl with a metal spatula approximately every 4 minutes as needed. The oil-sediment mixture was placed into four 20-L aquaria/treatment (1 kg/aquarium). Test sediment was placed into the analytical chemistry 8-oz glass sediment jars for analysis by ALS Environmental; approximately 50 g of sediment was placed in the jar after each replicate tank was filled. Sediments in the jars were stored at 4°C until shipment to ALS Environmental. Water was slowly added to each aquarium to a depth of 15 cm. The sediment was allowed to settle overnight under static conditions prior to beginning flow-through water, aeration, and the addition of the shrimp.

H.3.2 Analysis of water and sediment samples

Analysis of PAHs in the water of each treatment was done by ALS Environmental. Water samples were collected by pulling water directly from the tanks. Samples were not collected

from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment. This was done by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis, 3.5 mL of water was added to 3.5 mL of 100% ETOH, and sample vials were stored at 4°C until analysis within 4 days.

H.3.3 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a stainless steel scoop or similar utensil until uniform color and consistency was reached. Once sediment was thoroughly mixed, a scoop was used to fill the analytical chemistry sediment jar (glass 8-oz). Sediments were sent to ALS Environmental for PAH analysis.

H.4 Experimental Design

Grass shrimp were held in 5-gal (20-L) aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each replicate aquarium with sediment on the bottom contained 12 female and 6 male grass shrimp. Ovigerous females were placed in clean brooding aquaria (maximum volume of 28 L) containing 15 cages; females were placed in brooding tanks following a randomized block design. Additionally, there was a single egg incubation aquarium [48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D) with an overflow drain of 19 cm providing a maximum water volume of 34.4 L] containing 24 incubation cups with no sediments (100-mm petri dish bottoms with a 15-cm tall 40-µm mesh nylon collar) for incubating grass shrimp embryos prior to hatch.

H.5 Test Design

A flow-through test system was used in this evaluation. Clean ASW (15-ppt salinity) flowed from a head box partitioner into 7 splitter boxes and then into the aquaria. The flow rate for brooding aquaria was 1,000 mL/aquarium/cycle, and flow rate for the embryo incubation aquaria was 2 L/cycle. To ensure test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

H.5.1 Shrimp exposure

The exposure was initiated with 12 female grass shrimp introduced into each of 16 study aquaria (4 replicates of 4 sediment treatments), and after 7 days, 6 male grass shrimp were added to each tank. Ovigerous females were removed from the aquaria and placed into individually isolated cages in brooding aquaria; females were added to brooding aquaria following a randomized block design. Ovigerous females were sacrificed after incubating their embryos for 9 days and embryos were transferred to incubation cups with no sediment in an incubation aquarium until 24 hours post-hatch. After 28 days exposure, all unmated females from each aquarium were removed and sacrificed; the hepatopancreas was collected and placed in RNAlater for possible subsequent RNA extraction. Hepatopancreas tissue was also collected from ovigerous females at time of sacrifice. Temperature was maintained at 27 ± 1°C, and salinity maintained at a nominal 15 ppt.

H.5.2 Test initiation

Test shrimp were distributed to the 16 test aquaria in 2 rounds of 6 shrimp each, such that all treatment aquaria contained 6 shrimp before the next 6 shrimp were added. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

H.5.3 Diet

During exposure, shrimp were fed *Artemia* nauplii and/or commercial pellet food twice daily.

H.5.4 Temperature and lighting

Temperature was regulated at 27°C and for each single test the temperature was constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

H.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment, such that each aquarium was monitored every 5 days.

H.5.6 Biological data

A representative subsample of female shrimp from the culture aquaria was weighed and measured at test initiation. All female shrimp were weighed and measured upon sacrifice. Observations of mortality and presence/absence of molts and eggs were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead shrimp were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. Ovigerous females were observed daily, and were sacrificed 9 days after the first appearance of embryos; embryos were counted and a subsample of 20 was isolated until 24 hours post-hatch.

I. Testing Protocol 9: Effects of HEWAF, CEWAF, and Corexit on Gill and Gut Microflora of Sheepshead Minnow

I.1 Test Organisms

Sheepshead minnow from GCRL laboratory culture.

I.2 Acclimation/Holding

Adult fish used in this study were in 100% dilution/culture water for 4 days prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs for details). Fish were fed *Artemia* nauplii and a commercial fish flake food twice daily during the holding and test periods.

I.3 Procedure

I.3.1 Preparation of stock solutions

The stock solution was prepared following standard protocols from the QAPP. New stock solution was prepared every 48 hours.

I.3.2 Sampling

Samples were sent to ALS Environmental for possible analyses of PAHs and DOSS.

Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface. This was done by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis once weekly at 2 and 46 hours post new stock; 3.5 mL of water was added to 3.5 mL of 100% ETOH and sample vials were stored at 4°C until analysis within 4 days.

I.4 Experimental Design

Adult female and male sheepshead minnow were held in 5 gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions.

Thirty-two fish (16 males, 16 females) from the culture population were individually weighed (0.001 g) and measured (SL) at test initiation to obtain time 0 data for growth. Males and females were impartially assigned to the test aquaria, and then exposed for 14 days to the selected treatments and controls. Test day 0 began with the initial exposure of the test organisms to the test substance. After 7 days of exposure, 2 males and 2 females from each aquarium were removed and sacrificed with MS-222. Fish were weighed (0.001 g) and measured (SL), and gonadal tissues removed and weighed (0.001 g). Gill and gut (entire stomach and intestine) tissues were placed in Eppendorf tubes with 1 mL of RNAlater for possible subsequent microflora analysis. Additionally, liver and gonadal tissues were removed from each fish and placed into Eppendorf tubes with 1 mL of RNAlater for possible RNA extractions. Tissues were stored at -80°C until processing. The remaining fish carcasses were given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. After 14 days of exposure, 2 female and 2 male fish from each replicate of each treatment were sacrificed, weighed (0.001 g), measured (SL), and sampled as described above for gill, gut, liver, and gonadal tissues. Tissues were stored at -80°C until processing. The remaining fish carcasses were given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

I.5 Test Design

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of HEWAF, CEWAF, or Corexit into the dilution water (see test-specific TCTs). New stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps. Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber then delivered 500 mL of the appropriately diluted study concentration to each replicate aquarium through a glass delivery line. To ensure test aquaria maintain adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 4 days prior to addition of fish to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

I.5.1 Fish exposure

The exposure was initiated with 8 sheepshead minnow (4 males, 4 females) introduced into each aquarium. After 7 days exposure, 2 male and 2 female fish from each aquarium were removed and sacrificed for possible microflora and gene expression analyses as described above. After 14 days of exposure, the remaining 2 female and 2 male fish from each aquarium were removed and sacrificed for potential microflora and gene expression analyses as described above. Temperature was maintained at 27 ± 1°C, and salinity maintained at a 15 ppt.

I.5.2 Temperature and lighting

Temperature was regulated at 27 ± 1°C for each test and a 12-hour light and 12-hour dark photoperiod was maintained.

I.5.3 Test initiation

Test fish were distributed to the test aquaria in rounds of 4 fish each, such that 4 males were placed into 1 aquarium in all treatments prior to placement of the 4 females into each replicate aquarium. The maximum loading rate in the treatment aquaria was greater than 1.0 g tissue/L.

I.5.4 Diet

During the exposure, fish were fed *Artemia* nauplii and commercial flake food twice daily.

I.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment, such that each aquarium was monitored every 5 days.

I.5.6 Biological data

A subsample of culture fish were weighed and measured at test initiation, and all fish were weighed and measured during sampling at days 7 and 14. Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a

minimum of once daily during the exposure. If dead fish were discovered they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

J. Testing Protocol 10: Effects of Field Collected Sediment on Survival, Growth, and Gene Expression of Grass Shrimp

J.1 Test Organisms

Grass shrimp from Ocean Springs, Mississippi, marshes.

J.2 Acclimation/Holding

Shrimp used in this study were fully acclimated to 100% dilution/culture water for at least 4 days prior to test initiation. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Shrimp were fed *Artemia* nauplii and a commercial flake food twice daily during the holding and test periods. The diet was supplemented with frozen adult *Artemia*.

J.3 Procedure

J.3.1 Preparation of sediments

Frozen, field-collected sediments (contaminated and reference) were used for this exposure. Frozen sediment (2,000 g) was weighed and placed in a 5-L glass container with 500 mL of ASW (15 ppt) and allowed to thaw for 18 hours at room temperature in the dark. The sediment/water solution was stirred to thoroughly mix into a homogeneous solution, and equally distributed into each replicate aquarium in 100–150 g portions (total of ~ 500 g sediment/aquaria). Any water that was remaining in the glass container was divided equally among the replicates. The remaining sediment (50–100 g) was put into a glass sample jar for analysis by ALS Environmental. The aquaria with sediments were placed in a water bath and 8 L of 15 ppt seawater was gently added to each aquarium so that the sediment was not stirred up. The sediment settled for 24 hours prior to the addition of shrimp to the system.

J.3.2 Analysis of water

Analysis of PAHs in the water of each treatment was done by ALS Environmental. Water samples were collected from each replicate aquarium and combined into 1 sample/treatment at

each collection time point. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Water samples were collected from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis. Next, 3.5 mL of water was added to 3.5 mL of 100% ETOH, and sample vials stored at 4°C until analysis was performed within 1 week.

J.4 Experimental Design

Adult grass shrimp were held in 10-gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Six different sediment types were used for the exposure with 4 replicates each and 15 shrimp in each replicate (see test-specific TCTs). A minimum total of 360 grass shrimp were needed to initiate the experiment.

J.5 Test Design

Clean ASW (15-ppt salinity) flowed from a head box partitioner into 6 splitter boxes and then into the aquaria at a rate of 500 mL/aquarium/cycle. To ensure test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of compressed oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

J.5.1 Test initiation

Test shrimp were distributed to the 24 test aquaria. Each individual shrimp was weighed in a beaker of water prior to being added into a treatment aquarium; a mean weight of the 15 shrimp/aquarium was calculated. The addition of shrimp was done in a random manner until the required numbers were distributed into each test aquarium. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

J.5.2 Shrimp exposure

The exposure was initiated with 15 grass shrimp introduced into each of 24 study aquaria (4 replicates of 6 sediment treatments). At the end of the exposure period, shrimp were removed from the aquaria and sacrificed by immersion in ice water. Shrimp were weighed (0.001 g),

measured (mm CL), and hepatopancreas tissue from 6 shrimp/replicate were placed in Eppendorf tubes with 0.5 mL of RNAlater for possible subsequent RNA extractions. Tissues were stored at -80°C until processing. All remaining shrimp were wrapped in acetone-rinsed foil, labeled, and frozen at -20°C.

J.5.3 Temperature and lighting

Temperature was maintained between 26°C and 28°C. A 12-hour light and 12-hour dark photoperiod was maintained.

J.5.4 Diet

During the exposure, shrimp were fed commercial flake food once daily and a mixture of *Artemia* nauplii and frozen adult *Artemia* shrimp once daily.

J.5.5 Water quality

Temperature was measured in 1 test aquarium continuously throughout the test. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was measured in 1 replicate aquarium per day such that each aquarium per treatment was measured every 5 days.

J.5.6 Biological data

Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead shrimp were discovered, they were removed, with each shrimp given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

K. Testing Protocol 11: Effects of Exposure to HEWAF on the Immune Function Fish

K.1 Organisms for Exposure

1. Red Snapper (*Lutjanus campechanus*) were obtained from the Cedar Point aquaculture facility at GCRL

2. Atlantic croaker (*Micropogonias undulates*) were obtained from the Cedar Point aquaculture facility at GCRL

3. Red drum (*Sciaenops ocellatus*) were obtained from the Texas Parks and Wildlife aquaculture facility in Lake Jackson, Texas.

K.2 Procedures

K.2.1 Preparation of stock solutions

HEWAF stock solutions were prepared according to the QAPP.

K.2.2 Experimental design

These experiments were designed to analyze how exposures to oil in aqueous solutions affect fish immune response to acute pathogenic insults. Oil exposures were performed for the duration of the experiments, except for the period when fish were removed for pathogen exposure and immediately placed into a bacterial exposure tank containing ASW and bacteria. After bacterial exposure, the fish were removed and placed in their original WAF exposure tanks. All fish were monitored for the times specified in test-specific TCTs. At regular intervals post-bacterial exposure, some fish were removed from each tank and sacrificed for tissue sampling (see test-specific TCTs). At each sampling event, fish were weighed and measured, visually assessed for the presence of skin lesions, and dissected aseptically according to the *General Immunotoxicity Testing* SOPs. During necropsies, blood and tissue samples were collected for assessing various endpoints (see test-specific TCT).

Juvenile fish were separated into different treatment groups: no oil/no pathogen, oil only (no pathogen, see test-specific TCTs for oil concentrations), pathogen only (no oil), and oil +

pathogen. The number of replicates per treatment and the number of fish per replicate are described in the test-specific TCTs.

For each test, pertinent information concerning fish species, life stage, numbers of organisms, treatment levels, exposure durations, oil loading rates, bacteria species, inoculation loading rates, and analytical sampling requirements is included in the test-specific TCTs.

K.2.3 Endpoints

Various endpoints were assessed throughout the course of each test. Some endpoints, such as survival, growth, and gross pathology observations were performed on live fish on a regular basis. Other endpoints, such as internal gross pathology observations and histopathology sampling required sacrificing individual fish and thus were conducted only once per fish. Fish were sacrificed at various time points after the pathogen challenge and sampled. These time points were specified in test-specific TCTs. A description of potential endpoints for each test is below (see test-specific TCTs).

Survival: Survival of each fish was assessed daily by observing movement in response to gentle prodding or similar external stimuli. Mortality observations were recorded on the appropriate bench sheets.

Growth: Growth was assessed by measuring the total weight and length of each fish before being exposed to oil and at the end of the experiment just before it was euthanized. Initial weights (to the nearest tenth of a gram) and lengths (to the nearest millimeter) were taken from un-anesthetized fish immediately after removal from the tank. All dead fish were removed, weighed, and measured as above, and preserved as archived tissue samples, as described in the QAPP. Growth data were recorded on the appropriate bench sheet.

Gross pathology: A general analysis of fish health was conducted regularly on live fish during the course of the exposure (external) and during necropsies (internal). Gross external assessments of live fish were conducted twice daily. Observers looked at fish eye and skin color, as well as the surface of the skin and fins for presence of lesions or other external abnormalities. Behavior observations included lethargy, erratic swimming, and feeding vigor. External gross pathology assessment results were record on the appropriate bench sheet. Fish were not handled during these external assessments. Internal gross pathology assessments were conducted on euthanized fish during necropsies. Internal organs were assessed for gross pathological changes. These changes included changes in organ size, texture, shape, or color as described in the *Fish Dissection and Sampling* SOP. Internal gross pathology assessment results were record on the appropriate bench sheets.

Immune response: Blood was collected according to the *Bacterial Killing Assay* SOP. Blood sampling was documented on the appropriate bench sheets. Blood was analyzed for bactericidal potential using the BKA in accordance with the *Bacterial Killing Assay* SOP and for cytokine levels in accordance with the *Cytokine Analysis of Plasma SOP*.

Bacterial infection: Some tissues from kidney, spleen, liver, gills, intestine, and any samples of opportunity (e.g., ascites and lesions) were aseptically sampled for microbiomic response. Aseptic samples were taken immediately after a fish was necropsied and blood was taken using freshly decontaminated dissection equipment. Sample collection procedures are described in the *Bacterial analysis of tissue* SOP. Aseptic sampling was documented on the appropriate bench sheets.

Gene expression: Some tissues from liver, kidney, and spleen were collected for gene expression using transcriptomics or qPCR. If collected, tissue samples were preserved in RNALater as described in the *Fish Dissection and Sampling* SOP. For sample containers and storage requirements, refer to the QAPP. Gene expression sampling was documented on the appropriate bench sheets. Gene expression analyses followed the *RNA extraction and qPCR for gene expression analyses* SOP.

Histopathology: Some tissues from the spleen, liver, kidney, and any samples of opportunity (e.g., lesions) were collected for histopathological analyses according to the *Fish Dissection and Sampling* SOP. Histopathology samples were collected after all blood, bacterial infection, and gene expression samples were taken. The QAPP provides details on histology sample containers, fixative, and storage requirements. Sample collection information was recorded using the appropriate bench sheets. Histological analyses were done using the *Gill Histology Image Analysis Methods* SOP and the *Liver Histology Image Analysis Methods* SOP.

Raw seawater bacterial characterization: The bacterial content of the water in the circulating system was tested before adding fish and at the end of the observatory period. This was accomplished via bacterial plating (see the *Bacteriological Analysis of Sediment and Water* SOP), qPCR quantification (see the *RNA extraction and qPCR for gene expression analyses* SOP), and microbiomics (see the *Microbial Diversity Analysis* SOP).

L. Testing Protocol 12: Effects of Exposure to Spiked Sediment on the Immune Function Fish

L.1 Organisms for Exposure

Southern flounder were obtained from UTMSI.

L.2 Procedures

L.2.1 Sediment preparation/exposure

Oil was mixed into uncontaminated sediments using a KitchenAid stand mixer (*Protocol for Preparation of Oil-Spiked Sediments*). For each replicate, the appropriate amount of sediment was weighed and thawed overnight (see test-specific TCTs). Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs. Oil was mixed into the sediment for 30 minutes at moderate speed (4.5 on the mixer); the sides of the bowl were scraped with a metal spatula every 2–4 minutes as needed. For test 900, the oil mixture was placed directly into the tanks. For test 904, the oil-sediment mixture was weighed and placed into mesh cages (10-cm petri dish with a 20-cm column of 2-mm nylon mesh) for each treatment (approximately 75-g sediment/cage). When preparing the tanks, sediment samples for chemical analysis from each treatment group were prepared by taking 50-g aliquots of sediment into the analytical chemistry sediment jar, in-between filling each replicate tank. Samples were stored at 4°C until shipment. Cages were placed in treatment aquaria and sediment was slowly added to each aquarium. The sediment was allowed to settle in the cages overnight under static conditions before beginning flow-through water and adding the fish.

L.2.2 Experimental design

These experiments were designed to analyze how exposures to spiked sediment affect fish immune response to acute pathogenic insults. Oil exposures were performed for the duration of the experiments, except for periods when fish were removed from the oil exposure tanks and placed into a bacterial exposure tank containing ASW and bacteria or a control tank containing no bacteria. After exposure, the fish were removed and placed back into their original WAF or control tanks. All fish were monitored for the times specified in test-specific TCTs. At regular intervals post-bacterial exposure, fish were removed from each tank and sacrificed for tissue sampling (see test-specific TCTs). At each sampling event, fish were weighed and measured, visually assessed for the presence of skin lesions, and dissected aseptically according to the

General Immunotoxicity Testing SOPs. During necropsies, blood and tissue samples were collected for assessing various endpoints.

For each test, there were the following treatment groups: no oil/no pathogen, oil only (no pathogen, see test-specific TCTs for oil concentrations), pathogen only (no oil), and oil + pathogen. The number of replicates per treatment and the number of fish per replicate are described in the test-specific TCTs.

For each test, the pertinent information concerning fish species, life stage, numbers of organisms, treatment levels, exposure durations, oil loading rates, bacteria species, inoculation loading rates, and analytical sampling requirements is included in the test-specific TCTs.

L.2.3 Endpoints

Various endpoints were assessed throughout the course of each test. Some endpoints, such as survival, growth, and gross pathology observations were performed on live fish on a regular basis. Other endpoints, such as internal gross pathology observations and histopathology, sampling required sacrificing individual fish and thus were performed only once per fish. Fish were sacrificed at various time points after the pathogen challenge and sampled. These time points were specified in test-specific TCTs. A description of potential endpoints is below (see test-specific TCTs).

Survival: Survival of each fish was assessed daily by observing movement in response to gentle prodding or similar external stimuli. Mortality observations were recorded on the appropriate bench sheets.

Growth: Growth was assessed by measuring the total weight and length of each fish before being exposed to oil and at the end of the experiment just before fish were euthanized. Initial weights (to the nearest tenth of a gram) and lengths (to the nearest millimeter) were taken from un-anesthetized fish immediately after removal from tank. All dead fish were removed, weighed, and measured as above, and preserved as an archived tissue sample, as described in the QAPP. Growth data was recorded on the appropriate bench sheet.

Gross pathology: A general analysis of fish health was conducted regularly on live fish during the course of the exposure (external) and during necropsies (internal). Gross external assessments of live fish were conducted twice a day. Observers looked at fish eye and skin color, as well as the surface of the skin and fins for presence of lesions or other external abnormalities. Behavior observations included lethargy, erratic swimming, and feeding vigor. External gross pathology assessment results were record on the appropriate bench sheet. Fish were not handled during these external assessments. Internal gross pathology assessments were conducted on euthanized fish during necropsies. Internal organs were assessed for gross pathological changes.

These changes included changes in organ size, texture, shape, or color as described in the *Fish Dissection and Sampling* SOP. Internal gross pathology assessment results were record on the appropriate bench sheets.

Immune response: Blood was collected according to the *Bacterial Killing Assay* SOP. Blood sampling was documented on the appropriate bench sheets. Blood was analyzed for bactericidal potential using the BKA in accordance with the *Bacterial Killing Assay* SOP or for cytokine levels in accordance with the *Cytokine Analysis of Plasma* SOP.

Bacterial infection: Some tissues from kidney, spleen, liver, gills, intestine, and any samples of opportunity (e.g., ascites and lesions) were aseptically sampled for microbiomic response. Aseptic samples were taken immediately after a fish was necropsied and blood was taken using freshly decontaminated dissection equipment. Sample collection procedures are described in the *Bacterial analysis of tissue* SOP. Aseptic sampling was documented on the appropriate bench sheets.

Gene expression: Some tissues from liver, kidney, and spleen were collected for gene expression using transcriptomics and/or qPCR. If collected, tissue samples were preserved in RNALater as described in the *Fish Dissection and Sampling* SOP. For sample containers and storage requirements, refer to the QAPP. Gene expression sampling was documented on the appropriate bench sheets. Gene expression analyses followed the *RNA extraction and qPCR for gene expression analyses* SOP., the *RNA sequencing analysis* SOP, and the *Bioinformatics and Transcriptomics* SOP.

Histopathology: Some tissues from the spleen, liver, kidney, and any samples of opportunity (e.g., lesions) were collected for histopathological analyses according to the *Fish Dissection and Sampling* SOP. Histopathology samples were collected after all blood, bacterial infection, and gene expression samples were taken. The QAPP provides details on histology sample containers, fixative, and storage requirements. Sample collection information was recorded using the appropriate bench sheets. Histological analyses were done using the *Gill Histology Image Analysis Methods* SOP and the *Liver Histology Image Analysis Methods* SOP.

Raw seawater bacterial characterization: The bacterial content of the water in the circulating system and exposure sediment was tested before adding fish and at the end of the observation period. This was accomplished via bacterial plating (*Bacteriological Analysis of Sediment and Water* SOP), qPCR quantification (*RNA extraction and qPCR for gene expression analyses* SOP), and microbiomics (*Microbial Diversity Analysis* SOP).

L.2.4 RNA sequencing analysis SOP (National Center for Genomic Research)

Total RNA samples were processed by taking an aliquot for QC analysis to determine the amount of RNA and the integrity of the RNA using Qubit and Bioanalyzer, respectively. All samples passed QC and were made into sequencing libraries using the Illumina TruSeq RNA Sample Preparation Kit. Total RNA went through poly-A selection reaction, in which the mRNA is pulled down using poly-T oligo-attached to magnetic beads. The pulled-down mRNA was fragmented and randomly primed in a one-step reaction. The randomly primed mRNA was then taken through first-strand synthesis using a reverse transcriptase enzyme. The product then underwent a second strand synthesis using a second strand master mix that contained DNA polymerase I and RNase H. The synthesized second strand was end repaired using End Repair Mix (converted overhangs generated from fragmentation into blunt ends), followed by the addition of an A-base on the 3' end of the double-stranded cDNA molecule. The addition of A-base prepared it for the ligation of Illumina adapters, which had a T-base on its 3 end. After the ligation of the sample with uniquely barcoded adapters, the resulting product was taken through 15 cycles of PCR amplification. QC was performed on the Nanodrop to determine the amount and on the Bioanalyzer for fragment size determination of the library and for any adapter dimers.

L.2.5 Bioinformatics and Transcriptomics SOP (National Center for Genomic Research)

Bioinformatics was performed on the Illumina HiSeq 2000 50-nt paired-end sequencing results of the 52 RNA flounder samples (average reads per sample was 24.5 M). First, the sequence reads were processed to create an optimized *de novo* transcript assembly. Using an iterative approach, an annotated assembly was created containing a minimum amount of contigs, or transcripts. The reads were then aligned to this newly created reference and counted to perform differential expression analysis. The differential expressed transcripts were than filtered using standard criteria, and pathway analysis was performed. The details of each step are explained below.

1. *De Novo* Transcriptome Assembly

The National Center for Genome resources *de novo* assembly pipeline has three main components: the assembly of transcripts or contigs, the annotation of genes and transcripts, and peptide annotation.

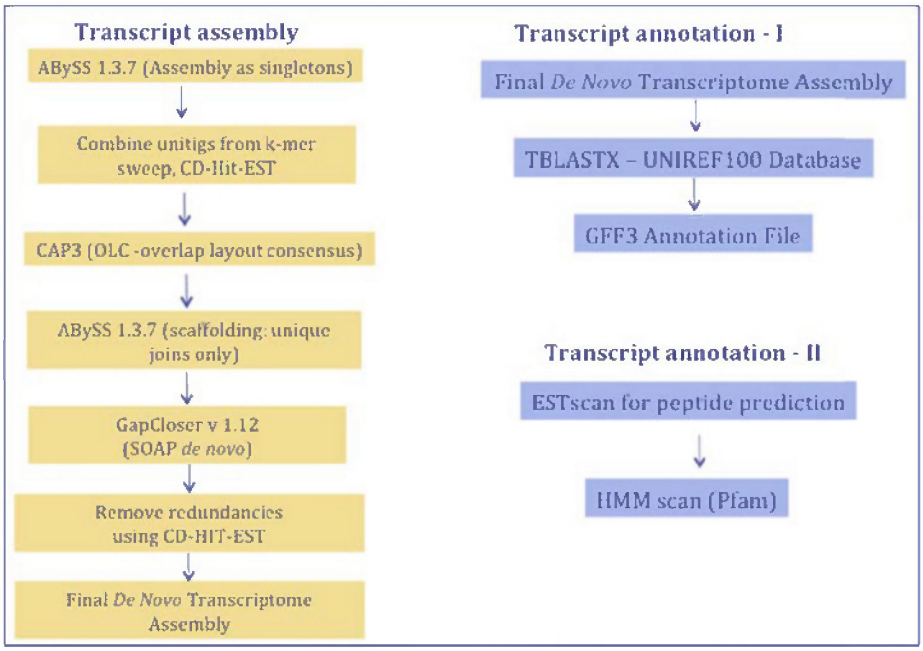

Figure L.1. *De novo* Assembly pipeline.

2. Differential Expression Analysis

Following *de novo* assembly of the Southern flounder transcriptome, differential expression analysis of flounder transcripts for experiments 904, 900, 113, and field-collected samples was performed using NCGR's Bioinformatics-in-a-Box™ (BiB) tool with default parameters (http://www.lumenogix.com/de-novo-rna-seq-expression-analysis). Differential expression analysis using BiB began with a quality check of the data using the FastQC program (Andrews, 2010), followed by mapping/alignment of the raw reads, in fastq format, to an annotated transcriptome using Bowtie (Langmead et al., 2009). After mapping of the raw reads to the transcriptome, the mapped/aligned reads were quantified using the RSEM (Li et al., 2011) algorithm that is built into BiB. The read counts generated by RSEM were used to perform differential expression analysis using the built-in EBSeq (Leng et al., 2013) algorithm. Differential expression results generated by EBSeq were filtered using BiB by a posterior probability of differential expression (PPDE) of greater than or equal to 0.95: transcripts with a PPDE > = 0.95 made up the list of differentially expressed transcripts, with a target false discovery rate (FDR) controlled at 5% (FDR < = 0.05).

3. Pathway Analysis of Differentially Expressed Transcripts

The filtered lists of differentially expressed transcripts (PPDE > = 0.95) contained uniref100 IDs that served as transcript identifiers. The uniref100 IDs were converted to Ensembl transcript IDs or associated gene names using the UniprotKB database (UniProt Consortium, 2015). The Ensembl transcript IDs and associated gene symbols were converted to Danio rerio (Zebrafish) Ensembl gene IDs using the Ensembl biomart website (Cunningham et al., 2015). The Danio rerio Ensembl gene IDs were used to perform pathway analysis (Biological Processes and Reactome or Molecular Functions) using the ClueGo app (Bindea et al., 2009) in Cytoscape (Shannon et al., 2003).

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4. Hopkins Marine Station of Stanford University and Northwest Fisheries Science Center General Laboratory Procedures and Practices

4.1 Methods

Tuna or mackerel or both were exposed to different concentrations of whole oil water accommodated fractions (WAFs) under laboratory conditions. Physiological data were generated from both whole organisms and tissue samples. Water chemistry analyses were carried out by ALS Environmental and Northwest Fisheries Science Center (NWFSC).

All organisms and tissues from organisms collected for and used during testing were archived according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

4.1.1 Test organism sources and husbandry

Pacific bluefin and yellowfin tuna (*Thunnus orientalis* and *Thunnus albacares*)

Pacific bluefin and yellowfin tuna were field collected by hook and line from off the coast of California and brought back to the Tuna Research and Conservation Center (TRCC) at Hopkins Marine Station of Stanford University (Hopkins), where they were housed in one of three holding tanks at the facility, each with its own life support system. Two of the tanks (T2 and T3) are 30,000 gal each (109 m^3) and the third (T1) is 90,000 gal (327 m^3). The tanks are matched to pumps, high-speed sand filters, aeration/degassing towers, and protein fractionator towers that provide high-quality seawater. The life support system supplies water to each tank at 100% oxygen saturation at a temperature range from 20 to 25°C, a pH range of 7.8–8.0, and unionized ammonia at less than 0.01 mg/L. In captivity, fish were typically fed three times weekly (30 kcal/kg) with a mix of squid, sardines, and an enriched gelatin diet.

Pacific mackerel (*Scomber japonicus*)

Pacific mackerel were field collected by hook and line, and brought back to the TRCC, where they were housed at 20°C in tanks T4 and T5. Another tank (T6) was the dedicated oil exposure tank for mackerel. The turnover rate for T4, which is 5,900 gal (22.3 m^3), was one volume per hour through two sand filters for biological filtration and then through a packed column for aeration and off-gassing any nitrogen. There was approximately 5 gpm of new seawater added to the tank continuously. There was a nightlight over the tank that was very dim but gave enough

light for the mackerel to see the tank walls. They were fed a mixture of chopped squid and sardines typically every other day.

Tanks T5 and T6 were each 1,000 gal (3.78 m^3), and each tank had its own dedicated life support system consisting of a sand filter for biofiltration. After filtration, the seawater returned to each tank through an aeration system consisting of a cascade arrangement of aeration biorings. Temperature was controlled by either immersion heaters or cooling coils; both temperature systems were controlled by thermostats. The turnover rate in both of these tanks can be adjusted to 1–1.5 volumes per hour. T5 was dedicated as the tank to hold mackerel that were trained to swim in the respirometer.

4.1.2 Exposure media preparations

All seawater used for exposure studies and used in the TRCC holding tanks was sterilized, filtered seawater from Monterey Bay and supplied to the TRCC by the Monterey Bay Aquarium.

WAFs for electrophysiological experiments were prepared in Ringer's solution (all mM: 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES; and pH adjusted to 7.7 via NaOH), which is a normal physiological salt solution. Ringer's solution was made fresh for each experiment using Millipore filtered freshwater.

High energy water accommodated fraction preparation

Note: This protocol was applicable only for mixing oil with volumes of water equal to 4 L or less.

High energy water accommodated fractions (HEWAFs) were made for tissue exposures with each of four oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A). Control exposure solutions were made using the same protocols, but without any oil.

The protocol used for making the HEWAF is found in the QAPP with the following modifications:

- Ringer's solution, rather than seawater, was used for preparing all electrophysiology experiment HEWAFs.
- A Gilson Microman positive displacement pipette with positive displacement tips was used to transfer the liquid oil samples, rather than a gastight syringe.
- For Slick A and B oils, a small amount of the viscous oil was transferred to an amber vial that was then heated in a 65°C water bath for approximately 20 minutes, reducing the

viscosity enough to allow the use of the Gilson Microman positive displacement pipette. Once this oil was pipetted onto the surface of the water in the blender, it was then gently pushed onto the blender blades with a metal spatula, ensuring a more uniform dispersion.

- Slick A and B HEWAF preparations were blended for 2 minutes, rather than 30 seconds, on the low setting in the Waring CB15 commercial blender.
- 4-L glass carboys with bottom spigots were used for the 1-hour separation step, rather than separatory funnels.

Globular high energy water accommodated fraction preparation

Note: This protocol was designed for mixing oil with volumes of water between 30 and 50 L. This concentrated globular high energy water accommodated fraction (GWAF) is then mixed with a larger volume of water (1,000–2,500 L) to reach the desired final concentration.

GWAFs were made for scombrid species exposures in dedicated exposure tanks in the TRCC for each of four oil types: Slick A, Slick B, source oil, and weathered source oil. A control without oil added was also made.

4.1.3 Testing methods

Electrophysiology experiments

A. Cardiac myocyte electrophysiology methods

The following procedures were used for these experiments and can be found in Hopkins and NWFSC General Laboratory Procedures and Practices (GLPP):

- *Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy*
- *Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique.*

For confocal experiments:

1. Cells were incubated in Ringer's solution plus oil (at various concentrations) for at least 1 hour. The maximum incubation time was 3 hours.
2. Measurements were taken after 1–2 hours of incubation, sometimes 2–3 hours if the cells were also incubated with a sarcoplasmic reticulum (SR) inhibitor.
3. Cells from at least two fish were used for each oil type tested. At least 10 cells (number of reps) were used for each oil type tested.

For the patch clamp experiments:

1. Each cell was in Ringer's solution for at least 5 minutes (control), then HEWAF was added to reach a HEWAF:Ringer's solution concentration of 1/20,000, followed by 2–3 minutes exposure to reach steady state before measurements were taken, then additional HEWAF was added to increase the nominal concentration to 1/10,000 followed by another 2–3 minutes exposure, then again HEWAF was added to increase the nominal concentration to 1/5,000 followed by 2–3 minutes exposure before final measurements were taken.

2. Measurements were taken the entire time, before and during oil exposure. Measurements were taken until a new steady state was reached, typically in 2–3 minutes.

3. Three replicate cells, from at least two different fish, were used for each oil type.

Respirometry experiments

The following protocols and test conditions were used to determine the impacts of crude oil or dispersant exposure or both on metabolic and behavioral responses of adult scombrid fish and to characterize the toxicity of crude oil on whole organism metabolic rates from Hopkins and NWFSC GLPP:

- *Testing Protocol 3: Fish Respirometry within 30-L Respirometer Chambers with or without Exposure to Oil.*

A. Respirometry methods

- Pacific mackerel were placed individually in a 30-L respirometer to measure baseline metabolic rate at 20°C water temperature, swimming at one body length per second (BL/s).
- Mackerel were exposed to specific concentrations of oil for specific durations (2–4 days) in a 1,000-gal exposure tank (Tank T6).
- Mackerel were then individually placed in a 30-L respirometer to measure metabolic rate during oil exposure at 20°C water temperature, swimming at 1 BL/s.
- Tailbeat frequencies were measured.
- Baseline metabolic rates were compared to post-exposure metabolic rates to determine if there is a metabolic response to oil exposure.

B. Respirometry water chemistry sampling

No WAF stock, archive water, or fluorescence samples were taken during respirometry exposures. Samples from the exposure tank after mixing in oil GWAF were taken at multiple time points during the 24-, 48-, 72-, and 96-hour exposures and analyzed as follows:

- All samples were analyzed for polycyclic aromatic hydrocarbons (PAHs)
- Source oil GWAF was analyzed for benzene, toluene, ethylbenzene, and xylenes (BTEX) in addition to PAHs.

Sample bottle, shipping, and handling requirements were performed as described in the QAPP.

4.1.4 Water quality monitoring

A. Water quality monitoring for electrophysiology experiments

Protocols in the Hopkins and NWFSC GLPP provide detailed descriptions of solutions used for the electrophysiological experiments. HEWAFs for electrophysiological experiments were prepared in Ringer's solution (made fresh daily), and pH was measured/recorded before cardiomyocyte exposures:

- *Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy*
- *Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique.*

B. Water quality monitoring for respirometry experiments

1. Temperature

Temperature in the exposure tank (T6) and in the respirometry reservoir was obtained by using a thermometer or a YSI Pro ODO meter with an oxygen and temperature probe. When this probe was used, it was calibrated prior to every test.

2. Salinity

Salinity was obtained using a refractometer. This instrument is commonly used in the laboratory and field. Accuracy of the refractometer is checked once monthly using salinity standards.

3. Dissolved oxygen (DO)

A YSI Pro series sonde with an optical DO probe was used to measure DO in the exposure tank (T6) and in the respirometry reservoir. The probe was calibrated according to manufacturer's specifications prior to every experiment.

4. pH

The pH was measured using litmus paper, within the exposure tank (T6) as well as in the respirometry reservoir.

5. Ammonia

Ammonia in the exposure tank (T6) and the respirometry reservoir was measured with a Hach Ammonia Test Kit (model NI-SA).

For definitive tests using Deepwater Horizon (DWH) oil, these water quality samples were taken and recorded at the beginning of each individual exposure to oil and each subsequent 24 hours until the end of the test.

4.1.5 Analytical chemistry

Analyses of PAHs and other compounds were conducted by ALS Environmental, with some samples analyzed at NWFSC. For the collection procedure, see the QAPP. Bile PAHs were measured at NWFSC.

4.2 Reporting and Testing Documentation

Reporting of the data followed procedures described in the QAPP.

4.3 General Testing Standard Operating Procedures

4.3.1 Preparation of GWAFs with DWH oils

This method was used for generating dispersed oil for large-scale (1,000-L) exposures of adult/subadult mackerel for respirometry studies.

Glassware/mixer decontamination procedure

Rinse all glassware and GWAF mixer components with three rinses of acetone, followed by three rinses of dichloromethane (DCM) before and in-between each prep. Allow sufficient time for full evaporation of final solvent rinse. Inspect the inside parts of the drywall paddle prongs for visible oil and scrub with solvent-soaked Kimwipes. Use gloved hands throughout all preparation steps, and use common-sense laboratory safety for handling solvents, especially DCM. Refer to appropriate material safety data sheets if necessary. All other decontamination efforts during GWAF preparation follow the *Decontamination SOP* found in the QAPP.

Recordkeeping

The *Water Accommodated Fraction Preparation and Sampling Table* bench sheet provided by Stratus Consulting in the QAPP was used to record data during GWAF preparations.

Procedure

A. Prepare negative control

1. Measure appropriate volume of seawater into pre-cleaned GWAF mixer (40 L).
2. Close mixer lid.
3. Blend for the same amount of time that the treatment WAF is blended.
4. After mixing, collect water samples immediately for analytical chemistry from below the surface using disposable glass pipettes. Store water samples and ship according to the QAPP.

B. Prepare oil GWAF

1. Measure appropriate volume of water into pre-cleaned GWAF mixer (40 L).
2. Measure desired volume of oil. The source oil and artificially weathered source oil samples are fluid enough to use a graduated cylinder. For the thick surface slick oils (i.e., Slick A and Slick B), warm a large-enough volume at 65°C in a water bath for 30 minutes to decrease the viscosity for measuring in a graduated cylinder.

   Common dilutions of oil include the following:

   - 1:10,000: 250 mL oil into 40 L of water, mixed, then added to 2,500 L
   - 1:100,000: 25 mL oil into 40 L of water, mixed, then added to 2,500 L.

3. Close GWAF mixer lid.

4. Blend 4 hours or until the oil and water looked sufficiently mixed.

5. Take appropriate water samples as indicated in #A.4 above.

6. Transfer remaining contents in GWAF mixer to the exposure tank, T6.

7. Note time of transfer. Details regarding water samples taken for analytical chemistry are in the *Water Accommodated Fraction Preparation and Sampling Table* bench sheet provided in the QAPP.

A. Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy

A.1 Isolated Myocyte Preparation

Myocytes from mackerel and tuna were obtained by adaptation of the isolation protocol (Shiels et al., 2004). Each fish was euthanized by pithing, and the heart was excised. The heart was perfused with isolating solution until it had stopped beating and was cleared of blood. This was accomplished by retrograde perfusion through the ventricular lumen for ~ 10 minutes in mackerel and ~ 20 minutes in tuna. In tuna, the coronary artery was additionally perfused to clear blood from the arteries and compact myocardial tissue. Proteolytic enzymes were then added to the isolating solution, and retrograde luminal perfusion continued for ~ 12 minutes in mackerel and ~ 40 minutes in tuna. After enzymatic treatment, the atrium and ventricle were placed in separate dishes containing fresh isolating solution. Tissues were cut into small pieces with scissors and then triturated through the opening of a Pasteur pipette to free individual myocytes. Myocytes were stored in fresh isolating solution for up to 8 hours at 20°C.

A.1.1 Solutions

All chemicals were obtained from Sigma unless otherwise indicated.

Laboratory notebooks were used to record solution preparation dates, parameters, and standard operating procedure (SOP) modifications.

The isolating solution contains (mM) 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose, and 10 HEPES, with pH adjusted to 6.9 with NaOH at 20°C. For enzymatic digestion, collagenase (type IA), trypsin (type IX), and fatty acid-free Bovine Serum Albumin (BSA) were added to this solution (0.75 mg/mL, 0.25 mg/mL, and 0.75 mg/mL, respectively). For small mackerel (< 200 g), BSA was increased to 1.125 mg/mL. Isolating solution was made fresh daily.

The extracellular (Ringer's) solution used for recording myocyte Ca^{2+} transient contains (mM) 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES, and pH is adjusted to 7.7 via NaOH. Ringer's solution was made fresh daily.

To assess the role of the SR, myocytes were incubated for at least 30 minutes with SR inhibitors (5 μm ryanodine and 2 μM thapsigargin) prior to experimentation.

In some experiments, a pulse of caffeine (20 mM, 5–10 seconds) was applied to the cell (via a local perfusion system) to assess SR Ca load. If a caffeine pulse was applied, it was noted in the designated laboratory notebook.

A.1.2 Oil incubation

HEWAFs were prepared using Ringer's solution, rather than seawater, following the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Myocytes were incubated for at least 1 hour with an oil preparation prepared with an equal volume of cell suspension and HEWAF (for example: 1 mL of cell suspension is added to 1 mL of HEWAF). The maximum incubation time was 3 hours. Exposure concentrations and exposure times were recorded in the designated laboratory notebook. All oil exposures were at 20°C.

A.1.3 Ca^{2+} transient recording

Measurements were taken after 1–2 hours of incubation, or sometimes 2–3 hours if the cells were also incubated with an SR inhibitor. All experiments were performed at 20°C. Myocytes were incubated with 5 µM Fluo-4 [stock solution 1 mM in with 20% Pluronic F127 in dimethyl sulfoxide (DMSO) solution] for 20–30 minutes. An Olympus 100x water-immersion objective was used in all measurements. The pin-hole aperture was set to the size of the Airy disk to optimize z-axis resolution. Images were collected using repetitive line scans (2,500 lines of 512 pixels) every 3 or 5 ms across the width of the cell. Start times of oil exposures, as well as oil exposure concentrations, were recorded in the designated laboratory notebook. The protocol is below.

- A sample (80–160 µL) of ventricular myocytes was added to the recording chamber (volume ~ 610 µL, filled with Ringer's solution or Ringer's solution with HEWAF oil preparation) and allowed to settle on the bottom.
- Ca^{2+} transients were elicited by field stimulation through a pair of platinum electrodes, at a frequency of 0.5 Hz.
- Line-scan images were acquired at a sampling rate of 3 or 5 ms to get the best signal-to-noise ratio.
- Cells were replaced by fresh ones, which were within either the control solution or an oil exposure solution, after several minutes of recording (< 15 minutes total). The chamber was cleaned with distilled water and 100% ethanol when changing cells from one experimental solution to the other.

B. Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique

B.1 Isolated Myocyte Preparation

Myocytes from mackerel and tuna were obtained by adaptation of the isolation protocol (Shiels et al., 2004). Each fish was euthanized by pithing, and the heart was excised. The heart was perfused with isolating solution until it had stopped beating and was cleared of blood. This was accomplished by retrograde perfusion through the ventricular lumen for ~ 10 minutes in mackerel and ~ 20 minutes in tuna. In tuna, the coronary artery was additionally perfused to clear blood from the arteries and compact myocardial tissue. Proteolytic enzymes were then added to the isolating solution, and retrograde luminal perfusion continued for ~ 12 minutes in mackerel and ~ 40 minutes in tuna. After enzymatic treatment, the atrium and ventricle were placed in separate dishes containing fresh isolating solution. Tissues were cut into small pieces with scissors and then triturated through the opening of a Pasteur pipette to free individual myocytes. Myocytes were stored in fresh isolating solution for up to 8 hours at 20°C.

B.2 Solutions

All chemicals were obtained from Sigma unless otherwise indicated.

Laboratory notebooks were used to record solution preparation dates, parameters, and SOP modifications.

The isolating solution contained (mM) 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose, and 10 HEPES, with pH adjusted to 6.9 with NaOH at 20°C. For enzymatic digestion, collagenase (type IA), trypsin (type IX), and fatty acid-free BSA were added to this solution (0.75 mg/mL, 0.25 mg/mL, and 0.75 mg/mL, respectively). For small mackerel (< 200 g), BSA was increased to 1.125 mg/mL. Isolating solution was made fresh daily.

The experimental solutions designated for each specific recording are listed below. All experimental solutions were made fresh daily.

B.2.1 Action potential

When recording the action potential of myocytes, Ringer's was used as an extracellular solution (in mM): 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, 10 HEPES, and pH adjusted to 7.7 via NaOH and the internal (pipette) solution contained (in mM): 10 NaCl, 140 KCl,

5 MgATP, 0.025 EGTA, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with KOH. A low concentration of EGTA was included to achieve near physiological Ca^{2+} buffering capacity.

B.2.2 K^+ current

To avoid contamination of overlapping currents, the external solution contained (in mM) 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 0.005 Tetrodotoxin (TTX), 0.010 nifedipine, 0.01 glibenclamide, 10 glucose, and 10 HEPES, and pH was adjusted to 7.7 via NaOH, and the internal (pipette) solution contained (in mM) 10 NaCl, 140 KCl, 5 MgATP, 5 EGTA, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with KOH. High concentration of EGTA was included to block Na-Ca exchanger current. TTX was included to block fast Na^+ current, nifedipine to block Ca^{2+} current, and glibenclamide to block ATP-sensitive K^+ current.

B.2.3 Ca^{2+} current

To avoid contamination of overlapping currents, the external solution contained (in mM) 150 NaCl, 5.4 CsCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES, and pH was adjusted to 7.7 via NaOH; the internal (pipette) solution contained (in mM) 130 CsCl, 15 TEACl, 5 MgATP, 0.025 EGTA, 5 NA_2Phosphocreatine, 0.03 NaGTP, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with CsOH. Cs^+ was included to avoid contamination by K^+ currents. In some experiments, TTX was added to block I_{Na}; otherwise a pulse to -40 mV was used to inactivate this current. The EGTA concentration was designated to mimic physiological Ca^{2+} buffering properties of fish myocytes (see Shiels et al., 2004 for details).

B.2.4 Oil exposure

A single cardiac myocyte was exposed to control (external solution) until the recording had reached steady state (at least 5 minutes). Then external solution with oil (HEWAF prepared in Ringer's according to the QAPP) was applied to the same myocyte, via bath perfusion (~ 100 µL final volume) for typically 2–3 minutes, starting from the lowest concentration and then moving up to the highest concentration, in order to determine the dose response curve (all dilutions should reach steady state effect). In some cases (e.g., K^+ current recording), a specific blocker was included in the final solution to inhibit the specific current. All exposure times and concentrations were recorded in the designated laboratory notebook.

B.2.5 Electrophysiological recordings

Myocytes were studied in a chamber mounted on the stage of an inverted microscope (Nikon DIAPHOT 200, Japan). Cells were initially superfused with Ringer's solution. All experiments were performed at room temperature (~ 20°C).

The membrane potential and currents were recorded using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981; Brette et al., 2006). An Axopatch 200B (Axon Instruments, Union City, California) amplifier was used, controlled by a Pentium PC connected via a Digidata 1322A A/D converter (Axon Instruments), which was also used for data acquisition and analysis using pClamp software (Axon Instruments). Signals were filtered at 2–10 kHz using an 8-pole Bessel low-pass filter before digitization at 10–20 kHz and storage. The resistance of the patch clamp pipettes was typically 1.5–2.5 MΩ when filled with intracellular solution (above).

In all experiments, at least 5 minutes was allowed for cell dialysis by the pipette solution before experiments were initiated. The cell membrane capacitance was measured by integrating the capacitance current recorded during a 10-mV hyperpolarizing pulse from -80 mV.

Action potentials were evoked by 2.5 ms subthreshold current steps. Trains of pulses were applied at 0.5 Hz.

In all voltage clamp experiments, cell capacitance and series resistance were compensated (> 60%) so that the maximum voltage error was < 2.5 mV. The holding potential was -80 mV, to be near to physiological resting membrane potential. I_K was elicited by a rectangular step to +40 mV (peak of activation of K^+ current in fish) (Galli et al., 2009) and then the tail current at -20 mV was analyzed (stimulation frequency 0.1 Hz). I_{Ca} was elicited by a rectangular step (300-ms pulse) to 0 mV (peak of Ca^{2+} current, current-voltage relationship; Brette and Cros, unpublished data) after a 100 ms prepulse to -40 mV to inactivate I_{Na}. Current-voltage relationship for I_K was obtained by a 4-second pulse starting from -40 mV up to +50 mV (10-mV step) and activation curve (tail current) at -20 mV (4 seconds). The current-voltage relationship for I_{Ca} was obtained by a 300 ms pulse from -40 mV up to +50 mV (10-mV step). Action potential clamp experiments were performed by averaging action potential from control conditions and one oil type. In this case TTX was added to the external solution to block I_{Na}.

B.2.6 Protocols

- A sample of ventricular myocytes was added to the recording chamber and allowed to settle on the bottom. Myocytes were initially perfused with Ringer's solution.
- Once gigaseal was achieved, the current or voltage stimulation was induced as described above.
- Once recording for the cell was achieved, the whole bath was changed after washing with pure ethanol.

C. Testing Protocol 3: Fish Respirometry within 30-L Respirometer Chambers with or without Exposure to Oil

C.1 Procedure

C.1.1 TRCC tank and respirometer setup

1. Tanks T4 and T5 were dedicated to holding untrained and respirometer-trained mackerel, respectively, and tank T6 was used solely for oil exposures of fish.

2. The TRCC holds two respirometers; R1 was used for respirometry related to non-oil-exposed fish, while R2 was used for respirometry related to oil-exposed fish and had oiled water from tank T6 running through the system.

3. Fish were trained to swim in respirometer R1 for 24–72 hours.

4. All fish swam for a baseline measurement. Baseline data were logged into a notebook and the video archives were kept on both the dedicated computer hard drive and the backup external hard drive.

C.1.2 Preparation of oil exposure tank (T6)

1. Prepare a GWAF according to the *Preparation of GWAFs with DWH oils* SOP in the Hopkins and NWFSC GLPP.

2. While the GWAF is mixing, fill exposure tank T6 with an appropriate volume of sterilized, filtered seawater (typically 2,500 L) from the Monterey Bay Aquarium seawater supply pumps.

3. Always wear dedicated elbow-length rubberized gloves, boots, and surgical apron when handling oil-exposed specimens or working with oil-contaminated water.

4. Check that the life support system is set to by-pass:

    a. Turn off pump #1
    b. Close valves #3 and #4
    c. Open valve #5
    d. Open valve #6
    e. Turn on pump #1 (check flow-rate and pressure gauge on top of the filter – it should read zero pressure).

5. After the GWAF has mixed for the appropriate amount of time and a sample has been collected for analytical chemistry (refer to the *Preparation of GWAFs with DWH oils* SOP in Hopkins and NWFSC GLPP), add the entire mixture to T6.

6. Once the exposure water in T6 has been thoroughly mixed (via pumping action) for 30 minutes, proceed to take water samples for analytical chemistry using a glass transfer pipette submerged below the surface. Store and ship water samples according to the QAPP.

7. Add the appropriate number of fish to T6 (typically 3) and record the water quality measurements on the TRCC *Water Quality Monitoring* bench sheet (see Section C.1.3 below).

C.1.3 Fish transfer to exposure tank (T6)

1. Ensure that the T6 environment is prepared according to the protocol above.

2. Use the crowder net to isolate specific fish (tagged with small Floy tags) in tank T4 or T5, depending on specimen location.

3. Catch fish with the rubberized net and carefully walk fish to T6. Typically three fish will be placed into T6.

4. Leave fish in T6 for 24, 48, or 72 hours, depending on the length of exposure that is being run.

5. After fish are exposed to oil in T6, a portion of the water will be used to fill the respirometer for the respirometry experiment (see Section C.1.4). After filling the respirometer and its reservoir, transfer the test fish to the respirometer (see Section C.1.5).

6. Record exposure times and concentrations in the TRCC in a dedicated notebook.

7. Obtain water quality data from T6 when fish are first moved into the tank, on a 24-hour cycle, and when fish are transferred to the respirometer (see *Water quality monitoring* in Hopkins and NWFSC GLPP) and recorded on the *Water Quality Monitoring* bench sheet.

8. Take exposure water samples for chemical analysis using a glass transfer pipette submerged below the surface every 24 hours. Store and ship water samples according to the QAPP.

9. Transfer any remaining oily seawater in the tank to the Baker Tank designated for oil waste.

10. Thoroughly wipe down T6 and rinse with seawater, and then transfer that water to the Baker Tank as well.

C.1.4 Respirometer preparation

1. Fill the reservoir to the 1,000-L line with water pumped from T6 (~ 20°C) for exposed fish or T5 for unexposed fish.

2. Plug in the heater and set to 20°C to maintain a constant temperature.

3. Plug the sump pump into the computer control unit to fill 30-L respirometer (ensure the return tube properly drains back to the reservoir).

4. Turn on the flume propeller and remove any air bubbles from the interior chamber of respirometer (the speed and direction may need to be varied to dislodge all trapped bubbles).

5. Set up the video camera to monitor (open circuit) the swim chamber.

6. Roll down the black-out tarp, turn off overhead lights, and turn on the clip-in light.

7. Set speed to 1 BL/s.

8. Place O_2 and temperature sensors in the swim chamber (visually inspect to ensure sensors are clean and no air bubbles remain in the chamber).

9. Press the "measure" button on the sensor computer to ensure the temperature (~ 20°C) and oxygen (~ 95% air sat.) are favorable.

10. Take water quality measurements from the swimming chamber (see *Water quality monitoring* in Hopkins and NWFSC GLPP).

11. Water samples to be sent for analysis must be taken according to the water sampling guidelines in the QAPP.

C.1.5 Fish transfer from tank to respirometer

1. Use the crowder net to isolate specific fish in the tank (either T5 or T6).

2. Catch fish with the rubberized net and carefully walk fish to the respirometer.

3. Place fish in the swim chamber and close the lid (do not screw shut at this time).

4. Once the fish swims unaided in the correct location (forepart of the chamber), seal the lid closed.

5. Use the Excel spreadsheet that calculates speed versus length (file location and name: \\Mola\topp\TRCC\TRCC\Calibrations\Respirometers\TRCC_30L_OilSpill_respo_callibration_0411011.xls) to determine the correct motor setting and adjust it so that the speed is 1.0 BL/s.

6. On the data logging computer, press the "measure and data" button and give the file a name, including "year month day – fish identification (ID) – type of run – flume – species" (e.g., 2011 0711 – fish024 – exposure X – oil 30L – PacMack).

7. Set the oxygen drop-down button to record in mg/L, if not already set.

8. Go to the computer control box and set the interval for desired on/off flush in seconds (610 for flush, 605 for closed, if cycle is for 10 minutes).

9. Start the flush/closed cycles on a 5-minute interval (e.g., 01:05, 01:10, 01:15).

10. Record the following in a dedicated notebook: time fish entered the flume, electric speed setting used, and time flush/closed cycles were started. Also record any deviations from SOP or other relevant details (e.g., observations concerning fish swimming patterns, difficulty catching while in holding tank).

11. Record all digital data (water quality monitoring, video footage, etc.) on the dedicated oil spill computer. Data will also be backed up on the dedicated TRCC external hard drive.

12. Run the fish for 24 hours in the flume.

C.1.6 During run

1. Turn oxygen from the compressed oxygen tank on and off in reservoir as needed (maintain DO between 5.9 and 6.5 mg/L). Use O_2 flow settings "1" or "2" for up to 20 minutes to increase DO concentration.

2. Conduct tailbeat frequency counts hourly by watching the live video feed and counting how many times the tail beats in 1 minute during the closed cycle. Record these data into the dedicated notebook.

3. Record the time of any disturbances in the Tuna Center (e.g., slammed doors, accidental changes in lighting), including the time at which they occur and any changes in the fish's swimming patterns.

4. Record any changes in the swimming behavior of the fish.

5. Record any entrances or disturbances to the respirometry black-out area.

6. At the end of the respirometer run, wipe down and rinse the reservoir and respirometer, transferring all oily wastewater to the Baker Tank for disposal.

C.1.7 Fish removal

1. Slowly enter the black-out area.

2. Quickly unseal the chamber.

3. Remove the fish with a small net and return it to the appropriate holding tank or, if tissue sampling is to be performed, sample appropriate tissue (Section C.1.8). If bile is to be collected, follow the instructions in Section C.1.9.

4. Take water quality sample from swimming chamber.

5. Turn off the data logging on the data logging computer.

6. Upload the file to a secure network location.

7. Copy all digital files onto the TRCC external hard drive.

C.1.8 Tissue collection

After the fish was euthanized by pithing, the body cavity was opened with a clean scalpel. Using a new scalpel blade the internal organs were severed at the esophagus and the entire internal organ mass was removed and placed on aluminum foil.

C.1.9 Fish bile collection and storage

1. Euthanize/sacrifice animal.

2. Open the body cavity using scissors and forceps, or a clean knife if necessary on large fish. Use one set of tools to cut open the animal and a separate set for cutting tissue sections inside the animal.

3. Note the gender of the fish, if possible, based on the presence of testes or ovaries. Disconnect the esophagus and pull the entire internal organ mass gently away from the fish cavity. Place the excised internal organ mass on a clean sheet of aluminum foil on the cutting board.

4. If there is blood on the outside of the gall bladder, rinse the gall bladder with distilled water (contained in squirt bottle).

5. Separate the gall bladder (sac-like organ that is a green to yellow color, and either bulbous or elongate depending on species) from the liver, being sure to grip it by the bile duct or nearby connective tissue to prevent bile from flowing out of the bladder.

6. Hold the gall bladder at the mouth of the amber vial, and puncture the bladder with the scalpel blade, thus directing the bile fluid into the vial.

    a. The volume of bile collected into the individual 4-mL vial should be > 50 µL, but as much bile as possible should be collected, leaving some space at the top of the 4-mL vial to allow for expansion during freezing.

        i. With small fish that do not yield 50 µL, form a composite sample from fishes for up to 200 µL. These composites should be collected into inserts provided with the amber vials. Document in the notebook how many fish were used to make the composite sample and each fish's weight and length.

        ii. For large gall bladders (> 1.5 cm diameter), use a 1-cc tuberculin syringe to draw bile from the gall bladder; high-volume samples may be stored in 20-mL scintillation vials; however, those samples must be wrapped in foil

to avoid any exposure to light. Do not reuse syringes, and when disposing of them, do not re-cap the needles.

7. Verify the proper labeling of the sample vial, and place on ice in a covered cooler. Samples must be transferred to -20°C for longer-term storage.

8. Replace the viscera into the body cavity. Wrap fish in aluminum foil, and place in a zip-loc freezer bag that is labeled with a unique Stratus Consulting Sample ID (example: HS-B0926-TA-451-101).

9. Rinse the scalpel blade and dissecting tools with isopropanol and shake dry between each fish. If tools become fouled with tissue, wash them with soap and water, and rinse with isopropanol. Replace scalpel blades instead of washing them.

10. Change scalpel blades when switching to a different species of fish, or between sites or treatment groups.

    a. Hold the scalpel by the handle with the blade pointing away from any person, and ensure that the blade and blade lock face upward with the slanted blade end facing your hand.

    b. Grip the slanted edge with forceps, ensuring a secure hold to avoid slipping.

    c. Lift the blade from the slanted end until the lock hole of the blade separates from the lock. The blade should be loose at this time.

    d. Pull blade away from the tip to remove. Dispose of blade in a sharps container.

Appendix References

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5. Miami University of Ohio General Laboratory Procedures and Practices

5.1 Methods

Miami University of Ohio (MUO) conducted laboratory studies with low levels of simulated sunlight under constant controlled conditions. Laboratory tests simulated the diminished levels of ultraviolet (UV) radiation present at water depths up to 10 m. These experiments were used to establish the lower range of photo-induced toxicity. In addition, holding other environmental variables constant (e.g., intensity of light over time, temperature, photoperiod) provided greater control of test conditions.

5.1.1 Test organism sources and husbandry

This section describes the sources and husbandry for sheepshead minnow (*Cyprindon variegatus*) and mahi-mahi (*Coryphaena hippurus*) used in the toxicity tests. The test organisms were provided by independent culture facilities and transported to test laboratories at MUO.

Sheepshead minnow

Sheepshead minnow adults were obtained from Aquatic Biosystems Inc. in Ft. Collins, Colorado. The MUO protocol for maintenance and spawning of sheepshead minnow can be found in Section 5.3.1.

Mahi-mahi

Mahi-mahi embryos used in MUO testes were shipped from the University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS). The RSMAS General Laboratory Procedures and Practices (GLPP; Section 7.1.1, *Test organism sources and husbandry – cobia and mahi-mahi*) contains information regarding the source and husbandry of these organisms.

5.1.2 Exposure media preparations

Test media were prepared according to established protocols. See *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The synthetic saltwater used in all exposure studies was prepared with Fritz's Sea Salt or Instant Ocean mixed with dechlorinated water (DCW) from MUO.

The experimental design was factorial and included two oil types [Slick A (CTC02404-02) and weathered source oil (072610-W-A)], two water accommodated fraction (WAF) preparation methods [high-energy (HEWAF) and chemically enhanced (CEWAF)], and two UV radiation treatments. Each test included a control and four WAF concentrations.

5.1.3 Whole organism exposures

Test procedures

Details regarding test procedures and testing apparatuses are outlined in the testing protocols in the appendix of the MUO GLPP. In short, organisms were exposed to treatment media in glass crystallizing dishes (exposure chambers). Loading of organisms into exposure chambers never exceeded 0.5 g/L. Each test used different WAF concentrations, as well as negative (i.e., no oil) controls [specific concentrations can be found in test-specific test conditions tables (TCTs)]. Exposures were carried out as static renewals with daily water changes. Treatments included different WAF concentrations and two UV treatments, with or without UV. Information regarding the light intensity used during each test can be found in the test-specific TCTs. Concentrations of oil, determined in consultation with Stratus Consulting and the National Oceanic and Atmospheric Administration, can be found in the test-specific TCTs.

Each test included WAF stock samples, archive water samples, and fluorescence water samples. WAF stock water samples were sent to ALS Environmental for chemical analysis. The types of analyses were recorded on Chain of Custody (COC) forms when samples were sent to ALS, and depended on the WAF preparation method used. If the same stock solution in which samples were previously drawn was used to renew test media, then WAF stock water sampling was not repeated on this solution. This would only occur with WAF stock solutions that were less than 24 hours old and had been securely stored in a cool, dry, and dark location. See the QAPP for more details.

Fluorescence water samples were collected from each WAF stock and dilution series. These samples were used to generate standard curves for analyzing dilution series samples. See the QAPP for more details.

When possible, all organisms used in toxicity tests were retained as archive tissue samples. These samples included dead organisms removed from test chambers when making daily observations or renewals, and any remaining organisms left at the end of each test. Archive tissue samples were placed in sample containers with as little water as possible; this sometimes required blotting the sample on a clean Kimwipe prior to placing it in a sample container.

Equipment used in processing archive tissue samples was made of stainless steel, anodized aluminum, borosilicate glass, polytetrafluoroethylene, ceramic, or quartz (U.S. EPA, 2000). New, certified pre-cleaned borosilicate glass or polytetrafluoroethylene bottles were used to store tissue. Some tissue samples were wrapped in aluminum foil and placed into a properly labeled sample containers or plastic bags. All retained samples were securely stored at -20°C. Sample labeling, storage, and shipping methods followed requirements outlined in the QAPP.

5.1.4 Phototoxicity model development

A global model of polycyclic aromatic hydrocarbons (PAHs) phototoxicity was developed over the past 25 years (Jeffries et al., 2013). Photo-enhanced toxicity is a function of (1) a particular PAH's ability to generate reactive oxygen species upon absorption of actinic UV radiation, (2) the extent to which a PAH is taken up by an organism (i.e., combination of bioavailability and bioaccumulation potential – aka fugacity), and (3) the intensity of actinic UV exposure. Model input is thus a combination of structure-activity data on compound-specific levels of phototoxicity, bioaccumulation data, and quantitative information on intensity and duration of UV exposure. Model output is the predicted time-to-death for an organism exposed to combinations of PAH levels that are taken up by an organism, and UV intensity and duration. This output can be used to estimate site-, time-, season-, or depth-specific phototoxic injury for specific mixtures of PAH. The model takes the form of:

$$TTD = f\left\{\sum_{i=1\text{-}n}([PAH]_i * RPA_i) * (UV)\right\}, \qquad \text{Equation 1}$$

where TTD = predicted time to death; $[PAH]_i$ = molar body burden of PAH "i" over PAH "i" to "n;" RPA_i = relative photodynamic action of PAH_i (ratio of phototoxicity intensity of PAHi and anthracene – a PAH with a median level of phototoxicity); and UV = dose of actinic UV radiation exposure (typically expressed as broad-band UV-A radiation in the range of 320–400 nm).

This model can be used to predict time-to-death under specific conditions or can be used to determine predicted no-effect levels of PAH and UV exposure on a site-specific basis. Data from tests conducted under the MUO GLPP were modeled using these concepts to predict levels of toxicity and areas of injury within the Deepwater Horizon spill zone. In addition, the data were incorporated into the global model of phototoxicity, and model comparisons served as one form of validation of both the model and its predictions.

5.1.5 Water quality monitoring

Water quality was monitored as described in the QAPP and using the *MUO protocol for water-quality monitoring during Deepwater Horizon toxicity testing standard operating procedures*

(SOP) in Section 5.3.4. Dissolved oxygen (DO), pH, conductivity, salinity, ammonia, and temperature were measured once daily from each test medium stock. Temperature, DO, salinity, and ammonia were also measured once over the course of the test in each of five randomly selected test chambers prior to renewal. In addition, solar radiation was measured using a calibrated biospherical radiometer, which takes continuous measurements of UV during the entire test period. Calibration was performed and certified by Biospherical Instruments in San Diego, California.

5.1.6 Analytical chemistry

Analytical samples were taken as described in the QAPP.

Analytical chemistry was performed on WAF stock water samples by ALS Environmental. A set of water samples was collected from each WAF stock solution. Sample type, bottle type, and requested analyses were specific to the test oil type and WAF preparation method as described in the QAPP. Sample collection, labeling, shipping, and custody procedures followed guidelines specified in the QAPP.

All archive water samples were unfiltered and collected in 250-mL amber glass bottles and stored at 4°C. When taken, all archive samples were sent to ALS Environmental for extraction and preservation. COC forms indicated that these samples were to be extracted and archived by marking the "Extract and archive only" box for each sample.

Archive tissue samples were collected and preserved according to methodologies required for organic analysis outlined in the QAPP. The samples were securely stored at -20°C at the testing laboratory. COC forms were used to document the transfer of archive tissue samples to the storage facility.

5.2 Reporting and Testing Documentation

All documentation of test procedures, results, etc., was carried out as described in the QAPP. Documentation was provided to Stratus Consulting via test-specific bench sheets and electronic and hard copies were stored at MUO.

5.3 General Testing SOPs

5.3.1 Laboratory culture and maintenance of the sheepshead minnow SOP

Purpose

This section provides an outline of procedures for the successful culture of sheepshead minnow.

Procedures

A. Source of organisms

Sheepshead minnows were obtained from Aquatic Biosystems Inc. An in-house laboratory culture facility served as the source for eggs and other organism developmental stages for use in toxicity tests, or to increase the broodstock supply.

B. Laboratory culture facility

Sheepshead minnows were maintained in aquaria in the MUO Animal Facility. The culture facility comprises the following main components:

1. Water supply
2. Holding aquaria in which young or adult broodstock were held
3. Egg incubation units in which fertilized eggs were placed to hatch
4. Water baths and culture containers for the care of fry.

Water

A. Synthetic saltwater was mixed from Instant Ocean or Fritz's Sea Salt and DCW from the MUO Animal Facility. The DCW was City of Oxford (Ohio) well water conditioned by carbon filtration and blended with reverse-osmosis deionized water to a hardness of 150 mg Ca/L, stored and aerated in a 7,600-L recirculating [closed, high-efficiency particulate absorption (HEPA)-filtered, UV-sterilized] tank.

The synthetic saltwater was held in a recirculating 400-L polyethylene holding tank with UV sterilization. Salinity was generally maintained between 20 and 25 ppt.

B. Adult broodstock were maintained in static tanks. Water was continuously aerated and biologically filtered with recirculating power filters (e.g., Penguin Biowheel and Aqueon Quiet Flow models), and a 20–30% water change was effected twice weekly. DCW was added as needed to compensate for evaporation.

Water quality parameters (i.e., salinity, pH, temperature, and DO) were regularly measured and recorded.

General culture conditions

A. Adult fish were generally maintained at a density of approximately 0.5 g/L on a 16-hour light:8-hour dark photoperiod, at a temperature of 21–25°C, and a salinity of 15 ppt in 20-gal aquaria (Figure 5.1).

B. Adult fish were fed *Artemia* nauplii and Tetramin dry-flake food twice daily.

Figure 5.1. Broodstock tanks for adult sheepshead minnows in Room 47B of the MUO Animal Facility.

Spawning

Individuals selected for use in egg production were those that were actively feeding, exhibited normal swimming patterns, and reflected no outward indications of stress or abnormality. Brood fish ranged in age from 3–24 months. Adult broodstock obtained from Aquatic Biosystems were generally 7–8 months old.

A. Natural egg production

Females produced 10–25 eggs per day. At culture temperatures (~ 25°C), fertilized eggs hatched in 5–6 days. At times embryo production was enhanced by maintaining the temperature in the broodstock tanks at closer to 30°C. Supplemental feedings were also used to improve embryo production.

The egg collection method follows:

1. When embryos were required, broodstock were transferred from their holding tanks to spawning tanks prepared with "spawning rings" (generally 5–7 days prior to the initiation of a test). The spawning tanks were 20-gal glass aquaria equipped with recirculating power filters. Generally, six females and four males were placed in each tank. The males selected a territory (i.e., spawning ring) and attracted females to spawn over them.

2. The spawning rings were a modified "Schesny ring" design consisting of a 4" diameter polyvinyl chloride (PVC) ring fitted with a coarse (3–5 mm) Nitex mesh above and a fine (200 micron) mesh below (Figures 5.2 and 5.3). Two to three spawning rings were placed in each tank (Figure 5.4). Spawning tanks were generally 1°C warmer than holding tanks (~ 25°C). Spawning generally began within 24 hours or less. Embryos fell through the coarse mesh and onto the collecting screen. They were collected for 24 hours and then transferred to crystallizing dishes for incubation. Spawning rings were replaced in the spawning tanks until a sufficient number of embryos had been collected. To help keep the embryos clean, the adults were fed while the spawning rings were removed.

Figure 5.2. Spawning ring for inducing natural spawning in sheepshead minnows.

Figure 5.3. Spawning ring with coarse mesh top removed.

Figure 5.4. Spawning tank with spawning rings in place.

3. Alternatively, spawning chambers placed into designated tanks were used. The spawning chambers were rectangular boxes, approximately 20 × 35 × 22-cm high, with sides made of polycarbonate. Windows on two sides of the box increased water circulation through the chamber. If parents were selected, male and female fish were isolated in spawning chambers placed into designated tanks or raceways. Generally, three females and two males were sequestered in each spawning chamber that met these specifications.

4. During the egg collection period, each spawning chamber sat atop an egg collection tray, which consisted of a Nitex screen attached to a polycarbonate frame.

Embryo/fry care

A. Egg collection

Each spawning ring was checked daily for the presence of eggs (prior to cleaning or feeding).

1. If eggs were present, they were quickly transferred to glass crystallizing dishes (700-mL capacity) for incubation. Care was taken to keep the eggs submerged in water whenever possible.

    a. A crystallizing dish was filled with ~ 250 mL of synthetic saltwater from the recirculating synthetic saltwater holding tank.

    b. The spawning ring was inverted over the crystallizing dish and the underside of the spawning ring was sprayed vigorously (using a squirt bottle with synthetic saltwater) to transfer the eggs into the crystallizing dish. The mesh and the area around the silicone bead on the top of the spawning ring were checked carefully for eggs, and any remaining eggs were sprayed into the crystallizing dish.

    c. Eggs were transferred at a density of 1–4 per 10 mL of water (i.e., ≤ 200 eggs per dish).

2. When eggs were used for toxicity testing, an initial count of the eggs was required. The crystallizing dish was placed on a light table and the embryos were counted.

3. Incubation dishes were labeled with the DATE, the TANK # from which the eggs were collected, and the # of EGGS in the dish. The water level was also marked on the side of the dish as a reference for water renewal.

4. The dishes were placed on a shaker table at a moderate speed to promote aeration and minimize fungal growth.

B. Egg care (through hatching)

1. Egg incubation units were checked daily on a light table or with a dissecting scope. Unfertilized eggs and eggs that had become infected by fungus were removed and discarded.

2. Hatched fry were removed daily, placed in larval containers (generally, ~ 10-L square plastic containers) with approximately 6 L of synthetic saltwater, and maintained in a water bath at 25°C. Fry were maintained at a density not exceeding 100 per 2 L.

    a. Larvae were transferred with a wide bore plastic transfer pipette. Larvae were counted during transfer and containers were labeled with the HATCH DATE and # OF LARVAE.

3. Containers were aerated using a disposable glass pipette attached to the air supply line with silicone tubing. The air flow was high enough to generate a constant stream of air bubbles, but not high enough to cause ripples on the water surface.

C. Larval care

1. A 75% water change was effected daily in each bowl or tank, and water quality (i.e., temperature, salinity, pH, DO) was monitored daily. Water was poured from the culture vessel into a second container. In this way, any larvae accidentally poured out were recovered and placed back into the culture vessel. Synthetic saltwater (from the holding tank) was added to reach the 6-L mark, and containers were returned to the water bath and aerated.

2. During their first week post-hatch, fry were fed *Artemia* nauplii twice daily. During the third week post-hatch, a dry-flake food feeding was introduced. Two or three weeks post-hatch, fry were transferred to a larger aquarium or chamber (10-gal).

Post-hatch fry care and growth

Post-hatch fry were maintained in aquaria until their use in testing or their release into the broodstock pool. When fry reached an average length of approximately 15- to 20-mm (standard length), 50 fish were loaded into 10-gal aquaria, which offered rapid growth potential. After reaching an average size of 28- to 30-mm (standard length), the tanks were thinned to approximately 25 fish. Optimal growth was attained at a temperature of 25 to 30°C and a 16-hour light:8-hour dark photoperiod.

5.3.2 Protocol for the culture and collection of *Artemia* for sheepshead minnow SOPs

1. The air supply was removed from the appropriate *Artemia* hatching chamber. The eggs (cysts) and hatched *Artemia* were allowed to separate, which took 5–10 minutes.

2. A 1-L glass beaker was filled with approximately 100 mL of saltwater from the 20-L carboy in the sheepshead minnow fish culture area.

3. Hatched *Artemia* were captured by pouring the hatching jar contents (excluding the very top layer of unhatched eggs) into a fine-mesh round sieve. *Note:* The round sieve was suspended inside a square plastic collecting container so that the water that passed through the sieve could be returned to the hatching jar after the *Artemia* were filtered out.

4. Using a squirt bottle filled with DCW, the sieve was sprayed to concentrate the *Artemia*. The *Artemia* were then poured into the 1-L beaker containing 100 mL of synthetic saltwater, and the sieve was thoroughly rinsed in the beaker using the squirt bottle and spraying vigorously.

5. The 1-L beaker (filled with *Artemia* and some remaining cysts) was placed near a light source for 3–5 minutes.

6. Water in the square plastic collecting container was poured back into the hatching jar. It was poured down the edges of the hatching jar to rinse *Artemia* eggs caught on the sides back into the solution.

7. After 3–5 minutes, the *Artemia* in the 1-L beaker had congregated near the light source. They were then separated from the layer of cysts on the bottom of the beaker. A plastic transfer pipette was used to carefully siphon out the *Artemia* nauplii. The nauplii were then placed into a 100-mL glass beaker.

8. The remaining cysts in the 1-L beaker were poured back into the hatching jar.

9. The air hose was then returned to the chamber.

10. Using a graduated disposable glass pipette, the *Artemia* were thoroughly stirred to distribute them evenly in the water. Sheepshead minnow larvae were fed 0.5 mL per test dish. The remaining nauplii were fed to adults.

11. The collecting container, the sieve, and the 100-mL and 1-L glass beakers were thoroughly rinsed with tap water.

5.3.3 Protocol for the daily care of sheepshead minnow SOPs

Daily care overview

1. The temperature and salinity of each tank were measured and recorded on the International Animal Care and Use Committee (IACUC) #838 daily care log. Temperature was between 21 and 25°C and salinity was between 20 and 25 ppt.

2. Fish were fed twice daily at 7–8 a.m. and 3–4 p.m. (see details below).

3. Aquaria were cleaned if necessary. Larval tanks received a minimum 50% water change once daily. Adult tanks were changed once weekly (see details below).

4. Any abnormal changes of tank systems or fish health/behavior were reported. Dead fish were removed and recorded on the daily care log.

Detailed instructions

Feeding: Each tank received food as follows:

Tank #	Food
1–4 (adult broodstock)	***2 large pinches of Tetramin fish flakes*** – Males in tanks 1 and 2 may have taken 3 pinches but fish were fed no more than what would be consumed in 5 minutes. ***Artemia*** – Adults were fed whatever remained after feeding larvae evenly across tanks with a plastic transfer pipette.
1 (larva)	***Artemia*** – Fed 4 full pipettes.
2 (larvae)	***Artemia*** – Fed 2 full pipettes.

Cleaning

A. Larval tanks – once daily

1. After water quality was checked, at least 50% of the tank water (~ 1.5 L) was poured into a separate container. Larvae that were accidentally poured out of the culture tank were retrieved.

2. The culture tank was refilled to the 3-L mark with saltwater from the 20-L carboy in the sheepshead culture area.

B. Adult tanks – once weekly

1. Water was siphoned from the tank to remove any remaining fish waste and food. Siphoning generally removed approximately 25% of the water from each tank (~ 15 L).

2. Tanks were refilled with water from the synthetic saltwater holding tank.

5.3.4 MUO protocol for water-quality monitoring during Deepwater Horizon toxicity testing SOPs

Temperature, pH, and conductivity/salinity were measured with a portable VWR sympHony multimeter (model SP90M5) and probes. DO was measured with the Mettler Toledo SevenGo pro optical DO meter. Total ammonia was measured with a Hann Instruments 93700 photometer.

pH

Test solution pH was measured with the VWR sympHony multimeter and a gel-filled 3-in-1 pH/ATC electrode with an epoxy body (Model 14002-860).

A. Electrode calibration

1. pH calibration with two buffers (performed once monthly or daily whenever one-point calibration checks were outside the acceptable range as described below)

   a. Press the power key to turn on the meter.
   b. Select two buffers that bracket the expected sample pH and are one to four pH units apart (e.g., 7.00 and 10.01).
   c. Press the calibrate key.
   d. Rinse the electrode with deionized water and blot dry with a lint-free tissue. To avoid static buildup, do not wipe or rub the electrode.
   e. Insert the electrode into the first buffer and gently stir.

f. Wait for the pH icon to stop flashing and the pH value to appear in the meter window. The meter should display the temperature-corrected pH buffer value. If the displayed buffer value is incorrect, enter the value by pressing the up/down arrow keys to adjust each digit and the digits key to move to the next digit.
g. Press the calibrate key.
h. Rinse the electrode with deionized water and blot dry with a lint-free tissue.
i. Insert the electrode into the second buffer and gently stir.
j. Wait for the pH icon to stop flashing and the pH value to appear in the meter window. The meter should display the temperature-corrected pH buffer value. If not, enter the correct value as above.
k. Press the calibrate key and then press the measure key to save and end the calibration. The slope will be displayed and the meter will proceed to the measurement mode.

2. pH one-buffer calibration check (to be performed daily prior to measurements)

a. Choose a buffer near the expected sample pH (e.g., 7.00).
b. Rinse the electrode first with distilled water and place it into the buffer.
c. When the reading is stable, observe the measured pH. If the measured value is within 5% of the pH value of the buffer, proceed with measurements. If the reading is greater than 5% ± of the expected pH, proceed with the two-buffer pH calibration described above.

B. pH measurements

1. Calibrate the electrode as described in the "Electrode calibration" section below.

2. Rinse the electrode with deionized water, blot dry with a lint-free tissue, and insert the electrode into the sample.

3. With the meter in AUTO-READ mode, press the measure key to start the reading. The auto-read icon will flash until the reading is stable. Once the reading is stable, record the pH value on the appropriate data form.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

Soak the electrode in a pH electrode storage solution (Model 14002-828).

Conductivity/salinity

Test solution conductivity and salinity were measured with the VWR sympHony multimeter and a VWR epoxy body two-cell platinum conductivity probe (Model 11388-372).

A. Electrode calibration

1. Conductivity calibration was performed daily prior to measuring test solutions.

   a. Press the power key to turn on the meter. Use the line select key to move the arrow icon to the conductivity measurement line.
   b. Select the VWR conductivity standard that has the closest conductivity to the expected sample value (e.g., 12.9 mS/cm).
   c. Enter the nominal cell constant value for the conductivity probe as follows:

      i. Press the setup key.
      ii. Press the up arrow key until COND is displayed on the top line.
      iii. Press the line select key to move the arrow icon to the middle line. Press the up arrow key until CELL is displayed.
      iv. Press the line select key to move the arrow icon to the bottom line. Enter the nominal cell constant by pressing the up/down arrow keys to adjust each digit and the digits key to move to the next digit. 0.475 cm-1 is the default setting.

   d. Rinse the conductivity probe with deionized water and blot dry with a lint-free tissue.
   e. Insert the conductivity probe into the conductivity standard and gently stir.
   f. Press the calibrate key. The meter will show the manual calibration display for about five seconds. During this time, do not press any keys.
   g. After about 5 seconds, the meter will proceed to the autocalibration display. Wait for the µS/cm or mS/cm icon to stop flashing and the arrow icon to start flashing. The meter should display the conductivity standard value at 25°C.
   h. Press the measure key to save and end the calibration.

B. Conductivity/salinity measurements

1. Calibrate the electrode as described in the "Electrode calibration" section in the DO section below.

2. Rinse the conductivity probe with deionized water, blot dry with a lint-free tissue, and insert the probe into the sample.

3. With the meter in AUTO-READ mode, press the measure key to start the reading. The auto-read icon will flash until the reading is stable. Once the reading is stable, record the conductivity and salinity values on the appropriate data form.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

The conductivity electrode should be stored clean and dry.

DO

DO in test solutions was measured with the Mettler Toledo SevenGo pro optical DO meter.

A. Electrode calibration

Calibration was performed weekly. The first point of a DO calibration was always done in water-saturated air (100% O_2).

1. Remove the OptiOx calibration tube cap and then the sponge from the cap.

2. Saturate the sponge with distilled water and squeeze the excess water out of the sponge.

3. Reassemble the OptiOx calibration tube.

4. Ensure that no water droplets are on the surface of the OptiOx sensor cap.

5. Slide the calibration tube over the front of the sensor until the calibration tube is firmly connected to the sensor.

6. Allow at least 5 minutes for the temperature to stabilize prior to calibration.

7. Press and hold MODE for 3 seconds to switch to the single-channel measurement screen when in dual-channel measurement.

8. Press CAL. Cal 1 will appear on the display. The meter calibrates according to the preselected endpoint mode automatically after the signal has stabilized or after pressing READ. The standard value is shown on the display.

9. Press End to accept the calibration and return to sample measurement. The calibration result is shown on the display.

10. Press Exit to reject the calibration.

B. DO measurements

1. Place the sensor in the sample. The InLab OptiOx must be immersed in a minimum 3.5-cm solution so that the temperature sensor is covered.

2. Press READ to start a measurement. As soon as the measurement is stable according to the selected stability criterion, the Stability icon will appear.

3. Press READ to manually stop the measurement and record the DO value.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft-bristled brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

Place the DO sensor in the calibration tube, making sure to wet the sponge with distilled water.

Ammonia

Total ammonia was measured using a Hanna Instruments 93700 photometer. This instrument uses an adaptation of the ASTM International Manual of Water and Environmental Technology, D1426-92, Nessler method.

A. Measurement procedure

1. Turn the meter ON.

2. Remove the cuvette cap. Fill the cuvette to 1.5 cm below the rim with 10 mL of unreacted sample and replace the cuvette cap.

3. Place the cuvette into the photometer and ensure that the notch on the cap is positioned securely into the groove.

4. Press ZERO and "SIP" will appear on the display.

5. Wait for 5 seconds and the display will show "-0.0-," which means that the meter is zeroed and ready for measurement.

6. Remove the cuvette.

7. Remove the cuvette cap and add 6 drops of Reagent #1. Replace the cap and swirl the solution for 5 seconds.

8. Remove the cap and add 10 drops of Reagent #2.

9. Replace the cap and swirl the solution for 5 seconds.

10. Reinsert the cuvette into the instrument.

11. Press READ TIMED and the display will show the countdown prior to the measurement (e.g., 3 minutes and 30 seconds).

12. The instrument directly displays the concentration in mg/L of ammonia nitrogen (NH_3-N). To convert the reading to mg/L of ammonia, multiply the display reading by a factor of 1.214.

13. *Note*: The cuvette should be cleaned between each test solution as follows: Rinse the cuvette with a 1% Alconox solution, gently clean with a cotton-tipped applicator, and follow with three rinses of distilled water.

5.4 MUO Test Media Disposal Guidelines

1. All oil-containing solutions were disposed of by draining through an activated charcoal bed, with outflow leading into a sink drain.

2. Any solid pieces (e.g., weathered oil) were removed from the charcoal bed after use and placed in a waste vessel stored in a fume hood.

3. At the completion of the project, both the activated charcoal bed and solid waste were disposed of under the chemical safety and disposal regulations of the Environmental Health and Safety Office at MUO.

References

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A. Testing Protocol 1: Assessing Photoenhanced Toxicity of Deepwater Horizon Oil to Mahi-mahi (*Coryphaena hippurus*)

A.1 Testing Apparatus

The indoor testing apparatus consists of a bank of solar-simulating light bulbs and a water table covered in either UV transparent (Aclar) or UV opaque (Courtgard) plastics. Courtgard (CP Films, Inc., Martinsville, VA, USA) is a long-wave-pass plastic that in water transmits photosynthetically active radiation (PAR; 95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm, with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ, USA; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both photosynthetically active radiation (PAR; 100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acts simply as a cooling bath and organisms are never in contact with cooling water.

Feed water into the water table and vary the rate of flow to maintain a constant temperature during the test. Provide simulated sunlight by a bank of 84 solar-simulating fluorescent bulbs (Durotest Vitalite, 40W, and fluorescent blacklight bulbs), suspended from a grid above the laboratory, with the height above tanks adjustable between 0.5 and 2.5 m. This setup provides a spectrum that is > 90% equivalent to natural sunlight at an intensity approaching 10% of natural sunlight.

A.2 Test Procedure

A.2.1 Prior to test initiation

1. Prepare WAF according to the *Protocols for Preparing Water Accommodated Fractions* SOP as described in the QAPP. Retain samples as specified in the *Analytical Sample Shipping and COC* SOP as described in the QAPP.

2. Bench sheets should be filled out as described in the QAPP (e.g., ”WAF preparation”).

A.2.2 ~ 12 hours prior to test initiation

1. Turn on water to water tables to cool to desired temperature.

2. Prepare appropriate dilutions of test media from stock WAF. The dilution series should include a synthetic saltwater blank and span the working range of WAF to be used in the test (see test-specific TCTs). Dilutions should be mixed in clean, large (≥ 4 L) glass flasks using the same synthetic saltwater source used for WAF preparations. Mix well between each serial dilution. Retain samples as described in the QAPP.

3. Sample water quality in test media dilutions (as described in the MUO GLPP).

4. Obtain clean, glass crystallizing dishes. Label each dish with the test treatment and tank # (replicate number) according to QAPP guidelines.

5. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately (as described in the MUO GLPP).

6. Place organisms into each replicate dish (see test-specific TCTs for the total number of organisms to add to each replicate).

7. Place test dishes randomly on the water table under appropriate plastic according to UV + or UV- treatment.

8. Start UV monitor. UV will be monitored continuously using a Biospherical Instruments multi-wavelength radiometer. The radiometer measures wavelengths in the UV-B, UV-A, and visible light spectra.

9. Bench sheets should be filled out as described in the QAPP.

10. Leave dishes in a secure laboratory area in the dark until test initiation.

11. After approximately a 12-hour uptake period, turn on UV lights (48-hour test period begins).

12. After 14 hours of exposure to artificial sunlight, turn off UV lights.

13. Measure temperature and DO content of five randomly selected dishes from each UV treatment.

14. Prepare WAF and retain sample according to protocols described in step 2. Prepare appropriate dilutions of test media from WAF. Retain samples as described in the QAPP.

15. Sample water quality in test media dilutions (as described in the MUO GLPP).

16. Remove at least 90% of the test medium (~ 180 mL) from each replicate dish using a syringe. Dispose of test media appropriately (as described in the MUO GLPP).

17. Using a glass graduated cylinder and the appropriate WAF dilution, replace the test media in each replicate dish with a volume equal to the volume extracted.

18. Return test dishes on the water table to respective location under appropriate plastic according to UV + or UV- treatment.

19. After 10 hours of "dark," turn on UV lights. Repeat steps 12 and 13.

20. At the conclusion of the test, count the dead and living organisms. All dead test organisms should be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

B. Testing Protocol 2: Assessing Photoenhanced Toxicity of Deepwater Horizon Oil to Embryonic and Larval Sheepshead Minnow (*Cyprinodon variegatus*)

B.1 Testing Apparatus

The indoor testing apparatus consists of a bank of solar-simulating light bulbs and a water table covered in either UV transparent (Aclar) or UV opaque (Courtgard) plastics. Courtgard (CP Films, Inc., Martinsville, VA, USA) is a long-wave-pass plastic that in water transmits PAR (95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ, USA; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both PAR (100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acts simply as a cooling bath and organisms are never in contact with cooling water.

Feed water into the water table and vary the flow rate to maintain a constant temperature during the test. Simulated sunlight is provided by a bank of 84 solar-simulating fluorescent bulbs (Durotest Vitalite, 40W, and fluorescent blacklight bulbs), suspended from a grid above the laboratory, with the height above tanks adjustable between 0.5 and 2.5 m. This setup provides a spectrum that is > 90% equivalent to natural sunlight at an intensity approaching 10% of natural sunlight.

B.2 Test Procedure

B.2.1 Prior to test initiation

1. Prepare WAF according to the *Protocols for Preparing Water Accommodated Fractions* SOP as described in the QAPP. Retain samples as specified in the *Analytical Sample Shipping and COC SOP* as described in the QAPP. Prepare WAF daily for subsequent renewals.

2. Bench sheets should be filled out as described in the QAPP (e.g., "WAF preparation").

B.2.2 ~ 12 hours prior to test initiation

1. Turn on water to water tables to cool to desired temperature.

2. Prepare appropriate dilutions of test media from stock WAF. The dilution series should include a synthetic saltwater blank and span the working range of WAF to be used in the test (see test-specific TCTs). Dilutions should be mixed in clean, large (≥ 4 L) glass flasks using the same synthetic saltwater source used for WAF preparations. Mix well between each serial dilution. Retain samples as described in the QAPP.

3. Sample water quality in test media dilutions (as described in the MUO GLPP).

4. Obtain clean, glass crystallizing dishes. Label each dish with the test treatment and tank # (replicate number) according to QAPP guidelines.

5. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately (as described in the MUO GLPP).

6. Place fertilized embryos or larvae into each replicate dish (see test-specific TCTs for total number of organisms to add to per replicate). Ensure that embryos are less than 24 hours post-fertilization at test start.

7. Place test dishes randomly on the water table under appropriate plastic according to UV + or UV- treatment.

8. Start UV monitor. UV will be monitored continuously using a Biospherical Instruments multi-wavelength radiometer. The radiometer measures wavelengths in the UV-B, UV-A, and visible light spectra.

9. Fill out bench sheets as described in the QAPP.

10. Leave dishes in a secure laboratory area in the dark until test initiation.

B.2.3 After ~ 12 hour uptake period (early morning of day 1)

1. Prepare appropriate dilutions of test media from stock WAF. Retain samples as described in the QAPP.

2. Sample water quality in test media dilutions (as described in the MUO GLPP).

3. Count the dead and living organisms in each replicate dish. Remove dead organisms and retain according to the QAPP guidelines. Do not replace organisms. Fill out bench sheets as described in the QAPP.

4. Measure temperature and DO in five randomly selected dishes from each UV treatment (as described in the MUO GLPP).

5. Remove at least 90% of the test medium (~ 180 mL) from each replicate dish using a syringe. Dispose of test media appropriately (as described in the MUO GLPP).

6. Using a glass graduated cylinder and the appropriate WAF dilution, replace the test media in each replicate dish with a volume equal to the volume extracted.

7. Return test dishes on the water table to respective location under appropriate plastic according to UV + or UV- treatment.

8. Feed Artemia nauplii (larvae only) ad libitum (as described in the MUO GLPP).

9. Turn on UV lights (larvae-only 96-hour test period begins).

B.2.4 After 14 hours of exposure to artificial light

1. Turn off UV lights.

B.2.5 Early morning of day 2

1. Repeat procedures from Section B.2.3. Note that WAF is prepared daily (as in Section B.2.1) for subsequent test solution renewals.

Carry out test until the last organism in the lowest WAF concentration has died or for a maximum of 7 days. Retain all remaining test organisms as archive tissue samples following the methodology described in the Status Consulting QAPP. At the conclusion of the test, count the dead and living organisms. All dead test organisms will be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

6. Mote Marine Laboratory General Laboratory Procedures and Practices

6.1 Introduction

Mote Marine Laboratory (Mote) conducted toxicity tests on two fish species [red drum (*Sciaenops ocellatus*) and inland silverside (*Menidia beryllina*)] to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil. This chapter describes the General Laboratory Procedures and Practices (GLPP) used at Mote.

6.2 Methods

This section describes sources and respective husbandry/maintenance procedures for test organisms that were used for toxicity evaluations.

6.2.1 Fish culturing

Fish that were produced for the toxicity studies were obtained from appropriate fish culturing facilities for each species. Each species had its own set of rearing conditions, which are described below. Juvenile fish were produced in 3,300-L nursery tanks to the desired size for the trials.

Red drum

Red drum were obtained from the Florida Fish and Wildlife Conservation Commission Stock Enhancement Research Facility. Red drum were provided to Mote as fertilized embryos at about 12 hours before hatching and were collected within a few hours of fertilization. These embryos were used for toxicity trials or reared to older early life stages used for larval or juvenile trials. Larvae were grown to the desired size (see Testing Protocols) for the trials at 26°C in either 100-L or 129-L rearing tanks maintained using dedicated recirculating filtration systems. Larval feeding, using enriched rotifers, was initiated at 3 days after hatch (DAH) and continued until 11 DAH. Algae paste was added to the rotifer culture vessels to ensure that the rotifers met the nutritional needs of the larvae. Live food (rotifers) to feed the larval fish was produced either in a high-density rotifer production system or in a batch culture system. A dry diet was initially introduced with the rotifers at 3 DAH. Larvae were fully weaned off of rotifers and fed *Artemia* nauplii and a commercially prepared dry diet by 12 DAH. Larvae began to metamorphose to juvenile red drums at 24 DAH.

Inland silverside

Silversides were obtained from Marinco Bioassay, Sarasota, Florida. Both larvae (7 DAH) and juveniles (12 DAH) were transported to the Mote Aquaculture Park for 24 hours of acclimation before toxicity tests began. Larvae were placed in acclimation beakers (1 L) in 28 ppt salinity, 7.5–8.0 pH, 26°C water for a 24-hour acclimation period. Silversides were fed concentrated *Artemia* nauplii.

6.2.2 Toxicity test exposure media preparations

Media preparation

Protocols for test solution preparation are found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The types of toxicity testing solutions are as follows:

- Two different water accommodated fraction (WAF) preparations, high energy and chemically enhanced WAFs (HEWAF and CEWAF, respectively) using two oil types: Slick A (CTC02404-02) and weathered source oil (072610-W-A). Oils were provided to Mote by Stratus Consulting.
- Corexit-only dispersant exposures – as a definitive test (dose-response) without the addition of oil. Corexit was provided to Mote by Stratus Consulting.
- Control water – prepared similarly as WAFs but without the addition of oil.

Seawater used for all exposure studies was obtained from Mote Aquarium's supply of natural seawater from the Gulf of Mexico. It was sand-filtered, purified by ozonation, and monitored for proper dissolved oxygen (DO), pH, temperature, and salinity.

Mote ozonation process for seawater purification

Mote's seawater ozonation process included seawater intake at the New Pass (Sarasota Bay, Florida) dock that collected water from the coastal Gulf of Mexico, average salinity 34 to 36 ppt. The water was pumped to one of four treatment tanks (ranging in volume from 8,000 to 30,000 gal), where it was ozonated for 1 to 2 hours to attain an oxidation-reduction potential (ORP) of 650 mV. Ozone was produced from compressed air with a PCI WedCo ozonation unit, 7 lb O_3/hour capacity, and injected through a Mazzei induction venturi. Following ozonation, air continued to be injected for 24 hours to complete degassing and ORP reduction to attain seawater background levels of less than 300 mV. The stored water was continuously passed through a sand filter at ~ 150 gpm to clean and polish the water. Each storage tank had a

dedicated venturi, pump, and filter system for complete isolation and redundancy. The treated water was continuously monitored for ORP and temperature. The water was sampled and tested for bromine residue before putting the tank on line for use. Cleaned seawater was transported to the aquarium through a dedicated one-way directional pipe. The salinity was tested and adjusted with reverse osmosis (RO) water as needed for specific uses.

6.2.3 Water quality monitoring

Water quality measurements (temperature, pH, DO, salinity, ammonia) were taken as follows (see the QAPP and Table 6.1).

Table 6.1. Water quality constituents and monitoring schedule

Temperature	At initial preparation of stock solution and daily in water bath
pH	At initial preparation of stock solution and from two random test chambers at the end of each 48-hour period
DO	At initial preparation of stock solution and then daily from each test chamber
Salinity	At initial preparation of stock solution and from two random test chambers at the end of each 48-hour period
Total ammonia	Initially for source water for preparation of stock solution and then from two random test chambers at the end of each 48-hour period

6.2.4 Analytical chemistry

Final confirmatory analytical chemistry was conducted offsite at ALS Environmental as described in the QAPP.

6.2.5 Standard operating procedure: Mote standard operating procedures for pH, DO, temperature, ammonia, and salinity

Meters were bench-calibrated or their accuracy was verified against standards or alternative methodologies prior to use, and the accuracy was assessed. Bench-calibration (verification) records were maintained in a laboratory file specific to instrument type and traceable to individual units by serial number. Calibration or verification protocols were based on the manufacturer's methods. Calibrations and calibration verifications were performed prior to sampling. For measurements using probes in the test chambers, measurements began with deionized (DI) water and proceeded to the highest concentrations. This limited the possibility of cross-contamination of chambers. The analyses for pH and DO took place directly in the

exposure chambers. Ammonia was collected from subsamples of test solution during the solution renewal at 48 hours or at the end of the test for analysis. Similarly, drops of test solution from selected chambers were collected with a pipette for the refractometer measurements. Temperature measurements were taken within the water bath table and also during the in-chamber DO measurements.

pH: VWR sympHony meter

1. Record serial number of meter and probe
2. Verify that the batteries are not low
3. Turn on pH meter, then press CALIBRATE to go to CAL 1
4. Rinse probe with DI water; shake and put in either pH 7 or 10 buffer solution
5. When pH display stops flashing, record values of pH and temperature (e.g., 7.00 pH and 24.2°C)
6. Press CALIBRATE while the triangle on right side of screen is still blinking; rinse probe with DI water; shake probe to shake off water and place in next buffer
7. With display of CAL 2, record values of pH and temperature (e.g., 10.02 pH and 24.4°C), and record slope while triangle is still blinking (e.g., SLP 95.4%)
8. On the screen of the pH meter, both 7.00 and 10.00 buffers should be displayed
9. Rinse probe with DI water, shake and place into water sample, and press the measure button
10. Record the value of the pH after the pH display stops flashing
11. Rinse the probe between each of the three triplicate test samples, and record readings from lower concentrations to higher (e.g., C1-3, T4-6)
12. After all the pH parameters are recorded, clean probe with warm water and Simple Green cleaning solution, rinse in DI water, then store in the electrode storage solution until the next sampling.

DO and temperature: YSI ProODO

A. DO

To accurately air calibrate, ensure that the altitude setting is set to the approximate altitude of the region in which the meter will be used and the approximate salinity of the water being analyzed.

For both ease of use and accuracy, YSI recommends performing a DO % water-saturated air calibration as described below prior to sample analysis.

B. Calibrating DO % in water-saturated air; air calibrate (prior to sample analysis)

Note: Air calibration must take place within ± 10°C of the sample temperature.

1. Moisten the sponge in the storage sleeve with a small amount of clean water. (*Note*: the sponge must be clean and free of bacterial growth, which may consume oxygen and interfere with calibration.)

2. Make sure that there are no water droplets on the sensor cap and temperature sensor, then install the storage sleeve over the probe.

3. Wait 15 minutes for the storage container to become completely saturated and to allow the temperature and DO sensors to stabilize.

4. Press CAL (calibration).

5. Highlight DO % and press ENTER to confirm.

6. The instrument will use the value from the internal barometer during calibration, and will display this value in brackets at the top of the display.

7. Wait for the temperature and DO % values under "Actual readings" to stabilize, then highlight Accept Calibration and press ENTER to calibrate.

8. Press CAL to complete the calibration.

9. Press ESC to cancel calibration.

C. DO measurement

Always analyze starting with the lowest concentration of oil or dispersant (control) and work up to the highest concentration.

To take readings, insert the probe into the sample. The DO and temperature sensors should be immersed in the sample.

Allow the temperature readings to stabilize, and wait approximately 20–25 seconds for the DO readings to stabilize.

1. Record each sample's DO directly from the meter display
2. Rinse probe in DI water and repeat
3. To clean probe, wash with warm water and Simple Green cleaning solution.

D. Temperature

All ProODO probes have built-in temperature sensors. Temperature calibration is not required nor is it available. To set the units, press the Probe key on the ProODO instrument and select Display. Highlight temperature and press ENTER. Highlight the desired temperature units of °F, °C, or K and press ENTER to confirm the selection. Record each sample's temperature directly from the meter display as you read the DO measurement.

Ammonia: Hach DR2800 using Method 8155 salicylate method

- Reagents
  - Ammonia Cyanurate Reagent Powder Pillows
  - Ammonia Salicylate Reagent Powder Pillows
  - Sample Cells, 1-in. Square, 10 mL.

A. Calibration verification

Calibration is incorporated into the software and is not adjusted by the user. In lieu of a calibration, a weekly "Accuracy Check" is performed to verify that the instrument is working properly.

1. Create a sample blank as described in the procedure or use sample blank from previous test.

2. Place the blank in the cell holder with the fill line facing right.

3. Press OPTIONS > MORE. Press STANDARD ADDITIONS. A summary of the standard additions procedure will appear.

4. Press OK to accept the default values for standard concentration, sample volume, and spike volumes.

5. After values are accepted, the unspiked (BLANK) sample reading will appear in the top row.

6. Open an Ammonia Nitrogen Standard Solution, 10 mg/L as NH_3N provided by Hach.

7. Prepare three sample spikes by filling three mixing cylinders with 25 mL of sample water.

8. Use a calibrated pipette to dispense 0.2 mL, 0.4 mL, and 0.6 mL of standard to the three respective cylinders and mix each thoroughly.

9. Analyze each sample spike as described in the procedure above, starting with the 0.2-mL sample spike.

10. Accept each standard addition reading by pressing READ.

11. Each addition should reflect approximately 100% recovery.

12. After completing the sequence, press GRAPH to view the best-fit line through the standard additions data points accounting for matrix interferences.

13. Press IDEAL LINE to view relationships between the sample spikes and the "Ideal Line" of 100% recovery.

B. Ammonia measurement

1. Turn on instrument. Press STORED PROGRAMS.

2. Select the test (385 N. Ammonia, Salic).

3. Prepare the sample by filling a 1-in. square sample cell to the 10-mL mark.

4. Prepare the blank by filling a second square sample cell with RO water to the 10-mL mark.

5. Add the contents of one Ammonia Salicylate Reagent Powder Pillow to each cell.

6. Stopper each cell and shake to dissolve.

7. Press TIMER > OK, and a 3-minute reaction period will begin.

8. When the timer expires, add the contents of one Ammonia Cyanurate Reagent Powder Pillow to each cell.

9. Stopper each cell and shake to dissolve.

10. Press TIMER > OK, and a 15-minute reaction will begin. (A green color will develop if ammonia-nitrogen is present.)

11. When the timer expires, insert the BLANK into the cell holder with the fill line facing right.

12. Press ZERO. The display will show 0.00 mg/L NH_3-N.

13. Wipe the sample cell and insert it into the cell holder with the fill line facing right.

14. Press READ. Results are given in mg/L NH_3-N.

Salinity: VEE GEE STX-3

The VEE GEE STX-3 has an automatic temperature range of 10°C to 30°C. It has a range of 0–100 ppt salinity with an accuracy of ± 1 ppt.

A. Zero verification

1. Prior to use, place two drops of RO water on the prism and "zero" the unit using the ZERO RING directly behind the prism. It is best to zero at or near the temperature of the sample.

2. Record time and location of zeroing.

B. Salinity measurement

1. Rinse prism with DI water and blot dry between each sample
2. Place two drops of sample directly on the center of the prism (hold prism level)
3. Close the clear plastic prism cover
4. Read the salinity in ppt from the view scope to the nearest ppt
5. Record salinity
6. Rinse with DI water, clean with Simple Green solution, rinse with DI water again, and blot dry prior to storage.

Dispersant measurement spectrophotometer

The instrument used for the analysis of the absorbance of the Corexit 9500 samples is a Perkin Elmer Lamda 35, UV/VIS Spectrometer with double-beam operation. All samples were recorded at 240 nm wavelength in the absorbance mode with the 1-nm slit using the UV WinLab software.

A five-point calibration curve was developed appropriate for the range of concentrations to be measured in the samples. Samples were then analyzed relative to the calibration curve for Corexit concentration confirmation.

A. Testing Protocol 1: Aquatic Toxicity Procedure

A.1 General Considerations

Decontamination of all glassware, spatulas, etc., followed the QAPP.

WAFs were prepared with the appropriate treatment oil [see test-specific test conditions tables (TCTs)] according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A. WAFs were prepared daily for subsequent renewals.

A.2 Experiment

Static and static-renewal exposures were conducted using standard guidance and the basic testing protocols of the QAPP and the Mote GLPP.

Static and static-renewal tests were carried out in clean decontaminated glass beakers for up to 96 hours. After preparation, the test solutions were added to the exposure containers holding the proper number of test organisms. An air tube was placed in each container, and air was bubbled at a rate of approximately 1–2 bubbles/second for aeration. Containers were covered by aluminum foil. Test organisms, with the exception of embryos, were fed during exposures.

All controls and the various concentrations of test solutions for each exposure test were in triplicate. Control treatments were prepared using the same methods as exposure treatments, but did not contain any contaminants. All sample and control preparations, as well as basic laboratory chemistry and standard cleaning procedures, followed the QAPP and the Mote GLPP for Toxicity Testing Protocols.

1. Set up water bath with an aquarium heater to achieve appropriate temperature for test organisms.

2. Transfer test organisms into test beakers. Test organisms should be transferred in seawater to each container for a species-specific acclimation period prior to the addition of test solution. The organisms must remain suspended in water during the transfer and be allowed to acclimate to minimize stress. This acclimation period is specific to each organism; e.g., red drum larvae have an acclimation period of 3 days, which begins at 15 DAH of the test species. Silversides have an acclimation period of 24 hours that begins at 8 DAH for larvae and 15 DAH for juveniles. There is no minimum acclimation period for red drum embryos. The number of test organisms placed in the beakers for acclimation exceeds the final number used for the test. Remove organisms that have deceased during this acclimation period from the beakers daily by siphon. On day 1 of the

test, remove excess organisms. Only the required number of test organisms for each exposure remains in the exposure chambers, and the test commences.

3. Keep beakers in water bath to maintain constant temperature.

4. Attach aeration stones to tubing attached to aquarium air pumps.

5. Place one air stone in each beaker, bubbling at a rate of approximately 1–2 bubbles/ second.

6. Cover beakers with foil.

7. After the acclimation period, add WAF at t = 0 by gently removing approximately 95% of the seawater from the container and replacing it with appropriate test solutions.

8. Collect and archive required samples for analytical chemistry according to the QAPP.

9. Measure water quality parameters (temperature, salinity, DO, ammonia, and pH) as described in the Mote GLPP document.

10. Feed test organisms with the appropriate food type and frequency determined for the organism's life stage. Remove unconsumed food daily from the bottom of beakers by a minimal-flow, small-diameter siphon.

11. Remove dead organisms from each test chamber each day, and record and archive according to the QAPP.

12. After 48 hours, repeat steps #7–10. Note: This step pertains only to tests that require a WAF renewal (see test-specific TCTs).

13. At the end of the exposure study, count the number of live organisms remaining in each exposure chamber. Record and archive all live and dead organisms according to the QAPP.

A.3 Analytical Testing Methods

All analytical testing methods followed the QAPP.

All sample and control preparations, as well as basic laboratory chemistry and standard cleaning procedures, followed the QAPP and the Mote GLPP for Toxicity Testing Protocols.

B. Testing Protocol 2: Red Drum Juvenile Behavioral Oil Toxicity Studies

B.1 General Considerations

Decontamination of all glassware, spatulas, etc., followed the QAPP.

WAFs and dispersant-only test solutions were prepared with the appropriate treatment oil (see test-specific TCTs) according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A.

B.2 Experimental Design

The study used a before-after control-impact (BACI) experimental design to measure pre-stimulus and post-stimulus reactions of juvenile red drum contained in experimental chambers filled with combinations of treatment water as follows:

1. Control tanks that contained seawater (no oil or dispersant) before and after alarm pheromone stimulus.

2. Tanks that contained CEWAF exposures before and after an alarm pheromone stimulus.

3. Tanks that contained dispersant (DISP) exposures before and after alarm pheromone stimulus.

Behavioral tests were carried out in decontaminated 1.9-L glass aquaria with blacked-out backgrounds for up to 16 hours. One to five experimental replicates were set up for each treatment. For a specific test, all of the experimental replicates from a specific treatment were conducted on the same day in combination with paired controls. See test-specific TCTs for treatment designations and number of replicates used for each treatment.

The behavioral responses of juvenile red drum were assessed just prior to and after the addition of an alarm pheromone cue to the exposure chambers. Cue additions and response observations were conducted at approximately 2- and 16-hours post-oil/dispersant exposure (see test-specific TCTs). Responses were video-recorded and focal observations quantified using computerized event recorder software (JWatcher™). All trials were conducted between 0900 and 1300 hours on each exposure day. Observations consisted of a 10-minute pre-stimulus and a 10-minute post-stimulus observational period. Each behavioral experiment used 30-day-old red drum. Each trial was stocked with new fish because the fish were sacrificed and preserved for every trial. No fish

was ever used more than once. All experimental tanks were custom-made from inert window glass. Aquaria sealant was used to seal joints.

B.3 Pre-trial Preparations

- Randomly arrange the chambers and place labels on each chamber appropriately. Insert visual barriers around and between the tanks so that the tank inhabitants cannot see any other tanks or researchers in the area.
- Arrange individual chamber air lines and cue addition lines with respective labeling.
- Hang a tarp barrier around the exposure chamber.
- Set up the overhead light timer appropriately (12 hours light, 12 hours dark).
- Prepare video cameras; stands, data storage disks, mobile table for cameras, charge batteries in all cameras, have A/C power chargers setup.
- Print appropriate bench sheets for data collection.

B.4 Test Setup

1. Prepare test exposure water and collect analytical chemistry samples according to the QAPP.
2. Fill chambers with 1,500 mL of the appropriate treatment.
3. Insert air stones and adjust aeration to a gentle rate so that juveniles do not have to swim against currents.
4. Calibrate water quality instruments (pH meter, DO meter) and measure pH, salinity, DO, and temperature. Record measurements on the "Water Quality Monitoring" bench sheet (QAPP).
5. Record all exposure data on the appropriate bench sheets.
6. Harvest red drum juveniles from Mote rearing tanks as follows:
    a. Do not feed juveniles on harvest day.
    b. Set up the harvester bucket.

c. Secure 3/4" silicone siphon hose to tank outflow and into harvester bucket.

d. Turn off bypass for drain water allowing all water to enter harvester bucket.

e. Open the spare tank to capture any extra water and prevent sump overflow.

f. Pull the standpipe from the source tank and drain into the harvester bucket. Flush thoroughly until all of the juvenile red drum are in the harvester bucket.

g. Collect approximately 50 individuals using a small nylon aquarium net.

h. Place fish into a bucket and transport them to the behavior room.

7. Stock the appropriate number of juvenile drum per individual chamber (see test-specific TCTs). Make sure to select healthy and vigorous-looking individuals for all chambers; however, do not select by size. Aerate the bucket while transferring fish to exposure chambers.

8. Record the harvest and stocking times on the appropriate bench sheet.

B.5 Initial Behavior and Acclimation

1. Using a hand-held video camera, record approximately 2 minutes of video per chamber and make initial observations of fish performance. Record start and stop times on the appropriate bench sheet.

2. Drop a tarp barrier around the exposure chambers.

3. Set up five video cameras with tripods focused on the first round of tanks to be recorded. Record tank/camera combinations on appropriate bench sheet.

4. Leave the behavior room for 2 hours to allow red drum to acclimate to the tanks.

B.6 Chemical Alarm Cue and Behavioral Response

1. After 2 hours and 16 hours, return to the behavior room and start filming with the 5 cameras 10 minutes prior to the addition of alarm cue.

2. Record the respective video start times on the appropriate bench sheets.

3. Leave the room and prepare the alarm cue solution from a donor fish as follows:

    a. Harvest a large a red drum individual (approximately 100–160 mm total length).

    b. Record the donor fish's length, weight, source tank, and condition at harvesting on the appropriate bench sheet.

    c. Lightly dab the fish with a paper towel to remove any excess water or slime.

    d. Sever the spine and homogenize the brain.

    e. Using a sharp tip scalpel (#11 blade), make 10 superficial and parallel cuts into the skin of each side of the donor fish. Make sure urine does not contaminate the work area.

    f. Pour 60 mL of seawater over the red drum and collect wash water in a 250-mL beaker.

    g. Fill a second beaker with an additional 60 mL of clean source water.

    h. Draw 10 mL of cue water into a pre-cleaned 60-mL syringe (cue syringe).

    i. Draw 10 mL of seawater water into a second 60-mL syringe (wash syringe).

4. After 10 minutes of filming, return to the behavior room and quietly connect the cue syringe to the respective delivery hose and slowly add the entire contents of the syringe into the delivery hose. Remove the cue syringe, connect the wash syringe, and deliver the contents into the delivery hose.

5. Fill the wash syringe with air and slowly purge the line to ensure that all the cue water has entered the tank.

6. Record the time the chemical alarm cue was added on the appropriate bench sheets.

7. Repeat the steps listed above for each replicate tank where cameras are set up.

8. Keep filming for 10 minutes post exposure to cue.

B.7 Post-behavioral Observational Period

1. Calibrate the water quality instruments.

2. Collect water quality measurements in each chamber according to the Mote GLPP: Test for pH, salinity, DO, and temperature.

3. Record the water quality measurements on the "Water Quality Monitoring" bench sheet found in the QAPP.

4. Collect water for ammonia analysis and place the water in a pre-labeled storage container and refrigerate.

5. Collect water for PAH analysis and place in pre-labeled storage container and ship to ALS Environmental.

6. Harvest all fish from each tank and record state (alive or dead) at harvest.

7. Place dead fish in labeled weigh dish. If fish are alive, euthanize fish by placing them in 200 ppm of MS-222 anesthesia. Then place each fish in a labeled weigh dish.

8. Dab the fish dry with paper towels and individually weigh (± 0.01 g) and measure total length (mm) using calipers. Record the data on the appropriate bench sheets.

9. Prepare archive tissue samples according the QAPP by placing all fish from one treatment group into a single sample container. Record sample information in the *Analytical Sample Inventory Bench Sheet* in the QAPP.

7. University of Miami Rosenstiel School of Marine and Atmospheric Science General Laboratory Procedures and Practices

7.1 Methods

All fish experiments, except for ones performed on yellowfin tuna, were conducted with embryos or larvae from broodstocks of fish maintained at the Rosenstiel School of Marine and Atmospheric Science (RSMAS), University of Miami, Miami, Florida. Copepod experiments were performed with copepod cultures purchased from AlgaGen (Vero Beach, FL), and maintained at RSMAS.

7.1.1 Test organism sources and husbandry – cobia, mahi-mahi, and yellowfin tuna

This section describes the sources and husbandry for test organisms used for toxicity tests, which were conducted with cobia, mahi-mahi, and tuna. Currently, the University of Miami Experimental Hatchery (UMEH) maintains broodstock populations of cobia stocked in semi-recirculating maturation tanks at the UMEH facility on Virginia Key, Florida.

Cobia

The primary group of F1 broodstock cobia (*Rachycentron canadum*) were stocked in an 80-ton semi-recirculating maturation system at the UMEH facility (Benetti et al., 2008b). Additional broodstock fish were maintained in 15-ton flow-through holding tanks. For cobia, a sex ratio of 1:1 (male:female) was maintained.

Fertilized eggs were obtained by allowing the fish to spawn in the tanks. When natural spawns were obtained from conditioned fish, fertilized eggs were harvested from the egg collectors outside the maturation tanks and incubated in 1-ton cylindrical-conical tanks with flow-through filtered seawater and constant aeration. Eggs were disinfected using 100 ppm formalin (Paracide) for 1 hour during the first cleavages. After hatching, yolk-sac larvae were transferred to the larval rearing tanks using 15-L buckets with water to avoid the use of nets.

Larval rearing: The intensive methods used for larval rearing described previously (Benetti, 1997; Benetti et al., 1998; Feeley and Benetti, 1999; Watanabe et al., 2005) have been refined and implemented at the UMEH (Benetti et al., 2007, 2008a, 2008b). These larval rearing techniques used ultraviolet (UV)-sterilized flow-through seawater ranging in temperature from 24°C to 32°C and a daily turnover rate of 100–800%. Newly hatched larvae were stocked at 5–

10/L. The standard protocol consisted of live feeds added 3–5 times a day as needed to maintain proper concentrations of microalgae (*Isochrysis galbana* at 10,000 cells/mL), rotifers (*Brachionus plicatilis* at 5/mL), and *Artemia* (at 0.1–1.0/mL). Cobia larvae were fed *ad libitum* using a pulse feeding technique. Microalgae and rotifers were added between 2 and 10 days post-hatch (DPH), with *Artemia* added from 7 DPH through 22 DPH. From 16 to 23 DPH, the post-larvae were weaned onto starting diets. Larvae were sampled daily and dissected under a microscope to look for any signs of pathogenic organisms. For information about acceptable control performance and other test success criteria, refer to the specific testing protocols for individual tests.

Mahi-mahi

Mahi-mahi (*Coryphaena hippurus*) broodstock were collected from coastal waters off Miami using hook and line fishing equipment. Once caught, fish were held in 250-gal cylindrical transport tanks mounted on the fishing vessel, and water quality was maintained by pumping in fresh seawater.

Upon arrival at UMEH, fish were quarantined for 5 to 7 days. As part of this process, the fish were weighed, measured, tagged for identification (ID) purposes, and sampled for assessment of sexual maturity (Benetti et al., 2007). All fish were injected with 1.5 mL of 100 mg/mL oxytetracycline to mark otoliths for posterior age and growth ID purposes, and to prevent infections that may occur as a result of bruising and stress during capture, transport, and handling (Wexler et al., 2003). Selected broodfish received prophylactic treatments according to standard quarantine protocols used at the UMEH for a variety of pelagic fish (Benetti et al., 2008a). The fish were treated for 1 hour each in a solution of 100 ppm formalin, or treated in a 200 ppm formalin bath for 2–5 minutes followed by a freshwater bath for 5–10 minutes. The freshwater/formalin treatments removed any ectoparasites from the gills and skin of the fish that may have accompanied the fish from the wild and that could later persist and proliferate after transfer to the maturation tank (Benetti et al., 2007). At times, a low dose of clove oil or MS-222 (10 ppm) was used during the freshwater bath to anesthetize the fish and ensure easier handling (Benetti et al., 2007, 2008a). After completion of the 5–7 day quarantine, the fish were stocked in maturation tanks.

Sex ratios (male:female) in nearly all mahi-mahi broodstock tanks were maintained between 1:1 and 1:4. In general, the mahi-mahi spawned in the early morning; fertilized embryos were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of embryos was the late blastula period or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

When natural spawns were obtained from conditioned fish, fertilized eggs were harvested from the egg collectors outside the maturation tanks and incubated in 1-ton cylindrical-conical tanks with flow-through filtered seawater and constant aeration. Eggs were disinfected using 100 ppm

formalin (Paracide) for 1 hour during the first cleavages. After hatching, yolk-sac larvae were transferred to the larval rearing tanks using 15-L buckets with water to avoid the use of nets.

Yellowfin tuna

Yellowfin tuna (*Thunnus albacares*) embryos were obtained from the standing broodstock of the Achotines Laboratory in Las Tablas, Panama. Embryos were collected within an hour of spawning and were treated in formalin for 1 hour followed by a 15-minute rinse before being placed in exposure chambers. Tests were conducted on-site at Achotines.

7.1.2 Test organism sources and husbandry – *Acartia tonsa* copepods

In addition to pelagic fish, the calanoid copepods *Acartia tonsa,* (*A. tonsa*) were used to investigate the toxicity of DWH oil. Live adult *A. tonsa* were purchased from AlgaGen (Vero Beach, FL) and transferred to 1-gal glass jars filled with 0.45-µm filtered seawater at the same temperature and salinity as the water the copepods were shipped in (typically 25 ppt and 24°C); the jars were gently aerated at approximately 1 bubble per second. Copepods were held at a density of one animal per 10 mL of water (Gentile et al., 1976). Salinity was adjusted in the jars at a rate of 4 ppt/d (Gentile et al., 1976) until all jars were at 35 ppt. Copepods were fed 1 x 10^3 cells/mL (Gentile et al., 1976) of *Isochrysis galbana* (TISO) and *Chaetocerous muelleri* (CHGRA). To set up synchronous cultures, mature adults were collected and transferred to a 2-L dish in which a generation cage was submerged (Gentile et al., 1976). The generation cage used a 100-µm filter screen that allowed copepod eggs to pass through, while retaining cannibalistic adults. After 24 hours, the eggs were collected and used to start a synchronous culture where all animals were the same age. For mass production, *A. tonsa* were grown in three 3,000-L static semi-continuous cultures in uncovered tanks outside and fed live TISO at concentrations of 40,000 to 100,000/cells per mL. Water temperature was maintained between 24°C and 30°C, and salinity was typically maintained at 20 ppt; these conditions assisted with better egg production: 17–40 eggs/day/female or 4–9 million eggs/day, assuming a 1:1 male to female ratio. For the oil exposures, a salinity of 30 ppt was used, so before tests began, the culture was slowly acclimated to this higher salinity. Two 250-L tanks were used as the hatching tanks for nauplii production. Live algae (TISO and CHGRA) were added to reach a concentration of 50,000 cells/mL at a 1:1 ratio, providing a stronger nutritional profile in the nauplii.

To maintain live algae cultures for feeding to copepods, primary microalgae inocula were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Working stocks were aseptically transferred weekly to fresh f/2 media (Guillard, 1975) and prepared with autoclaved 32 ppt ambient seawater. Stocks were aerated and housed at 22°C under continuous light from a single, cool-white fluorescent bulb. Cell concentrations were determined by hemocytometer/fluorometer.

7.1.3 Test organism sources and husbandry – Gulf toadfish

Finally, Gulf toadfish (*Opsanus beta*) were used to investigate the effects of DWH oil on adrenal function and stress response. Toadfish were collected from Biscayne Bay, Florida, using commercial shrimp trawlers (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-12-0729-SR). Upon arrival, toadfish were treated with 10 L of distilled water and 500 mL of seawater for 5 minutes, followed by a treatment of formalin (15 mg/L in seawater. Toadfish were held in 60-L glass aquaria with recirculated, aerated seawater (32 ppt) and maintained at approximately 20–25°C for at least seven days before the start of experimentation. Toadfish were fed raw squid weekly.

7.1.4 Exposure media preparations

Seawater used for all exposure studies at RSMAS was obtained from RSMAS' supply of natural seawater from Biscayne Bay, which has been sand-filtered and monitored for proper dissolved oxygen (DO), pH, temperature, and salinity.

Exposures took place in accord with the testing protocols and test conditions tables (TCTs) specific for each species and test. In all cases, water accommodated fractions (WAFs) of oil were prepared as outlined in the standard operating procedure (SOP) *Protocols for Preparing Water Accommodated Fractions* found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

Generally, three different WAF preparations were tested: high-energy, low-energy, and chemically enhanced WAFs (HEWAF, LEWAF, and CEWAF, respectively) using the following four oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A), see test-specific TCTs for details. In addition, Corexit 9500-only exposures were performed as a definitive test (dose-response). In all cases, control waters were prepared as the WAFs but without the addition of oil.

7.1.5 Water quality monitoring

See the QAPP and TCTs for required monitoring.

7.1.6 Analytical chemistry sampling

See the QAPP, related testing protocols, and TCTs for sampling and processing of water and tissue collected for chemical analyses.

7.2 Reporting and Testing Documentation

Reporting and testing documentation was done as outlined in the QAPP.

7.3 Water Quality Protocols – General Laboratory SOPs

7.3.1 Fluorescence measurements

Measuring oil in water using fluorescence spectroscopy SOP

This protocol is adapted from the *Standard Operating Procedure – Fluorescence Spectroscopy to Verify Dilutions of Water Accommodated Fraction for Toxicity Testing* available in the QAPP. The modifications herein are due to the difference of equipment between RSMAS and Queen's University, Kingston, Ontario. *Note:* Prior to any definitive testing, the emission and excitation settings were established for source and weathered source oil, with identical settings being optimal. The excitation wavelengths were typically 224 and 258 nm, with emission ranges of 275–425 nm and 275–475 nm, respectively. Definitive scans were still performed to verify these ranges for all definitive tests.

Determining excitation and emission wavelengths of sample oil

1. Turn on the LS-45 fluorescence spectrometer at least 1 hour prior to use. The lamp for the LS-45 turns on automatically with the machine. Turn on the computer and open the FL Winlab software package.

2. To deduce the excitation and emission wavelengths, run an excitation scan. This scan monitors the emission spectrum over a range of excitation wavelengths. To run an excitation scan, click on the scan.mth method on the startup page of FL Winlab. Once inside the scan method, click the multiple scan icon on the top toolbar (rainbow-colored spectral icon). Under setup parameters, select the excitation tab. Set the scan range from 200–500 nm and set the initial emission value to 200 nm. Under the 3D scan range heading, set the number of scans to 15 and the emission increment to 20 nm, which will result in an emission range of 200–500 nm. Input the results file name at the bottom. Name the file with the test ID# and the letters "es" (emission scans).

Note: The resulting files consisted of one .sp3 file, which is a 3D representation of all 15 scans. The software automatically adds a #01 to the end of the file name. Each individual scan was also saved as a .sp file, starting with the number #01 and ending at #15.

3. Load sample (see sample preparation section below) and press the start scan button (traffic light icon with the green light highlighted). Perform this scan with an intermediate-high WAF dilution. The scan takes approximately 30–45 minutes.

4. To evaluate the excitation and emission spectrums, open the main FL Winlab window and click the data handling tab on the toolbar. Select 3D view and open the test .sp3 file. Two background emission spectra are apparent on the 3D view, which are the result of the excitation light and not the sample. Typical scans have shown two dose-dependent peaks at approximately 224 and 258 nm. To identify the exact wavelength of the respective peaks, click on the excitation cut icon (last icon in the toolbar), and place the red line over the center of the peak. The vertical cut will appear in a new window with the excitation wavelength. To determine the emission wavelength range, look at the range of emission peaks in the excitation vertical cut window. Do not incorporate any of the background peaks into the emission range.

5. Once the excitation and emission wavelengths have been verified, proceed to standard curve and sample analysis.

Standard preparation and standard curve

Note: The procedures regarding sample and standard storage are also described in the QAPP, testing protocols, and RSMAS General Laboratory Procedures and Practices (GLPP).

1. Preserve all samples and standards immediately in 50% ethanol, and store at 4°C until analyzed. It is important that the curve be made in duplicate, with one set for analysis and the other for archive. Label samples as describe in the QAPP.

2. Make the standard curve during the test setup.

3. Perform standards curve dilutions in bulk test seawater to a 10 mL volume, after which add 10 mL of ethanol (ex. 10 ppt standard = 10 µL of HEWAF, 9.99 mL of seawater, 10 mL ethanol). It is best practice to serially dilute the following series: 100 ppt, 50 ppt, 10 ppt, 5 ppt, 1 ppt, 0.5 ppt, 0.1 ppt, and 0.05 ppt. Vortex the WAF/standard prior to proceeding to the subsequent dilution step. Not all standards are necessary, depending on the working range of the test.

4. The working range of the LS-45 is relatively small. For example, the detectable working range for preliminary weathered source HEWAF is 10 to 0.1 ppt. It is possible to change the detection sensitivity by changing the emission attenuator; however, a standard curve and samples must be performed using the same attenuator. Usually only 4–6 samples of the serial dilution curve can be measured. Any test samples that do not fit into this standard curve must be diluted appropriately.

5. To analyze standards, first sonicate at low intensity for 3 minutes. Make sure to sonicate on ice, as the sample will get extremely hot if not on ice. Following this, transfer 3 mL of the sample to two 1.5 mL microcentrifuge tubes and spin at 10,000 RPM for 10 minutes, which will remove salts. Carefully pipette the clear water layer from both microcentrifuged tubes into a pre-cleaned quartz cuvette. Do not pipette any of the precipitated salt.

6. Place the standard into the LS-45 and open a scan method in FL Winlab. Under the scan method, make sure the single wavelength scan is highlighted (this is the default setting). Click the single excitation wavelength tab and enter the appropriate excitation wavelength and emission wavelength range (as previously determined by the user). Also enter the desired file name. Name the file for standard scans with the test ID# followed by "sc" (standard curve), followed by the dilution (i.e., 333sc10). Scan all standards as well as the seawater blank. Between replicates, clean the quartz cuvette with Simple Green and distilled water, and dry with a Kimwipe®.

7. To analyze the data, go to the main page of FL Winlab and click the data handling tab and select area. Multiple files can be opened at once with this tool, so open all standards and the blank. The tool allows for optimizing the analysis wavelengths within this window; however, the conditions should be optimized so the entire read will be analyzed. Ensure that the emission wavelengths at the bottom of the window reflect this. Click analyze; a new text window will open with the areas under the curve within the respective windows. Record the values on the run data sheet. This file does not need to be saved, as it is readily reproducible from the saved raw data file.

8. To calculate the standard curve, first subtract the blank value from all standards (using Excel). Plot the values as an x-y scatter graph, with the peak fluorescence area values on the x-axis and the WAF concentration (ppt) on the y-axis. After plotting the graph, click on one data point and then right-click. Select add trend-line from the menu. Ensure that the trend-line is linear and that the options to show R^2 and equation are selected. Record both the R^2 and curve equation on the data sheet. Save this Excel spreadsheet (test ID# followed by data analysis). Ensure that the raw input numbers are double-checked against the data sheet by a second individual.

9. If the R^2 value is below 0.95, the procedure should be repeated from the beginning.

Test replicate analysis

1. The steps for analyzing unknowns are the same as steps 5–7 of the standards, including sonicating and centrifugation procedures. The standard curve is valid only over the working range of the serial dilutions, so if values come out higher than the curve, they must be diluted. If a value comes in lower than the curve, the curve must be extended on

the low end. A sample that comes in lower than the standard curve in practice cannot be differentiated from zero.

2. All unknowns files (.sp files) must be saved in duplicate, with one as an unopened archive file and one as a working file in the test folder. Name all files with the test ID#, replicate chamber number, and day (e.g., 333.T7.4 = test 333, test chamber 7, day 4).

3. Use the same procedure as for the standards to analyze the area under the curve. Record all values on the data sheets.

4. To calculate the actual value of oil (WAF) in the test replicate, open the test data analysis spreadsheet and input the raw unknown values. Using Excel, input the calculated area into the standard curve calculation (x-value) and solve the equation. Record all these values on the data sheet. Make sure that all numbers and the standard curve equation are double-checked by another individual. Once the final analysis is complete, save a second copy of the spreadsheet in the archive folder.

5. The final values are in ppt WAF, but this will be converted to units of total polycyclic aromatic hydrocarbon (PAH) by RSMAS personnel after the mass spectroscopy analysis is provided by ALS Environmental.

Note: At the end of the test, all data sheet files were checked by a third person against the raw data. This included checking the data sheet values against the area calculation .txt file, and checking that all values in Excel were input on the data sheets and that the standard curve calculation in Excel was correct. After the data were triple-checked, a second copy of the Excel spreadsheet was saved in the archive folder. This file was not accessed after this point.

7.3.2 Temperature measurement SOP

1. Measure temperature using a standard laboratory thermometer (VWR; 61028-080).
2. Place thermometer in solution and wait for 1 minute. Record value.
3. Clean the thermometer with absolute ethanol and distilled water between measurements.

7.3.3 Measurement of salinity SOP

1. Measure salinity using a refractometer (VWR SW series, #12777-992). Using a glass Pasteur pipette, place one or two drops of test solution on the measurement window and close the lid. Point toward the light, keep the refractometer level.

2. Record value.

7.3.4 pH measurement SOP

1. Measure pH using a combination glass electrode coupled to a PHM220 pH meter (Radiometer). Calibrate the meter each day according to the automated manufacturer procedures and using IUPAC buffers for pH 7 and 10 (expected sample pH of approximately 8.1).

2. Perform calibration in the environmental chamber and with temperature-acclimated buffer solutions.

3. Record the calibration performance on the log, with acceptable calibration at values at or above 95%.

4. For testing, immerse the electrode into the center of the test beaker and allow it to equilibrate for 1 minute, record the value. Clean the electrode between samples using Simple Green cleaning solution and distilled water.

7.3.5 Measurement of total ammonia SOP

Note: All samples that were tested for total ammonia were stored at -20°C until assayed. All water samples from a given toxicity test were tested for ammonia at the same time using the same standards. If multiple plates were needed, the same diluted standards were used; however, each plate was treated as a distinct assay that contained a standard curve. This took into account variation in development time between plates.

1. Measure total ammonia using the colorimetric assay described by Verdouw et al. (1978).

2. Run the assay on a flat-bottomed polystyrene 96 well plate (Model # 9017; Corning), with each sample/standard run in triplicate.

3. Prepare the standard curve from an acidified 50 mM stock solution of ammonium sulfate. Make a 0, 10, 25, 50, and 100 uM dilution series using seawater.

4. Add 160 µL of each standard/unknown to the respective wells.

5. Using a repeat pipette, add 20 µL of sodium salicylate solution to each well. Sodium salicylate solution = 40 g of sodium salicylate/80 mL Milli-Q. Make fresh daily.

6. Using a repeat pipette (different tip), add 20 µL of catalyst citrate solution to each well. Catalyst citrate solution = 0.02 g, sodium nitroprusside, 35 g sodium citrate, volume to 100 mL using Milli-Q water. Protect the solution from light, and store in the refrigerator.

7. Using a repeat pipette (different tip), add 20 µL of alkaline hypochlorite solution to each well. Alkaline hypochlorite solution = 4 g NaOH, 14 mL sodium hypochlorite, volume to 100 mL using Milli-Q. Store solution in the refrigerator.

8. Allow to develop for at least 1 hour and no more than 24 hours in a dark place at room temperature.

9. Read samples at 650 nm using the microplate spectrophotometer. Standard curve should have an $R^2 \geq 0.95$. Because the assay is run in triplicate, it is acceptable to remove obvious outlying assay replicates, but make careful notes of any removed outliers. Keep all raw data files, and also record all mean replicate ammonia values on data sheets, as well as the standard curve R^2 value.

7.3.6 Measurement of DO SOP

1. Measure DO using a hand-held YSI ProODO DO meter and probe (#626279).

2. Calibrate the oxygen meter daily. Fill out the calibration logs daily.

3. Calibration:

   a. *Salinity*: Because the solubility of oxygen in water decreases as salinity increases, it is important to input the test salinity into the meter prior to calibration and use. To input the salinity value into the meter, push the probe button and select salinity from the menu. Enter the appropriate value. Input the mean value of the measured salinity for the test replicates (salinity should not vary much between replicates). Record the calibration salinity used in the calibration log.

   b. *Temperature*: This meter does not need temperature calibration, although the meter reading should be recorded on the calibration log.

   c. *One-point calibration in water-saturated air*: This procedure is as described in the manufacturer's manual. Moisten the calibration sponge with a small amount of water, and ensure that no water droplets are present on the sensor/temperature caps; do not immerse the sensors in water. Place sensors in calibration sleeve and allow to equilibrate for 5 minutes. Press calibration button, highlight DO%, and press enter. Highlight the barometer value and press enter. (Although the value can be changed, the value measured by the meter should be used. If the barometer value is off, then a barometric pressure calibration should be performed.) Record the barometric pressure value on the calibration log. Wait for temperature and DO% values to stabilize, highlight accept calibration, and press enter. No

calibration efficiency rating is provided, but the user should check off on the calibration log that the meter was calibrated.

d. *Barometric pressure*: This value does not need to be routinely calibrated. If the value appears to be inaccurate, press the calibrate button and select barometer. Input an acceptable value and select calibration.

4. To test the DO in the test replicates, first set the meter to auto-stabilize by pressing probe and selecting auto-stable (the sensitivity should be set to the middle value, which allows a data variance of 1.275%). Place probe in the sample and briefly move to release air bubbles from the sensor. Continued movement is not necessary due to the optical luminescent measurement. Allow value to stabilize and record value on the data sheet.

5. Between replicates, clean the probe using Simple Green cleaning solution followed by distilled water.

7.3.7 WAF and toxicity test water disposal procedures SOP

1. Dispose of all oil-containing solutions by draining through an activated charcoal bed, with outflow leading into a sink drain at the University of Miami.

2. Remove any solid pieces (weathered oil) from the charcoal bed after use and place in a waste vessel stored in a fume hood.

3. At the completion of the project, dispose of both the activated charcoal bed and solid waste under the chemical safety and disposal regulations of the University of Miami.

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A. Testing Protocol 1: Yellowfin Tuna (*Thunnus albacares*) Embryo Acute Toxicity Test – Static Exposure

Note: In general, the animals spawn in the evening and are available for testing a few hours after fertilization. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that have entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test seawater collected at the Achotines Laboratory was filtered down to 0.35 µm, UV-sterilized, and temperature-matched to the water in which the eggs were collected prior to use.

A.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary chain-of-custody (COC) documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

3. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is UV-sterilized seawater. Seawater volume should be measured using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Preform dilution of WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis (see test-specific TCTs). Add both water and WAF volumes to 5-L aspirator bottle(s) equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting, as the

user deems appropriate. Note that at least 50 mL of volume should be allowed to drain from the outflow tubing before sampling. Collect 250 mL of each bulk solution, acidify with hydrochloric acid (HCl), and ship on ice to ALS Environmental, as described in the QAPP.

4. Take initial measurements of water temperature, pH, DO, and salinity, within each test chamber, as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Also remove a 5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Collect embryos as soon as possible after a spawn, recording the time.

6. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo's quality. At a 45x magnification, the developing embryonic morphology should be clearly discernible. Discard any embryos that look unfertilized or malformed. Since more embryos are obtained than the number needed for a test, it is best to remove any embryos that are at all questionable.

7. Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count the embryos. Count the embryos both as they come out of the eye dropper and after they have all been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

8. After all replicates are set up, cover exposure chambers with glass panes or inverted glass petri dishes to limit evaporation.

9. Observe and record survival, mortality, missing, and non-test mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Remove and archive dead animals unless they have decomposed and ensure that all COC documentation is filled out as described in the QAPP. Retain all dead animals to the extent possible and archive according to the QAPP. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP. Make the final survival count after 72 hours. Retain all remaining live and dead animals, to the extent possible, and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue samples, tissue samples were sometimes stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all dead animals were not counted or collected. An animal was considered dead if absent from the test chamber unless there was evidence of non-treatment mortality, such as jumping from the tank.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before hatch or immediately post-hatch.

10. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take a 5-mL sample for final PAH quantification by fluorescence spectroscopy as described in the RSMAS GLPP. Also remove a 5 mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

11. Remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anaesthetize all surviving animals with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers drop-wise until the animals stop moving. Collect animals and archive as outlined in the QAPP.

12. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

13. The test will be said to pass test criteria if the average control survival exceeds 70% across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

B. Testing Protocol 2: Yellowfin Tuna (*Thunnus albacares*) Embryo Acute Toxicity Test – Static Recirculating

Note: In general, the animals spawn in the evening and eggs are collected within 2 hours of the spawning event. The approximate developmental stage of the eggs at the initiation of testing is the late blastula or early gastrula period.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test seawater collected at the Achotines Laboratory was filtered down to 0.35 µm, UV-sterilized, and temperature-matched to the water in which the eggs were collected prior to use.

B.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the afternoon of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Acidify each sample with several drops of concentrated HCl to increase holding time. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

B.1.1 Embryo exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone

using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Seawater volume should be measured using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Dilution of WAF to treatment concentrations should be performed in bulk, with enough volume for all replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental for extraction and archiving, as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

B.1.2 Initial water quality measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

B.1.3 Embryo collection and experimental setup

1. Collect embryos the evening of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35 µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish

and improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1 hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the exposure room (set to 27°C) at the Achotines Laboratory for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 20–60 embryos into each test replicate using a large-bore Pasteur pipette (see test-specific TCTs).

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Replicates are maintained in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

B.1.4 Larvae collection and measurements

1. Following hatch [approximately 24–36 hours post-fertilization (hpf)], collect larvae for imaging or preserve in RNAlater for potential future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Proceed with imaging as outlined in Testing Protocol 4 of the RSMAS GLPP, *Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna* (Thunnus albacares) *Yolk-sac Larvae Static Recirculating Exposure*. Observe mortality visually and verify by prodding the animal with a pipette. Remove and archive numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Yellowfin tuna were not large enough for cannibalism at this life stage, and the static test setup made this a valid conclusion.

2. Take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

3. Anesthetize all surviving animals with clove oil by adding a concentrated solution to the exposure beakers. Collect and archive the animals as outlined in the QAPP.

4. Discard remaining test solutions as outlined in the *WAF and the toxicity test water disposal procedures SOP* in the RSMAS GLPP.

5. The test will be said to pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

C. Testing Protocol 3: Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna (*Thunnus albacares*) Yolk-sac Larvae – Static Exposure

These tests were performed in collaboration with the Northwest Fisheries Science Center/University of Miami RSMAS/Hopkins Marine Station of Stanford University.

C.1 WAF Preparation

1. Prepare the WAFs in advance according to *the Protocols for Preparing Water Accommodated Fractions* SOP, as described in the RSMAS GLPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

C.2 Embryo Exposure

Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. Measure seawater/WAF treatment volume using a graduated cylinder. Decant seawater/WAF into all replicate beakers for each treatment (see test-specific TCTs for number of replicates).

1. Transfer 20 fertilized embryos, screened at 2–3 hpf (~ 64-cell stage), into each exposure vessel using wide-bore glass pipettes.

2. Place vessels on a reciprocating shaker in a temperature controlled room set at 27°C.

3. Expose embryos until hatch (~ 24 hours at 27°C).

4. Monitor water quality and record before hatch. Water quality monitoring includes measurements of pH, DO, salinity, ammonia, and temperature.

C.3 Day 2: Morphological Assessment after Hatch

Image and process larvae in a temperature controlled room set to the same temperature as the exposure room (27°C).

A. Digital videomicroscopy

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed via Steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater in a 15-mL conical tube and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all the larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater with 10-ppm clove oil. Repeat process of capturing and mounting two or three larvae at a time until all larvae from the treatment vessel have been imaged, then move on to next beaker.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

C.4 RNA Preservation (RNAlater)

1. Label tubes using standardized nomenclature.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater with 10-ppm clove oil.

3. Using a wide-bore glass pipette, collect larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

Day 2 alternative: Collecting the majority of the replicates for RNA preservation

A. Digital videomicroscopy

1. Randomly select one replicate per treatment group.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, discard larvae.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

B. RNA preservation (RNAlater)

1. Label tubes using standardized nomenclature.

2. For the remaining replicates that were not imaged, anesthetize the fish using the same protocol above.

3. Using a wide bore glass pipette, collect anesthetized larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

C.5 RNA Sequencing Analysis* – Performed at the National Center for Genomic Research

RNA sequencing was performed at the National Center for Genome Research in Santa Fe, New Mexico. Total RNA samples were processed by taking an aliquot for quality control (QC) analysis to determine the amount sent and the integrity of the RNA using Qubit and Bioanalyzer, respectively. All samples passed QC and were made into sequencing libraries using the Illumina TruSeq RNA Sample Preparation Kit. Total RNA went through poly-A selection reaction in which the mRNA is pulled down using poly-T oligo attached to magnetic beads. The pulled-down mRNA is fragmented and randomly primed in a one-step reaction. The randomly primed mRNA is then taken through first strand synthesis using reverse transcriptase enzyme. The product then undergoes second strand synthesis using a second strand master mix that contains DNA polymerase I and RNase H. The synthesized second strand is end repaired using End Repair Mix (converts overhangs generated from fragmentation into blunt ends), follow by the addition of an A-base on the 3' end of the double-stranded cDNA molecule. The addition of A-base prepares it for the ligation of Illumina adapters that has a T-base on its 3' end. After the ligation of sample with uniquely barcoded adapters, the resulting product was taken through 15 cycles of PCR amplification. A QC is then performed on the Nanodrop for the amount and the Bioanalyzer for fragment size determination of the library, and also to check for any adapter dimers.

*Protocol applies to definitive test 543 only.

D. Testing Protocol 4: Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna (*Thunnus albacares*) Yolk-sac Larvae – Static Recirculating Exposure

These tests were performed in collaboration with the Northwest Fisheries Science Center/University of Miami RSMAS/Hopkins Marine Station of Stanford University.

D.1 WAF Preparation

1. Prepare the WAFs in advance according to *the Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental, as specified in the QAPP. Acidify each sample with several drops of concentrated HCl to increase holding time. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

D.1.1 Embryo exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to

treatment concentrations in bulk, with enough volume for all replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal aliquot from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental, as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

1. Collect embryos the evening of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone, and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35 µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, and remove them using a soft mesh net and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the exposure room (set to 27°C) at the Achotines Laboratory for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Replicates are maintained in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

D.1.2 Larvae collection and measurements

Following hatch (approximately 24–36 hpf), collect larvae for imaging or preserved in RNAlater for potential future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening the stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining.

D.2 Day 2: Morphological Assessment after Hatch

Image and process larvae in a temperature-controlled room set to the same temperature as the exposure room (27°C).

A. Digital videomicroscopy

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed by steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater in a 15-mL conical tube and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on the cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater with 10 ppm clove oil. Repeat process of capturing and mounting two or three larvae at a time until all larvae from the treatment vessel have been imaged.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

D.3 RNA Preservation (RNAlater)

1. Label tubes using standardized nomenclature described in the QAPP.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater with 10 ppm clove oil.

3. Using a wide-bore glass pipette, collect larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on the bench sheet and fill out the COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

Day 2 alternative: Collecting the majority of the replicates for RNA preservation

A. Digital videomicroscopy

1. Randomly select one replicate per treatment group.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and the dorsal to the top of the frame.

   a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

   b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, discard larvae.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

B. RNA preservation (RNAlater)

1. Label tubes using standardized nomenclature described in the QAPP.
2. For the remaining replicates that were not imaged, anesthetize the fish using the same protocol above.
3. Using a wide-bore glass pipette, collect anesthetized larvae from a replicate into a microcentrifuge tube.
4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.
5. Add 1 mL of RNAlater.
6. Store sample tubes at 4°C.
7. Record samples on the bench sheet and fill out the COC form.
8. Pack samples for shipment and store at 4°C until shipping.
9. Follow shipping guides written in the QAPP.

E. Testing Protocol 5: Embryo Acute Toxicity Test: Mahi-mahi (*Coryphaena hippurus*) and Cobia (*Rachycentron canadum*)

Note: In general, mahi-mahi spawn in the early morning, and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. In general, cobia spawn in the early evening and eggs are collected by 6:00–8:00 p.m. and are ready to use by 12:00 a.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV-sterilized seawater system at UMEH.

E.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the morning or evening of embryo collection, while LEWAFs and CEWAFs should be prepared 18-24 hours prior and the latter allowed to settle the morning or evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

4. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment

concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis. Add both water and WAF volumes to a 5-L aspirator bottle equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting, as the user deems appropriate. Note that at least 50 mL of volume should be allowed to drain from the outflow tubing before sampling. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

E.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Take 5 mL for initial PAH quantification, as described in the RSMAS GLPP. Also remove a 1.5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

E.3 Embryo Collection and Experimental Setup

1. Cobia – collect embryos the evening of the spawn (within 2 hpf; approximately 8:00 p.m.) and place in 100 ppm formalin for 1 hour.

   Mahi-mahi – collect embryos the morning of a spawn and place in 100 ppm formalin for 1 hour.

   Formalin acts as an antifungal/antibacterial agent increasing embryo survival. Rinse the embryos with three chamber volumes of UV-treated seawater (approximately 30 minutes) and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because the embryos are obtained well in excess of what is needed for a test, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count out the embryos. Count the embryos both as they come out of the eye dropper and after all have been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

4. After all replicates are set up, cover exposure chambers with glass panes to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with 16:8 light/dark photoperiod.

E.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-test mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Perform daily measurements of water chemistry and physical parameters, as outlined in QAPP and RSMAS GLPP. Make the final survival count after 96 hours. Retain all remaining live and dead animals to the extent possible and archive according the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the tank. Cobia and mahi-mahi are not large enough for cannibalism at this life stage, and the static test setup made this a valid conclusion.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

2. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the RSMAS GLPP. Take 5 mL for final PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP. Also remove 5 mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

3. Remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers drop-wise until the animals stop moving. Collect and archive the animals as outlined in the QAPP.

4. Discard remaining test solutions as outlined in *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

5. The test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates, as described in the QAPP and RSMAS GLPP.

F. Testing Protocol 6: Morphological Assessment of Crude Oil Cardiotoxicity in Mahi-mahi (*Coryphaena hippurus*) Yolk-sac Larvae

These tests were performed in collaboration with the Northwest Fisheries Science Center/University of Miami RSMAS/Hopkins Marine Station of Stanford University.

F.1 Day 0: Exposure

1. Prepare WAF and dilutions according to the QAPP.
2. Transfer 1 L of each WAF dose into a 1-L glass beaker.
3. Transfer 20 or 40 fertilized eggs into the 1-L glass beaker.
4. Expose embryos for 48 hours.

F.2 Day 2: Morphological Assessment after Hatch

Digital video microscopy:

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed in Steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. For each beaker, without anesthetic capture two or three larvae at a time using a plastic transfer pipette or a wide-bore glass Pasteur pipette.

3. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

4. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

5. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame. In some earlier tests, iMovie was used for image acquisition at one microscope station due to issues with the operating system on the available MacBook Pro laptop.

a. Image capture

i. At 3x, focus on cardiac/pericardial region and frame it from the head to the posterior of the yolk sac.
ii. At 3x, for two or three larvae per beaker, take composite shots of the entire larvae.

b. Video capture – 10-second video clips of the heart and pericardial area are captured for each larva at the highest magnification (6.3x).

6. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater. Repeat procedure until all larvae from the beaker have been imaged. Once all larvae from a replicate have been imaged and transferred to clean water, fix larvae in paraformaldehyde (PFA) or snap freeze (see below).

7. Save and copy files to two back-up hard drives.

8. Process image according to the NWFSC GLPP.

F.3 Fixation

1. Label 1.7-mL microcentrifuge tubes using standardized nomenclature.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater.

3. Using a plastic transfer pipette or a wide-bore glass pipette, transfer and pool larvae of one replicate into a microcentrifuge tube (i.e., one microcentrifuge tube per beaker).

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1.5 mL 4% Millonig's buffered PFA to tube and cap tube.

6. Fix overnight at 4°C or at room temperature for 4 hours.

7. After fixation, wash embryos into phosphate buffered saline (PBS) with one fast wash and then several longer washes (minimum three); store at 4°C.

8. Wash larvae into methanol with 10-minute washes of methanol/PBS

a. 25% methanol
b. 50% methanol
c. 75% methanol

d. 100% methanol
e. Store at -20°C.

9. Record samples on bench sheet and fill out COC form.

10. Pack samples for shipment and store at 4°C until shipping.

11. Follow shipping guides written in the QAPP.

F.4 Cryopreservation

1. Label vials using standardized nomenclature described in the QAPP.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater.

3. Using a plastic transfer pipette or a wide-bore glass pipette, transfer and pool larvae of one beaker into a cryo vial (i.e., one cryo vial tube per beaker).

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Immediately place sample tube in liquid nitrogen.

6. Store samples at -80°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

G. Testing Protocol 7: Juvenile (~ 30–45 DPH) Mahi-mahi (*Coryphaena hippurus*) Swim Performance Following Acute Embryonic Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

G.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs are made the morning of embryo collection, while CEWAFs are prepared 24 hours prior and allowed to settle the morning of embryo collection.

2. Perform acute embryo exposures in 2,500-L cylindrical fiberglass tanks at UMEH with a total test solution volume of approximately 2,200 L. The test medium is natural, filtered, and UV-treated seawater. Because oil/PAHs can adhere to plastic, use glass graduated cylinders to add WAF. Fill treatment tank with seawater and add WAF under inflow at approximately one-half to two-thirds final volume to achieve adequate mixing, and continue to fill to final volume. Use a single exposure chamber for each concentration.

3. After exposure chambers are filled and all WAF has been added, collect an initial water sample from the control and treatment tanks for chemical analysis by ALS Environmental as specified in the QAPP. Collect a water sample from each tank at 24 and 48 hours after WAF addition. Collect samples taken 48 hours after WAF preparation just prior to initiating flow-through conditions. Collect additional water at 24 and 48 hours following initiation of flow-through conditions from each treatment tank. Send all water samples for chemical analysis by ALS Environmental. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

G.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

G.3 Embryo Exposure

1. Expose embryos for 48 hours under static conditions with light aeration (see test-specific TCTs for details). Make initial egg counts using a volumetric method, whereby eggs are allowed to settle in oxygen-saturated water under static conditions in a graduated cylinder or beaker. Non-viable eggs will sink out under these conditions and be removed, so as to ensure that all embryos used in the trials are viable. From the mass of floating (i.e., viable) eggs, remove three 1-mL subsamples and count to obtain an egg-per-mL count. Obtain the final number of embryos using the mean egg-per-mL count and multiplying by the total volume (mL) of floating eggs. Thus each tank will be stocked with a specific volume of eggs to obtain the desired stocking density (see test-specific TCTs for details). Make effort to stock similar densities of embryos in control and treatment tank. Following the 48-hour exposure period, return flow of clean seawater to all tanks and maintain under flow-through conditions with gentle aeration for the remainder of the experiment until the fish reach adequate size for swimming (approximately 30–45 days, depending on rearing temperature). During this growout period, rear fish at UMEH using hatchery protocols.

2. During the 48-hour oil exposure, perform daily measurements of water quality and physical parameters, as outlined in RSMAS GLPP and test-specific TCTs. Remove 1.5 mL water sample for ammonia analysis daily for first 48 hours, and store at -20°C until assayed.

3. Keep notes throughout the larval rearing process, indicating any major drops in the number of larvae in each tank and being careful to note the time and date when these drops occurred. Additionally, note any signs of health impairment or distress should these conditions occur during the growout period.

G.4 Swimming Performance

1. Once fish are approximately 30–45 DPH, select fish to be used for swimming experiments at random using a slow scooping motion from below the fish in a way that avoids detection and stress. Transfer collected fish in buckets filled with seawater from the UMEH to RSMAS and hold in an environmental chamber overnight without access to food until ready to initiate experiments.

2. Every morning prior to swimming experiments, calibrate each swim tunnel setup for oxygen concentration and flow velocity. Open the AutoResp2 software. Oxygen is calibrated by locking a "high" value using vigorously aerated water (typically 1 hour) and by locking a "low" value using a 10 g/L solution of sodium sulfite. Calibrate flow velocity by adjusting the voltage using the speed-dial on the motor controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Adjust water temperature to 27°C using a submersible aquarium heater. Record calibration values on Swim Tunnel Calibration and Data Collection Table.

3. Collect each fish by gently corralling into a small transfer vessel (e.g., 500 mL beaker) and place into one of four swim chambers (every attempt should be made to randomize selection of swim chamber). Measure mass and total body length (BL) post-swimming to minimize handling stress.

4. Once placed in the swim tunnel, monitor the fish using a small video camera connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe.

5. Perform automated intermittent flow respirometry using up to four miniature Blazka-type variable speed respirometers with a DAQ-M control device and the AutoResp2 software.

6. Initiate a low flow velocity within the swim tunnel (~ 1 cm/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 4 hours.

7. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 4 cm/second followed by consistent increments in flow every interval (~0.5 BL/second) until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the back screen and does not regain activity after briefly decreasing flow and then

returning to the last speed achieved. Record the duration (T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed, and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

8. Euthanize fish according to the University of Miami animal care protocol using an overdose of MS-222. Record mass and length.

9. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

10. Clean each swim tunnel system in its entirety daily with a mix of mild detergent (Simple Green) and 10% bleach and rinse thoroughly two or three times with deionized water.

11. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

H. Testing Protocol 8: Sub-adult Mahi-mahi (*Coryphaena hippurus*) Swim Performance Following Acute Sub-adult Stage Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

H.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening prior to sub-adult fish exposure.

2. Perform sub-adult fish exposures in 2,500-L cylindrical fiberglass tanks at UMEH with a total test solution volume of approximately 360–900 L. The test medium is natural, filtered, and UV-sterilized seawater. Fill treatment tank with seawater. After treatment tank is filled, turn on the circulation pump to generate a directional current within the treatment tank for the ram ventilating fish species. Add WAF to treatment tank (do not expose control fish to WAF but otherwise treat as the exposed fish). Because oil/PAHs can adhere to plastic, use glass graduated cylinders to add WAF. Use a single treatment tank for each concentration.

3. Following a short period of circulation (~ 5 minutes) to achieve adequate mixing, collect an initial water sample from the control and treatment tanks for chemical analysis by ALS Environmental as specified in the QAPP. Collect additional water samples at 24 hours after WAF addition from each treatment tank. Send all water samples for chemical analysis by ALS Environmental. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

H.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO*

SOP, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

H.3 Sub-adult Exposure

1. Expose one sub-adult fish (0.15–1 kg) for 24 hours under static conditions (i.e., no new water inflow) with light aeration. Generate a directional water flow in the treatment and control chambers using a small pump to recycle the treatment media. Following the 24-hour exposure period, transfer the fish to the swim tunnel.

2. During the 24-hour oil exposure, perform measurements of water quality and physical parameters, as outlined in the QAPP and RSMAS GLPP. Remove a 1.5-mL water sample for ammonia analysis at the start and end of the 24-hour exposure period and store at -20°C until assayed.

3. Keep notes throughout the exposure period indicating any loss of fish in control and treatment tanks. Additionally, note any signs of health impairment or distress should these conditions occur during the exposure period.

H.4 Swimming Performance

1. Sub-adult fish used for swimming experiments are either hatchery-raised or from wild populations. Use only fish that are deemed to be in good health for swimming experiments, and select individuals randomly from the available fish at the RSMAS aquaculture facility. Feed fish on the day of their removal from their holding tanks, and withhold food from individuals during the exposure period.

2. Remove fish from their holding tanks (2,500–8,000 L each) using soft mesh nets, a water-filled vinyl sling, or water-filled plastic bags depending on the size being captured. Select fish randomly using a slow scooping motion from below the fish in a way that avoids detection and stress.

3. Calibrate the swim tunnel setup for oxygen concentration and flow velocity every morning prior to swimming experiments. Open the AutoResp2 software; oxygen is calibrated by locking a "high" value using vigorously aerated water (typically 20 minutes) and by locking a "low" value using a 10 g/L solution of sodium sulfite. Flow velocity is calibrated by adjusting the voltage/RPM using the speed-dial on the motor

controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Record water temperature (ambient) for calibration purposes and throughout the experiment. The swim tunnel will have filtered and UV-sterilized water flowing through the buffer chamber for the duration of the experiment to eliminate build-up of metabolic wastes. Water temperature will either be the ambient temperature of the RSMAS aquaculture facility or will be controlled by an inline heat pump to maintain a specific temperature (see test-specific TCTs). Record calibration values on the Swim Tunnel Calibration and Data Collection Table.

4. Transfer collected fish to the swim tunnel following treatment using the aforementioned methods (nets, sling, or bags). Generate a slow water current in the swim tunnel to replicate the water flow in the treatment tank from which the fish came. Place fish into the test section of the swim tunnel and secure the lid. Keep the test section on a "flush" cycle until fish become acclimated to the swim tunnel, during which time the fish will be swimming at a slow and steady speed. Confirm acclimation by the occurrence of two consecutive routine oxygen consumption (MO_2) readings of similar value at a level known to be indicative of low-exertion swimming.

5. Measure mass, total BL, and fork BL post-swimming to minimize handling stress.

6. Once placed in the swim tunnel, monitor the fish using two small video cameras connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe.

7. Perform automated intermittent flow respirometry using a 90-L swim tunnel variable-speed respirometer with a DAQ-M control device and the AutoResp2 software.

8. Initiate a low flow velocity within the swim tunnel (~ 1–3 BL/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 1 hour or longer if necessary until two consecutive 20-minute measurements of MO_2 are approximately the same.

9. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 80 cm/second (depending on size of test organisms) followed by consistent increments in flow every interval until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the back screen and does not regain activity after briefly decreasing flow and then returning to the last speed achieved. Record the duration

(T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

10. At the conclusion of the test, either sedate using MS-222 and transfer to a recovery tank or euthanize according to the University of Miami animal care protocol using an overdose of MS-222; mass and length should be recorded.

11. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

12. Clean the swim tunnel system in entirety after each testing period using a 10% bleach solution and rinse thoroughly two or three times with clean tap water. Drain all water from the swim tunnel system following each testing period to allow for drying of system components.

13. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

I. Testing Protocol 9: Juvenile Mahi-mahi (*Coryphaena hippurus*) Swim Performance Following Acute Juvenile Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

I.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the morning of the juvenile fish exposure.

2. Perform juvenile fish exposures in 20-L glass jars with a total test solution volume of approximately 12 L. The test medium is natural, filtered, and UV-sterilized seawater. Because oil/PAHs can adhere to plastic, use glass graduated cylinders or Hamilton syringes to add WAF. After adding WAF to the seawater, mix by gently stirring the solution using a Teflon coated stir rod.

I.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. Also, collect initial samples for total PAH analysis by ALS Environmental and store samples at 4°C. All SOPs can be found in the RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

I.3 Juvenile Exposure

1. Collect juveniles just after first feeding so that the animals are starved for at least 24 hours prior to swimming, and immediately transfer to the University of Miami environmental chamber (set to 27°C or an alternative temperature, as specified in the test-specific TCT) for exposure set-up. Juveniles should be transferred in at least 15 L of pre-aerated saltwater.

2. Transfer juveniles into each replicate chamber in random order 24 hours before transfer to swim tunnels (see test-specific TCTs for total number of individuals). Note that exposure initiation may be staggered to accommodate the lag time (timing effect) associated with swim performance tests.

3. After all replicates are set up, cover exposure chambers with a glass lid to limit evaporation. Maintain exposures in the environmental control chamber at 27°C with 16:8 light dark photoperiod and gentle aeration with an air stone.

4. Note that some WAF exposure durations may be less than the actual 24 hour holding time (e.g., the second 12 of the 24 hours). In such cases, fish will be held in a chamber containing clean seawater for a given time period and then transferred to another chamber containing the desired WAF dilution for the remaining time period. Initial and final measurements for water temperature, pH, salinity, and DO will be collected from the WAF dilution exposures, as outlined in the QAPP and RSMAS GLPP. Initial and final samples for total PAH analysis by ALS Environmental will also be collected and stored at 4°C. Alternatively, exposures may occur within the actual swim tunnel apparatus following a 24 hour holding period in clean seawater in the environmental chamber as described above. Such exposures will be initiated just following the introduction of the fish to the swim tunnel by adding the appropriate volume of WAF directly to the surrounding swim tunnel reservoir and mixed well with a glass stir rod to obtain the desired WAF dilution. Exposures will proceed during the acclimation period (typically 4 hours) and continue through the swim trial itself. Initial and final measurements for pH and salinity will be collected from the swim tunnel reservoir, as outlined in the QAPP and RSMAS GLPP (temperature and DO are measured continuously in the swim tunnel using the Fibox system used for measuring respiration). Initial and final samples for total PAH analysis by ALS Environmental will also be collected from the swim tunnel reservoir and stored at 4°C. Total PAH samples should be shipped on ice to ALS Environmental as soon as possible.

5. Observe and record mortality daily. Observe mortality visually and verify by prodding the animal with a Teflon coated stir rod. Remove and archive dead animals and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

6. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Also remove 1.5-ml water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out. Collect final samples for total PAH analysis by ALS Environmental and store at 4°C. Ship samples on ice to ALS Environmental as soon as possible.

I.4 Swimming Performance

1. Every morning prior to swimming experiments, calibrate each swim tunnel setup for oxygen concentration and flow velocity. Open the AutoResp2 software. Oxygen is calibrated by locking a "high" value using vigorously aerated water (typically 1 hour) and by locking a "low" value using a 10 g/L solution of sodium sulfite. Calibrate flow velocity by adjusting the voltage using the speed-dial on the motor controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Adjust water temperature to 27°C using a submersible aquarium heater. Record calibration values on Swim Tunnel Calibration and Data Collection Table.

2. Collect each fish by gently corralling into a small transfer vessel (e.g., 500 mL beaker) and place into one of four swim chambers (every attempt should be made to randomize selection of swim chamber). Measure mass and total BL post-swimming to minimize handling stress.

3. Once placed in the swim tunnel, monitor the fish using a small video camera connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe. For hypoxia exposures (see test-specific TCTs), only measure oxygen concentrations during the acclimation period. Because the oxygen levels are likely very near the survival threshold, and measuring oxygen consumption requires a closed interval during which ambient oxygen is depleted by the fish, conduct the hypoxia swim trials with a continuous flush cycle and no additional oxygen measurements.

4. Perform automated intermittent flow respirometry using up to four miniature Blazka-type variable speed respirometers with a DAQ-M control device and the AutoResp2 software.

5. Initiate a low flow velocity within the swim tunnel (~ 1 cm/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 4 hours. For hypoxia exposures, slowly decrease oxygen levels to the desired level (~ 37.5% O_2 saturation) during the acclimation period by gently bubbling nitrogen gas into the reservoir water surrounding the swim chamber (see test-specific TCTs).

6. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 4 cm/second followed by consistent increments in flow (~0.5 BL/second) every interval until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the

back screen and does not regain activity after briefly decreasing flow and then returning to the last speed achieved. Record the duration (T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

7. Euthanize fish according to the University of Miami animal care protocol using an overdose of MS-222. Record mass and length.

8. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

9. Clean each swim tunnel system in its entirety daily with a mix of mild detergent (Simple Green) and 10% bleach and rinse thoroughly two or three times with deionized water.

Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

J. Testing Protocol 10: Mahi-mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Exposure to WAF – Static Recirculating

Note: In general, mahi-mahi spawn in the early morning and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV sterilized seawater system at UMEH.

J.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP and RSMAS GLPP. For tests designated as an "unsettled HEWAF exposure" make the following modifications concerning Step C (Separation) in the *Protocols for Preparing Water Accommodated Fractions* SOP in the QAPP. For all other WAF exposures proceed to Step 2.

    a. Make HEWAFs the morning of the exposure initiation and do not allow them to settle before use. Instead, maintain the HEWAF under agitation for 1 hour on the stir plate before use.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

J.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

J.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette. Embryos should be added 1 cm below the water surface to avoid the oil slick forming on surface.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

6. For each treatment, at 96 hours (or final time point; see test-specific TCT), collect an equal volume sub-sample from each replicate beaker and composite. The composite sample volume must be at least 250 mL to ensure enough solution is available to fill the sample bottle for PAH analysis. Ship samples on ice overnight to ALS Environmental, as described in the QAPP.

J.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. The total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only use glass graduated cylinders or Hamilton syringes to add WAF. Perform the dilution of WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental, as described

in the QAPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

J.5 Larvae Collection and Measurements

1. At end of test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Record survival, mortality, missing individuals, and non-test mortality. Remove and archive numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. All surviving animals should then be anaesthetized with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222) and retained and archived according to the QAPP or preserved for possible histology analysis using a histological fixative (e.g., Zfix).

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will pass the test criteria if the average control survival meets or exceeds 80% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

K. Testing Protocol 11: Mahi-mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Differential Exposure Intervals – Static Recirculating

Note: In general, mahi-mahi spawn in the early morning and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV sterilized seawater system at the UMEH.

K.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP and RSMAS GLPP. HEWAFs should be made the morning of exposure initiation.

2. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

K.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

Additional temperature, pH, DO, and salinity measurements should be taken whenever WAF is switched to clean seawater or clean seawater to WAF. These measurements should be taken just following the washout or mixing period.

K.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

K.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment concentrations should be performed in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis.

To prepare WAF dilutions, add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times (see test-specific TCT).

1. Depending on the test-specific conditions, different exposure intervals (e.g., 2, 6, 24 hours) are initiated at different time points during 96-hour tests (e.g., 0-, 24-, 48-. and 72-hour time points). Thus, tests are either started with WAF in the exposure chamber or they are started with clean seawater in the exposure chamber. See test-specific TCTs for details on exposure interval length and exposure starting point for each individual test.

2. If the WAF exposure interval is at the start of the test, bulk WAF dilutions should be prepared at the desired nominal treatment concentrations (i.e., 1x) per description above. Once dilutions are prepared, dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8 L total). After the specified exposure interval, replace the WAF with clean seawater by briefly stopping flow to the Imhoff cone. Once flow is stopped, connect tubing from the 1-L beaker to a pre-filled intake tube drawing from a large reservoir of fresh seawater. Place a second larger tube on the outflow spout to drain into a bucket for disposal. Return flow to the Imhoff cone and allow fresh seawater to flush the Imhoff cone for 0.5 hour. During this time, discard the water in the beaker, rinse and wipe the beaker clean, and add 1 L of fresh seawater. After the 0.5-hour flush period, return tubing from the Imhoff to the 1-L beaker and remove the outflow tubing to allow for over flow to the 1-L beaker as it was set up before. Replace water in the control replicates to ensure that handling stress does not impact survival.

3. Tests with exposures initiated at the later time points begin with embryos placed in control seawater for each replicate at 0 hour. At the specified time, add WAF to the exposure chamber by replacing the 1-L beaker of clean seawater with 1 L of WAF that is 1.8x the desired WAF treatment concentration. Pause the flow from the peristaltic pump and the stopcock closed (simultaneously) during the exchange. Once the beaker is replaced, reinitiate flow to the Imhoff cone, allowing the 1.8x WAF in the beaker to mix with the clean seawater in the Imhoff cone. It is expected that the WAF in the beaker will be fully mixed with the clean seawater in the Imhoff cone within 1 hour of initiating flow.

4. Collect 250 mL from each initial bulk solution (the 1x or the 1.8x WAF) as well as a 250-mL composite sample for each dilution at the end of the exposure period. For the tests where clean seawater is replaced with WAF, also collect a 250-mL composite sample for each dilution after a 1-hour mixing period (i.e., the period after which the beaker water and Imhoff cone water are expected to be completely mixed). Ship all samples on ice overnight to ALS Environmental for total PAH analysis as described in the QAPP. Discard all WAF solutions according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

5. In addition, take a 10-mL composite sample from each treatment either after the 1-hour mixing period (when switching from clean seawater to WAF) or after the 0.5-hour washout period (when switching from WAF to clean seawater) for fluorescence measurements. Immediately dilute the 10-mL composite sample with 10 mL of ethanol for total PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and the *RSMAS GLPP*.

K.5 Larvae Collection and Measurements

1. At the end of the test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect the tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Record survival, mortality, missing individuals, and non-test mortality. Remove and archive

numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Euthanize all surviving animals with an overdose of sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222) and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will pass the test criteria if the average control survival meets or exceeds 80% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

L. Testing Protocol 12: 48-hour Exposure for RNA Analysis: Mahi-mahi (*Coryphaena hippurus*)

Note: In general, mahi-mahi spawn in the early morning, and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV-sterilized seawater system at UMEH.

L.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. Prepare HEWAFs the morning of exposure initiation.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice overnight to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

L.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

L.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

L.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment concentrations in bulk, with enough volume for all replicates and ALS Environmental sample analysis.

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect a composite 250-mL sample from each treatment and ship on ice to ALS Environmental as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

L.5 Larvae Collection and Cryopreservation

1. Take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Following hatch [approximately 48 hpf], collect larvae for cryopreservation for future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining.

3. Concentrate larvae by gently pouring through a fine-mesh filter and then transfer to a petri dish by squirting seawater through the opposite side. Score survival. Collect all live larvae with a large bore transfer pipette and transfer to a 2-mL cryotube. Remove as much excess water as possible with a Pasteur pipette, close tube, and immediately transfer to a dewar filled with liquid nitrogen. Once all animals are collected, transfer tubes immediately from liquid nitrogen to -80ºC freezer. Ship samples to the National Oceanic and Atmospheric Administration's Northwest Fisheries Science Center overnight on dry ice.

4. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. When possible, the volume was limited.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi are not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

5. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

6. The test will pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

M. Testing Protocol 13: Mahi-mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Differential Exposure Intervals – Static

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

M.1 LEWAF Preparation

1. Prepare the LEWAF 18–24 hours in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP.

2. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add LEWAF. Dilute LEWAF to desired treatment concentrations in bulk, with enough volume for all treatment replicates and for ALS Environmental sample analysis. Prepare LEWAF dilutions in a 5-L glass bottle and spin the solution for 5 minutes on a stir plate with a 50% vortex. Aliquot the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental, as described in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling should be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and the RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible. Discard unused LEWAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

M.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP, Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling the test chamber and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Use 10 mL for initial PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP.

M.3 Embryo Collection and Experimental Setup

1. Collect the embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain the water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water; place eggs in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a common paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface; remove them using a soft mesh net and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because the need for extra embryos is accounted for and excess embryos are on hand, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order using a wide-bore Pasteur pipette. Count the embryos, both as they come out of the pipette and after all have been transferred. Depending on the test, replicate beakers may have WAF treatment solutions or clean water at the start of the test. Exposure times to LEWAF will vary depending on the test and will involve one to two transfers to fresh treatment water (i.e., LEWAF dilution or clean seawater). The transfer of embryos (or larvae if hatched) to LEWAF or clean seawater should also be performed using a wide-bore Pasteur pipette. See test-specific TCTs for details on the exposure interval length and the exposure starting point for each individual test.

4. After appropriate duration, transfer the embryos from the WAF treatment solution to clean source water (or vice versa for tests started with clean water). See test-specific TCT for exposure duration and interval within the test.

5. If the exposure interval is in the middle of the test duration (e.g., during a 96-h test embryos start out in clean water, get transferred to WAF at 24 h, then exposed for 24 h, and then transferred back to clean water from the final 48 h), perform one more transfer of embryos to clean source water. See test-specific TCT for exposure duration and interval within the test.

6. Following set up or transfer of embryos, cover exposure chambers with glass lids to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with a 16:8 light/dark photoperiod.

M.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-test mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

2. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The volume of test water was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the tank. Cobia and mahi-mahi are not large enough for cannibalism at this life stage, and the static test set-up made this a valid conclusion.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

3. Collect a 250-mL composite of each LEWAF dilution at the end of the exposure period after embryos have been transferred out of each of the corresponding replicate vessels and ship the composite overnight on ice to ALS Environmental as described in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

4. Take daily measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the RSMAS GLPP. Initial measurements should also be taken for each transfer solution before transferring ring embryos. Also remove a 1-mL water sample for ammonia analysis at the end of the exposure and store it at -20°C until assayed. Ensure that all meter calibration logs are filled out.

5. At the end of the test, remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers using a dropper until the animals stop moving. Collect, count, and archive the animals as outlined in the QAPP.

6. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Note: A test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates.

N. Testing Protocol 14: Mahi-mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Different Exposure Intervals to Oil Slicks – Static

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

N.1 Oil Slick Preparation

1. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

2. Oil slicks are prepared by evenly spreading 2 g of Slick A or Slick B oil around the internal circumference of a 2.5-inch diameter PVC pipe coupler fitting and suspending the pipe for 4 hours below the surface of the water so that the oil is completely submerged but near the water surface. At the end of 4 hours, the pipe is slowly removed and the embryos added carefully beneath the slick, as described in Section N.3.

N.2 Initial Water Quality Measurement

Before slick formation, take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP, Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP.

N.3 Embryo Collection and Experimental Set-up

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and

rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This allows non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water before placing them in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, then remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because excess embryos are on hand, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order using a wide-bore Pasteur pipette. Count the embryos, both as they come out of the pipette and after all have been transferred. Transfer embryos by placing the tip of the Pasteur pipette several millimeters beneath the slick and then expelling embryos. Avoid disrupting the slick.

4. Exposure times to slick oil will vary depending on the test and will involve one to two transfers to fresh treatment water (i.e., from slick oil to clean water or from clean seawater to slick oil). Transfer of embryos (or larvae if hatched) to slick oil or clean seawater should also be performed using a wide-bore Pasteur pipette. See test-specific TCTs for details on exposure interval length and exposure starting point for each individual test.

5. Following set-up or transfer of embryos, cover exposure chambers with glass lids to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with a 16:8 light/dark photoperiod.

N.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-test mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

2. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The test water volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the tank.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

3. Take daily measurements of water temperature, pH, salinity, and DO for each treatment from a "dummy" beaker as outlined in the RSMAS GLPP. Do not take daily water quality measurements from actual exposure chambers, as this may disrupt the oil slick. Initial measurements should also be taken for each transfer solution before slick formation and transfer of embryos. Also remove 1 mL of water sample for ammonia analysis at the end of the exposure and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

4. At the end of the test, remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers using a dropper until the animals stop moving. Collect, count, and archive the animals as outlined in the QAPP.

5. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Note: The test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates.

O. Testing Protocol 15: Mahi-mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Exposure to Oil Slicks – Static Recirculating

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

O.1 Oil Slick Preparation

1. Perform acute embryo toxicity tests in 0.8-L glass Imhoff cones with a total test solution volume of 1.8 L (includes a 1-L glass overflow beaker). The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

2. Oil slicks are prepared by evenly spreading 2 g of Slick A or Slick B oil around the internal circumference of a 2.5-inch diameter PVC pipe segment and suspending the pipe for 4 hours below the surface of the water so that the oil is completely submerged but near the water surface. Note that flow through the cones is paused during the 4-hour incubation period. At the end of 4 hours, the pipe is slowly removed and the embryos are carefully added beneath the slick, as described in Section O.3.

O.2 Initial Water Quality Measurement

Take initial measurements (following the 4-hour incubation period) of water temperature, pH, DO, and salinity from each overflow beaker, as outlined in the *Temperature Measurement SOP, pH Measurement SOP, Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP.

O.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater.

Maintain the water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable embryos float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water before placing them in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a common paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, then remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test set-up.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette.

5. Transfer embryos by placing the tip of the Pasteur pipette several millimeters beneath the slick and then expelling the embryos. Avoid disrupting the slick.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with a 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters from overflow beakers, as outlined in the RSMAS GLPP.

O.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct the pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

1. Depending on the test-specific conditions, the water underlying the slick may be first replaced by flushing with clean seawater for 30 minutes before introducing the embryos. The flushing procedure is as follows: while the flow is paused, connect the tubing that is drawing from the 1-L beaker, to a valve attached to a pre-filled intake tube that is drawing from a large reservoir of fresh seawater. Place a second, larger tube on the outflow spout to drain into a bucket for disposal. Return the flow to the Imhoff cone and allow fresh seawater to flush the Imhoff cone for 0.5 hour. After the 0.5-hour flush period, return the tubing from the Imhoff to the 1-L beaker and remove the outflow tubing to allow for overflow to the 1-L beaker, as it was set up before.

2. Just before introducing the embryos, collect a 250-mL composite sample for each set of four replicates by sampling from the corresponding overflow spouts. Ship all samples on ice overnight to ALS Environmental for total PAH analysis, as described in the QAPP.

O.5 Larvae Collection and Measurements

1. At the end of the test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt the pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect the tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening the stopcock below the surface and allowing the beaker to slowly drain to a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

Record survival, mortality, missing individuals, and non-test mortality. Remove and archive numbers of dead animals unless the animals have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Euthanize all surviving animals with an overdose of sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222) and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume of test water was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test set-up made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will be said to pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates.

P. Testing Protocol 16: Copepod Acute Toxicity Test

Note: Experiments are set up using a synchronous culture, where all animals are the same age (within 24 hours). Adult copepods will be approximately 17 days old at test initiation.

Before beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. Obtain all test water from the UV-sterilized seawater system at UMEH.

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Prepare HEWAF the morning of experimental setup.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental, as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be overnight shipped on ice to ALS Environmental as soon as possible.

Note: Samples collected for the State of Louisiana tasks must be sent with a separate COC form and in a different shipment from samples collected under tasks conducted under the National Oceanic and Atmospheric Administration (NOAA) contract.

3. Use the bulk WAF solution to make the standard curve dilution series described in *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP. This dilution series should include a seawater blank and span the concentration range of WAF to be used in the test. Ensure that all documentation is kept.

4. Perform acute toxicity tests in 250-mL glass beakers with a total test solution volume of 200 mL. The test medium is UV-sterilized, 0.45-µm filtered seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to treatment concentrations with enough volume for five treatment replicates and ALS Environmental sample analysis (total volume 1.5 L). Add both water and WAF volumes to a 2-L glass beaker. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental for analysis, as described in the QAPP. Take 5 mL for initial PAH quantification, as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS

GLPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

5. Add 200 mL of WAF treatment or clean source water to replicate beakers.

6. Set up one non-treatment beaker as described above, without oil, and monitor for representative water quality daily, including dissolved oxygen, pH, and salinity.

7. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

8. Concentrate age-synchronized, adult copepods and place in a clean crystallizing dish. To concentrate copepods, place the dish containing the copepods on the counter in a dark room, and use a small light-emitting diode (LED) light placed against the side of the dish.

9. Using a glass Pasteur pipette, remove 20 copepods from the concentrated adults. Confirm under a dissecting microscope that the correct number of copepods have been removed, and gently transfer animals to the test beaker using a Pasteur pipette, taking care to not allow the pipette to come into contact with the treatment water.

10. Continue to transfer 20 copepods into each test replicate in random order.

11. After all replicates are set up, cover exposure chambers with aluminum foil to limit evaporation. Replicates are maintained in an incubator, with low aeration, at 25°C with a 24-hour light photoperiod. Aeration is accomplished using PE60 tubing with a 22G x 1.5-inch hypodermic needle (plastic hub removed), set to ~ 1 bubble/second.

12. For tests where copepods are fed, feed each beaker daily with 1 x 10^4 cells/mL *Isochrysis* (TISO) and 1 x 10^4 cells/mL *Cheatocerous* (CHGRA). See test-specific TCT for feeding.

13. The test is complete after 48 or 96 hours (see test-specific TCT). At the end of the test, record water quality (dissolved oxygen, pH, temperature and salinity) for each beaker. Take 5 mL for the final PAH quantification, as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP.

14. Gently pour each beaker through a 40-µm nylon filter to concentrate animals and eggs, and then place them into a clean glass crystallizing dish with a small amount of clean source water.

15. Determine the sex for all adult copepods by microscopic examination of uropod segments and antennae morphology using an inverted compound scope.

16. Determine the number of live and dead adults and nauplii under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. Remove dead animals and archive according to the QAPP.

17. For some tests, hatching success will be measured after adult survival counts have been determined. See test-specific TCT for list of endpoints. If hatching success is measured, a subset of 20 eggs from each replicate beaker will be pipetted out and placed in a 60 x 15 mm plastic Petri dish with clean seawater and returned to the incubator. The remaining animals from each replicate (live adults and nauplii) will be fixed using formalin and stained with the addition of Rose Bengal to make counting nauplii easier.

18. Count all nauplii from each replicate.

19. Hatching success will be determined after 72 hours using the 20 eggs set aside in Step 12. After 72 hours, samples will be fixed with formalin and stained using Rose Bengal, and all hatched nauplii and unhatched eggs will be counted and hatching success determined.

20. Retain all adult animals and nauplii, to the extent possible, by preserving in 10% buffered formalin. Archive according to the QAPP.

Note: Because attempts to separate water and dead animals will mostly result in destruction of the tissue sample, tissue samples may be stored with small amounts of test water. The volume of test water will be limited where possible.

21. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

22. The test will be said to pass test criteria provided that the average control survival of adult copepods exceeds 85% across the four replicates, and that there are no unacceptable deviation in water quality among replicates.

Q. Testing Protocol 17: Copepod Nauplii Acute Toxicity Test

Note: Experiments are set up using a synchronous culture, where all animals are approximately the same age (within 24 hours). Nauplii will be approximately 12–24 hours old at test initiation.

Before beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP and the RSMAS GLPP. All test water is obtained from the UV-sterilized seawater system at UMEH.

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the RSMAS GLPP. Prepare HEWAF the morning of experimental set up.

2. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

Note: Samples collected for the State of Louisiana tasks must be sent with a separate COC form and in a different shipment from samples collected under tasks conducted under the NOAA contract.

3. Perform acute toxicity tests in 250-mL glass beakers with a total test solution volume of 200 mL. The test medium is UV-sterilized, 0.45-µm filtered seawater. Measure the seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass-graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to treatment concentrations with enough volume for 5 treatment replicates and ALS Environmental sample analysis (total volume 1.5 L). Add both water and WAF volumes to a 2-L glass beaker. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental for analysis, as described in the QAPP. Take 5 mL for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

4. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Concentrate age-synchronized copepod nauplii and place in a clean crystallizing dish. To concentrate copepods, place the dish containing the copepods on the counter in a dark room, and use a small LED light placed against the side of the dish.

6. Using a glass Pasteur pipette, remove 20 copepods from those that are concentrated by the light. Confirm under a dissecting microscope that the correct number of copepods have been removed, and gently transfer the animals to the test beaker using a Pasteur pipette, taking care to not allow the pipette to come into contact with the treatment water.

7. Continue to transfer 20 copepods into each test replicate in random order.

8. After all replicates are set up, cover exposure chambers with aluminum foil to limit evaporation. Replicates are maintained in an incubator, with low aeration, at 24°C with a 24-hour light photoperiod. Aeration is accomplished using PE60 tubing with a 22 G x 1.5-inch hypodermic needle (plastic hub removed), set to ~ 1 bubble/second.

9. If the test includes feeding, transfer 1 x 10^4 cells/mL *Isochrysis* (TISO) and 1 x 10^4 cells/mL *Cheatocerous* (CHGRA) to each beaker daily.

10. The final survival count is made after 48 hours. At the end of the test, record water quality (dissolved oxygen, pH, temperature, and salinity) for each beaker. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out. Remove 5 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP.

11. Gently pour each beaker through a 40-µm nylon filter to concentrate animals, and then place into a clean glass crystallizing dish. Water from the replicate beakers will be pooled and then sampled for PAH analysis by ALS Environmental. See the QAPP for sampling and shipping procedures. Determine the number of dead animals under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. Once the dead animals are counted, fix using formalin. and then stain with the addition of Rose Bengal.

12. Count all animals in the fixed solution. Repeat for each replicate beaker. The total number of live animals will be determined from the total count of all stained animals minus the number of animals that were deemed dead before fixation and staining.

13. Retain all animals, to the extent possible, by preserving in 10% buffered formalin. Archive according to the QAPP.

Note: An animal will be considered dead if it is absent from the test chamber unless there is evidence of non-treatment mortality.

14. Discard remaining test solutions as outlined in the *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

The test will be said to pass test criteria provided that the average control survival exceeds 80% across the four replicates, and that there are no unacceptable deviations in water quality among replicates.

R. Testing Protocol 18: Toadfish Adrenal Study

This protocol describes four separate but related toadfish adrenal study trials.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontaminating Glassware* SOP, as described in the *QAPP*. All test water is obtained from the UV-sterilized seawater system at UMEH. Tests were performed using the following protocols:

Adrenal stress response study with large cannulated toadfish, exposed to oil only:

1. One week before exposure, prepare the cannulated toadfish. First, ensure that all toadfish to be cannulated are greater than 100 g in size. Next, anesthetize the toadfish using 1 g/L MS-222 buffered with sodium bicarbonate. Insert cannulas (Clay-Adams PE 50 tubing) filled with heparinized saline into the caudal vein. Secure the cannulas to the fish using 3-0 silk sutures and heat-seal the tip. Allow the fish to recover for up to one week in flow-through chambers.

2. After one week, take a post-recovery blood sample. To collect the blood sample, cut off the heat-sealed tip of the cannula, attach a heparin-filled disposable 1-mL syringe with a 23-gauge needle, and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µl "pre-sample" and set aside. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µl of blood (i.e., 0-h blood sample). Return the 100-µl pre-sample and then refill the catheter with heparizined saline to prevent the cannula from clotting. Keep the heparin- and saline-filled syringe attached to the cannula and carefully place the syringe and cannula in front of the glass chamber to ensure easy access for the next series of blood samples. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen for later analysis of cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

3. Prepare the WAFs in advance according to the *Protocols for Preparing Water* Accommodated *Fractions SOP*, as described in the QAPP. Prepare the HEWAFs less than 24 hours before exposure.

4. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

5. On the day of exposure, fill 1-L glass chambers with 0.75 L of test solution. Shield the sides and top of chambers to avoid evaporation and disturbance. Aerate each chamber using glass Pasteur pipettes.

6. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

7. Transfer the cannulated toadfish to their respective exposure chambers, one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil treatment were sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

8. After one hour of exposure, take blood and tissue samples from one control and one oil treatment fish.

9. Sample the control fish before the oil treatment fish. To collect a blood sample, first use a heparin-filled, disposable 1-mL syringe with a 23-gauge needle and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µl "pre-sample" and discard. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µl of blood and temporarily store it on ice.

10. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections, plus the section remaining, in liquid nitrogen. This will be analyzed later for corticotropin releasing factor (CRF) mRNA expression by quantitative real-time PCR (qPCR).

11. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

12. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental (ALS; formerly Columbia Analytical Services) as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the Analytical Sample Shipping and COC SOP, found in the QAPP and RSMAS Work Plan. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS Work Plan. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

13. Repeat steps 9 through 12, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

14. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Remove 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS Work Plan. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

15. Discard remaining test solutions as outlined in the WAF and *Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with large cannulated toadfish that were exposed to oil and air:

1. One week before exposure, prepare the cannulated toadfish. First ensure all toadfish to be cannulated are greater than 100 g in size. Next, anesthetize the toadfish using 1 g/L MS-222 buffered with sodium bicarbonate. Insert cannulas (Clay-Adams PE 50 tubing) filled with heparinized saline into the caudal vein. Secure the cannulas to the fish using 3-0 silk sutures and heat-seal the tip. Allow the fish to recover for up to one week in flow-through chambers.

2. After one week, take a post-recovery blood sample. To collect the blood sample, cut off the heat-sealed tip of the cannula, attach a heparin-filled disposable 1-mL syringe with a 23-gauge needle, and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µl "pre-sample" and set it aside. Then, using a new glass EDTA-rinsed

Hamilton syringe, collect 200 µL of blood (i.e., 0-hour blood sample). Return the 100-µl pre-sample and then refill the catheter with heparizined saline to prevent the cannula from clotting. Keep the heparin- and saline-filled syringe attached to the cannula and carefully place the syringe and cannula in front of the glass chamber to ensure easy access for the next series of blood samples. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

3. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Make HEWAFs less than 24 hours before exposure.

4. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

5. On the day of exposure, fill 1-L glass chambers with 0.75 L of test solution. Shield the sides and the top of the chambers to avoid evaporation and disturbance. Bubble air into each chamber using glass Pasteur pipettes.

6. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS Work Plan. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for the initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and the meter calibration logs are filled out.

7. Transfer the cannulated toadfish to their respective exposure chambers; one fish per chamber. Note that 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil

treatment are sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

8. After one hour of exposure, conduct air exposures by removing one control and one oil treatment fish from their chambers and place onto a clean damp towel for 5 minutes. After 5 minutes, return each fish to their respective chambers.

9. After 30 minutes post-air exposure, remove fish from the exposure chambers to sample blood and take tissue samples from one control and one oil treatment fish.

10. Sample the control fish before the oil treatment fish. To collect a blood sample, first use a heparin-filled disposable 1-mL syringe with a 23-gauge needle and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µl "pre-sample" and discard. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µL of blood and temporarily store on ice.

11. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections, plus the section remaining, in liquid nitrogen. These will be analyzed later for CRF mRNA expression by quantitative real-time PCR (qPCR).

12. Centrifuge the blood sample at 16,000 g for 5 minutes, retain plasma, and freeze immediately in liquid nitrogen for later analysis of cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

13. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental, [MK1]as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the Analytical Sample Shipping and COC SOP, found in the QAPP and RSMAS GLPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

14. Repeat steps 8 through 13, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

15. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

16. Discard remaining test solutions as outlined in the *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with small non-cannulated toadfish exposed to oil only:

1. Two days before exposure, place the non-cannulated toadfish (< 40 g) directly into 1-L glass exposure chambers, one per dish, with clean source water and allow to acclimate for 36–48 hours before starting the test.

2. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Prepare HEWAFs less than 24 hours before exposure.

3. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

4. On the day of exposure, after a 36 to –48-hour acclimation period, siphon the source water out of glass chambers and immediately replace it with appropriate WAF treatment or control water to start the exposure.

5. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the

respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

6. Immediately before transferring toadfish to exposure chambers, collect blood and tissue samples from one toadfish.

7. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

8. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

9. Immediately after collecting the blood sample, over-anesthetize the fish using a 1-g/L solution of MS-222 and remove the kidney for in-vitro analysis of spontaneous and ACTH-stimulated cortisol secretion. To prepare the kidney for analysis, finely chop into 1-mm^3 cubes and place in a 24-well cell culture dish with 1 mL of L-15 media. Place the culture dish on an orbital shaker and incubate for 2 hours with periodic L-15 media changes until spontaneous cortisol release is minimal. After the 2-hour pre-incubation period, replace the media with L-15 media + 3.3 x 10-6 M ACTH. Take an initial 35-µl sample of the media and subsequent samples at 30, 60, and 120 minutes after ACTH treatment for analysis of ACTH-stimulated cortisol secretion.

10. To start the test, transfer the toadfish to their respective exposure chambers; one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil treatment are sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

11. After one hour of exposure, sample blood and tissue samples from one control and one oil treatment fish.

12. Sample the control fish before the oil treatment fish. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

13. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP

Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

14. Immediately after collecting the blood sample, over-anesthetize the fish using a 1-g/L solution of MS-222 and remove the kidney for in vitro analysis of spontaneous and ACTH-stimulated cortisol secretion. To prepare the kidney for analysis, finely chop into 1-mm^3 cubes and place in a 24-well cell culture dish with 1 mL of L-15 media. Place the culture dish on an orbital shaker and incubate for 2 hours with periodic L-15 media changes until spontaneous cortisol release is minimal. After the 2-hour pre-incubation period, replace the media with L-15 media + 3.3 x 10-6 M ACTH. Take an initial 35-µL sample of the media and subsequent samples at 30, 60, and 120 minutes post-ACTH treatment for analysis of ACTH-stimulated cortisol secretion.

15. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental, as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP and RSMAS GLPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

16. Repeat steps 12 through 16, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

17. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Remove 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* in the RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

18. Discard remaining test solutions of WAF and toxicity test water as outlined in the *Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with small non-cannulated toadfish that were exposed to oil and air:

1. Two days before exposure, place non-cannulated toadfish (< 40 g) directly into 1-L glass exposure chambers with clean source water and allow to acclimate for 36–48 hours before the start of the test.

2. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Make the HEWAFs less than 24 hours before exposure.

3. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

4. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Before transferring the toadfish to exposure chambers, collect blood and tissue samples from one pre-exposure toadfish.

6. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

7. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

8. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections plus the section remaining in liquid nitrogen for later analysis of CRF mRNA expression by quantitative real-time PCR (qPCR).

9. After collecting the pre-exposure samples and prior to transferring the toadfish to the exposure chambers, remove another pre-exposure toadfish and place onto a clean damp towel for 5 minutes. After 5 minutes, place the fish into a 1-L beaker with clean seawater.

10. After 30 minutes post-air exposure, remove the fish and take blood and tissue samples by repeating steps 6 through 8.

11. To start the test, transfer the toadfish to their respective exposure chambers; one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for the control fish and the other 10 were used for the oil exposure treatment, so that one control and one oil treatment were sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

12. After one hour of exposure, conduct air exposures by removing one control and one oil treatment fish from their chambers and place onto a clean damp towel for 5 minutes. After 5 minutes, return each fish to their respective chambers.

13. After 30 minutes post-air exposure, remove the fish from exposure chambers to sample the blood and tissue samples from one control and one oil treatment fish.

14. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

15. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

16. Immediately after collecting the blood sample, over-anesthetize (3-g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections plus the section remaining in liquid nitrogen. They will be analyzed later for CRF mRNA expression by quantitative real-time PCR (qPCR).

17. Repeat steps 12 through 16, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

18. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

19. Discard remaining test solutions as outlined in the *Disposal Procedures SOP* found in the RSMAS GLPP.

8. University of North Texas General Laboratory Procedures and Practices

8.1 Introduction

The goal of these studies was to produce data to parameterize a model to assess the effects from photo-enhanced toxicity based on polycyclic aromatic hydrocarbon (PAH) concentration and site-specific variation in light intensity.

8.2 Methods

Both indoor and outdoor experiments were conducted to establish the full range of toxicity responses of fish and invertebrates to PAH under a wide range of ultraviolet (UV) radiation conditions likely to occur in natural habitats.

Laboratory tests conducted at the Miami University of Ohio (MUO) were used to simulate the diminished levels of UV radiation present at water depths of up to 10 m. These studies were conducted indoors with low light levels; see the MUO General Laboratory Procedures and Practices (GLPP) for details.

Outdoor tests were conducted by University of North Texas (UNT) personnel at the following facilities: the University of Miami, Rosenstiel School of Marine and Atmospheric Science (RSMAS); Florida Gulf Coast University (FGCU); Louisiana University Marine Consortium (LUMCON); and Auburn University (Auburn). Some tests were conducted at Sea Center Texas in collaboration with Stratus Consulting. Additionally, some tests were conducted onsite at UNT. Sunlight was used as a source of UV, and plastic shielding was used to alter exposure wavelengths and intensity.

Outdoor testing apparatus consisted of a water table covered in either UV transparent (Aclar recommended) or UV opaque (Courtgard recommended) plastic sheeting. Courtgard (CP Films, Inc., Martinsville, VA; http://www.plasticstoday.com/sourcebook/cpfilms-inc) is a long-wave-pass plastic that in water transmits photosynthetically active radiation (PAR; 95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm, with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both PAR (100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, and 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acted simply as a temperature control, and

organisms were never in contact with water from the table. Water was fed into the water table and the rate of flow varied to maintain a certain temperature during the test; see test-specific test conditions tables (TCTs) for details. Plastic sheeting was attached to the water table to allow some airflow over the table for cooling. Test dishes were placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in the water table. The water table was placed in an unshaded location between 8:00 a.m. and 6:00 p.m.

UNT also conducted studies in an indoor UV testing apparatus using high-intensity lights as a source of UV light and fluorescent lights as a source of light without UV. The lights were suspended over laboratory tables, and the height of the high-intensity UV lights was adjusted so that the intensity at the table's surface mimicked the UV intensity of a sunny summer day in the Gulf of Mexico.

8.2.1 Test organism sources and husbandry

This section describes the sources and husbandry for test organisms used for toxicity tests, which were conducted with sheepshead minnow, mahi-mahi, blue crab, oyster, fiddler crab, grass shrimp, red drum, and speckled seatrout.

Sheepshead minnow

Sheepshead minnow were shipped to UNT from MUO; see the MUO GLPP for information regarding sources and husbandry. Sheepshead minnow tests were conducted with artificial seawater (ASW) prepared with a mix of Instant Ocean and Milli-Q water.

Mahi-mahi

Mahi-mahi tests were conducted onsite at RSMAS; see the RSMAS GLPP for information regarding mahi-mahi sources and husbandry.

Blue crab

Blue crab tests were conducted onsite at UNT. For tests conducted prior to 2013, blue crab zoeae were shipped to UNT by personnel at the University of Southern Mississippi Gulf Coast Research Laboratory (GCRL); see the GCRL GLPP for blue crab source and husbandry information. For tests conducted in 2013, blue crab zoeae were purchased from the University of Maryland's Institute of Marine and Environmental Technology blue crab aquaculture facility and shipped to UNT. Blue crab tests were conducted with ASW prepared with a mix of BioSea Marine Mix salts and Milli-Q water.

Oyster

Oyster tests were conducted onsite at FGCU; see the FGCU GLPP for information regarding oyster sources and husbandry.

Fiddler crab

Fiddler crab tests were conducted onsite at Auburn; see the Auburn GLPP for information regarding fiddler crab sources and husbandry.

Grass shrimp

Grass shrimp tests were conducted onsite at UNT. All adult grass shrimp were obtained from the NOAA-Charleston laboratory or GCRL. Animals were shipped overnight to UNT and held in glass aquaria containing ASW (15 ppt; aeration) before testing (generally 48 hours; see individual testing protocols in the UNT GLPP for differences in holding times). Animals were fed fine-ground commercial fish food daily and monitored for mortality/disease before testing. Any animals not used in tests were euthanized and disposed of.

Red drum and speckled seatrout

Red drum and speckled seatrout tests were conducted onsite at Sea Center Texas; see the Stratus Consulting GLPP for information regarding sources and husbandry of red drum and speckled seatrout.

Bay anchovy and red snapper

Bay anchovy and red snapper tests were conducted onsite at LUMCON; see the LUMCON GLPP for information regarding sources and husbandry of bay anchovy and red snapper.

Mysid shrimp

Mysids were shipped to UNT from Aquatic BioSystems (Ft. Collins, CO) and held overnight before testing began.

8.2.2 Exposure media preparations

For water exposures, test media were prepared according to established protocols for preparing high-energy and chemically enhanced water accommodated fractions (HEWAF and CEWAF, respectively). See the *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Each test included at least two UV radiation treatments (full-spectrum solar and

solar with UV screened out). For some tests, a 50% full-spectrum solar UV light treatment was also included. Each test included a control [prepared similarly to water accommodated fractions (WAFs) without the addition of oil or dispersant] and different concentrations of WAF.

8.2.3 Phototoxicity model development

Over the past 25 years, a global model of PAH phototoxicity has been developed with major contributions to the area of study coming from Principal Investigators at MUO and UNT (Jeffries et al., 2013). Photo-enhanced toxicity is a function of (1) a particular PAH's ability to generate reactive oxygen species upon absorption of actinic UV radiation, (2) the extent to which a PAH is taken up by an organism (i.e., combination of bioavailability and bioaccumulation potential – also known as fugacity), and (3) the intensity of actinic UV exposure. Model input is thus a combination of structure-activity data on compound-specific levels of phototoxicity, bioaccumulation data, and quantitative information on intensity and duration of UV exposure. Model output is the predicted time-to-death for an organism, exposure to combinations of PAH levels that are taken up by an organism, and UV intensity and duration. This output can be used to estimate site-, time-, season-, or depth-specific phototoxic injury for specific mixtures of PAH. The model takes the form of:

$$TTD = f\left\{\sum_{i=1\text{-}n}([PAH]_i * RPA_i) * (UV)\right\}, \qquad \text{Equation 1}$$

where TTD = predicted time-to-death; $[PAH]_i$ = molar body burden of PAH "i" over PAH "i" to "n;" RPA_i = relative photodynamic action of PAH_i (ratio of phototoxicity intensity of PAH_i and anthracene – a PAH with a median level of phototoxicity); and UV = dose of actinic UV radiation exposure (typically expressed as broad-band UV-A radiation in the range of 320–400 nm).

This model can be used to predict times-to-death under specific conditions or can be used to determine predicted no-effect levels of PAH and UV exposure on a site-specific basis.

Data from tests conducted under the UNT GLPP were modeled using these concepts to predict levels of toxicity and areas of injury within the *Deepwater Horizon* oil spill zone. In addition, the data were incorporated into the global model of phototoxicity, and model comparisons served as one form of validation of both the model and its predictions.

8.2.4 Water quality monitoring

Water quality was monitored as described in the *Water quality measurement standard operating procedure* (SOP) in Section 8.4.1. Dissolved oxygen (DO), pH, conductivity, salinity, ammonia,

and temperature were measured in each test dilution stock just prior to renewal. Temperature and DO were measured hourly during the test procedure. Solar radiation was measured using a calibrated, BioSpherical profiling ultraviolet (PUV) radiometer, which took continuous measurements of UV during the entire test period. Calibration was performed and certified by BioSpherical Instruments (San Diego, California).

8.2.5 Analytical chemistry

Water samples were collected as described in the QAPP.

8.3 Reporting and Testing Documentation

All documentation of test procedures, results, etc., was carried out as described in the QAPP. Documentation was provided to Stratus Consulting and electronic and hard copies were kept on file at UNT.

8.4 General Protocols

8.4.1 Water quality measurement standard operating procedure

A. Temperature (YSI ProDO)

1. Using traceable thermometer associated with the optical DO probe, place probe in one replicate of an exposure concentration.
2. Hold probe in replicate test solution until reading stabilizes for 5 seconds.
3. Record reading.
4. Clean probe using analytical detergent and soft sponge with deionized (DI) water.
5. Repeat steps 1 through 4 on one replicate per exposure, per UV exposure (UV exposed and UV shielded).

B. pH (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

C. DO (YSI ProODO)

1. Using saturated solution, verify calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

D. Conductivity (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

E. Salinity (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using detergent and soft sponge with DI water, and store as per manufacturer's instructions.

F. Total ammonia (Hach colorimetric test)

1. Obtain 5 mL of exposure media for titration.

2. Add test strip according to kit instructions, and compare to colorimetric reference chart to obtain ammonia concentration.

3. Record concentration and dispose of test strip.

4. Repeat for all exposure stocks.

Reference

Jeffries, M.K.S., C. Claytor, W. Stubblefield, W.H. Pearson, and J.T. Oris. 2013. Quantitative risk model for polycyclic aromatic hydrocarbon photo-induced toxicity in Pacific herring following the *Exxon Valdez* oil spill. *Environmental Science and Technology* 47(10):5450–5458.

A. Testing Protocol 1: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Sheepshead Minnow (*Cyprinodon variegatus*)

The survival and hatch success of sheepshead minnow embryos and larvae life stages exposed to WAFs were assessed. For each treatment concentration, there were five replicates exposed to full intensity (100%) UV light and five replicates exposed to approximately 10% of the full UV light. Each replicate contained 200 mL of WAF and 10 organisms.

A.1 Testing Apparatus

Testing apparatus for outdoor UV tests with sheepshead minnow was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment media as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Embryo and larvae tests were started in the evening with an overnight soak in their respective treatment WAFs before being exposed to UV the next day. Mortality and hatch success were assessed daily, and tests were carried out until the last organism died or for a maximum of 168 hrs for embryos and a maximum of 96 hrs for larvae.

A.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10 organisms in each test dish and feed as appropriate (specified on test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning.

6. In the morning, turn on the water to water tables to cool to desired temperature.

7. Obtain new, clean 250-mL crystallizing dishes and label with appropriate treatment and replicate numbers.

8. Fill clean dishes with 200 mL of appropriate test medium using glass graduated cylinder.

9. Transfer organisms from old crystallizing dish to new dish containing renewed test medium. Note any mortality (do not replace organisms). All dead test organisms will be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Collect old dishes, wash, and prepare for afternoon water change. Cover unused test media and place in a refrigerator at 4°C.

11. Label blueboard floats as "UV+" or "UV-" with a marker. Place test dishes in appropriate floats by treatment.

12. By 9:00 a.m., place floats in the water table under appropriate plastic (either UV transparent for UV+ or UV opaque for UV-). Dishes should be floated to maximize direct contact with the cooling water.

13. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

14. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

15. Check temperature and DO hourly, and adjust flow of cooling water as needed.

16. In the afternoon, remove WAF preparations from the refrigerator and place in water bath to equilibrate them to test temperature.

17. After approximately 8 hrs of exposure to sunlight, collect dishes and return indoors.

18. Count the number of dead and living organisms and transfer living organisms to new, clean crystallizing dishes containing appropriate test medium (as described above). Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

19. Leave dishes in secure location overnight.

20. Next morning, repeat procedure from step 6.

21. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 168 hrs for embryos and a maximum of 96 hrs for larvae.

B. Testing Protocol 2: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Mahi-mahi (*Coryphaena hippurus*)

The survival and hatching success of mahi-mahi embryo life stages exposed to WAFs were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10–20 organisms.

B.1 Testing Apparatus

Testing apparatus for outdoor UV tests with mahi-mahi was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment media without renewal (static) in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table that was placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Due to the typical timing of a mahi-mahi spawn and the rapid development of their embryonic life stage, tests began mid-day with the organisms going out into the sun as soon as the test dishes were prepared. Mortality and hatch success were assessed at the end of test, and tests were carried out for 48 hrs.

B.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10–20 organisms in each test dish (as specified in test-specific TCTs).

4. Complete test forms as described in the QAPP.

5. Leave dishes in a secure laboratory area until outside water tables are ready.

6. Turn on water to water tables to cool to desired temperature.

7. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

8. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check the temperature and DO hourly, and adjust flow of cooling water as needed.

11. After approximately 6–8 hrs of exposure to sunlight, count the dead and living organisms. Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

12. After counts, return dishes to blueboard floats in water table and cover test dishes with a tarp to shelter from overnight weather and prevent further sunlight exposure.

13. The next morning, remove tarp and repeat procedure from step 8.

14. Carry out tests for 48 hrs.

C. Testing Protocol 3: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Blue Crab (*Callinectes sapidus*) and Fiddler Crab (*Uca longisignalis*) Zoeae

The survival of blue crab and fiddler crab zoeae exposed to WAFs was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light. In addition, some tests included a 50% UV exposure, with three to five replicates, for each WAF treatment (see test-specific TCTs for details). Each replicate contained 200 mL of WAF and 10–20 organisms.

C.1 Testing Apparatus

Testing apparatus for outdoor UV tests with blue crab and fiddler crab zoeae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Tests began in the evening with an overnight soak in their respective treatment WAFs before being exposed to UV the next day. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hrs (see test-specific TCTs).

C.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.
2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.
3. Place 10–20 organisms in each test dish and feed as appropriate (see test-specific TCTs for details).
4. Complete test-specific bench sheets as described in the QAPP.
5. Leave dishes in a secure dark laboratory area overnight until the next morning.
6. In the morning, turn on water to water tables to cool to desired temperature.

7. If renewal, use a pipette to remove ~ 80% of the test solution out of the chamber and dispense the water through a nylon mesh to avoid inadvertently removing organisms during WAF renewal. If no renewal, skip to step 8.

8. Before replacing WAF, note any mortality (do not replace organisms). Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

9. Refill dish with appropriate test medium so that there is approximately 200 mL of WAF in each dish. Unused test media should be covered and placed in fridge at 4°C.

10. Label blueboard floats with appropriate UV treatment. Place test dishes in appropriate floats by treatment.

11. By 9:00 a.m., place floats in water table under appropriate plastic (e.g., UV transparent for 100% UV). Transfer dishes to blueboard floats making sure to maximize direct contact with the cooling water.

12. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

13. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer, or when unavailable, make manual measurements every 5 to 15 minutes using an Ocean Optics JAZ-EL200 spectrometer. Both the PUV and the JAZ-EL200 measure UV-A, UV-B, and visible light.

14. Check the temperature and DO hourly, adjust the flow of cooling water as needed.

15. After approximately 8 hrs of exposure to sunlight, count dead and living organisms in each dish per step 8 above. WAF in dishes should not be renewed at this time.

16. Leave dishes in a secure location overnight.

17. The next morning, repeat procedure from step 6.

18. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48–96 hrs.

D. Testing Protocol 4: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Oyster (*Crassostrea virginica*) Fertilization, Embryos, Veligers

The survival of eastern oyster embryos and veligers as well as fertilization success (gametes) exposed to WAFs was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light. In addition, some tests included a 50% UV exposure, with three to five replicates, for each WAF treatment. Each replicate contained 20–200 mL of WAF and 1,000–4,000 organisms (see test-specific TCTs).

D.1 Testing Apparatus

The outdoor testing apparatus consisted of a water table covered in either UV transparent or UV opaque clear plastic sheeting (Aclar and Cortgard recommended). The water table acted as a cooling bath only and organisms were never in contact with cooling water. Water was fed into the water table and the rate of flow varied to maintain a constant temperature during the test (see test-specific TCTs). Plastic sheeting was attached to the system to allow some airflow over the table for cooling. Test dishes were loaded according to test-specific TCTs. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in the water table. The water table was placed in an unshaded location between 8:00 a.m. and 6:00 p.m.

D.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 20–200 mL of the corresponding treatment medium using a glass cylinder (see test-specific TCTs). Unused test media should be disposed of appropriately.

3. Place organisms in each test dish and feed as appropriate; the total number of organisms included per dish varied between tests and is specified in test-specific TCTs.

4. Complete bench sheets as described in the QAPP.

5. For all gamete tests, and for embryo and veliger tests prepared in the morning, move prepared dishes immediately to water table floats for 1 hour exposure to UV. For embryo and veliger tests prepared in the late afternoon/evening, leave dishes in a secure area in the dark until next morning.

6. Turn on water to water tables to cool to desired temperature.

7. Label blueboard floats according to the UV exposure treatments included in test. Place test dishes in appropriate floats by treatment.

8. Place floats in water table under appropriate plastic (either UV transparent for UV+ or UV opaque for UV-). Dishes should be floated to maximize direct contact with the cooling water.

9. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

10. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. Check temperature and DO hourly, and adjust the flow of cooling water as needed.

12. After ~ 8 hrs of exposure to sunlight (or 1 hour in the case of gamete tests), collect dishes and return indoors.

13. For gametes, following the one-hour UV exposure, assess fertilization rates by counting the number of fertilized and unfertilized embryos in a sample of 100 organisms. For embryos and veligers tested before 2013, assess mortality by counting the number of live and dead organisms in a sample of 100 organisms. For embryos and veligers tested in 2013, count the number of live and dead organisms in a 250 µL subsample. Retain all dead test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

14. Leave dishes in a secure location overnight.

15. The next morning, repeat procedure from step 6.

16. Carry out tests for a total of 1 hour sunlight exposure (gametes) or a maximum of 96 hrs (embryos, veligers); see test-specific TCTs.

E. Testing Protocol 5: Assessing Photo-Enhanced Toxicity of Maternal Exposure to *Deepwater Horizon* Oil Spiked Sediment or WAF to Fiddler Crab (*Uca longisignalis*) Zoeae

Survival of zoeae hatched from gravid females from the test described in Auburn GLPP Testing Protocol 1 or Testing Protocol 4, depending on the matrix of the maternal exposure, was followed to assess the percentage of zoeal survival in relation to their exposure to oil during egg development. The zoeal survival studies were done outdoors with and without UV exposure. For each female crab, there were three replicates exposed to full UV light and three replicates exposed to approximately 10% of the full UV light. Each replicate contained 200 mL clean ASW at 20-ppt salinity with 20 organisms.

E.1 Testing Apparatus Design

Testing apparatus for outdoor UV tests with fiddler crab zoeae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hrs (see test-specific TCTs).

E.2 Outdoor (UV exposure) Experimental Methods

Aquatic Habitats (AHAB) tanks were checked at least twice a day, first thing in the morning and again in the evening to determine if gravid females had hatched their eggs. If zoeae were found in the morning, they were collected from the filter cups and a subsample was immediately transferred to test dishes to be place outside for the day (see steps 1–4 below). If zoeae were found in the evening, test dishes were prepped and kept in the dark until the following morning. Detailed steps are provided below.

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL clean ASW using a glass cylinder.

3. Place 20 organisms in each test dish.

4. Complete bench sheets as described in QAPP.

5. If morning, go directly to step 6. If later in the day or evening leave dishes in a secure laboratory area in the dark until following morning.

6. First thing in the morning, turn on water to water tables to cool to desired temperature.

7. If test dishes were kept overnight, continue with steps 8–10, otherwise skip to step 11.

8. Pipette out ~ 80% of ASW from each dish.

9. Before replacing water, note any mortality (do not replace organisms). Remove and retain all dead test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Refill each dish to ~ 200 mL with fresh ASW.

11. Place test dishes outside in blueboard floats in water bath under appropriate UV plastic covering. Label blueboard floats as "UV+" or "UV-" with a marker.

12. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

13. After approximately 8–10 hrs of exposure to sunlight, bring in all dishes, renew water and count mortalities (see steps 8–10 above).

14. Turn off water to water tables and turn off and bring in UV monitor.

15. Leave dishes in a secure location overnight.

16. The next morning, repeat procedure from step 6.

17. Continue test until the last organism in the lowest WAF concentration has died or for the maximum number of hours specified in test-specific TCTs.

F. Testing Protocol 6: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil Following Different Exposure Periods Using Fiddler Crab (*Uca longisignalis*) Zoeae

The survival of fiddler crab zoeae exposed to WAFs was assessed following different WAF exposure periods. For these tests, the testing apparatus and testing procedures were the same as described in Testing Protocol 3 in the UNT GLPP, except that the WAF exposure period was an additional variable across WAF treatment concentrations (see test-specific TCTs). After a predetermined duration of WAF exposure, zoeae from the dishes were transferred to clean seawater. Survival in the clean seawater, following different exposure durations in WAF was then followed until the end of the test. For each treatment concentration, there were three to five replicates for each WAF treatment, UV dose (10%, 50%, and 100% full spectrum solar), and exposure duration (e.g., 2, 4, and 8 hour) combination (see test-specific TCTs). Each replicate contained 200 mL of WAF and 10–20 organisms. Specific test design details are found in the TCTs for each test. The total test duration was 24–48 hrs.

G. Testing Protocol 7: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Mahi-mahi (*Coryphaena hippurus*) Using Imhoff Cones

The survival and hatch success of mahi-mahi embryos exposed to WAFs in customized Imhoff cones were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light (see test-specific TCTs).

G.1 Testing Apparatus

Acute embryo exposures were performed in customized 1-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume in this testing apparatus was 1.8 L and was circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Retain embryos/larvae in the cone using a glass excluder extending from the overflow drain with nylon mesh fastened on both sides with silicone o-rings. The test medium was UV sterilized seawater. Seawater volume was measured using a graduated cylinder.

The Imhoff cones were placed in an unshaded location between 8:00 a.m. and 6:00 p.m. and covered with different clear UV plastic sheeting that either let 100% or 10% of the full spectrum solar UV light through (see test-specific TCTs). To control water temperature, the 1-L beaker reservoirs for each Imhoff cone were placed in a water bath where water was fed into the water bath and the rate of flow varied to maintain temperature. Mortality and hatch success were assessed daily, and tests were carried out until the last organism died or for a maximum of 96 hrs.

G.2 Test Procedure

1. Label each Imhoff cone/beaker reservoir with test treatment and tank number.

2. Fill with 1.8 L of the corresponding treatment medium using a glass cylinder. Dispose unused test media appropriately.

3. Place organisms in each test dish (see test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave Imhoff cones in a secure laboratory area until water table is ready.

6. Turn on water to water bath to cool to desired temperature.

7. Label UV plastic sheet as "UV 100" or "UV 10" with a marker. Place Imhoff cones under appropriate UV plastic (see test-specific TCTs). Place beakers in a water bath.

8. Measure temperature, pH, salinity, and DO content of one randomly selected beaker from each PAH/UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check temperature and DO hourly, and adjust flow of cooling water as needed.

11. After approximately 6–8 hrs of exposure to sunlight, cover Imhoff cones by a steel frame outdoor canopy to shelter from overnight weather and prevent further sunlight exposure. During intense rain events, cover cones using the same outdoor canopy.

12. The next morning, remove canopy and repeat procedure from step 6.

13. Carry out tests until the last organism in the lowest WAF concentration has hatched/died, or for a maximum of 96 hrs.

14. At the end of the test, count all dead and living organisms. Retain all test organisms removed according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Because of the difficulty in scoring animals in the Imhoff cones, hatch success, survival, mortality, missing, and non-test mortality were recorded at 96 hrs only.

H. Testing Protocol 8: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Exposure to Juvenile Mahi-mahi (*Coryphaena hippurus*) Swim Performance

The swim performance of juvenile mahi-mahi exposed to WAFs was assessed.

H.1 Testing Apparatus

Testing apparatus for outdoor UV tests with mahi-mahi was the same as described in Section 8.2 (Methods) of the UNT GLPP except the exposure tanks were 10-gal glass aquaria. Two to three fish were added to each aquarium. Organisms were exposed to WAF treatment media without renewal (static) for 1 day (~ 4–6 hrs of UV). Loading of organisms into chambers never exceeded 0.5 g/L. Test chambers were placed in the water bath in an unshaded location between 8:00 a.m. and 6:00 p.m. Exposures were carried out for 1 day and the animals returned to the RSMAS laboratory where they are kept overnight. Swim performance was assessed the following day.

H.2 Test Procedure

1. Obtain clean 10-gal glass aquaria needed for test. Label each aquarium test treatment.
2. Fill each dish with ~ 8 gal of the corresponding treatment medium. Unused test media should be disposed of appropriately.
3. Place 2–3 organisms in each aquarium.
4. Complete bench sheets as described in the QAPP.
5. Leave aquaria in a secure laboratory area until water tables are ready.
6. In the morning, turn on water to water tables to cool to desired temperature.
7. Place aquaria in water tables. Care should be taken to prevent them from floating.
8. Measure temperature and DO content from each UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check temperature and DO hourly, and adjust flow of cooling as needed.

11. After approximately 4–6 hrs of exposure to sunlight, remove organisms from the test chamber and place in new WAF. Return aquaria to the RSMAS laboratory for an overnight period. Assess swim performance the following day per the RSMAS GLPP Testing Protocol 9.

I. Testing Protocol 9: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil HEWAF Exposure to Larval Grass Shrimp (*Palaemonetes pugio*)

Survival of larvae hatched from gravid females was determined following exposure to WAF/UV light; gravid females were obtained from collections made by NOAA (Charleston) or GCRL. The larval survival studies were done outdoors with and without UV exposure. Larvae were collected that hatched within a 24-hour period.

I.1 Testing Apparatus Design

Testing apparatus for outdoor UV tests with grass shrimp larvae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatments as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hrs (see test-specific TCTs).

I.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Dispose of unused test media appropriately.

3. Place 10 organisms in each test dish and feed as appropriate (see test-specific TCTs for details).

4. Complete test-specific bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight.

6. In the morning, turn on water to water tables to cool to desired temperature.

7. Note any mortality (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

8. Label blueboard floats with appropriate UV treatment. Place test dishes in appropriate floats by treatment.

9. By 9:00 a.m., place floats in the water table under appropriate plastic (e.g., UV transparent for 100% UV). Transfer dishes to blueboard floats, making sure to maximize direct contact with the cooling water.

10. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

11. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

12. Check the temperature and DO hourly, and adjust the flow of cooling water as needed.

13. After approximately 8 hrs of exposure to sunlight, count dead and living organisms in each dish and remove dead organisms per step 7 above. WAF in dishes should be renewed at this time.

14. Leave dishes in a secure location overnight.

15. The next morning, repeat procedure from step 6.

16. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48–96 hrs (see test-specific TCTs).

J. Testing Protocol 10: Assessing Photo-Enhanced Effects of Spiked Sediment Maternal Exposure to Larval Grass Shrimp (*Palaemonetes pugio*)

Survival of larvae that were exposed to UV, and that hatched from gravid females that were exposed to spiked sediments, was determined. The larval survival studies were done indoors (with and without UV) using a high-intensity lighting system. For each sediment concentration, there were three replicate adult exposure tanks. For each adult exposure tank, there were three replicate dishes of larvae under each UV intensity. Larvae were exposed to 100%, 50%, or 0% of the full UV light. Each replicate contained 200 mL clean ASW at 15-ppt salinity with 10 organisms.

J.1 Testing Apparatus Design

J.1.1 Sediment exposure

Sediments were spiked at UNT following the protocol listed in Section 10.6 of the Pacific EcoRisk GLPP (Protocol for Preparation of Spiked Sediment). Sediment tanks consisted of 20-gal glass aquaria with 1 kg of sediment per aquarium and 7 L of water on a 16:8 light/dark photoperiod. Water was renewed (80%) every other day and the water quality was checked daily. Five adult "early gravid" female shrimp obtained from NOAA (Charleston Laboratory) were placed in each sediment tank. Females were left on sediments for ~13 days and then moved to 2-L glass chambers containing ASW until the eggs hatched. Chambers were checked several times per day and hatched larvae were collected and held in separate glass containers for no more than 24 hrs for use in UV survival tests. Larvae from several females within an adult replicate tank were pooled, but larvae between replicate adult tanks were not pooled.

J.1.2 Indoor UV exposure

Testing apparatus for indoor UV tests with larval grass shrimp consisted of overhead high-intensity UV lights (100% UV) or fluorescent (0% UV) lights suspended over a laboratory table. Height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a "sunny" summer day. Because of the height of the lights and room temperature control, no additional water bath cooling was needed. Larvae were exposed in clean water as static renewal in 250-mL glass crystallizing dishes. The total density of organisms in a chamber never exceeded 0.5 g/L. Test dishes were placed on the tabletop under either 100% or 0% UV for

8 hrs followed by a 16-hour dark cycle. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hrs (see test-specific TCTs).

J.2 Spiked Sediment Exposure Experimental Methods

1. Obtain clean, 20-L glass aquaria needed for test. Label each aquarium with test treatment and tank number.
2. Fill each aquarium with 1 kg of corresponding spiked or control sediment and 7-L ASW.
3. Place five adult "early gravid" female shrimp in each test aquarium.
4. Complete bench sheets as described in the QAPP.
5. Every 24 hrs, monitor DO, temperature, ammonia, and salinity according to water quality SOPs described in UNT GLPP. Check adult shrimp for mortality. Record any mortalities and remove any dead shrimp and archive according to the QAPP.
6. After 13 days, remove shrimp from aquaria with sediments and place in 2-L tanks filled with 15-ppt ASW.
7. Check 2-L chambers several times per day for hatched larvae. If larvae are present, collect and hold larvae in separate glass containers with 2 L of clean 15-ppt ASW for no more than 24 hrs until transferring them to crystallizing dishes for the start of the UV survival test (see Section J.3). Larvae from several females within an adult replicate tank can be pooled. Do not pool larvae from different replicate tanks.
8. Once an adult female has hatched her eggs, collect and euthanize by placing in a freezer. Archive according to the QAPP.

J.3 Indoor (UV exposure) Experimental Methods

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.
2. Fill each dish with 200-mL clean ASW using a glass cylinder.
3. Place 10 organisms in each test dish.
4. Complete bench sheets as described in the QAPP.
5. Place test dishes under UV lights and turn lights on.

6. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After approximately 8 hrs of exposure to UV, turn off the lights, renew water, and count mortalities (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Leave test dishes in the dark overnight.

8. The next morning, assess mortality according to step 7 and then repeat UV exposure according to steps 5–7.

9. Continue test until the last organism in the lowest sediment concentration has died or for the maximum number of hours specified in test-specific TCTs.

K. Testing Protocol 11: Assessing Photo-Enhanced Effects of Spiked Sediment Exposure to Adult Grass Shrimp (*Palaemonetes pugio*)

Survival of adults exposed to UV following exposure to spiked sediments was determined. Survival studies were done indoors (with and without UV) using a high-intensity lighting system. For each sediment concentration, there were three replicate tanks. Adults were exposed to full UV light or 0% of the full UV light. Each replicate contained 19 L of clean ASW at 15-ppt salinity with five organisms per tank.

K.1 Testing Apparatus Design

Sediments were spiked at UNT following the protocol listed in Section 10.6 of the Pacific EcoRisk GLPP (Protocol for Preparation of Spiked Sediment). Sediment tanks consisted of 20-gal glass aquaria with 1 kg of sediment per aquarium. Ten female adult shrimp were placed in each sediment tank. Females were held on sediments for 6 days and then moved to 20-L test chambers containing ~ 19 L of clean water for UV exposure. The 10 females from each sediment replicate were split into two groups of 5, one group under 100% UV light and one group under 0% UV light.

The testing apparatus for indoor UV tests with grass shrimp consisted of overhead high-intensity UV lights or fluorescent lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a "sunny" summer day. Because of the height of the lights and room-temperature control, no additional waterbath cooling was needed. Adults were exposed in clean water. The density of organisms loaded into chambers never exceeded 0.5 g/L. Test tanks were placed on the tabletop under either 100% or 0% UV for 8 hrs followed by a 16-hour dark cycle. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hrs (see test-specific TCTs).

K.2 Spiked Sediment Exposure Experimental Methods

1. Obtain clean, 20-L glass aquaria needed for test. Label each aquarium with test treatment and tank number.

2. Fill each aquarium with 1 kg of corresponding spiked or control sediment and 7 L of water.

3. Place 10 adult female shrimp in each test aquarium.

4. Complete bench sheets as described in the QAPP.

5. Every 24 hrs, monitor DO, temperature, ammonia, and salinity according to water quality SOPs described in UNT GLPP. Check adult shrimp for mortality. Record any mortalities and remove any dead shrimp and archive according to the QAPP.

6. After 6 days, remove shrimp from the aquaria with sediments and place in a new 20-L glass aquaria filled with ~ 19 L of clean 15-ppt ASW for the start of UV exposure (see Section K.3).

K.3 Indoor (UV exposure) Experimental Methods

1. Obtain clean, 20-L glass tanks needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 19 L of clean ASW using a glass cylinder.

3. Place five organisms from the appropriate sediment tank into each aquaria.

4. Complete bench sheets as described in the QAPP.

5. Place aquaria under UV lights and turn the lights on.

6. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After approximately 8 hrs of exposure to UV, turn off the lights, renew the water, and count mortalities (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Leave aquaria in the dark overnight.

8. The next morning, assess mortality according to step 7 and then repeat UV exposure according to steps 5–7.

9. Continue the test until the last organism in the lowest sediment concentration has died or for the maximum number of hours specified in test-specific TCTs.

L. Testing Protocol 12: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF Exposure to Red Drum (*Sciaenops ocellatus*) and Speckled Seatrout (*Cynoscion nebulosus*)

The survival of red drum and speckled seatrout embryo and larval life stages exposed to WAFs were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10 organisms.

L.1 Testing Apparatus

The testing apparatus for outdoor UV tests with red drum and speckled seatrout was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media as static renewal in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

L.2 Test Procedure

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder (see test-specific TCTs). Prepare treatment medium according to the QAPP. Collect chemistry samples as described in the QAPP. Dispose of unused test media appropriately.

3. Place 10 organisms in each test dish (as specified in test-specific TCTs).

4. Complete test forms as described in the QAPP.

5. Leave dishes in a secure laboratory area until outside water tables are ready.

6. Turn on water to water tables to cool to desired temperature.

7. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

8. Measure the temperature and DO content of five randomly selected dishes from each UV treatment.

9. Place plastic sheets over appropriate UV treatments.

10. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. After approximately 6 to 8 hrs of exposure to sunlight, collect dishes and bring indoors. Hold organisms until ~ 24 hrs have passed since larvae were initially loaded into test dishes. For example, if larvae were loaded at 10 p.m. the previous evening and dishes brought inside at 4 p.m., do not proceed to step 12 until 10 p.m. Total test length is 24 hrs from the time of initial loading.

12. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

M. Testing Protocol 13: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Speckled Seatrout (*Cynoscion nebulosus*)

The survival of speckled seatrout embryos exposed to oil slicks was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10 to 20 organisms (see test-specific TCTs).

M.1 Testing Apparatus

The testing apparatus for outdoor UV tests with red drum and speckled sea trout was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media as static renewal in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

M.2 Slick Preparation

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the "Tank ID, Dilution, or Stock Code Definitions" datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place oiled PVC on a clean tray to store until start of test.

7. Repeat steps 1–6 for each replicate.

8. Place PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure 1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hrs.

Figure 1. Top view of the crystallizing dish slick exposure set-up.

M.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the QAPP and Stratus Consulting – Red Drum and Speckled Seatrout GLPP document. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary because of routine hatchery operations.

2. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Transfer a sub-sample of embryos from the beaker to Petri dishes for sorting.

3. Use a pipette to collect 10 embryos from the Petri dish. Note that all 10 embryos should be in the pipette simultaneously for transfer to the test dish. If needed, use a separate dish to transfer and count out embryos, and then collect them simultaneously.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously, expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into the test beakers. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and replace the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. Leave dishes in a secure laboratory area overnight until the outside water tables are ready the next morning.

7. The next morning, turn on water to water tables to cool to desired temperature.

8. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

9. Measure the temperature and DO content of five randomly selected dishes from each UV treatment.

10. Place plastic sheets over appropriate UV treatments.

11. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

12. After approximately 6 to 8 hrs of exposure to sunlight, collect dishes and return them indoors. Leave the organisms until approximately 24 hrs has passed since larvae were initially loaded into test dishes. For example, if larvae were loaded at 10 p.m. the previous evening and dishes brought inside at 4 p.m., do not proceed to step 12 until 10 p.m. Total test length is 24 hrs from the time of initial loading.

13. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

N. Testing Protocol 14: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Mahi-mahi, Red Snapper, and Bay Anchovy

The survival of fish embryos exposed to oil slicks with UV light was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV. Each replicate contained 200 mL of test solution and 10–20 organisms. For details, see test-specific TCTs.

N.1 Testing Apparatus

The testing apparatus consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day. For mahi-mahi testing, a temperature-controlled room was used to maintain temperature of exposure dishes; for bay anchovy and red snapper testing, a recirculating water bath was used to maintain temperature. Exposure dishes were placed under either 100% or 0% UV light for several hours followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 48 hrs (see test-specific TCTs).

N.2 Slick Preparation

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with the test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the "Tank ID, Dilution, or Stock Code Definitions" datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling, about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place oiled PVC on a clean tray to store until start of the test.

7. Repeat steps 1–6 for each replicate.

8. Place PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure 1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hrs.

Figure 1. Top view of the crystallizing dish slick exposure setup.

N.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the QAPP. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary (see test-specific TCTs).

2. Collect embryos from hatchery personnel (see Chapters 7 and 16). Transfer a sub-sample of embryos from the beaker to Petri dishes for sorting.

3. Use a pipette to collect 10–20 embryos from the Petri dish (see test-specific TCTs). Note that all 10–20 embryos should be in the pipette simultaneously for transfer to the test

dish. If needed, use a separate dish to transfer and count out embryos, and then collect them simultaneously.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into the test beakers. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and place the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. Either leave dishes in a secure laboratory area overnight or immediately place under UV lighting (see test-specific TCTs).

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off lights.

9. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless an analysis of individually identified organisms is required.

O. Testing Protocol 15: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF on Red Snapper and Bay Anchovy

The survival of fish embryos exposed to WAFs was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV. Each replicate contained 200 mL of test solution and 10–20 organisms. For details, see test-specific TCTs.

O.1 Testing Apparatus

The testing apparatus consisted of overhead high-intensity UV lights or fluorescent (no UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a sunny summer day. A recirculating water bath was used to maintain temperature for snapper and anchovy testing. Test tanks were placed under either 100% or 0% UV for several hours (see test-specific TCTs for UV exposure duration) followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 48 hrs (see test-specific TCTs).

O.2 Test Procedure

1. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10–20 organisms in each test dish (see test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning, or place immediately under UV lights (see test-specific TCTs).

6. Measure temperature and DO content of randomly selected dishes from each UV treatment.

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off UV lights.

9. Count the number of dead and living organisms. Sample all dead test organisms and retain them according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of the individually identified organisms is required.

10. Leave dishes in the dark and in a secure location overnight.

11. The next morning, repeat the procedure.

12. Carry out the tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48 hrs.

P. Testing Protocol 16: Acartia Copepod Acute Toxicity Test

The survival of adult copepods exposed to WAF was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10–20 organisms (see test-specific TCTs).

P.1 Testing Apparatus

The testing apparatus for outdoor UV tests with adult copepods was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontaminating Glassware Standard Operating Protocol*, as described in the QAPP. All test water was obtained from the UV-sterilized seawater system at the University of Miami Experimental Hatchery (see RSMAS GLPP; Chapter 7).

Note: Experiments were set up using a synchronous culture, where all animals were the same age (within 24 hs). Adult copepods were approximately 17 days old at test initiation.

P.2 Test Procedure

1. Prepare desired WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP and RSMAS GLPP. HEWAF is made the morning of experimental setup.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct all sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C;

record all necessary sample numbers and prepare necessary chain-of-custody (COC) documentation, as described in the QAPP. Ship samples overnight on ice to ALS Environmental as soon as possible

3. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

4. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

5. Before adding test organisms to the aforementioned dishes, take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each representative chamber, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*, respectively. All SOPs can be found in the QAPP and RSMAS GLPP. See the respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

6. Place 10–20 organisms in each test dish (see test-specific TCTs). To transfer copepods place clean crystallizing dish with adult, age-synchronized copepods on a counter in a dark room, and use a small light-emitting diode (LED) light, placed against the side of the dish to concentrate the animals. Using a glass Pasteur pipette, remove 20 copepods from those that are gathered by the light. Confirm under a dissecting microscope that the correct number of copepods has been removed, and gently transfer the animals to the test dish, taking care to prevent contamination by not allowing the pipette to come into contact with the treatment water.

7. Complete bench sheets as described in the QAPP.

8. After all the replicates are set up, hold them indoors in a secure location overnight.

9. The next morning, move the test dishes to a water bath tank outside. The water bath consists of an 80-gallon, 183 x 51 x 25 cm fiberglass trough, through which 25°C water is continuously run at approximately 50 gallons per minute. The water bath is located in an area where it will receive maximum sunlight throughout the day. The 250-mL beakers are floated in the water bath using a 0.5-inch Styrofoam sheet, through which holes have been punched that exactly fit the beaker diameter. Aeration for each beaker is accomplished using PE60 tubing with a 22G x 1.5-inch hypodermic needle (plastic hub removed), set to approximately 5 bubbles/second. Half of the water bath is covered with a UV-transparent plastic sheet (the UV-exposed treatment), while the other half of the water bath is covered with a UV-opaque plastic sheet (the UV-blocked treatment).

10. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off UV lights.

12. Perform daily measurements of water chemistry and physical parameters on representative chambers, as outlined in the QAPP and RSMAS GLPP. Feed the animals in each beaker daily with 5×10^3 cells/mL of *Isochrysis* (TISO) and 5×10^3 cells/mL of *Cheatocerous* (CHGRA).

13. Make the final survival count and reproductive output measurement after 96 hrs. Gently pour each test dish through a 40-µm filter to concentrate the animals and eggs, and then place the organisms into a clean, glass crystallizing dish. Retain the treatment water for final water quality analysis (see Step 15). Determine the number of live and dead adults under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. After adult survival counts have been determined, pipette eggs out and place them in clean seawater to determine hatching success. Fix the remaining animals in each beaker (live adults and nauplii) using formalin and stain with the addition of Rose Bengal to make counting nauplii easier.

14. Determine the sex of all adult copepods by microscopic examination of uropod segments and antennae morphology using an inverted compound scope.

15. Retain all adult animals and nauplii to the extent possible, preserved in 10% buffered formalin and archived according the QAPP.

16. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

17. The test will be said to pass test criteria if the average control survival exceeds 85% across the four replicates, and if there are no unacceptable deviations in water quality among replicates, as described in the QAPP.

Q. Testing Protocol 17: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF or Dispersant on Mysid Shrimp

The survival of mysid shrimp exposed to WAF or dispersant was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

Q.1 Testing Apparatus

The testing apparatus for mysid shrimp indoor UV tests consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under either 100% or 0% UV for several hours (see test-specific TCTs), followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hrs (see test-specific TCTs).

Q.2 Test Procedure

1. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10 organisms in each test dish.

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning, or place them immediately under UV lights (see test-specific TCTs).

6. Measure temperature and DO content of randomly selected dishes from each UV treatment.

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After several hours of exposure to UV (see test-specific TCTs for times), collect the dishes and return them indoors.

9. Count the number of dead and living organisms. Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Leave dishes in a secure location overnight.

11. The next morning, repeat the procedure.

12. Carry out the tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48 hrs.

R. Testing Protocol 18: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Mysid Shrimp

The survival of mysid shrimp exposed to oil slicks was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

R.1 Testing Apparatus

The testing apparatus for indoor UV tests with mysids consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under either 100% or 0% UV for several hours (see test-specific TCTs) followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hrs (see test-specific TCTs).

R.2 Slick Preparation

1. Obtain the clean, 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the "Tank ID, Dilution, or Stock Code Definitions" datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling, about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place the oiled PVC on a clean tray to store until the start of the test.

7. Repeat steps 1–6 for each replicate.

8. Place the PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure 1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hrs.

Figure 1. Top view of the crystallizing dish slick exposure set-up.

R.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the UNT GLPP. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary (see test - specific TCTs).

2. Use a pipette to transfer 10 mysids. Note that all 10 individuals should be in the pipette simultaneously for transfer to the test dish. If needed, use a separate dish to transfer and count out mysids, and then collect them simultaneously.

3. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

4. Simultaneously expel the mysids while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer into the test beakers. Record the

start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and replace the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain mysids.

5. Either leave the dishes in a secure laboratory area overnight, or immediately place under UV lighting (see test-specific TCTs).

6. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After the UV exposure is complete (see test-specific TCTs), collect the dishes and turn off the lights.

8. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

9. University of Maryland General Laboratory Procedures and Practices

9.1 Introduction

The University of Maryland conducted toxicity tests to identify the toxicological impacts of the 2010 Deepwater Horizon oil spill on blue crab. This chapter describes General Laboratory Procedures and Practices (GLPP) used at the University of Maryland.

9.2 Test Organism Source

Juvenile blue crab (*Callinectes sapidus*) were obtained from the University of Maryland Institute of Marine and Environmental Technology (IMET) blue crab aquaculture facility.

9.3 Exposure Media Preparations

The following field-collected sediments were obtained from Stratus Consulting:

1. South East Pass Campground 2011
2. South East Pass 2011
3. South Pass Spit 2011
4. Black Hole 2012
5. Black Hole 2011
6. Loomis II 2011.

Preparation procedures for sediments and blended sediments are described in the individual testing protocols.

9.3.1 Source water/dilution water

Seawater used for all exposure studies was prepared in bulk at the University of Maryland's Aquaculture Research Center (ARC) using a proprietary mix of salts and charcoal filtered tap water. Filtered tap water and seawater obtained from ARC were then blended to achieve desired salinity as specified on test-specific test conditions tables (TCTs).

9.4 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

9.5 Water Quality Standard Operating Procedures

9.5.1 Temperature and dissolved oxygen measurements

Temperature and dissolved oxygen (DO) were measured using a YSI ProODO meter with a ProBOD probe.

At the beginning of every day, the YSI ProBOD probe was calibrated for DO% in a moist environment with deionized (DI) water having a salinity of 0 ppt. For DO measurements in saline test solutions, the salinity was input into the meter so that an internal correction could be used.

Each tank was tested by placing the probe into the water deep enough to fully cover the temperature sensor. The sensor reading was allowed to stabilize before either parameter was recorded. The probe was thoroughly rinsed with DI water between treatments, always moving from least to most contaminated treatments, and then cleaned at the end of each day before returning to a moist environment.

The temperature (°C) and DO (mg L^{-1}) were recorded on the *Water Quality Monitoring* bench sheet found in the QAPP.

9.5.2 pH measurements

The pH of each tank was examined using an Accumet Research ARIS pH meter.

At the beginning of every day, the pH meter was calibrated using standard 4.0, 7.0, and 10.0 pH solutions. The calibration from the previous day was cleared and for each standard, the solution was allowed to fully stabilize before the standard was set. Only a calibration with a slope > 95% was accepted. The calibration was repeated until this was reached.

The pH measurements were done in conjunction with the YSI probe to maximize efficiency and time. Both probes were immersed in the water at the same time. The pH measurement of a tank was recorded once the reading had fully stabilized. If there was a problem with the pH meter probe, ColorpHast indicator strips (6.5–10.0 pH, 0.3 units) were used to measure pH levels. All

pH measurements were recorded on the *Water Quality Monitoring* bench sheet found in the QAPP.

9.5.3 Salinity measurements

The salinity for each tank was measured using a portable refractometer and disposable pipettes.

When measuring the salinity, a small water sample was pipetted out from each tank and a drop was added onto the lens of the refractometer, making sure that the lens was fully covered with water but not overflowing. Pipettes were changed between treatments to prevent cross-contamination. The refractometer was raised into direct light and the salinity was determined by the location of the junction of light and dark regions on the salinity scale.

The salinity of each tank was recorded in the *Water Quality Monitoring* bench sheet in the QAPP.

9.5.4 Ammonia measurements

The ammonia concentration was measured using an ammonia test kit (NH_3/NH_4^+) from Aquarium Pharmaceuticals. If ammonia levels were below 0.5 mg/L, additional tanks within a treatment were measured using a Hach DR/2400 portable spectrophotometer (Salicylate Method 8155 for Nitrogen, Ammonia).

Using the ammonia test kit (NH_3/NH_4^+), 2.5 mL of water from one tank was pipetted out with a disposable pipette and placed in a round bottom test tube. Pipettes were changed for each treatment to prevent cross-contamination. Four drops (from each bottle in the kit) were added to the sample and thoroughly mixed. After 5 minutes, the ammonia levels were read using a color chart for "Saltwater" samples that differentiates between 0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L ammonia. Any sample with a color that looked to be in-between two of the colors on the chart was designated to be between those two levels of ammonia.

Using the Hach spectrophotometer, 10-mL of sample was added to a flat-bottomed sample cell and Ammonia Salicylate Reagent Powder Pillow was added to the sample. The cell was capped, mixed thoroughly, and allowed to react for 3 minutes. Once the 3 minutes had ended, an Ammonia Cyanurate Reagent Powder Pillow was added to the cell. The cell was capped, mixed thoroughly, and allowed to react for 15 minutes. A seawater sample blank was prepared by the same method in conjunction with the first test sample. Once the 15 minutes had ended, the seawater control was measured using the Hach DR/2400 and zeroed to 0.00 mg/L NH_3-N. The test sample was then measured using the Hach DR/2400, internally normalized to the seawater

blank, and the ammonia level was recorded. All subsequent samples for 1 day were measured using the same seawater blank. A new seawater blank sample was used each day.

All ammonia levels measured were recorded in a daily *Water Quality Monitoring* bench sheet found in the QAPP.

A. Testing Protocol 1: Chronic Exposure of Juvenile Blue Crabs to Oil (*Callinectes sapidus*) Contaminated Sediments

A.1 Experimental Setup

A.1.1 Experimental design overview

Each sediment toxicity test consisted of six test sediment preparations, including five test sediments (contaminated) and a control (reference). Eight replicate aquarium tanks (8,000 mL total volume with standpipes for water changes set to 6,000 mL water volume) were used for each of the six sediment types (48 tanks in total). Contaminated and reference field-collected sediments were provided by Stratus Consulting. Each tank was divided into three sections and one organism was placed into each section. There were 24 total organisms per treatment (i.e., 8 × 3).

All exposures lasted 31 days. Water quality parameters including temperature, salinity, pH, ammonia, and DO were recorded daily. A partial flow-through system was used to maintain water quality conditions. Daily water changes were conducted for 40 minutes at a flow rate of 200 mL/minute using a pressurized pumping system (resulting in a change of 1.3x the volume of the tank). This system maintained all water quality parameters (including ammonia) at appropriate levels. During exposures, the light cycle was held at 14L:10D. Crabs were fed portions of squid tissue in the evening, and in the morning all excess food was removed and food consumption was recorded. To adequately maintain DO levels, oxygen delivered through air stones provided gentle aeration to the tanks.

During the exposure, the organisms were monitored daily for mortality and sublethal endpoints as described below. All endpoint data were recorded on the appropriate bench sheets provided by Stratus Consulting.

A.1.2 Tank design

The glass tanks (8,000 mL volume) used for the experiment had a circular hole cut in the bottom that was used to secure a stand pipe that allowed for water outflow that did not disturb the sediment (volume of water maintained in each tank set at 6,000 mL). Tanks were placed in two columns of 24 tanks (three treatments on each side). The tanks were propped up on rows of wood that allow for drainage from the stand pipe into a long trough.

The stand pipe included a polyvinyl chloride (PVC) male fitting adapter sealed to the bottom of the tank around the hole with professional aquarium grade 100% silicone. A PVC female adapter was fitted to the male adapter and a PVC pipe, cut to give each tank a standpipe level of 6,000 mL water volume, was tightly fitted into the female adapter. When additional sealing was needed to prevent leaks, a washer was added between the male and female adapters and polytetrafluoroethylene (PFTE) tape was used to seal connections. To prevent loss of any large particles of sediment, screens were added to cover the top of the stand pipe. All plastic pieces making up the stand pipe were properly cleaned according to the QAPP and conditioned with seawater (25 ppt salinity) for at least 24 hours prior to use.

The tanks were divided by perforated plastic pieces cut to the height and width of the tanks with holes large enough to allow water and small particle transfer between sections within a tank, but small enough to prevent organisms from moving between sections. Dividers were pre-conditioned with seawater at 25 ppt salinity for at least 24 hours before use. Professional aquarium grade 100% silicone was used to seal them to the bottom of tanks. The silicone was allowed to dry for 24 hours.

Once the silicone dried, the entire tank including dividers and standpipe was conditioned with 6,000 mL of seawater (25 ppt salinity) for at least 24 hours prior to the addition of sediment. This water was siphoned out and fresh seawater added before the sediment was added.

A suspended airline system was implemented in which a long pipe with separate air valves for each tank was suspended less than a foot above the tanks. Aeration tubing was attached to each valve and an air stone to each tube. The aeration took place in the middle section for each tank.

Tanks were covered by thick, translucent plastic coverings from the ARC algal culture facility. Plastic coverings were conditioned in seawater for at least 24 hours before use. Each row of eight treatments for both columns was covered by one plastic covering (three coverings total) that was creased in the middle to prevent movement from one column to another. Small holes were cut into the plastic covers for the airlines to be placed in the middle section of each tank.

To further prevent crabs from moving between tanks, permanently sealed translucent plastic tubes (same plastic as coverings above) filled with water were placed over top of the plastic cover to create a tight seal between tanks and sections while still allowing light penetration. These tubes did not come into direct contact with tanks or water in tanks, but only the tops of the plastic covers.

A.1.3 Sediment preparation

Each sediment type was prepared separately using a stainless steel mixing bowl. Mixing bowls were cleaned and solvent rinsed prior to use and between sediment types (the names and lettering of the different treatment types are listed below).

A. South East Pass Campground 2011
B. South East Pass 2011
C. South Pass Spit 2011
D. Blend 1:1.7 (Loomis II 2011: Black Hole 2012)
E. Black Hole 2012
F. Black Hole 2011.

For all treatments except D, 1 kg of sediment was weighed directly into the mixing bowl. One kg of seawater (25 ppt salinity) was added to the bowl (1:1 sediment to seawater ratio) and sediment was allowed to thaw. The sediment and seawater were mixed thoroughly by hand using a stainless steel spoon until well homogenized. These sediment mixtures were used to fill the tanks and 8-oz sediment sampling jars. Sediment sampling jars were sent to ALS Environmental for chemical analysis. Sediment preparation methods were documented in a laboratory notebook and proper bench sheets forms.

For the blend treatment (D), 629.7 mg of Black Hole 2012 and 370.4 mg of Loomis II 2011 were weighed in the mixing bowl to get a 1:1.7 ratio. One kg of seawater (25 ppt salinity) was added to the bowl with the sediments and the sediments were allowed to thaw. The sediments and seawater were mixed thoroughly until well homogenized.

A.1.4 Filling tanks with sediment

Each tank was prefilled with 4,800 mL of seawater (25 ppt salinity) prior to the addition of the sediment. The remaining seawater was added after a 24 hour settling period in order to minimize the risk of sediment exiting the drain pipe before settling.

Each tank and sampling jar was filled with 200 g of sediment total by four increments of 50 g. Tanks were filled with sediment using pre-cleaned stainless steel measuring cups that were cleaned and solvent rinsed between sediments.

The sediment was scooped from the mixture using a four-tiered layering method (see Figure 1).

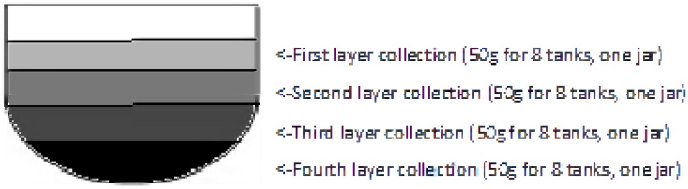

Figure 1. Four-tiered layering method.

The eight tanks and one jar were filled first by collecting from the top, "first" layer. After all the tanks and the jar had been filled once, they were filled again from the subsequent layer. This was repeated for a third and fourth time until all the tanks and jars had 200 g of total sediment/seawater mixture, always scooping from the top most volume. Each scoop contained 50 g of mixture.

The exact weight of all of the sediment scoops was measured and documented before pouring into the tanks. The measuring cups were rinsed with seawater between scoops to remove excess sediment and the measuring cup tared to 0.00 g before the weighing of each new scoop of sediment.

The 50 g of mixture in each scoop was distributed within the three sections of each tank (~ 1/3 of the scoop into each divided section). The scoop of sediment was added gently to make sure that no water/sediment went out of the drain pipe.

Any remaining sediment was placed into sample jars to be archived and stored at -20°C. Sample jars of sediment/seawater mixture were properly labeled as described in the QAPP, documented, and stored at -20°C.

The sediment was allowed to settle in the tanks for at least 24 hours covered with gentle aeration to keep DO levels high for the next day's addition of the blue crabs.

A.1.5 Addition of organisms to treatments

After the sediment had settled for at least 24 hours, composite water samples for chemical analysis were collected as described in Section A.5 and stored in a locked cooler at 4°C. After the composite water samples were collected, the water level for each tank was filled up gently to the 6,000 mL level (i.e., to the tip of the stand pipe) attempting to minimize the disruption of sediment.

After all the tanks had been filled, the juvenile blue crabs were added. The organisms were taken out of the holding tank in the ARC facility and separated by size. As much as possible, crabs of a

similar size were selected for use in the study so as to minimize the overall size range of the crabs in the study. The size range of the study depended upon the crabs available for use. Only healthy crabs with at least one cheliped and one swimming leg were chosen for the study, preferably two of each.

Before being added to the tanks, each crab was weighed, a photograph was taken on a grid for length/width measurements, and the sex was determined. All the measurements and the times that each crab was added to the tank were documented on an appropriate bench sheet.

The crabs were randomly selected from the holding buckets. The crabs were added to the "a" section (innermost) of each tank for every treatment first moving sequentially through treatment and tank number. After all the "a" sections were filled, the "b" sections (middle) for each tank for every treatment were filled. Lastly, all of the "c" sections (outermost) were filled.

Once all the crabs for a treatment were in their respective tank sections, seawater conditioned plastic covers were placed over the top of the tanks to protect the tanks from debris as well as to prevent crabs from moving between tanks. The air stones and tubing were placed into the water in the "b" (middle) section of each tank and kept at sufficient air pressure to keep DO levels high while not disturbing the sediment. To further seal tanks, permanently sealed translucent plastic tubes filled with water were placed over top of the plastic cover. Air lines were checked constantly to make sure that the air stones remained in the water.

A.1.6 Water change

A large reservoir of water was filled at the start of each day for the water change using a mixture of filtered tap water and seawater prepared at the ARC facility. The seawater and freshwater were mixed until the desired test salinity of 25 ppt was reached.

Daily water changes were conducted on eight tanks (one treatment) at a time until all treatments had received a water change. The flow rate of the water change was fast enough to provide an efficient tank exchange (approximately 1.3x the volume of the tank, i.e., 8,000 mL) of water, while not disturbing the sediment layer. The water change of each tank lasted 40 minutes at a flow rate of 200 mL/minute using a pressurized pumping system with individual valves for each tank.

The water being exchanged flowed out of the stand-pipe into a long drainage well and collected in an activated carbon filter apparatus. The treatment effluent percolated through the activated carbon, removing any contaminants before flowing into a sewer drain. The activated carbon filter was fully exchanged half way through the 31 day experiment and replaced with new activated carbon. All activated carbon used for filtration was packaged and given to the Environmental Health and Safety employees at IMET for hazardous waste disposal.

During the water change, the plastic sheets covering the tanks were moved so that only the front sections of each tank were exposed to minimize any potential movement of organisms from one tank to another. The front sections were also closely monitored during the water change. Individual water valves were monitored to make sure that all valves were releasing the standard flow rate and any valve with a flow rate that deviated was properly adjusted.

The times for each treatment's water change were recorded in the notes section of the *Water Quality Monitoring* bench sheet.

A.2 Daily Monitoring Endpoint

Every crab was checked daily for general-monitoring endpoints, including mortality, molting, feeding, and growth (weight and carapace length/width). Crabs were checked for general-monitoring endpoints one treatment at a time to minimize potential for crab movement between tanks. The water tube barriers were removed from the top of the plastic cover and placed in designated storage areas. The covers were lifted up and clamped into the air line system pipe to allow for access to all of the tanks for one treatment. The clamps were cleaned and solvent rinsed in-between treatments to prevent cross-contamination.

The checks for general monitoring were started before the start of the water changes, unless water quality prohibited visualization, in which case crabs were viewed during and/or after water changes. For example, treatments that contained finer sediments that reduce the water clarity were examined after the daily water change for that treatment to increase visibility.

Visibility of crabs could be difficult for middle and inner tanks, particularly for treatments that had finer sediments. Before checking crabs, the air stones were removed temporarily from the water. The air stones were replaced immediately after general monitoring endpoints had been examined for a treatment.

If the visibility was still reduced, a pre-cleaned glass beaker was used to reduce the path length and a head lamp or flashlight was used to aid visualization. The glass beaker was cleaned and solvent rinsed between treatments and after use.

All daily general monitoring endpoints were recorded on the *Test Performance Monitoring Bench Sheet,* provided in the QAPP, for mortality and sublethal endpoints.

A.2.1 Mortality

A *Test Performance Monitoring Bench Sheet* was filled out daily for all tanks, scoring crabs as alive or dead and indicating in the notes which individual crab number died within a tank. The

mortality was recorded as a treatment mortality if the crab died from the treatment and as a non-treatment mortality if the crab died from other sources (e.g., cannibalism). In tanks where the crabs were not visually moving or if they had buried themselves in the sediment, mortality was assessed by gently probing either the sediment (without disturbing and resuspending the sediment) or the actual crab. Crabs not responsive after gentle prodding were removed from tanks (see below) and scored as dead.

All dead crabs were removed from tanks as soon as possible. For each dead crab (when possible), a photo was taken for length and width measurements and recorded in the sublethal *Test Performance Monitoring Bench Sheet*. All dead crabs were individually wrapped in foil that was labeled on the inside and outside of the foil packet. The foil packet with crab was then placed into a labeled 8-oz glass jar or plastic bag. Labels included the date removed, individual crab identification (ID; treatment-tank-crab letter), and observer initials. Dead organisms from each treatment were kept in separate jars and all jars were stored in a locked freezer at -20°C.

A.2.2 Molting

All crabs were examined for molting daily and scored as molted (Yes or No) on the sublethal *Test Performance Monitoring Bench Sheet*. If the crabs buried themselves in the sediment, making them difficult to visualize, the sediment was gently probed to improve visibility of the crabs. Likewise, all thicker sediments were gently probed to help find any potential molted shells.

If a crab had molted, the molt shell was removed and photographed to validate the carapace size. If enough of the shell molt remained to determine the sex of the crab, the sex was recorded in the sublethal *Test Performance Monitoring Bench Sheet*.

Every crab that molted was examined for growth endpoints (weighed and photographed for carapace length and width) the following day.

A.2.3 Food consumption

The food consumption of the each crab was scored daily from 0 to 2 on the sublethal *Test Performance Monitoring Bench Sheet*. A score of 0 indicated No Feeding (all of the food remained). A score of 1 indicated Intermediate Feeding (some of the food has been consumed). A score of 2 indicated Complete Feeding (food was completely gone). As with the molt scoring, all thicker sediments were also probed to make sure all excess food was found.

Any remaining food in tanks was removed using pre-cleaned stainless steel tongs and placed in plastic bags for disposal. Tongs were cleaned and solvent rinsed between treatments.

A.2.4 Growth endpoints

Weight of crab

1. Crabs that had molted the previous day were gently removed from the tank using a pre-cleaned, large stainless steel serving spoon. From the spoon, the crab was picked up by the posterior region and placed into a paper towel to transport.

2. The crabs were removed as quickly and gently as possible attempting to minimize disturbance of sediment and stress to the crab. Any excess water that was brought up with the crab was drained back into the tank and the spoon was dipped multiple times back into the treatment water to remove any attached sediment. The spoon was cleaned and solvent rinsed between treatments.

3. A pre-cleaned 150-mL beaker containing approximately 50 mL fresh seawater (25 ppt salinity) was tared to 0.00 g on an analytical balance.

4. The crab was gently blotted with the paper towel to remove excess water and placed into the beaker on the analytical balance.

5. The weight and sex of the crab were recorded in the sublethal *Test Performance Monitoring Bench Sheet*.

Length and width of carapace

1. A camera was set up on a tripod prior to starting the measurements. The camera position and zoom were standardized for each daily group of crabs to allow for quicker analysis in Image J.

2. A grid of known distances was taped to a plate of glass with clear packaging tape and used to measure the length/width. The grid size was 0.25 in. (6.35 mm). The grid was placed under the camera and the focus was adjusted until the proper distance and clarity were reached.

3. For each treatment, tank number and crab letter ID labeled cards were created and used to identify the crab within each photograph. For each photograph, the appropriate card was placed in the top corner of the grid/photo.

4. The crab was taken out of the beaker used for measuring weight and gently blotted with a paper towel to remove excess water.

5. The crab was placed on the grid and held in place with a thin, wooden stick, making sure that the stick did not cover either of the lateral spines or the top/bottom of the carapace for the photograph.

6. Immediately after the photo was taken, the crab was placed back into its respective tank as it was important to minimize the time the crab spent out of the tank.

7. Analysis of the length and width of the carapace was done using Image J software. The length and width boxes on the sublethal *Test Performance Monitoring Bench Sheets* were left blank until those measurements were conducted. Once the length and width were measured, those data were then filled in on the original sheets for the day the crab was photographed.

A.3 Daily Water Quality Measurements

Water quality parameters, including temperature, salinity, pH, and DO, were recorded daily in all tanks when possible, or for a minimum of two tanks per treatment. Ammonia was recorded in at least two tanks per treatment every day (using a random sampling design). Ammonia levels were monitored in all tanks of a treatment at least once per week. All water quality parameters were measured in the outermost, front section of each tank. Water quality measurements, as well as the time each measurement was taken, were recorded on the *Water Quality Monitoring* bench sheet provided in the QAPP. See the University of Maryland GLPP document for details on water quality measurement procedures.

A.4 Feeding

Each crab was fed daily small pieces of squid tissue one treatment at a time to minimize the potential for movement of crabs between tanks. Squid tissue was pre-shredded and stored in a freezer at -20°C until they were thawed out in preparation for feeding them to the crabs.

After the water change, the pre-cut squid was taken out of the freezer and allowed to partially thaw in a thin layer of fresh seawater (25 ppt salinity) to the point where individual pieces could be removed. Feeding was the last task completed each day.

Each crab was fed one large pre-shredded squid or two smaller pre-shredded squid pieces depending on how the squid was shredded. Initially the total amount of food fed to the crabs was between 0.10 and 0.15 g. The amount of food fed to the crabs increased as the crabs molted.

The timing of feeding was documented in the laboratory notebook and in the notes section of the *Water Quality Monitoring* bench sheet. Any remaining food was documented and removed the following morning. See Section A.2.3 for more details.

A.5 Sampling

Both sediment and overlying water samples were collected throughout the experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP, with the following specifications:

- ***Initial sediment sampling:*** When preparing tanks for sediment exposures, sediment samples were collected from each treatment and control group. When preparing tanks, sediment samples from each treatment group and control were prepared as a "ninth" replicate during the aliquoting of sediment into the replicate tanks plus one analytical chemistry sediment jar (glass 8-oz). These initial sediment samples were sent to ALS Environmental for chemical analysis.

- ***Final sediment sampling:*** Composite sediment samples were collected from each treatment group and control tanks at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a pre-cleaned, large stainless steel spoon. Once sediment was thoroughly mixed, a pre-cleaned ¼ cup stainless steel scoop was used to fill the analytical chemistry sediment jar (glass 8-oz). This process was repeated for each treatment group and control using clean, decontaminated sample processing equipment. Final sediment samples were sent to ALS Environmental.

- ***Composite water sampling:*** Composite water samples were collected from each treatment group and control just before the crabs were added (time zero), every other day for 6 days, then once a week for the duration of the experiment. Time zero samples were taken just prior to adding the crabs; all other sampling was performed before the daily water changes. At each time point, a subsample from each of the eight replicate tanks within each treatment was collected and composited into a clean decontaminated glass mixing container. Subsamples were collected by dipping a clean, decontaminated ¼ cup stainless steel scoop into each tank. For consistency, the sample was always taken from the front section of each tank. The same transfer beaker was used for all tanks within a single treatment. A clean, decontaminated beaker was used when collecting subsamples from each additional treatment. The scoop was filled to the brim for each subsample to ensure that the same volume was added from each tank to the composite. After thoroughly mixing subsamples, the composite was used to fill the required analytical

chemistry containers for requested chemical analyses (see the QAPP). This process was repeated for each treatment group and control. Water samples were sent to ALS Environmental for analysis.

The standard chain-of-custody (COC) forms provided in the QAPP were used to request chemical analyses and relinquish samples to ALS Environmental.

A.6 Additional Endpoints

A.6.1 Dissection of tissue for molecular endpoints

Hemolymph and hepatopancreas tissues from one crab per treatment were extracted to examine DNA damage (i.e., single-strand breaks) with the Comet Assay. Remaining hepatopancreas tissue from each crab was extracted for genetic molecular biomarker analysis (see below). Before the experiment began, hemolymph and hepatopancreas tissues were also extracted from ten unexposed crabs (Time 0 hour crabs) collected from the same holding tanks as the test organisms were collected from. These samples were exposed to hydrogen peroxide to be used as a positive control for the Comet Assay.

From each crab, the hemolymph was extracted first to minimize the impacts of stress on the DNA of the hemolymph. Immediately after the crab was taken out of the water, approximately 50 µL of hemolymph was extracted directly into a syringe with a 23 gauge needle that had been coated with and contained approximately 50 µL of crustacean anticoagulant (1:1 hemolymph). The hemolymph was extracted from the crab's hypobranchial sinus through the arthrodial membrane between the chelae and the first walking leg. The crustacean anticoagulant contained 0.3 M NaCl, 0. 1 M glucose, 30 mM trisodium citrate, 25 mM citric acid, and 3 mM EDTA at a pH of 6. The hemolymph/anticoagulant solution was transferred into Eppendorf tubes and placed on ice. The tubes were then centrifuged at 800 xg for 10 minutes at 4°C to concentrate the cells. The supernatant of each tube was discarded and the pellet resuspended in 50 µL anticoagulant, which was kept on ice until processing for the Comet Assay. The exposure of the tubes to light was minimized.

After removal of the hemolymph, the crabs were placed on ice for at least 5 minutes (until torpid) before dissecting. Crabs were dissected one at a time with pre-cleaned and solvent rinsed dissection equipment. First, the hepatopancreas was dissected out and a small subsample taken for the Comet Assay. The subsample was immediately processed by mincing it in 300 µL of Hank's Balanced Salt Solution-HEPES buffer pH 7.6 (HBSS-HEPES) using a clean razor blade. To keep the sample cold, it was minced on a strip of parafilm on top of a cold metal aluminum sheet placed on ice. The rest of the hepatopancreas tissue was placed into pre-labeled cryo vials containing RNAlater. After allowing the RNAlater to permeate the tissues overnight, the cryo vials were placed into a sealed, labeled cryo vial box and stored in a locked -80°C freezer. If

RNAlater was not available, the cryo vials were flash frozen in liquid nitrogen and stored on dry ice during the remaining processing until they could be permanently stored in a locked -80°C freezer.

For the positive control samples, 10 µL of the resuspended hemolymph or the minced hepatopancreas tissue in HBSS-HEPES was placed into Eppendorf tubes containing a solution of 50 µM hydrogen peroxide in cold HBSS-HEPES and left on ice for 30 minutes before analysis with the other samples. These tubes were then centrifuged at 800 xg for 10 minutes at 4°C to concentrate the cells. The supernatant of each tube was discarded and the pellet containing the cells was resuspended in 50 µL anticoagulant for hemolymph or 50 µL HBSS-HEPES for hepatopancreas, kept on ice and covered with foil The positive controls were processed as quickly as possible to minimize potential impacts from residual hydrogen peroxide in the resuspension.

A.6.2 Alkaline single cell gel electrophoresis (comet) assay protocols

Microscope slides were coated with 1% normal melting point agarose (NMPA) in phosphate buffered saline (PBS) and allowed to dry at 37°C in the dark. These slides were stored in slide boxes within plastic bags containing desiccant.

From the hemolymph resuspensions, minced hepatopancreas tissues, or resuspended positive controls above, 10 µL was added to 100 µL of 0.6% low melting point agarose (LMPA; 37°C) in HBSS-HEPES pH 7.6 and layered over the NMPA layer on the slides. Two replicate slides were made for each crab sample. Coverslips were placed onto slides and the agarose allowed to polymerize for at least 5 minutes on a metal tray over ice. Following solidification, the coverslips were removed and the slides were placed into cold (4°C) lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, 1% sodium sarconsinate; pH 10) for at least 1 hour but not more than 24 hours at 4°C in the dark.

Once removed from the lysing solution, slides were rinsed with distilled water, placed on a horizontal gel electrophoresis tray, and covered with cold (4°C) electrophoresis buffer (0.20 M NaOH, 1 mM EDTA; pH > 12) for 10 minutes to allow the DNA to unwind. Electrophoresis was conducted at 25 V, 300 mA for 10 minutes. Slides were removed and placed in a cold (4°C) neutralization solution (0.4 M Tris, pH 7.5) for 5 minutes (repeated for three washes for a total of 15 minutes). Slides were then drained and placed in 100% ethanol (4°C) for 5 minutes, allowed to dry in a dark container overnight, and then placed in a desiccated slide box until processing by the image analysis system.

The slides were reconstituted with 2 µM/mL ethidium bromide in HBSS-HEPES and examined using an epifluorescent microscope (Olympus BX50) with a green filter at 40x magnification (Q Imaging Retiga 1300 camera). The Komet 5.5 Software's (Kinetic Imaging, Liverpool, UK)

image analysis package was used to score the cells. From each duplicate slide, 50 non-overlapping cells were randomly selected for quantification. The results were expressed as means ± standard error of the means in terms of the percentage DNA in tail (% tail DNA), tail length (μm), and Olive tail moment.

A.6.3 Gene expression protocols

The hepatopancreas tissue samples from all of the Time 0 hour and treatment crabs were homogenized using a Fast Prep Bead Breaker method, and the mRNA was extracted using an RNAqueous Kit (Life Technologies). The extracted mRNA for each crab was treated with DNase using a TURBO DNA-free kit (Life Technologies) to remove any genomic DNA contamination and then reverse transcribed to cDNA using a High Capacity RNA-to-cDNA Kit (Life Technologies) for absolute quantitative polymerase chain reaction (qPCR) analysis. For each sample, a no reverse transcription (No RT) control was also conducted in parallel to the reverse transcription (RT) reaction to examine genomic DNA contamination in the cDNA sample remaining after the DNase treatment.

Primers for each gene of interest were designed using the National Center for Biotechnology Information (NCBI) Primer-Basic Local Alignment Tool (BLAST) from either existing blue crab mRNA sequences in GenBank or from Expressed Sequence Tags database (EST) hits observed by using BLAST with known mRNA sequences for other crab species in a Blue Crab EST Library available in Dr. Al Place's laboratory at IMET.

PCR products for each gene of interest were run on a 1.5% agarose gel, extracted using a QIAquick Gel Extraction Kit (QIAGEN), and ligated into a TOPO pCR-II plasmid. These plasmids were transformed into chemically competent TOP10 *E. coli* (Invitrogen). The plasmids were then purified from the *E. coli* using a ZR Plasmid Miniprep-Classic Kit (Zymo Research) and the plasmid DNA were quantified on a NanoDrop 2000 Spectrophotometer. The purified products and plasmids were sequenced by the IMET BioAnalytical Services Laboratory and the orientation of the sense strand of product insert in relation to T7 promoter was verified. The plasmid DNA for each gene was linearized using a restriction digest (Hind *iii*) and complete digestion was verified by gel electrophoresis. The linearized plasmids were then appropriately diluted to a six-point serial dilution series and run on qPCR to examine the qPCR efficiency for all genes.

To conduct absolute quantification, the plasmid inserted products were transcribed into RNA using a TranscriptAid T7 High Yield Transcription Kit (Fermentas). The RNA concentrations for each gene were measured on a NanoDrop 2000 Spectrophotometer and six-point RNA serial dilutions were prepared to give a known copy number for the RT reaction. Each RNA dilution was reverse transcribed to cDNA of a known copy number. Absolute qPCR was conducted using the Power SYBR Green Kit on an Applied Biosystems 7500 Fast Real-Time PCR System. For

each gene, all the crab cDNA samples were run on qPCR in triplicate and the injunction with the six-point cDNA standards and no template controls were also run in triplicate. The No RT crab cDNA samples were run in duplicate for arginine kinase (AK) with AK cDNA standards in triplicate to examine genomic DNA contamination. A dissociation curve was produced for all samples and standards. A linear regression equation was produced for the cDNA standards of each gene and used to determine the copy number of each crab sample. The copy numbers were then normalized to the total RNA concentration (ng) and reported as copy numbering total RNA.

A.6.4 Histology

At the beginning of the experiment, five organisms were selected at random from the holding tank and dedicated for histology. At the end of the study, one crab from every tank, provided there were not two mortalities within a tank, was dedicated for histology (i.e., eight crabs per treatment).

Whole crabs were placed on ice until torpid (up to 15 minutes). Chilled crabs were injected with a few drops of fixative (Bouin's solution) using either a 26.5 ga or a 23 ga needle and a syringe of 1 or 3 mL. Fixative was injected under the carapace by putting the needle into the viscera through the ventral shell if it was thin (< 1 g crab) or through the membrane between the swimming leg and the carapace. Care was taken to avoid moving the needle tip laterally once it has been inserted into the viscera as this could tear the organs and tissues within. When necessary, it was acceptable to make two injections, one on either side, to ensure complete penetration of fixative. Penetration of fixative was visible as a change in opacity or color (if Bouin's was used) under the ventral shell.

Once the crab had been injected with fixative, it was placed in > 10 volumes of fixative in a sealable container. For example, a 0.5 g crab would be fixed in at least 5 mL of fixative. For crabs under 3 g, 50 mL conical polypropylene tubes were used. For crabs that were too large to fit into the 50 mL tube, medical specimen jars or histology jars were used. Each crab had its own 50 mL conical tube or jar. Jars were prepared ahead of time with all suitable labels to avoid mixing up treatments. Because labels could be rubbed off or erased by later solvents or acids, a label inside the jar was also included. This label was a piece filter paper that was written on in soft pencil (not pen) and accompanied the fixed crab through later steps. Crabs were stored at 4°C in fixative for at least 96 hours and up to 14 days.

The fixative was later decanted, the crabs were rinsed in distilled water for 1 hour, and then they were placed in 70% ethanol (10 volumes) for at least 1 day. Traces of fixative were rinsed from the crab and container with 3–5 mL of 70% ethanol. Then the containers were filled with the > 10 volumes of fresh ethanol for long-term storage.

A.6.5 Polycyclic aromatic hydrocarbons bioaccumulation

At the beginning of the experiment, ten unexposed organisms were selected at random from the holding tank to serve as controls for the analysis of polycyclic aromatic hydrocarbons (PAHs) bioaccumulation. At the end of the study, the remaining crabs from each treatment (i.e., any crabs not used for the histological or molecular endpoints) were collected for PAH bioaccumulation.

The crabs to be used for PAH bioaccumulation were first weighed and photographed for length/width of carapace as detailed in Section A.2.4. The crabs were then placed on ice for at least 5 minutes and then individually wrapped in foil, labeled, and stored in bags. All crabs from each treatment were stored in the same bag and placed at -20°C for potential bioaccumulation analyses.

10. Pacific EcoRisk General Laboratory Procedures and Practices

10.1 Background

Pacific EcoRisk (PER) is an environmental consulting firm conducting research and testing in the field of environmental toxicology. Stratus Consulting contracted with PER to perform standardized testing of whole sediment acute toxicity on *Leptocheirus plumulosus* using methods that were developed by the U.S. Environmental Protection Agency (EPA). PER was also contracted to perform standardized testing of water accommodated fractions (WAFs) on mysid shrimp and the diatom *Skeletonema costatum*. After the conclusion of these tests, PER provided Stratus Consulting with reports describing the procedures used to conduct each test. Considering that these tests followed EPA standardized guidelines, PER did not write a General Laboratory Procedures and Practices (GLPP) document. Stratus Consulting used information from PER reports to prepare a PER GLPP in the *General Laboratory Protocols and Procedures: Deepwater Horizon Laboratory Toxicity Testing* document.

10.2 Methods

10.2.1 Test organism sources

Leptocheirus plumulosus

These tests were performed on the amphipod *L. plumulosus*, which were obtained from a commercial supplier (Chesapeake Cultures, Hayes, Virginia). A sample of the same sediment used to culture the amphipods (termed "Lab Control") was also obtained from the amphipod supplier for use as one of the control sediment treatments.

Mysid shrimp (*Americamysis bahia*)

The *A. bahia* used in this test were obtained from a commercial supplier (Aquatic Indicators, St. Augustine, FL). Upon receipt at the laboratory, the test organisms were held in aerated tanks containing reverse osmosis, deionized (RO/DI) water adjusted to a salinity of 25 ppt via addition of an artificial sea salt (Crystal Sea® – bioassay grade). Test organisms were fed brine shrimp nauplii *ad libitum* during this pre-test holding period.

Diatom (*Skeletonema costatum*)

S. costatum were ordered from The Culture Collection of Algae at the University of Texas at Austin. Test cultures were ordered far enough in advance to ensure that the algae culture was in the log growth phase before set-up. Algae were cultured at PER at the desired test salinity for at least two weeks before test initiation.

10.2.2 Source of natural seawater for testing

The natural seawater used in the tests for all species was obtained from the University of California Davis Granite Canyon Marine Laboratory; this water was stored at the PER laboratory in a 3,000-gal insulated high-density polyethylene tank at 4°C. Prior to use in these tests, 150 gal of this water was archived in a large carboy (a sample of this 150-gal "batch" was collected and sent to the analytical laboratory for source water chemical analyses). A sample of every "batch" that was used was sent to ALS Environmental for source water analyses. This water was stored in a temperature-controlled room at 4°C for use in these tests in order to ensure that the water used in all of the Stratus Consulting testing was of the same origin. This seawater was adjusted to the desired test salinity [see test-specific test conditions tables (TCTs)] via the addition of Type I laboratory water (RO/DI water); these diluted natural seawaters are referred to using the adjusted salinity level (e.g., 20 ppt seawater).

10.2.3 Biological testing procedures

The methods used in conducting this testing followed established guidelines:

Leptocheirus

- Standard E1367-99: Standard Guide for conducting 10-day static toxicity tests with marine and estuarine amphipods (ASTM, 1999)
- Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

A. bahia

- Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition (EPA/821/R/02/012; U.S. EPA, 2002).

S. costatum

- Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae (ASTM E1218-97a; ASTM, 1997).

10.2.4 Solid-phase sediment toxicity testing

Solid-phase sediment tests were conducted to evaluate the potential adverse impacts of the sediments on the benthic community. These tests involved exposing a benthic amphipod (*L. plumulosus*) to test sediments under static conditions for 10 days, after which the survival of the amphipods was determined and evaluated.

10.2.5 Sediment samples

Tests were conducted with both field-collected sediments provided to PER by Stratus Consulting and reference sediment that was spiked at the University of Mississippi Gulf Coast Research Laboratory (GCRL) and shipped to PER. The methods used by GCRL to spike the sediment are in Section 10.6.

10.2.6 Reference toxicant testing of *L. plumulosus*

To assess the sensitivity of the amphipods used in these tests to toxic stress, a reference toxicant test was run concurrent with the solid-phase sediment testing. The amphipod reference toxicant test consisted of a 96-hour water-only exposure to KCl with survival (%) as the test endpoint. The resulting test response data were analyzed by PER to determine key dose-response point estimates [e.g., EC50 (the concentration for which 50% of the test organisms were affected)]; all of the statistical analyses were made by PER using the CETIS software. The test responses were compared to the "typical" response range established by the mean ± 2 standard deviations (SD) of the point estimates generated by the 20 most recent previous reference toxicant tests performed by this laboratory.

10.2.7 Acute toxicity of WAFs on mysid shrimp and *skletonema*

High-energy WAFs (HEWAFs) and low-energy WAFs (LEWAFs) were prepared at PER according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP; located in Attachment 3) for mysid shrimp and *S. costatum* testing. Routine water quality characteristics [pH, dissolved oxygen (DO), and salinity] were determined for each

treatment test solution before the start of the test. A subsample of each test solution was also collected for PAH analysis, and shipped to ALS Environmental for analysis.

10.3 Pore Water Characterization for Sediment Tests and Additional Sediment Sampling

Dummy test replicates for each sample/treatment were sacrificed at the time of test initiation and test termination for sediment pore water characterizations (pH, salinity, total sulfides, and total ammonia). The overlying water in each test replicate selected for the pore water characterization was carefully siphoned off to minimize any disturbance of the sediments, after which the remaining sediment was transferred to a 750-mL centrifuge bottle. The sample sediment was centrifuged at 2,500 G for 30 minutes, after which the overlying supernatant was carefully decanted from the centrifuge bottle and water quality characteristics were determined.

At the time of the pore water characterization (test initiation and test termination), additional test replicates were similarly sacrificed to provide bulk sediment for chemical analyses. The overlying water was siphoned out as before, after which the sediment was transferred into a 250-mL glass sample jar (provided by the analytical laboratory). The sample containers were wrapped in bubble wrap and securely packed inside a cooler with crushed ice. A temperature blank was included in each cooler. The original signed chain-of-custody (COC) forms were placed in a sealed plastic bag and taped to the inside lid of the cooler. Appropriate packaging tape was wrapped completely around the cooler. A "This Side Up" arrow label was attached on each side of the cooler, a "Glass – Handle with Care" label was attached to the top of the cooler, and the cooler was sealed with custody seals on both the front and the back lid seams. These samples were shipped via overnight delivery to ALS Environmental (Kelso, Washington) for the chemistry analysis.

10.4 Quality Assurance/Quality Control Review

The biological testing of these sediments incorporated standard quality assurance/quality control (QA/QC) procedures to ensure that the test results were valid, including the use of negative laboratory controls, positive laboratory controls, test replicates, and measurements of water quality during testing. QA procedures that were used for sediment testing are consistent with methods described in the EPA and the ASTM International (ASTM) guidelines. For tests performed with field-collected sediments, sediments for the bioassay testing were stored appropriately at -20°C at the request of Stratus Consulting and the aliquots used for testing were broken off of the frozen samples prior to thawing. For tests performed with spiked sediments, sediments for the bioassay testing were stored appropriately at 4°C at the request of Stratus

Consulting until they were processed prior to testing. Sediment interstitial water characteristics were within test acceptability limits at the start of the tests. The overlying water in these solid-phase sediment toxicity tests consisted of natural seawater diluted to the test salinity using Type I laboratory water.

All measurements of routine water quality characteristics were performed as described in Section 10.5 *Water Quality Laboratory Standard Operating Procedures*. All biological testing water quality conditions were within the appropriate limits. Laboratory instruments were calibrated daily according to PER standard operating procedures (SOPs), and calibration data were logged and initialed.

10.5 Water Quality Laboratory Standard Operating Procedures

10.5.1 Conductivity/salinity analysis SOP

This is the SOP for the Thermo Scientific Orion 3-Star meter.

Calibration of meter

1. Turn on Conductivity/Salinity meter.
2. Press the "Calibrate" button. There should be a number (i.e., 0.475) displayed below CELL. This is the cell constant. Record this number.
3. Place the probe in 100 µS standard at 25°C. The meter will automatically recognize the standard. When the reading stabilizes at 100.0, press "Calibrate Again."
4. Repeat with the 1,413 µS and 12.9 mS standards. After the reading for the 12.9 mS standard has stabilized, press MEASURE.
5. A new value for the cell constant will be displayed. Record this number. Verify as described in the next section before use.

Verification of calibration of meter (required minimum of daily)

1. Turn on Conductivity/Salinity meter.
2. Place the probe in a standard National Institute of Standards and Technology (NIST) salinity or conductivity solution, making sure that the standards are at 25°C.

3. Adjust the readout of the meter to display conductivity (µS/cm) and take the reading of the lowest standard (100 µS) and compare to the standard value. If readout is within 5% of the standard value, the meter is properly calibrated. If the reading is outside the 5% range, follow the steps in the previous section to calibrate the meter.

4. Record the reading into the Conductivity/Salinity logbook.

Measurement procedure

1. With the instrument prepared for use and the probe calibrated, select the appropriate measurement parameter (conductivity of salinity).

2. Place the probe into the test solution. There is no need to agitate the probe or the sample to measure.

    a. Hold the probe by the sensor-body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and to the probe reading incorrectly.

3. While the meter is measuring the solution, the units symbol (µS/cm) will blink. Once the reading has stabilized, the symbol will stop blinking and the value displayed can be recorded.

4. Rinse the electrode with Type I water and proceed to measure the next sample.

Storage

1. Rinse electrode with Type I water and place it in the holder.

2. Turn the meter off.

Maintenance

1. All conductivity verification standards must be changed out weekly. Replace conductivity standard bottles every 2 weeks.

2. Inspect all conductivity probes for damage at least once a week, but preferably every day during calibration.

3. Keep an eye on the bottles of conductivity standard solutions. If a bottle is getting low (less than one-fourth full) or the standard is going to expire, it must be replaced prior to calibration.

4. Record all standard changes and maintenances in the "Conductivity Log Book."

10.5.2 Dissolved oxygen analysis SOP

This is the SOP for the Thermo Scientific 3-Star RDO Meter.

Air calibration

1. Turn on the RDO Meter.

2. Place the probe in a chamber with 100% saturated air conditions, making sure that the probe membrane is not touching anything or has any water droplets on it.

3. Press CALIBRATE.

4. Record Pre-Calibration % Saturation and let stabilize. In a few seconds, it should stabilize to 100%, and this should be recorded.

5. Press the UP arrow, and record the air pressure in torr (barometric pressure). Press the DOWN arrow, to return to mg/L, and the meter is ready to use.

Measurement procedure

1. Submerge the end of the probe in the water sample. There is no need to agitate the probe or the sample to measure. Record the value in mg/L once the reading has stabilized.

    a. The probe should be held by the sensor-body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and the probe reading incorrectly.

Salinity adjustment

1. When measuring DO in salty samples (> 15 ppt), enter the sample salinity into the meter prior to measurement.

2. Press SETUP.

3. Press the bottom-right button (with three rectangles and an arrow) once. The screen should read RES.

4. Press the DOWN arrow three times. The screen should read SALF.

5. Press the bottom-right button again. The curser should be on the bottom row.

6. Use the bottom-left button (with the number 8 and arrows) to scroll between digits, and the arrow buttons to change the numbers until the value on the screen reflects the salinity of the sample.

7. Press the bottom-right button again to save. Press MEASURE to return to measurement mode.

8. Repeat the process to return salinity to zero after the measurement of salty samples is complete.

Maintenance

1. Change Type I water in the probe storage chamber weekly and refill to the fill line with new water.

2. Inspect probes for any damage at least once a week, but preferably daily during calibration.

3. Record all water changes and weekly maintenance in the "DO log book."

4. Replace probe caps annually.

10.5.3 pH analysis SOP

This is the SOP for the Beckman pHi 410/ISE/mV meter.

Three-point calibration of meter

1. Turn pH meter on and check that all electrodes are properly attached. The screen display should show the following:

    a. A "pH" symbol in the upper left hand corner. If not displayed, press the 1 button to place the meter in "pH" mode.

b. "ATC" indicating that automated temperature compensation is functioning properly. If no "ATC" is displayed, the probe is not properly attached, or the built-in thermometer is not functioning.

c. If a battery icon is displayed, the batteries should be changed out within 25 hours of the icon first appearing.

d. If the word "bat" is displayed, the batteries are too low to provide reliable measurements. The meter must not be used until batteries are changed out.

2. Press the pH button to enter pH mode.

3. Press CAL then DELETE. This will clear the previous calibration from the memory of the meter.

4. Place the probe in the first buffer solution (Pink pH 4.01).

5. Press CAL. The Cal icon will begin flashing.

6. If the buffer displayed on the screen is correct, press ENTER. The pH buffer value will flash until calibration is complete.

   a. If the pH buffer is incorrect, press the arrow keys to select another buffer.

7. When calibration is complete for the current buffer, the display will show a flashing Cal 2 and the next pH buffer value will display (e.g., 7.00).

8. Remove the probe from the PINK pH 4.01 buffer and rinse with DI water.

9. Place the probe in the YELLOW pH 7.00 buffer and repeat steps 6 through 8 above, but use the pH 7.00 buffer.

10. Place the probe in the BLUE pH 10.01 buffer and repeat steps 6 through 8 above, but use the pH 10.01 buffer.

11. Calibration is complete when the final calibration point has been entered. The meter will beep three times and display the slope (4.01–10.01), mV offset at pH 7.00 and the number of calibration points.

12. All slopes must be between 95% and 105%. If not, a flashing E04 will be displayed in the center of the screen. In this case, the calibration procedure must be repeated, beginning with step 2. If the slopes are within the acceptable range, proceed to step 13. If the slope

is not acceptable, proceed with recalibration and/or seek the guidance from the equipment manual for remedies.

13. Press the pH or ENTER button to return to the main screen. You are ready to measure pH values. The pH or ENTER button must be pressed in order to measure pHs correctly.

Procedure

1. Turn on the pH meter. Ensure that daily calibration is complete. If, not refer to the *Three-point calibration of meter* section.

2. Remove the pH electrode from the electrode holder, and rinse with DI water.

    a. The probe should be held by the sensor body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and the probe reading incorrectly.

3. Place pH electrode into a sample.

4. The pH value will rise or fall depending on the solution being measured. There is no need to agitate the probe or the sample to measure. When the pH value displayed on the meter is stable and the display reads "Ready," the meter has stabilized and the value displayed may be recorded onto the appropriate data sheet.

5. Remove the electrode from the sample and rinse with Type I water.

6. Proceed to the next sample.

7. When the pH analyses are completed, place clean electrode back into the electrode holder.

Storage

1. At the end of the day, store the pH probes in a storage solution in order to maintain a properly functioning probe.

2. Place the probe into the Erlenmeyer flask containing the storage solution and submerge the tip of the probe in the solution.

3. Turn the meter off.

Maintenance

1. All pH probe storage solutions must be changed out weekly.
2. All pH buffer solutions must be changed out weekly.
3. All pH probes should be thoroughly cleaned once a week.
   a. Soak probes in pH probe cleaning solution for 30 minutes.
   b. Rinse thoroughly with DI water before proceeding to calibration of meters.
4. Record all buffer changes and weekly maintenance in the "pH log book."

10.5.4 Sulfide analysis SOP

Theory of operation

The following SOP outlines the procedures for testing sulfide concentrations in wastewater. This spectrophotometric method is equivalent to EPA 376.2 for wastewater and is based on Hach Method 8131 for sulfide. This test can detect sulfide concentrations from 0 to 800 µg/L.

Supplies needed

1. Hach spectrophotometer DR/4000
2. Two matching 25 mL cuvettes
3. Type I water for sample blank
4. Sulfide 1 Reagent, sulfuric acid (Hach Cat. No. 1816-32)
5. Sulfide 2 Reagent, potassium dichromate (Hach Cat. No. 1817-32)
6. Small graduated cylinder
7. 50-mL Erlenmeyer flask (turbid samples only)
8. Bromine Water (turbid samples only; Hach Cat. No. 2211-20)
9. Phenol Solution (turbid samples only; Hach Cat. No. 2112-20).

Procedure

1. Switch on the spectrophotometer and allow it to self-calibrate.
2. Select Hach program #3500.
3. Pour 25 mL of sample into a cuvette (for turbid samples, please see next section). Pour 25 mL of Type I water into the matching cuvette.
4. Add 1 mL of Sulfide 1 Reagent to each cuvette and immediately swirl.
5. Add 1 mL of Sulfide 2 Reagent to each cuvette and immediately swirl. A pink color will develop, and the solution will turn blue if sulfide is present.
6. Press the START TIMER button on the spectrophotometer, which begins a 5-minute countdown. Allow the solutions to react during this period.
7. When the timer beeps, place the DI blank in the cell holder and close the lid. Press ZERO.
8. The display should read 0 µg/L S^2.
9. Place the sample cuvette in the cell holder and close the lid and press READ. The results will be displayed.

Turbid samples

1. If testing a turbid sample, prepare a sulfide-free sample blank to use in place of the Type I blank.
2. Measure 25 mL of sample into a 50 mL Erlenmeyer flask.
3. Add bromine water dropwise with constant swirling until a permanent yellow color just appears.
4. Add phenol solution dropwise until the yellow color just disappears. Use this solution in place of Type I in Step 3.0 above.

Interferences

1. Wipe cuvettes clean prior to reading
2. Turbidity can interfere with the spectrophotometer; follow the steps outlined in the *Turbid samples* section.

Quality control

1. Verify expiration date of all reagents prior to use
2. Verify and clean spectrophotometer quarterly at minimum
3. Analyze samples immediately.

Safety and disposal

1. Ensure appropriate personal protection equipment is used.
2. Sulfide 2 Reagent contains potassium dichromate, and should be handled appropriately. The final solution will contain hexavalent chromium at a concentration regulated as a hazardous waste. Collect final solutions into sulfide waste container and dispose of as hazardous material.

10.5.5 Thermometer calibration SOP

This SOP describes procedures for calibration of digital and mercury thermometers in compliance with the National Environmental Laboratory Accreditation Conference (NELAC) protocol for equipment quality of standards.

Calibration of liquid-in-glass thermometer

A certificate is provided to verify instrument calibration in accordance with NIST. Recalibration of the instrument is to take place annually, and any resulting correction factors will be recorded in the logbook and on the thermometer.

Range: -8 to 32°C in 0.1 graduations.

Accuracy: See reference NIST SP 250-23.

Calibration of laboratory thermometers

Laboratory thermometers are to be calibrated biannually against a thermometer that is traceable to NIST.

Water bath thermometers, digital

1. Three temperature standards are required for accurate calibration of digital thermometers used in water baths, at 12°C, 20°C, and 25°C
2. Place NIST-certified thermometer in protective casing
3. Fully immerse the bulb and liquid column into a water bath set at 12°C
4. Place digital thermometer in same bath as the NIST-certified thermometer
5. Allow thermometers to stabilize
6. Record the correction factor in the Thermometer Calibration logbook and on the thermometer
7. Record the NIST correction factors in the Thermometer Calibration Logbook and on the thermometer.

Refrigerator/freezer thermometers, mercury

One temperature standard, at 4°C, is required for calibration of refrigerators, and at 0°C for calibration of freezers.

1. Place a flask of water (ethanol if calibration is performed in a freezer) in refrigerator 24 hours prior to calibration
2. Place NIST-certified thermometer in the flask
3. Place the mercury thermometer in same refrigerator
4. Allow temperature adjustment with refrigerator doors closed
5. Open refrigerator and record NIST correction factor in Thermometer Calibration Logbook and on the thermometer.

10.5.6 Total ammonia analysis SOP

Theory of operation

The following SOP outlines the procedures for testing ammonia concentrations in wastewater. This spectrophotometric method is based on Hach Method 10205 for ammonia.

Supplies needed

1. Hach spectrophotometer DR/3800.
2. TNT 831 ammonia vials (blue caps), warmed to 20–23°C. TNT 830 (green caps) or TNT 832 (red caps) ammonia vials may be needed for follow-up should the ammonia concentration not fall within the 1–12 mg/L range of the TNT 381 vials.
3. 1 mL pipettor, set to 0.5 mL, and tips.
4. DI water for rinsing vials before discarding.

Procedure

1. Allow samples and reagent vials to warm to 20–23°C. Samples should be unpreserved and measured as soon as possible after receipt. However, if samples arrive above pH 8, they should be adjusted down with small amount of acid to around pH 7. If several samples arrive within a short period of time, it may be convenient to measure them together in one batch.
2. Turn on the spectrophotometer and allow it to self-calibrate.
3. Press the "Barcode Programs" button.
4. Obtain TNT 831 vials. Stand the vials upright in the plastic tray. Peel the foil off the cap of each vial and remove the cap.
5. Add 0.5 mL of sample to a vial. Repeat for each sample, being sure to keep vials and samples in the same order. Change pipettor tips between each sample to avoid contamination.
6. Replace the caps and firmly shake each vial three times. The solution should turn yellow.
7. Press the clock on the spectrophotometer screen and set the timer for 15 minutes (press the 1, 5, 0, 0 buttons). Also be sure to set a portable stopwatch to 14 minutes and keep it with you at all times in case you leave the room. It is essential to return to the

spectrophotometer to take measurements after the reaction is complete. Samples with ammonia will turn green.

8. When the timer beeps, wipe the vial clean with a Kimwipe and make sure there is no debris on the outside of the vial. Gently invert the vial two or three times to mix. Slide the door on top of the spectrophotometer open and gently place the vial in the hole. Release the vial to allow it to turn. Be sure that the arrow-shaped light shield is in place (it should never be removed).

9. The spectrophotometer will automatically read the vial and give a number within a few seconds. If the number is less than 1 mg/L, it will be shown in red as out-of-range, and should be recorded as "< 1" in the log book. If the value is between 1 and 12, the number will be displayed in black and should be recoded as-is. If the value is greater than 12, several red asterisks will be displayed with an out-of-range message.

10. Repeat the above procedures with the remaining samples. The color of the reaction will stay stable only for another 15 minutes after the timer goes off, so it is important to work efficiently to get everything measured quickly.

11. If the sample's value is greater than 12 mg/L, then the process must be repeated using the TNT 832 vial kit (with red caps). The process is identical, except 0.2 mL of sample rather than 0.5 mL is added to the vial. There is no need to change the program, because a TNT 832 vial can be measured with a batch of TNT 831 vials.

12. Typically, samples with a reading of < 1 mg/L do not need any follow-up. For special projects that require the reporting of a precise value less than 1 mg/L, TNT 830 vials (green caps) are used. The process is identical as above, except 5 mL of sample are added to each vial.

13. To dispose of the vials, empty them into the "ammonia test waste" bottle by the spectrophotometer; this waste can be disposed of down the drain once the bottle is full. The vials are made to not spill easily, so it may help to tap them on the inside of the bottle to get them to drain. Rinse the bottle three times with DI water into the waste bottle. Vials can then be disposed of in the sharps container. Caps should be rinsed with DI into the waste bottle, and disposed of into the trash can.

14. Turn off the spectrophotometer when finished, clean it with a wet paper towel (if dirty), cover it, and return any unused vials to the refrigerator for storage. Ensure that the work area around the spectrophotometer is completely cleaned.

10.6 Protocol for Preparation of Spiked Sediment

This protocol was used by GCRL to provide the spiked sediment samples that were used in PER testing.

10.6.1 General guidelines

1. Controls were prepared using the same technique used for spiking sediment, with the exception of adding oil.

2. Each sediment-oil concentration was made separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

3. For each step during the sample preparation and collection, all appropriate information was entered into the data entry sheets provided by Stratus Consulting; see Section B.4 in Appendix B of the QAPP.

4. Unused prepared sediments were placed into a zip-lock bag and stored in the dark at 4°C (short term) or in freezer at -20°C (long term).

10.6.2 Glassware preparation

Prepare all of the equipment in accordance to the *Decontamination SOP* in the QAPP.

10.6.3 Preparation of sediments

1. Allow the sediment to thaw

2. Remove all debris (grass, shells, etc.) from the thawed sediment and place in a mixer bowl

3. Using a Cuisinart SM-70 7-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

10.6.4 Mixing oil into sediment

1. Weigh out the appropriate amount of oil as outlined below:

   Slick oil should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and two or three Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Using the spatula, transfer the oil onto the sediment in the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

2. Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.

3. Mix the oil into the sediment at medium speed (5) with a Cuisinart SM-70 7-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with the putty knife.

4. Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the sediment from the mixing bowl into bags for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for shipment.

References

ASTM. 1997. Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae. ASTM Standard E1218-97a. ASTM International, West Conshohocken, PA.

ASTM. 1999. Standard Guide for Conducting 10-day Static Toxicity Tests with Marine and Estuarine Amphipods. ASTM Standard E1367-99. ASTM International, West Conshohocken, PA.

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods*. EPA 600/R-94/025. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

U.S. EPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. EPA/821/R/02. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

A. Testing Protocol 1: 10-Day Sediment Exposure *Leptocheirus plumulosus*

A.1 Biological Testing Procedures

The methods used in conducting these testing followed established guidelines:

- ASTM E1367-99: Standard Guide for conducting 10-day static toxicity tests with marine and estuarine amphipods (ASTM, 1999)
- Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

A.2 Solid-Phase Sediment Toxicity Testing

A.2.1 Field-collected sediment preparation

The frozen sediment samples were removed from frozen storage and chunks of sediment were broken off to provide sufficient volume for testing. Once a sufficient volume of sediment had been attained, the remaining bulk sediment was returned to freezer storage; the collected chunks were placed into polyethylene bags and held at 4°C storage and allowed to slowly thaw. The day prior to test initiation, the thawed sediments were removed from cold storage and homogenized in a stainless steel bowl using a stainless steel spatula; at the request of Stratus Consulting, the sediments were not sieved prior to testing.

A.2.2 Spiked sediment preparation

The day prior to test initiation, the sediments were removed from cold storage and homogenized in a stainless steel bowl using a stainless steel spatula; as per instructions from Stratus Consulting, the sediments were not sieved prior to testing.

A.2.3 Test initiation

For each sample, the sediment was transferred into each of nine test replicate 1-L glass beakers to a depth of ~ 2 cm, after which ~ 800 mL of 20 ppt salinity seawater was carefully poured into reach replicate so as to minimize the disturbance and resuspension of the sediment. A negative "Lab Control" control sediment consisting of the same sediment used to culture the test

organisms (provided by the test organism supplier) was similarly processed and tested. The test replicates were then placed into a temperature-controlled room at 25 ± 1°C under continuous illumination.

The tests were initiated the following day. At this time, one of the test replicates was sacrificed for the determination of the initial sediment pore water characteristics and a second replicate was similarly sacrificed for bulk sediment chemical analyses. Immediately prior to test initiation, routine water quality characteristics (pH, temperature, DO, salinity, and total ammonia) were measured in the overlying water in each replicate. The tests were then initiated with the random allocation of 20 amphipods into each test replicate.

Each day of the test, routine water quality characteristics (pH, temperature, DO, and salinity) were measured in the overlying water in one randomly selected replicate for each sediment treatment.

A.2.4 Test termination

The tests were terminated after 10 days of exposure. At this time, one of the remaining test replicates was sacrificed for the determination of the final sediment pore water characteristics and an additional replicate was again sacrificed for bulk sediment chemical analyses. For each of the remaining five replicates, the sediments were rinsed out into a 0.5 mm sieve and wet-sieved, and the number of surviving amphipods in each was determined. The resulting survival data for these sediments were evaluated by PER using the CETIS statistical software (TidePool Scientific, McKinleyville, California). The data were also sent to Stratus Consulting and included in their database.

B. Testing Protocol 2: Characterization of the Acute Toxicity of Oil (WAFs) to the Mysid Shrimp, *Americamysis bahia*

B.1 Biological Testing Procedures

The methods used in conducting these tests followed established guidelines:

- Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition (EPA/821/R/02/012; U.S. EPA, 2002).

B.2 Acute Toxicity Testing

B.2.1 WAF Preparation

The appropriate WAF was prepared by the addition of Slick A or Slick B oil to 25 ppt seawater at a nominal concentration of 1 g/L, after which the oil and 25 ppt seawater were mixed and processed according to the QAPP. The WAF was prepared twice: once to prepare the test solutions used to initiate the test (Day 0); and then again, on Day 2 of the test, to prepare fresh media for the test solution renewals. A WAF "Blank" consisting of Laboratory Water Control medium (25 ppt seawater) was similarly mixed, processed, prepared, and tested.

B.2.2 Test Initiation

The Laboratory Water Control medium and the prepared WAFs were used to create diluted test solutions at desired concentrations (see test-specific TCTs).There were four replicates at each test treatment, each replicate consisting of 200 mL of test solution in a 400-mL glass beaker. The test was initiated by randomly allocating 10 four- or five-day-old *A. bahia* into each replicate beaker. The beakers were randomly positioned in a temperature-controlled room at 20°C (temperature was monitored daily) under a 16 L:8 D photoperiod. The mysids were fed freshly hatched brine shrimp nauplii twice daily.

After approximately 48 hours, fresh WAF solutions were prepared, archived, and characterized as before. The test replicate beakers were removed from the temperature-controlled room and each replicate was examined; any dead animals, uneaten food, wastes, and other detritus was removed. The number of live mysids in each replicate was recorded. Then, approximately 80%

of the test media in each beaker was carefully poured out and replaced with fresh test solution, after which the test beakers were returned to the temperature-controlled room.

Using the test water that had just been removed during the water renewal, water quality characteristics (pH, DO, and salinity) were measured for each treatment on one randomly selected replicate.

B.2.3 Test Termination

After 96 (± 2) hrs of exposure, the test was terminated and the number of live mysids in each replicate beaker was recorded. Immobile mysids that did not respond to gentle prodding were considered dead. Live and dead organisms were collected according to the QAPP.

C. Testing Protocol 3: Characterization of the Chronic Toxicity of Oil (WAFs) to the Diatom *Skeletonema costatum*

C.1 Biological Testing Procedures

The methods used in conducting these tests followed established guidelines:

- Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae (ASTM E1218-97a; ASTM, 1997).

C.2 Acute Toxicity Testing

C.2.1 WAF Preparation

The appropriate WAF was prepared by the addition of Slick A or Slick B oil to 25 ppt seawater at a nominal concentration of 1 g/L, after which the oil and 25 ppt seawater were mixed and processed according to the QAPP. A WAF "Blank" consisting of a Laboratory Water Control medium (25 ppt seawater) that was similarly mixed and processed, was also prepared and tested.

C.2.2 Test Initiation

The Laboratory Water Control medium and the prepared WAFs were used to create diluted test solutions at desired concentrations (see test-specific TCTs). In some cases, additional auxiliary controls were added to the tests (see test-specific TCTs).There were four replicates at each test treatment, each replicate consisting of a 250-mL glass Erlenmeyer flask containing 100 mL of test solution; an additional replicate was established at each test treatment for the measurement of test solution water quality characteristics during the test and at test termination. Each flask was inoculated to an initial diatom cell density of 20,000 cells/mL from a laboratory culture of *S. costatum* that was maintained in a log-growth phase. These flasks were loosely capped and randomly positioned within a temperature-controlled room at 20°C, under continuous illumination from cool-white fluorescent bulbs.

Each day, the temperature and pH were determined from the designated "water quality" replicate for each treatment; each replicate flask was gently shaken and re-positioned within the

temperature-controlled room. All flasks were shaken by hand and re-randomized in the temperature-controlled room daily.

C.2.3 Test Termination

After 96 (± 2) hrs exposure, the flasks were removed from the temperature-controlled room and the diatom cell density in each was determined by microscopic analysis. At the end of testing, replicates were pooled by treatment and poured into graduated cylinders. The graduated cylinders were stored in a cold room for 2–3 days, after which the overlying water was decanted and all of the tissue that had settled to the bottom of the graduated cylinder was placed into a freezer-proof container and archived according to the QAPP.

Appendix References

ASTM. 1997. Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae. ASTM Standard E1218-97a. ASTM International, West Conshohocken, PA.

ASTM. 1999. Standard Guide for Conducting 10-day Static Toxicity Tests with Marine and Estuarine Amphipods. ASTM Standard E1367-99. ASTM International, West Conshohocken, PA.

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods*. EPA 600/R-94/025. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

U.S. EPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. EPA/821/R/02. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

11. Louisiana State University General Laboratory Procedures and Practices

11.1 Introduction

Louisiana State University (LSU) conducted toxicity tests to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil spill on gulf killifish. Dr. Fernando Galvez served as the Principal Investigator, and all tests were conducted in his laboratory at LSU. This chapter describes General Laboratory Procedures and Practices (GLPP) used at LSU.

11.2 Reporting and Testing Documentation

Experimental data and relevant activities were reported as described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Hard copies of these data were prepared according to the QAPP, and kept in the Galvez laboratory in a locked filing cabinet after preparation. A duplicate hard copy and an electronic version of the data were prepared according to the QAPP, and sent to Stratus Consulting on a timely basis for quality assurance.

11.3 Equipment Decontamination

All testing equipment used was decontaminated before use following the methods outlined in the *Decontamination SOP* (Standard Operating Procedure) of Appendix A described in the QAPP. After completing the *Decontamination SOP*, equipment was rinsed twice with reverse osmosis (RO) water. Care was taken to avoid any cross-contamination of testing equipment, test exposure chambers, laboratory glassware, and analytical samples. To the extent possible, new, certified, clean materials were used to conduct testing and sampling activities. All equipment and testing materials that could not withstand the decontamination procedure were not reused.

11.4 Exposure Media Preparations

The artificially formulated seawater (AFS) used for water accommodated fraction (WAF) exposures was prepared using RO water mixed with Instant Ocean sea salts to achieve the desired test salinity [see test-specific test conditions tables (TCTs) for the desired test salinity]. RO water was derived from dechlorinated tap water prior to RO in a Culligan Aqua-Cleer water treatment system. Exposure media were prepared as follows:

- WAFs were prepared as outlined in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP. WAFs were prepared using one of two preparation methods, high energy and chemically enhanced (HEWAF and CEWAF, respectively) and one of two oil types, Slick A (CTC02404-02) and Slick B (GU2888-A0719-OE701).

- Sediment exposures were conducted with clean field-collected sediment spiked with different amounts of the desired oil. Additional details regarding the sediments, the spiking protocol, and oil types used are provided in the testing protocols and test-specific TCTs.

- Oil slick exposures used one of two types of oil: Slick A or Slick B. Additional details regarding the slick formation protocol and oil types used are provided in the Testing Protocols and test-specific TCTs.

  Note: new jars of Slick B oil were pre-mixed over low heat to homogenize contents of the jar before oil was used in oil slick exposures. To mix, the entire content of the oil jar was placed into a large glass bowl, which was then set on a hotplate (FisherBrand Isotemp Model 1160049SH) on the lowest heat setting. The oil was mixed in the bowl on the hotplate until it had the consistency of taffy (this usually took around 5 to 10 min). Oil was then used to prepare polyvinyl chloride (PVC) rings or put back into its original jar for storage at 4°C. This step was only performed once per jar.

11.5 Test Organism Husbandry – Gulf Killifish (*Fundulus grandis*)

All experiments were conducted with fish held at LSU. Fish were held in AFS, prepared as described above, by dissolving Instant Ocean sea salts into water purified by RO.

1. During testing, water quality was monitored as described in the QAPP using the water quality SOPs described in Section 11.9. Dissolved oxygen (DO), pH, conductivity, salinity, total ammonia nitrogen (TAN), and temperature were recorded once every other day from one random container in each treatment. For the initial few tests, conductivity measurements were collected using a YSI 85 meter; for later tests, conductivity was replaced by salinity measurements taken using a refractometer. Information regarding when water quality measurements were taken can be found in the test-specific TCTs. DO, pH, salinity, TAN, and temperature were recorded for all of the brood stock systems at least once weekly.

2. AFS for the adult brood stock was kept at a salinity of 12 ppt. Holding tanks received continuous aeration to maintain DO levels above 6 mg/L. Holding tanks received continuous mechanical, biological, and ultraviolet (UV) filtration through recirculation.

Water exchanges of at least 20% were performed, mechanical filter media were cleaned, and the biological bead filtration system was back-flushed with water exchanges on a weekly basis. Tanks were siphoned and scrubbed weekly.

3. Adult gulf killifish were maintained in high-capacity recirculating holding systems in the Life Science Building (LSB) aquatic facility at LSU.

   a. Larval gulf killifish were held in 2.8-L tanks within a Marine Biotech multi-tank zebrafish-style holding system (Figure 11.1).

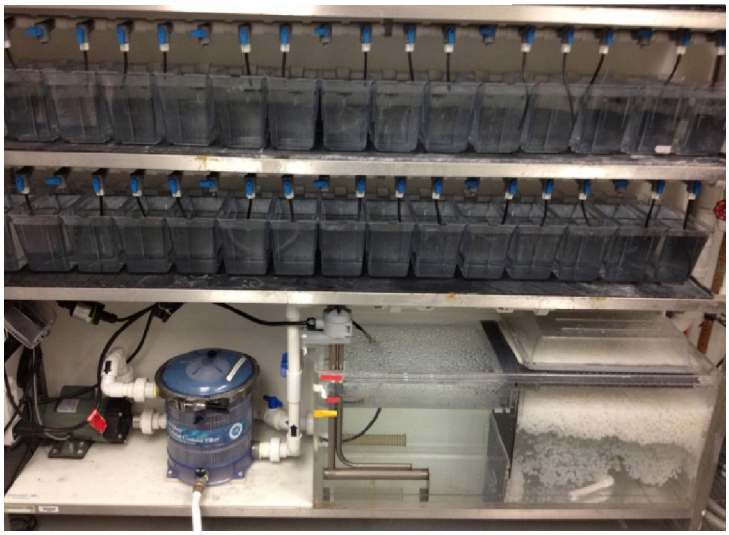

Figure 11.1. Marine Biotech zebrafish-style holding system.

   b. Embryos (pre-hatch) that were not used in larval acute toxicity tests were maintained in 950-mL glass Pyrex dishes under 50% static renewal at 48-hr intervals.

   c. Sexually mature fish used as a brood stock were held in a 1,200-L recirculating system.

4. All fish were maintained at 20°C on a 14-hr light:10-hr dark photoperiod.

5. Feeding:

   a. Adult fish were fed a formulated diet manufactured by AquaFeed (4010) of pellets containing 40% protein and 10% lipid until satiation, once daily.

b. Larval gulf killifish were fed an Otohime powdered diet daily. The diet was gradually switched to the AquaFeed pelleted food once juvenile fish reached a suitable size to ingest fish pellets.

6. Fish health:

All tanks were checked for fish mortality or signs of illness and stress daily. Signs of disease and abnormal rates of mortality were treated on a case-by-case basis in consultation with the State Fish Pathologist, Dr. John Hawke, from the Louisiana Aquatic Diagnostic Laboratory.

11.6 Test Organism Sources

11.6.1 Fish collection

1. Adult gulf killifish were collected in Cocodrie, Louisiana, adjacent to the Louisiana Universities Marine Consortium facility, for use as a brood stock (LUMCON; Figure 11.2).

2. Fish were collected using wire minnow traps baited with approximately 1/2 cup of Ol' Roy Puppy Feed. Traps were placed at the marsh edge and allowed to sit undisturbed for at least 45 minutes prior to checking for activity. As fish were collected, they were held in 20-L individually aerated containers at a density of no more than 2 fish per liter for transport back to LSU. Fish were collected for use as a brood stock for embryo production, as described in Section 11.6.2 (*Production of embryos for toxicity experiments*). *In vitro* fertilization occurred onsite at LUMCON or at the LSB following transport of the brood to LSU. If *in vitro* fertilization was performed at LUMCON, embryos were transferred to air-incubation containers (Figure 11.3) immediately upon fertilization, as described in Section 11.6.4.

3. Upon arrival at LSU, adult fish were treated with a buffered active copper treatment of Cupramine for 7–14 days and Praziquantel for 7 days, before being introduced into the brood stock holding tank.

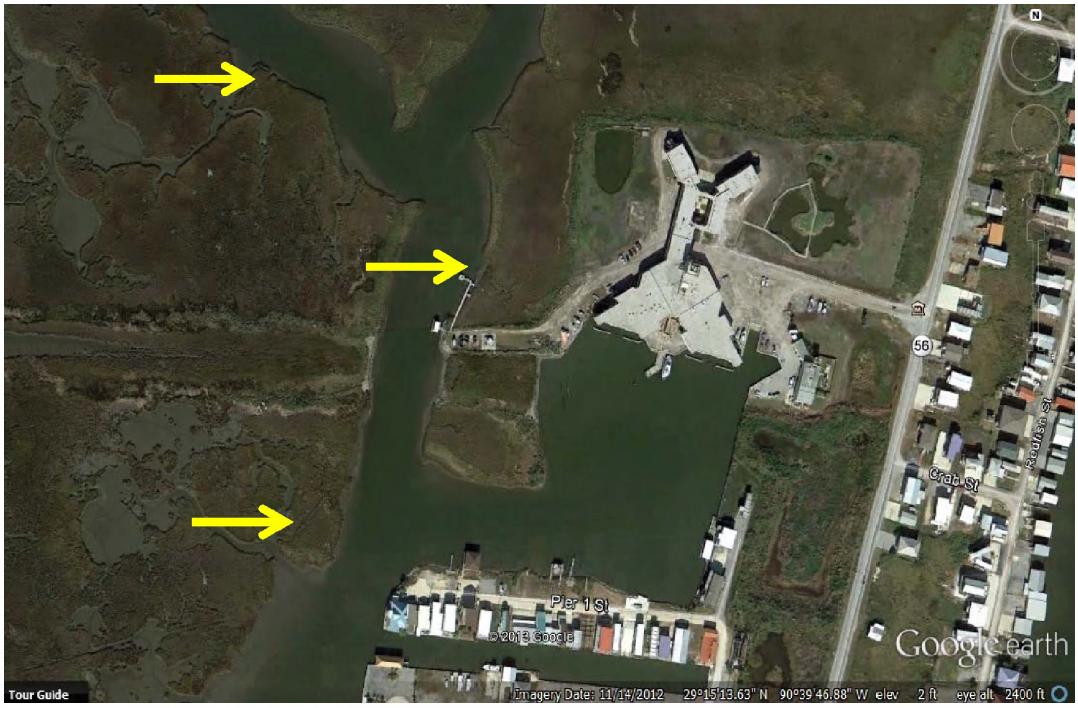

Figure 11.2. Field location of brood stock source population at the Louisiana Universities Marine Consortium facility in Cocodrie, Louisiana. Arrows indicate fish collection locations.

Source: 29°15'13.36" N, 90°39'46.88" W, Google Earth, November 14, 2012.

Figure 11.3. Example of plastic container with polyurethane foam used to air-incubate embryos.

Source: Photograph by Galvez Laboratory – LSU.

11.6.2 Production of embryos for toxicity experiments

1. Embryos were incubated in moist conditions (air incubated), but were not submersed in water until ready for hatching. This allowed for synchronization of hatch time and ensured uniform levels of developmental maturation of larvae upon hatch.

2. Fish selected for spawning were in good health and showed no signs of infection. Male fish that displayed reproductive readiness, as indicated by the darkening of the operculum and yellow coloring; and gravid females were selected to generate embryos.

3. Male and female fish were collected, and held separately in 20-L temporary holding tanks with aeration at a density of no more than 1 fish per liter. Care was taken to collect an adequate number of female fish to generate enough embryos for testing (large females can produce up to approximately 50 eggs per breeding cycle, but may not yield this many, or any at all).

4. To obtain eggs from a potentially gravid female, the female was held over a 950-mL Pyrex dish. A gentle digital pressure was applied at the anterior aspect of the abdominal cavity, behind the opercula, from both sides, sliding fingers posteriorly toward the cloaca. The eggs were deposited from the ovipositor, posterior to the cloaca. The eggs were collected in the 950-mL Pyrex dish. If eggs did not appear, the procedure was repeated twice. If eggs did not appear after the third digital pressure application, the fish was released into a 20-L temporary holding tank until egg collection was complete.

5. To obtain spermatozoa, males were euthanized by cervical severance and then testes were removed. Testes from three males were combined and held in glass Petri dishes covered in 20 mL of Hank's Balanced Salt Solution (350 mOsmol/L, 130.0 mM NaCl, 5.36 mM KCl, 0.08 mM $CaCl_2 \cdot 2H_2O$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.16 mM $NaHCO_3$, 5.55 mM $C_6H_{12}O_6$). To activate and release spermatozoa, testes were immediately forced through a cell sieve. Spermatozoa were rinsed in a 950-mL Pyrex dish with 12 ppt salinity water, and the sieve was also rinsed with 12 ppt water. This created a solution of sperm that was directly added to the eggs. Successful fertilization of the eggs was assessed by monitoring for an elevation of the fertilization envelope.

6. An appropriate number of females (depending on the number of embryos required for the experiment) and 3–5 males were used for each fertilization event.

7. If the yolk comprised greater than 50% of the volume of the egg, the embryo was considered valid for use in experimentation. Embryos with less than 50% yolk were terminated and not used in testing.

11.6.3 Assessing embryo viability for use in test

1. Embryos were obtained using the *in vitro* fertilization technique outlined in Section 11.6.2.

2. Embryonic exposures began either within 24 hr post-fertilization or following neurulation (2–3 days post-fertilization). Until the time of the exposure, embryos were air incubated as described in Section 11.6.4, and monitored once daily to ensure proper development and viability.

3. If the embryos were used for a test within 24 hr post-fertilization, embryo viability was determined by the elevation of the fertilization envelope. This indicated that the eggs had been fertilized.

4. If the embryos were used post-neurulation, embryo viability was determined by the formation of the optic vesicles (Figure 11.4). This signaled that the embryo had completed neurulation. After completion of neurulation, these embryos were either used immediately in exposures to oiled sediment or WAF, or were reared until hatch for use in larval toxicity tests.

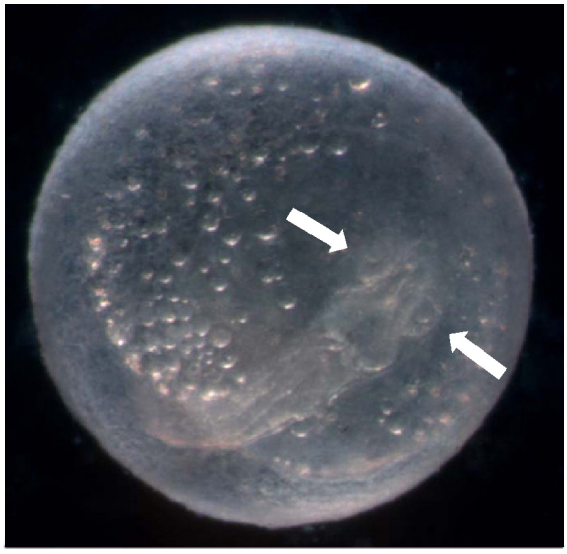

Figure 11.4. Gulf killifish embryo at approximately 2 days post-fertilization. Arrows point to optic vesicles.

Source: Photograph by Galvez Laboratory – LSU.

11.6.4 Air incubation of embryos

1. Embryos were placed in filter baskets and set on top of a disk of polyurethane foam that was saturated with freshly made 12 ppt AFS. The embryos in the basket were then covered with another layer of AFS saturated polyurethane foam prior to replacing the container lid (Figure 11.3; modified from Coulon et al., 2012). The containers were maintained at 21–24°C.

2. Embryo mortality was monitored daily under a stereomicroscope. Prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or take on an otherwise cloudy-white appearance. They were considered viable if they were clear and continued to develop.

3. After the onset of circulation, heart function and/or evidence of circulation determined the viability of embryos.

4. Once the embryos were mature, they were hatched by immersion in AFS.

5. To hatch, mature embryos were randomly distributed from air-incubation chambers to glass Petri dishes filled with 50 mL AFS. Each Petri dish contained the number of larvae required per exposure chamber as specified in the test-specific TCTs.

6. The time and date of immersion were recorded. Embryos were given 24 hr to hatch and acclimate prior to being added to the exposure chambers.

11.7 Analytical Chemistry

Water, tissue, and sediment samples were collected as described in the QAPP. Sampling frequency and any additional specific sampling were carried out as described in the testing protocols or test-specific TCTs.

11.8 Water Quality Monitoring

Water quality was monitored as described in the test-specific TCTs using the SOPs in Section 11.9.

11.9 Water Quality SOPs

11.9.1 Total ammonia

Procedure

All samples that were tested for total ammonia were stored at -20°C until assayed. All water samples from a given toxicity test were tested for ammonia at the same time using the same standards. If multiple plates were needed, the same diluted standards were used; however, each plate was treated as a distinct assay that contained a standard curve to account for any plate-to-plate variation.

1. Measure total ammonia using the colorimetric assay described by Verdouw et al. (1978).

2. Run the assay on a flat-bottomed polystyrene 96 well plate (Model # 9017; Corning) with each sample/standard run in triplicate.

3. Prepare the standard curve from an acidified 50 mM stock solution of ammonium sulfate. Make an appropriate standard curve dilution series using AFS.

4. Add 160 µL of each standard/unknown to the respective wells.

5. Using a repeat pipette, add 20 µL of sodium salicylate solution (40 g of sodium salicylate/80 mL Milli-Q) to each well.

6. Using a repeat pipette (different tip), add 20 µL of catalyst citrate solution (0.02 g sodium nitroprusside, 35 g sodium citrate, volume to 100 mL using Milli-Q water) to each well.

7. Using a repeat pipette (different tip), add 20 µL of alkaline hypochlorite solution (4 g NaOH, 14 mL sodium hypochlorite, volume to 100 mL using Milli-Q water) to each well.

8. Allow the samples to develop in a dark location at room temperature for at least 1 hr, but no more than 24 hr.

9. Read the samples at 570 nm using the microplate spectrophotometer. The standard curve should have an R^2 value of greater than or equal to 0.95. Because the assay is run in triplicate, it is acceptable to remove obvious outlying assay replicates, but make careful notes regarding any removed outliers. Keep all raw data files, and also record all mean replicate ammonia values on data sheets, as well as the standard curve R^2 value.

11.9.2 Temperature and DO (YSI ProODO)

Procedure

1. Verify instrument calibration daily. Ensure that the sponge in the storage sleeve is moistened with RO water and store the probe in the sleeve. Check to see that the DO% is reading a calibration value of 101% (associated with a barometric pressure of 30.22 in Hg).

2. Once the instrument is calibrated, remove the storage sleeve and all protective coverings on the probe. Place the probe into each treatment container to be tested. Be sure that the round metallic temperature probe is submerged below the liquid level (continuous movement is not required).

3. Allow temperature and DO readings to stabilize and record readings.

4. Repeat as necessary for each treatment container.

5. After use, decontaminate the probe with mild soap and water and then replace the storage sleeve and all protective coverings.

11.9.3 Conductivity (YSI 85)

Procedure

1. Using the manufacturer's standards, test the range and calibration of the probe. Recalibrate as per manufacturer's instructions if needed.

2. Place the probe in the test stock or control solution and continue to swirl until the reading stabilizes.

3. Record the reading.

4. Clean the probe using detergent and a soft sponge with deionized (DI) water, and store as per manufacturer's instructions.

5. Repeat steps 1 through 4 for each container.

11.9.4 Salinity (YSI 85 or refractometer)

YSI 85 procedure

1. Using the manufacturer's standards, test the range and calibration of the probe. Recalibrate as per manufacturer's instructions if needed.

2. Place the probe in the test stock or control solution and continue to swirl until the reading stabilizes.

3. Record the reading.

4. Clean the probe using detergent and a soft sponge with RO water, and store as per manufacturer's instructions.

5. Repeat steps 1 through 4 for each container.

Refractometer procedure

1. Check the refractometer for calibration at the start of each day using RO water as zero.

2. Place 1–3 drops of water on the viewing window of the refractometer and place the plastic hinged cover over the water.

3. Look through the eyepiece and read the number at the blue line.

4. Repeat as necessary for each treatment to be measured.

5. Decontaminate the refractometer after use with mild soap and water.

11.9.5 pH

Procedure

1. Obtain all pH readings on a Denver Instrument UltraBasic UB-10 pH/mV meter according to the manufacturer's instruction manual. Rinse the probe thoroughly with RO water between each buffer or sample solution and blot excess water lightly from the end of the probe with a Kimwipe®.

2. Before each use, calibrate the pH meter with all three reference buffer solutions.

    a. Prepare reference buffer solutions within 1 month; otherwise, prepare a new 50-mL conical tube with the reference pH and the date. Pour 20–40 mL of the corresponding reference buffer into the tube.

    b. To calibrate the pH meter:

        i. Insert the probe into the first reference buffer.

        ii. Press "Standardize" and allow the probe to equilibrate. When the meter has reached equilibrium, the meter reading will stop blinking. Press "Enter" to accept the buffer value. Repeat this step if the meter does not accept the value or if the buffer displays a slope error message.

        iii. Repeat steps a and b for all three reference buffers (pH 4, 7, and 10).

3. During a water change, remove between 30 and 40 mL of water and add to a 50-mL conical tube for testing.

4. Insert the probe into the sample water and allow the probe to reach equilibrium. Record this value.

Repeat steps as necessary for each sample to be tested.

References

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Verdouw, H., C.J.A. Van Echteld, and E.M.J. Dekkers. 1978. Ammonia determination based on indophenol formation with sodium salicylate. *Water Research* 12(6):399–402.

A. Testing Protocol 1: Gulf Killifish (*Fundulus grandis*) Larval Acute Toxicity Test – Static Exposure

A.1 Before Test Initiation

1. WAFs were prepared with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A subsample of the exposure solution for each treatment was collected for chemical analysis by ALS Environmental as specified in the QAPP. Sample collection, labeling, and handling were conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP.

3. Remaining treatment WAF was disposed of appropriately.

4. Appropriate forms (*WAF Preparation Table, Test Conditions Table, Water Quality Monitoring Table, Tank Identification Table*, etc.) were filled out as described in the QAPP.

5. Glass Pyrex containers (950 mL) were prepared and labeled for the test. Treatments consisted of a control (0% WAF) and a series of WAF dilution treatments. Before larvae were added to exposure chambers, initial water quality measurements were taken for each treatment.

A.2 Larval Exposures

1. Embryos were hatched according to the protocol described in the LSU GLPP.

2. At the end of the 24-hr acclimation period following embryo hatch, excess water and moisture were removed from the glass Petri dishes containing larvae. The glass Petri dishes were then held above replicate treatment containers holding 300 mL of treatment WAF. Using one transfer pipette per replicate treatment container, the glass Petri dish was gently rinsed with treatment WAF to transfer 20 larvae into each treatment container. The time and date were recorded upon transfer of larval groupings to replicate treatment containers. The lid was gently placed on top of each treatment container and then placed

on an orbital shaker set to 20 RPM. Replicates were maintained in an environmental control chamber at 22°C with a 12:12 light/dark photoperiod.

3. Larvae were checked once daily for mortalities and survivors, and observations were recorded. Mortality, missing individuals, and non-test mortalities were recorded on the appropriate bench sheets as described in the QAPP. Dead animals were removed and archived (unless they had decomposed), and all dead animals were retained to the extent possible and archived according to the QAPP. At this time, water was collected to evaluate water quality.

4. At the end of 96 hr, the final number of mortalities and survivors were counted for each treatment and recorded as described in step 3. For each treatment, dead animals were collected and archived according to the sample retention guidelines described in the QAPP.

5. Survivors were transferred to a Marine Biotech multi-tank zebrafish-style holding system maintained at 12 ppt salinity. Tanks were labeled with test number, treatment, and the date when larvae were first added to the tank. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If these fish were not used within 3 months, or if requested by Stratus Consulting, they were sacrificed using approved methods of euthanasia at LSU.

B. Testing Protocol 2: Gulf Killifish (*Fundulus grandis*) Embryo Acute Toxicity Test to Oiled Sediments – Partial Replacements

B.1 Preparation of Exposure Chamber

1. Reference sediments were oiled according to the *Protocols for Preparation of Oil-spiked Sediments* provided by Stratus Consulting and attached below (Section B.2).

2. Sediments were loaded with oil at different concentrations (see test-specific TCTs). Another treatment with no oil added was used as a reference exposure.

3. For each replicate, 280 g (~ 150 mL) of sediment was added to a 950 mL Pyrex container.

4. Nine glass marbles (diameter of approximately 1.5 cm) were placed inside each Pyrex container. The marbles were placed toward the glass wall where they aligned with the rim of the filter basket.

5. The polytetrafluoroethylene (PTFE) basket was placed in the treatment container so that it rested on the glass marbles.

6. Slowly 200 mL of 12 ppt AFS was added into each exposure chamber, taking care to not disturb the sediment. Suspended particles were allowed to settle prior to the addition of the organisms to the filter basket.

B.2 Protocols for Preparation of Oil-spiked Sediments

B.2.1 General guidelines

1. Controls were prepared using the same technique used for spiking sediment, with the exception of adding oil.

2. Each sediment-oil concentration was made separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

3. For each step during the sample preparation and collection, all information was entered into the appropriate bench sheets provided by Stratus Consulting.

4. Unused sediments were placed in a ziplock bag and stored in the dark at 4°C (short-term) or in a freezer at -20°C (long-term).

B.2.2 Preparation of sediments

1. A hammer and a chisel were used to break frozen sediment into pieces. The appropriate amount for each treatment was then weighed and placed into a clean glass container and covered (one container/treatment).

2. Sediments were allowed to thaw overnight at room temperature.

3. Once thawed, debris (grass, shells, etc.) was removed and sediment was placed in a mixer bowl.

4. Sediments were homogenized by mixing for 2 min at a low speed (1) using a Cuisinart SM-70BC 7-quart stand mixer.

B.2.3 Mixing oil into sediments

1. Oil was weighed as outlined below.

   A weigh boat and 2–3 Kimwipes were tared on a top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was added to the weigh boat. Then, using a clean spatula, the oil was transferred to the mixing bowl by placing it in several areas around the bowl. Any remaining oil was then wiped off the spatula with tared Kimwipes, and the weigh boat and Kimwipes were then reweighed to determine the actual mass of oil transferred.

2. After the oil was added, thawed sediments were added to the mixing bowl.

3. The sediments were then mixed at a medium speed (5) with the Cuisinart SM-70BC 7-quart stand mixer for 30 min. The mixer was stopped every 2–4 min to scrape the sides of the mixing bowl with the putty knife.

4. Once mixing was complete, the mixer paddle and bowl were scraped with a putty knife. The sediments were transferred from the mixing bowl to the test containers using a

stainless steel spoon. If the sediments were not used immediately, they were stored at 4°C until test initiation.

B.3 Embryo Exposures to Oiled Sediments

1. Ten to 20 embryos (number based on available numbers of viable embryos following *in vitro* fertilization; see test-specific TCTs) were transferred to the top of each PTFE mesh on each replicate exposure chamber.

2. Exposure chambers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Every other day, 65 mL of water was removed from the exposure chambers and replaced with new 12 ppt AFS.

5. Embryos were checked once daily for mortalities and hatched embryos. Mortalities, missing individuals, non-treatment mortalities, and hatches were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as dead if they were found to be opaque or had an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed; all dead animals were retained to the extent possible and archived according to the QAPP.

6. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate. These animals were selected randomly from the batch of 10–20 animals. To view embryos for heart rate measurements, embryos were carefully removed with a wide-bore transfer pipette and transferred to an empty glass Petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-sec interval.

7. The number of hatched and unhatched embryos was documented during mortality checks.

8. After counting hatched larvae, they were removed and archived at -20°C according to the QAPP.

9. Embryos were exposed to sediments for a predetermined duration (see test-specific TCTs). At the end of the exposure, all unhatched embryos were counted and then archived according to the QAPP.

Note: sampling baskets were constructed using virgin PTFE pipe stock (4B Plastics, Baton Rouge, Louisiana) and PTFE mesh with 250-µm openings (Macmaster-Carr). Two interlocking rings were machined (4B Plastics, Baton Rouge, Louisiana) and fitted together to clamp the PTFE mesh tightly across the rings to create a filter basket that rested on the sediment for the duration of the exposure, and elevated the embryos approximately 5 mm above the sediment-water interface.

B.4 Water and Sediment Sampling

1. One sediment sample (8-oz sediment jar) per treatment was collected and sent to ALS Environmental for analysis.

2. At each water renewal, a composite water sample (250-mL amber bottle) was collected from each treatment from the water removed during the renewal. The sample was sent to ALS Environmental.

C. Testing Protocol 3: Gulf Killifish (*Fundulus grandis*) Embryo Acute Toxicity Test to WAF – 96-hour Static Exposures

C.1 Preparation of Exposure Chamber

1. WAFs were prepared as described in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A total of 24 containers (950-mL Pyrex) were used for definitive tests containing 250 mL of 12 ppt salinity AFS (reference), or 250 mL of WAF in 12 ppt salinity AFS at differing concentrations (see test-specific TCT).

C.2 Embryo Exposures to WAF

1. Ten to 20 air-incubated embryos (see test-specific TCT) were transferred per replicate container.

2. Containers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Embryos were checked once daily at 24-hour intervals from the start of exposures for mortalities. Mortalities, missing individuals, and non-treatment mortalities were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or had taken on an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

5. Embryos were exposed to WAF for 96 hours under static conditions.

6. At 96-hours post-exposure to WAF, the embryonic heart rate was assessed in three embryos per replicate and the remaining animals were assessed for viability. To view embryos for heart-rate measurements, embryos were carefully removed with a wide-bore

transfer pipette and transferred to an empty glass Petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-sec interval.

7. Live embryos were transferred to 250 mL of clean 12 ppt AFS to be monitored for another 16 days. Embryos were first washed by placing them on a nylon mesh, and then passing approximately 100 mL of clean 12 ppt AFS over the top.

8. Embryos were monitored daily for mortality and hatch. The hatching of gulf killifish embryos typically occurred between days 10 and 14 post-fertilization at room temperature. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

9. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate as described in step 6. These animals were selected randomly from the batch of surviving animals.

10. At test termination, all unhatched embryos were counted and archived according to the QAPP and all hatched larvae were placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were sacrificed using approved methods of euthanasia at LSU.

D. Testing Protocol 4: Gulf Killifish (*Fundulus grandis*) Embryo Toxicity Test to WAF – 20-day Exposure with 48-hour Renewals

D.1 Preparation of Exposure Chamber

1. WAFs were prepared as described in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A total of 24 containers (950-mL Pyrex) were used for definitive tests containing 250 mL of 12 ppt AFS (reference) or 250 mL of WAF prepared with 12 ppt AFS at differing concentrations (details in test-specific TCTs).

D.2 Embryo Exposures to WAF

1. Ten to 20 air-incubated embryos were transferred per replicate container (see test-specific TCTs).

2. Containers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Embryos were exposed to WAF for a maximum of 20 days with 48-hour renewals.

5. Embryos were monitored daily for mortality and hatch. Hatching of gulf killifish embryos typically occurred between days 10 and 14 post-fertilization at room temperature. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or took on an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

6. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate. These animals were selected randomly from the batch of surviving animals. To view embryos for heart rate measurements, embryos were carefully

removed with a wide-bore transfer pipette and transferred to an empty glass petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-second interval.

7. At test termination, all unhatched embryos were counted and then archived according to the QAPP, and all hatched larvae were placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were sacrificed using approved methods of euthanasia at LSU.

E. Testing Protocol 5: The Effects of Direct Exposure of Gulf Killifish (*Fundulus grandis*) Embryos to an Oil Slick: Assessing the Influence of Time of Direct Slick Exposure

This protocol describes the procedure for exposing embryos directly to an oil slick each day for 2-14 hr per day during a 20-day period.

E.1 Preparation of PVC Rings

To prepare the PVC rings for slick exposures, researchers gathered one PVC ring per replicate test (see test-specific TCTs). PVC rings were 3" PVC pipes cut to lengths of 3.5 to 4.5 cm. The PVC rings were washed with soap and tap water, rinsed with RO water, and then dried before the test.

E.2 Preparation of Oil Slick in Exposure Containers

1. Before preparing the oil rings, oil was placed into a glass bowl and thoroughly mixed by hand at room temperature (see details in test-specific TCTs for the number of treatments and replicates).

2. Approximately 2 g of oil (± 0.2 g) were weighed in an aluminum weigh boat. Using a stainless steel spatula, as much of the oil from the weigh boat as possible was applied to a PVC ring. The oil was applied as a thin layer of oil to the inside of each PVC ring approximately 1 cm from one edge. This step was repeated for each PVC ring. Note that oil was added to the PVC rings no earlier than 2 days before being used in testing. Oiled PVC rings were kept at 4°C until use.

3. To prepare exposure containers, one PTFE basket (containing PTFE mesh from McMaster-Carr with 0.045 x 0.025" openings) was placed into each 950-mL Pyrex dish with 300 mL of 12 ppt seawater (see details in test-specific TCTs for the number of treatments and replicates).

4. One PVC ring was placed on top of the PTFE basket in each Pyrex dish. For oiled treatments, the PVC rings were placed within the PTFE basket with the oiled edge down so that the water surface intersected the oiled layer on the PVC ring. Clean, unoiled PVC rings were used for control treatments.

5. PVC rings were soaked for 4 hr to allow the oil slick to form.

6. After 4 hr, embryos were added to each treatment container (see test-specific TCT). To avoid disrupting the slick, embryos were placed between the outside of the PVC and the inside of the PTFE basket using a laboratory spatula, at an average distance of 1.67 cm from the water surface when resting on top of the PTFE mesh.

7. Immediately after the embryos were added, the oiled PVC rings were removed from the treatment container. This marked the beginning of the exposure. The date and time were recorded on the *Tank Identification Table* as described in the QAPP.

8. Every day at approximately the same time as the start of the exposure, partial water replacements were conducted (see Section E.2), followed by the renewal of the oil slick (steps 2 through 6). PVC rings were removed after a 4-hr soak.

E.3 Partial Water Replacements

1. Partial water replacements in exposure containers were performed daily.

2. During water replacements, the water level was dropped by removing the water, bringing the oil slick in direct contact with the embryos resting on the PTFE mesh. Water replacements were also conducted for the control treatments. Water replacements were conducted as follows:

    a. A 120-mL syringe and a long 18-gauge needle were used to draw water out of the treatment container.

    b. The needle was placed between the side of the Pyrex dish and the PTFE basket.

    c. 120 mL of water was pulled from each exposure chamber. This was enough water to ensure that the water level dropped below the embryos sitting on the PTFE mesh. Embryos were air exposed for 1, 2, 6, or 14 hr, depending on the treatment (see test-specific TCTs). Note that each experiment included control treatments with embryos that were air exposed for corresponding timeframes (i.e., 1, 2, 6, or 14 hr).

    d. After air exposures, water levels were returned to normal levels by adding 120 mL of fresh 12 ppt AFS back into the dish in the same manner that it was removed.

3. Following partial water replacement, the oil slick in each exposure chamber was renewed as described in Section E.2.

E.4 Exposure and Endpoint Measurements

1. During the test, exposure chambers were placed on an orbital shaker set to 20 RPM to prevent the formation of hypoxic boundary layers around the embryos.

2. Animals were kept at room temperature on a natural light cycle.

3. Mortality/hatch checks were performed daily just before water and oil slick renewals. All hatched larvae were removed and placed into a tank under recirculating conditions for long-term holding for possible future experiments. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note that before the onset of circulation, embryos were classified as dead if they had an opaque or cloudy-white appearance. Dead animals were removed and archived unless they were decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

4. At 7 days post-fertilization, heart rates were measured as follows:

   a. Measurements of heart rates were performed just before the oil slick renewal, so that the embryos could be removed with minimal disruption to the oil slick.

   b. Three animals from each replicate were selected randomly from the batch of surviving animals. To view embryos for heart rate measurements, embryos were carefully transferred to an empty glass Petri dish using a wide-bore transfer pipette. Using a stereomicroscope, heart beats for each embryo were counted for 30 sec.

   c. Embryos were then returned to their treatment container using a wide-bore transfer pipette.

5. Exposures continued for 20 days.

6. At test termination, all unhatched embryos were counted and then archived according to the QAPP; all hatched larvae were immediately placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of

development. If not used within 3 months, or if requested by Stratus Consulting, fish were euthanized at LSU and then archived according to the QAPP.

E.5 Imaging of Oil Slicks

1. Treatment containers were imaged using a digital camera on a solid black surface just after removal of PVC rings and at 3 or 4 additional time points during the test.

2. During imaging, ambient room lighting was turned off. Oil slicks were illuminated using a small lamp (with a Reveal bulb) clamped securely to a ring stand. The light was diffused through an opaque plastic tray to remove glare (see Figure E.1). The distance from the table to the opaque plastic was approximately 20 cm and the distance from the opaque plastic to the light was 10-20 cm.

3. Images were taken at an angle 30 to 60 degrees relative to the light source, approximately 15-25 cm away from the oil slick.

Figure E.1. Set-up for imaging oil slicks indoors.

F. Testing Protocol 6: The Effects of Direct and Indirect Exposure of Gulf Killifish (*Fundulus grandis*) Embryos to an Oil Slick with Subsequent Exposure to UV

This protocol describes the procedure for exposing embryos to an oil slick derived from Slick A or B oil (see test-specific TCTs) and subsequently exposing the embryos to UV light. Embryos that were 1-day post-fertilization were exposed to an oil slick for 14 hr with varying times of direct and indirect exposure to the oil slick (see test-specific TCTs). The oil-exposed embryos were then transferred to clean water and exposed to varying levels of ambient UV light for approximately 6 h. Embryos were monitored for 20 days following UV exposure for heart rate, mortality, and hatch.

F.1 Preparation of PVC Ring

To prepare the PVC rings for slick exposures, the required number of PVC rings were gathered (1 ring for each replicate was required; see test protocol TCTs). PVC rings were 3" PVC pipes cut to lengths of 3.5-4.5 cm. The PVC rings were washed with soap and tap water, rinsed with RO water, and then dried before the test.

F.2 Preparation of Oil Slick in Exposure Containers

1. Before the preparation of oil rings, oil was placed into a glass bowl and thoroughly mixed by hand at room temperature (see details in test-specific TCTs for number of treatments and replicates).

2. Approximately 2 g of oil (± 0.2 g) were weighed in an aluminum weigh boat. Using a stainless steel spatula, as much of the oil from the weigh boat as possible was applied to a PVC ring. The oil was applied as a thin layer of oil to the inside of each PVC ring approximately 1 cm from one edge. This step was repeated for each PVC ring. Note that oil was added to the PVC rings no earlier than 2 days before being used in testing. Oiled PVC rings were kept at 4°C until use.

3. To prepare exposure containers, one PTFE basket containing PTFE mesh from McMaster-Carr with 0.045 x 0.025" openings was placed in each 950-mL Pyrex dish

with 300 mL of 12 ppt seawater (see details in test-specific TCTs for number of treatments and replicates).

4. One PVC ring was placed on top of the PTFE basket in each Pyrex dish. For oiled treatments, the PVC rings were placed with the oiled edge down so that the water's surface intersected the oiled layer on the PVC ring. Clean, unoiled PVC rings were used for control treatments.

5. PVC rings were soaked for 4 hr to allow the oil slick to form.

6. After 4 hr, embryos were added to each treatment container (see test-specific TCT). To avoid disrupting the slick, embryos were placed between the outside of the PVC and the inside of the PTFE basket using a laboratory spatula at an average distance of 1.67 cm from the water's surface when resting on top of the PTFE mesh.

7. Immediately after the embryos were added, the oiled PVC rings were carefully removed from the treatment container by hand. This marked the beginning of the exposure. The date and time were recorded on the *Tank Identification Table* as described in the QAPP.

F.3 Direct Exposure of Embryos to Oil Slick

For all treatments with direct exposure to the oil slick, the procedures described below were followed. For each oil treatment, a no-oil control treatment was also run using the same methods. For treatments with no direct exposure to the oil slick (i.e., the indirect treatments), see Section F.4. See test-specific TCTs for treatments.

1. Immediately after embryos were added to Pyrex dishes, the water level was dropped, bringing the oil slick in direct contact with the embryos resting on the PTFE mesh. Direct exposure to the oil slick continued for 2–14 h depending on the treatment (see test-specific TCTs). Water removal was conducted as follows:

    a. A 120-mL syringe and a long 18-gauge needle were used to draw water out of the treatment container.

    b. The needle was placed between the side of the Pyrex dish and the PTFE basket.

    c. 120 mL of water was pulled from each exposure chamber. This was enough water to ensure that the water level dropped below the embryos sitting on the PTFE mesh.

2. After the appropriate amount of time of direct exposure to the oil slick (2–14 h, depending on the treatment), 120 mL of clean AFS was added back into each Pyrex dish using a clean, 120-mL syringe with an 18-gauge needle. As with water removal, water was added back in by placing the needle between the side of the Pyrex dish and the PTFE baskets.

3. All direct exposures were run for a total of 14 h.

F.4 Indirect Exposure of Embryos to Oil Slick

1. For indirect exposure treatments, the embryos were never in direct contact with the oil slick during exposure. The purpose of the indirect exposure is to investigate potential differences between direct exposure to slick oil, and exposure to water just beneath. For these exposures, as 120 mL of water was slowly removed from each Pyrex dish containing an oil slick, an equal volume of clean water was added back to the dish at the same rate, keeping the water level constant throughout the exchange. This was done immediately after the embryos were added to the Pyrex dish, using the same methods described above for the removal and addition of water in the direct exposures. However, to carefully control the rate that water was added and removed, two people were required for each water exchange, one to operate a syringe to remove the water, and one to operate a syringe to add water back into the Pyrex dish. For each oil treatment, a no-oil control treatment was also run using the same methods

2. Following water exchange, indirect exposure continued for 14 h.

F.5 UV Exposure

1. After the oil slick exposure, an equal number of embryos from each Pyrex dish were immediately transferred using a stainless steel spatula to one of three glass crystallizing dishes (corresponding to 10%, 50%, and 100% ambient UV) containing 200 mL of fresh 12 ppt water.

2. Embryos were exposed outdoors to 10%, 50%, or 100% ambient UV light for up to 6 hr for one or more consecutive days (see test-specific TCT). These exposures were performed on the rooftop of the Life Sciences Annex building at LSU. See step 5 below for description of water bath.

3. Crystallizing dishes were covered with fiberglass trays during transport to and from the outdoor water bath for UV exposure.

4. UV and manual temperature measurements were performed at 15-min intervals during embryonic UV exposures. Additionally, water temperature and light intensity were measured using automatic HOBO temperature monitors. These data loggers were placed in 6 locations (listed below) within the water bath or within dummy chambers placed within or outside of the water bath. Dummy treatment containers had an identical amount of water as test treatment containers and were used to monitor temperature in the following locations:

    a. Inside the northwest corner of the water bath
    b. Inside the southeast corner of the water bath
    c. Inside the middle of the water bath
    d. Outside the north side of the water bath to collect air temperature
    e. Inside the dummy treatment container on the east side of the water table
    f. Inside the dummy treatment container on the west side of the water table.

5. A water bath was prepared the day before the start of experiments to ensure that the water cooled overnight. If necessary, during the day a chiller was used to maintain proper water temperature (Figure F.1).

    a. The water table was oriented with its longest dimension facing southward. The water table was placed on cinder blocks to allow for drainage.

    b. The water table was then sectioned into thirds, with different plastic sheeting covering each section to allow 10%, 50%, or 100% of the ambient UV light to reach the crystallizing dishes underneath. See the UNT GLPP for further description of plastic sheeting.

    c. Tap water was used to fill the water table to within 2" of the top.

    d. Tubing (3/4" interior diameter) connected the pump and chiller, and a submersible pump situated inside the northwest corner of the water table pumped water to the chiller. The chilled water was returned to the water table using tubing situated inside the southeast corner of the water table. If needed, an additional pump was located inside the southeast corner of the water table to circulate water inside the water table, maintaining a more uniform temperature throughout the water table during the UV exposure.

Figure F.1. Water table set-up for UV exposure to embryos.

6. Before the crystallizing dishes were moved outdoors, the water in the water bath was brought to the correct temperature.

7. By mid-morning, crystallizing dishes were placed outdoors in the water bath under the different plastic covers corresponding to their UV treatment.

8. At the end of the day (approximately 6 hr of UV exposure), crystallizing dishes were brought indoors and placed on orbital shakers set to 20 RPM, in a room maintained at 20 to 22°C.

9. If repeat UV exposures were conducted, the following day crystallizing dishes were transported back to the outdoors water bath and placed under their respective UV plastic covers by mid-morning for up to 6 hr of UV exposure (see test-specific TCTs).

F.6 Post-exposure Monitoring and Endpoint Measurements

1. Embryos were monitored for heart rate, mortality, and hatch up to 20 days following UV exposure.

2. During this monitoring period, 100% water replacements were performed weekly immediately after water quality monitoring. For each container, embryos were removed using wide-bore transfer pipette to a glass Petri dish for mortality and hatch checks.

While embryos were removed, a total water replacement was performed. Embryos were then placed back into the crystallizing dish wide-bore transfer pipette.

3. Exposure chambers were maintained at 20 to 22°C with a natural light cycle on an orbital shaker set to 20 RPM to prevent the formation of hypoxic boundary layers around the embryos.

4. Mortality/hatch checks were performed daily. All hatched larvae were removed and placed into a tank under recirculating conditions for long-term holding for possible future experiments. Mortalities, missing individuals, non-treatment mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note that before the onset of circulation, embryos were classified as dead if they had an opaque or cloudy-white appearance. Dead animals were removed and archived unless they were decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

5. At 4, 7, and 10 days post-fertilization, heart rates were measured as follows:

   a. Measurement of heart rates was performed just before oil slick renewal, so that the embryos could be removed with minimal disruption to the oil slick.

   b. Three animals from each replicate were selected randomly from the batch of surviving animals. To view embryos for heart-rate measurements, embryos were carefully transferred to an empty glass Petri dish using a wide-bore transfer pipette. Using a stereomicroscope, heart beats for each embryo were counted for 30 sec.

   c. Embryos were then returned to their treatment container.

6. Exposures continued for 20 days.

7. At test termination, all unhatched embryos were counted, and then archived according to the QAPP; all hatched larvae were immediately placed into a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were euthanized at LSU and then archived according to the QAPP.

12. U.S. Army Engineer Research and Development Center General Laboratory Procedures and Practices

12.1 Introduction

The U.S. Army Engineer Research and Development Center (ERDC) conducted sediment toxicity tests using the estuarine amphipod, *Leptocheirus plumulosus*, to identify toxicological effects of the 2010 *Deepwater Horizon* oil spill on benthic species. This chapter describes general laboratory practices and procedures used at ERDC.

12.2 Testing

12.2.1 Test organism sources and husbandry – *Leptocheirus plumulosus*

All experiments were conducted using the amphipod *Leptocheirus plumulosus*. Experimental organisms were cultured on site at the ecotoxicology laboratory of the Environmental Laboratory, ERDC, in Vicksburg, Mississippi, according to Environmental Ecotoxicology and Risk Assessment Team (EERT) standard operating procedure (SOP) M-003: Culture, Care and Maintenance of *Leptocheirus plumulosus*. Cultures were maintained in 42 × 24 × 15 cm polyethylene (PE) tote boxes containing 2 to 3 cm of sediment purchased from Sequim Bay, Washington, and 3 L of artificial seawater at 20 ppt salinity. Cultures were held under a 16:8 hour light:dark cycle at 23°C and with trickle flow aeration. Amphipod cultures were fed ground TetraMin (purchased from Tetra Sales, Blacksburg, Virginia), and 40–60% of the overlying water was renewed three times weekly.

12.2.2 Source water

Artificial seawater at 20 ppt was prepared by mixing Crystal Sea Marinemix Bioassay Laboratory Formula, purchased from Marine Enterprises International, Inc., Baltimore, Maryland, in reverse-osmosis (RO) water.

12.2.3 Oil-spiked sediment preparation

Exposures took place in accordance with test-specific protocols and test conditions tables (TCTs). In all cases, oil-spiked sediments were prepared as outlined in the SOP: *Protocol for Preparation of Spiked Sediment*, in Section A.3. Sediment and slick oil were provided by the National Oceanic and Atmospheric Administration (NOAA).

12.2.4 Biological testing

The chronic effects of oil-spiked sediments on survival, growth and reproduction of the amphipod *Leptocheirus plumulosus* were assessed according to ERDC Testing Protocol 1. The protocol was developed using the following established guidelines:

- ASTM Method E1367-03 (Reapproved 2008): Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates (ASTM, 2008)
- Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-associated Contaminants with the Amphipod *Leptocheirus plumulosus* (U.S. EPA and USACE, 2001)
- Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

12.2.5 Overlying water quality monitoring

See the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP; located in Attachment 3) and SOPs listed in Section 12.4 for required monitoring and associated bench sheets.

12.2.6 Analytical chemistry sampling

See QAPP and test-specific TCTs for sampling and processing of sediment and water collected for potential chemical analyses.

12.3 Reporting and Testing Documentation

Reporting and testing documentation was performed as outlined in the QAPP.

12.4 Water Quality

12.4.1 Temperature measurement

1. Temperature measurement in the water baths and environmental chamber was performed with a Thermco Products Model ACC895 certified triple-digit thermometer

2. The water temperature of the overlying water in the test chambers was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 Multi Probe System (MPS)

3. Temperature in pore water was taken prior to pH and ammonia measurement and performed with the Orion 9107 BN pH low-maintenance triode according to EERT SOP I-006: Calibration and use of Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter.

12.4.2 Measurement of salinity

1. Salinity in the artificial seawater preparation and in pore water was measured using a temperature compensating refractometer made by Aquamarine, model ABMTC. Using a glass Pasteur pipette, one to two drops of test solution were placed on the measurement window and the lid was closed. The refractometer was pointed toward the light, making sure that it was kept level. The value was recorded.

2. The salinity of the overlying water was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.4.3 pH measurement

1. pH in pore water was measured using the Orion 9107 BN pH low-maintenance triode according to EERT SOP I-006: Calibration and use of Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter

2. The pH of the overlying water was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.4.4 Measurement of total ammonia

1. Total ammonia as nitrogen in pore water was measured with the Orion 9512 HPBNWP ammonia probe following the procedures outlined in EERT SOP I-001: Procedure for calibration and use of Orion 9512 HPBNWP Ammonia Probe.

2. The water ammonia in overlying water was measured with the LaMotte model R-5864 colorimetric kit. Five milliliters of sample were added to the colorimetric test tube. Then, eight drops of reagent #1 were added to the test tube. The tube was capped and shaken. Then, eight drops of reagent #2 were added to the tube. The tube was capped and shaken. The tube was placed in the color comparison module. The module was held up to light and the reading closest to color in the test tube was recorded.

12.4.5 Measurement of dissolved oxygen

1. Overlying water-dissolved oxygen (DO) was measured using the YSI model 556 Multiprobe System, according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.5 Sediment Toxicity Test Sediment and Water Disposal Procedures

1. Overlying water from the tests was disposed of in accordance with ERDC guidelines

2. At the completion of the project, solid waste was disposed of under the chemical safety and disposal regulations of the ERDC.

12.6 General Laboratory SOPs

12.6.1 Culture, care, and maintenance (*Leptocheirus plumulosus*) EERT SOP: M-003

Purpose: To ensure proper handling and care of the estuarine amphipods to yield healthy animals for toxicological testing.

Leptocheirus plumulosus is a gammarid amphipod native to estuarine waters of eastern North America (Bousfield, 1973). This species is cultured for use in acute and chronic sediment toxicity tests (DeWitt et al., 1992).

This SOP outlines procedures for maintaining cultures under relatively uniform conditions. It is divided into the following sections: Culture Overview, Culture Set Up, Culture Breakdown, Food Preparations, Feeding, Water Changes, Water Quality Monitoring, Population Monitoring, and Cadmium Reference Testing. The SOP uses the following definitions:

"Size or size class"	Unless otherwise indicated, determined by sieve pore diameter, rather than animal length
"Neonate"	Animals $\geq$ 300 µm but $\leq$ 425 µm in size
"Juvenile"	Animals $\geq$ 425 µm but $\leq$ 600 µm in size
"Sub-adult"	Animals $\geq$ 600 µm but $\leq$ 1 mm in size
"Adult"	Animals $\geq$ 1 mm in size
"Culture sediment"	300 µm sieved sediment from Sequim Bay, Washington; this sediment was frozen prior to initial sieving
"RO"	Reverse-osmosis water
"Culture water"	5 or 20 ppt salinity artificial salt water generated from Crystal Sea and RO water (aged $\geq$ 5 days)
"Culture container"	PE tote box [45 cm (L) ×24 cm (W) × 15 cm (D) (approximate internal dimensions); surface area approximately 1,073 cm^2]
"Cultures"	Culture containers containing populations of *L. plumulosus*

Culture overview

The ERDC *L. plumulosus* culture was initially derived from animals collected from James Pond on the Magothy River of the Chesapeake Bay, Maryland, in October 1994. To increase diversity, additional laboratory cultures from other commercial vendors were added to the laboratory cultures in February 1997 and approximately every 2 years afterward. The culture was maintained in the ERDC culture facility at 20 ± 2 ppt, 23 ± 2°C, at a radiation intensity of approximately 6 µE S^{-1} m^{-2} and under 16:8 hours light:dark cycle. Animals were cultured in plastic tote bins placed in temperature-regulated water baths. All animals were maintained on culture sediment with overlying culture water. Culture containers were routinely broken down and set up again to avoid undue crowding and to provide animals for testing purposes. Culture "health" or sensitivity was monitored through cadmium reference tests. Water quality was

monitored weekly and maintained by frequent water changes. Cultures were fed three times per week.

Culture set up

Overview

Culture containers were set up to provide a regular supply of animals for test purposes. If a constant culture was desired, the number of new culture containers was approximately equal to the number of culture containers broken down. New culture containers were generated on the same day as culture containers were broken down.

Materials

- Squirt bottle
- Culture book
- Indelible black ball-point pen
- Pasteur pipets
- Pipet bulb
- Culture container
- ~ 3 L culture sediment
- Air stone and air line
- ~ 8 L culture water
- Dispensing container (100–250 mL glass beaker)
- 200 animals (67 juveniles, 67 sub-adults, 66 adults)
- *L. plumulosus* food (see below for composition).

Methods

1. Rinse culture container with culture water.

2. Add culture sediment to each culture container to a depth of 2.0 ± 0.5 cm.

3. Add ~ 8 L culture water (to ≥ 6 cm depth). Aerate with air stones.

4. Label each culture with the date of the sediment addition and the sediment code.

5. Allow the sediment to settle for > 12 hours and < 3 days.

6. Before adding animals, and also within 12 hours after adding animals, replace 60% of the culture water. This action may reduce ammonia and hydrogen sulfide levels in the overlying water.

7. Stock culture containers with animals generated from breakdowns conducted the same day (i.e., do not leave animals overnight before adding them to a culture container).

8. Pool animals from each size class; pooling animals from separate culture containers may increase homogeneity of animals in the new culture containers.

9. For each new culture container, count 67 juveniles, 67 sub adults, and 66 adults into a dispensing container. If insufficient animals are available from any size class, substitute animals from another class. Record deviations in the culture record book.

10. Gently add the 200 animals into the culture container. Be sure to add animals from below the water level of the culture container. Rinse the dispensing beaker thoroughly with culture water to ensure that no animals are retained in the dispensing container. Gently push animals trapped on the water surface into the water column using drops of culture water from a pipet or squirt bottle. If the water in the dispensing container is > 2°C different from the culture container, then the dispensing container should be allowed to equilibrate with the culture water before the animals are added. This can be accomplished by placing the dispensing container in the culture container for 1 hour.

11. See *Feeding*, below, for feeding instructions. Only feed cultures on regular feeding days.

12. Replace the culture container label with a label containing the following culture code "L" followed by: a number, one number larger than the one on the previously created culture container; the number of animals added, usually "200;" size class, or "mixed;" sediment code; and the date animals were added.

13. Record culture code, number animals added and size class, animal source (i.e., culture codes from which the animals were derived), culture container (if not standard), salinity (20 ppt), temperature (23°C), module number, and any additional comments in the culture record book.

Culture breakdown

Overview

Culture containers were broken down when they were 28 to 35 days old. Older culture containers were broken down before younger culture containers. When culture containers were used to generate animals for testing purposes, including reference tests, juveniles from three culture containers were counted; if only one or two culture containers were broken down, juveniles in all containers were counted. During the culture breakdown process, animals received air exposure of only 3 to 4 minutes and remained moist during the entire air exposure; animals were never allowed to dry out.

Materials and personnel

- One 12-in. sieve of each of the following sizes: -300, 425, and 600 µm; 1 mm
- One 5 L or equivalent pitcher labeled "20‰"
- RO water
- Two squirt bottles each labeled "20‰"
- One fire-polished Pasteur pipet with narrow end > 1.5 mm diameter; wide end inserted in pipet bulb
- "Catch-pan" to place under bucket
- Laboratory apron
- *L. plumulosus* culture record book
- One person for culture breakdown; if culture animals were counted, a second person to assist with counting; if multiple breakdowns in one month, animals counted only once
- Mechanical counters
- In addition, for each culture to be broken down:
    - One 5-gal bucket with outer diameter slightly less than 12-in. sieve
    - *L. plumulosus* culture container
    - 12 L culture water
    - Glass culture bowls (4 and 8 in.).

Methods

1. Place catch-pan in a convenient working area and next to a work bench; place the bucket inside the pan. Stack the sieves sequentially on the bucket: 300, 425, 600 µm, then the 1 mm sieves – smaller-diameter sieve on bottom, largest on top.

2. Fill a pitcher and squirt bottles with culture water. Label four culture bowls with the four sieve sizes. For counting juveniles, label an additional three culture bowls with "425 µm" and the culture identification number.

3. Don a laboratory apron.

4. Move the culture container to work bench.

5. Using a gloved hand, gently stir the sediment into the overlying water for approximately 30 seconds. Once overlying water becomes a relatively thick suspension of sediment, pour the suspension gently and from as low a height as possible onto the stacked sieves. Do not pour more than 200 mL of suspended sediment onto sieves at one time.

6. Examine the 300-µm sieve to ensure it is not retaining water. Correct a vapor lock by gently lifting one side of the 300-µm sieve away from the bucket. Correct accumulation of sediment by tapping the underside of the 300 µm sieve with your fingers until water passes through sieve.

7. If sediment remains in the culture container, pour approximately 1 L culture water into the culture container. Repeat steps 5 and 6.

8. Repeat step 7 until < 200 mL of unsuspended sediment remains in the culture container.

9. Using a squirt bottle, remove sediment attached to walls of culture container and pour onto stacked sieves. The culture container should now be free of sediment.

10. If there is a need to count juveniles from a particular culture container, ensure that sediment is not spilled directly onto the catch-pan or allowed to leak from any sieve other than from the 300-µm sieve. This will help prevent the loss of individual animals.

11. Using a squirt bottle, gently rinse the sediment through the top sieve, avoiding undue pressure on the animals. Do not use a brush to push sediment through the sieve; this method would injure animals by forcing them against the metal sieve.

12. Using a squirt bottle, transfer animals from the 1-mm sieve to the appropriate culture bowl. Squirt water from behind the sieve, and venially downward along the sieve plane; this method will help ensure that animals are transferred to the culture bowl rather than to the work bench. If the water in the culture bowl appears too cloudy for counting, animals may be sieved again using a clean 300-µm sieve; use a 300-µm sieve for all but the initial sieves to ensure that all animals are retained within the original size class.

13. Repeat steps 10 and 11 for the remaining sieves, using the appropriately labeled culture bowl.

14. Label the bucket containing the sieved sediment with sediment type (e.g., "SC9501a"), source (*L. plumulosus* culture), sieve diameter (300 µm), date, and generator's initials.

15. Rinse sieves and tray thoroughly with RO water.

16. Using fire-polished pipet, count juveniles from the designated cultures. Record counts and breakdown dates in the *L. plumulosus* culture record book.

17. Animals not needed for new culture containers or for tests are euthanized by exposing them to hot tap water and pouring them down a drain.

Food preparation

Materials

- TetraMin
- 0.5-mm screen
- Food mill from Cyclone Sample Mill, UDY Corp., Fort Collins, Colorado
- ~ 100-mL sample vial
- Sealable, water-impermeable food container.

Methods

1. Attach sample vial to mill output. Place the 0.5-mm screen in the food mill. Firmly attach mill cover and turn on mill. Pour TetraMin flakes slowly into the food mill.

2. When desired amount of food is grinded, clean mill. Clean detachable components with paper towel or soap and water. Attach clean sample vial to mill output.

3. Place food in food container and refrigerate.

Feeding

Materials

- Food (see above)
- Weighing balance
- Weighing boat
- Beaker (size determined by volume of food solution)
- Stir bar
- Stir plate
- Culture water
- 10-mL pipet.

Methods

1. Round the number of culture containers plus three to a convenient number. Multiply by 0.8 g. (For example, you count 10 culture containers: 10 + 3 = 13; 13 culture containers × 0.8 g/culture = 10.4 g). Weigh this amount of food into the weighing boat.

2. Multiply the number of culture containers by 10 mL to give the total dilution volume (for example, 13 culture containers × 10 mL/culture = 130 mL). Add this volume of culture water to a beaker. Place the beaker on the stir plate and add the stir bar.

3. Add the food in the weighing boat to the beaker. Stir for ≥ 30 minutes.

4. Pipette 10 mL food suspension into each culture. Attempt to evenly distribute food across culture container surface. Ensure that food particles remain suspended during the pipetting process by stirring the food suspension with the pipet tip before and during the pipetting process.

Water changes

Overview

Water quality in cultures containers is likely improved by frequent water changes.

Materials

- Culture water at 20 ± 2 ppt salinity and at 23 ± 2°C
- Siphon hose of ≥ 1 m length and containing a ≤ 300 µm screen over one end
- Bucket
- 5-L or equivalent pitcher
- RO water.

Methods

1. Three times a week, on Monday, Wednesday, and Friday, siphon 40–60% of overlying water from all culture containers into a bucket or other container. Pour the water into a drain.

2. Test the salinity and temperature of culture water. If salinity is 20 ± 1 ppt and the temperature is 23 ± 3°C, proceed to step 4. Otherwise, adjust culture water salinity or temperature.

3. Gently pour new culture water from a pitcher directly into the culture container.

4. Rinse the siphon hose, pitcher, and bucket with RO water.

Water quality monitoring

Overview

To ensure that water quality remains within prescribed limits, water quality parameters are measured once per week.

Materials

- DO and temperature meter and probe
- pH meter and probe
- Refractometer
- Culture book.

Methods

1. On a new page in the culture book, create a table for entry of water quality parameters.

2. Calibrate meters.

3. Measure DO, pH, temperature, and salinity in culture containers, record data in the water quality table mentioned above. If high ammonia levels > 1 mg/L are suspected, take a 30-mL sample of the overlying water and check it for ammonia.

4. The following actions should be taken by the water quality measurer when water quality parameters deviate from the following "norms:"

Parameters	Norm	Action by measurer if parameters differ from norm	Contact laboratory QA/QC officer if:
DO	≥ 6 mg/L	Increase aeration	> 10% of culture containers ≤ 4 mg/L
Temperature	23 ± 2°C	Carefully adjust Remcor temperature control	> 2 culture containers are ± 5°C from norm; > 50% culture containers are ± 3°C from norm
pH	6.5–9	Contact QA/QC Officer	pH differs from norm
Salinity	20 ± 2‰	Change water and, if aeration and salinity are both high, reduce aeration	Salinity in > 50% of culture containers is outside normal limits for > 2 weeks

Parameters	Norm	Action by measurer if parameters differ from norm	Contact laboratory QA/QC officer if:
Ammonia	< 5 mg/L (total ammonia)	Increase aeration	Ammonia level in > 10% culture containers is > 5 mg/L
QA/QC: quality assurance/quality control.			

12.6.2 Procedure for calibration and use of the YSI Model 556 MPS EERT SOP: M-002

Purpose

- To ensure accurate and reliable water quality measurements (DO, conductivity, temperature, and pH) in aqueous solutions.

Materials

- YSI 556 MPS
- pH buffers in small specimen cups (4.01, 7.0, and 10.0)
- Conductivity standards in small specimen cups (1.413, 12.9, 15, 28 ms/cm^2)
- 2-mL PE (blue) bonded DO membrane caps (YSI # 5909)
- DO electrolyte filling solution
- Rinse bottle with RO or deionized (DI) water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

The YSI Multi probe should be calibrated each day before initial use and calibrations should be logged into the calibration book for that instrument.

Procedure

1. ***Probe setup:*** When the probe is first received, install each of the sensors. Periodically a sensor or DO membrane may need to be replaced. Follow these instructions for installing sensors and DO membranes. For information on setting up the meter for the proper sensors installed and the measurement units reported, refer to the YSI 556 MPS Operations Manual (YSI, 2002).

    a. Remove the transport/calibration cup and thoroughly dry the entire probe. Water must not get into the sensor ports during the changing of the sensors. Use compressed air to dry the probe before removing old sensors.

b. Using the sensor installation tool supplied in the maintenance kit, unscrew and remove the old sensor or the sensor port plugs.

c. Locate the port with the connector that corresponds to the sensor that is to be installed. Apply a thin coat of o-ring lubricant to the o-ring on the connector side of the sensor.

d. To prevent the o-ring from leaking, make sure there are no contaminants between the o-ring and the sensor. Again, be sure the probe is free of moisture and dry further if necessary with compressed canned air.

e. Align the sensor with the sensor port so that the two connectors will fit together properly. With the connectors aligned, screw down the sensor nut using the sensor installation tool. Tighten the nut until it is flush with the face of the probe module bulkhead. Do NOT over tighten.

f. Caution: Do not cross thread the sensor nut. This may damage the port threading, causing the entire probe to leak and malfunction.

g. Repeat the above procedures for each sensor to be installed.

h. Install the DO membrane cap onto the DO sensor by removing the old cap. Filling a new cap half full of electrolyte solution, tap the cap lightly on the table to remove any bubbles from the walls of the cap and screw the membrane cap onto the sensor moderately tightly. A small amount of electrolyte should overflow. Observe the probe for bubbles inside the cap. If bubbles are present, remove the cap and repeat the process.

2. ***Calibrations:*** Each of the sensors in the probe must be calibrated separately. The typical order of this calibration is: DO, conductivity, and then pH.

a. *DO calibration:* To begin the calibration of the DO sensor, obtain information on barometric pressure from the following websites. http://www.wunderground.com/weather-forecast/39180 will provide the current Vicksburg barometric pressure. This measurement is provided in inches of mercury and must be converted to mm of mercury. Enter the measurement from the weather underground site into the conversion program at http://www.onlineconversion.com/pressure.htm and select inches of Hg to mm of Hg. Subtract 5 from the value provided to correct for the elevation of the ERDC laboratory and use that number in the calibration set-up. The aforementioned process must be completed each time the instrument is calibrated.

i. Turn on the instrument by pressing the power button (green).

ii. Access the calibration menu by pressing the <Esc> button, and using the down arrow key to scroll down to the line that says Calibrate. Press the Enter button that looks like a left facing arrow <←>.

iii. From the Calibration Menu, scroll down using the down arrow button to the DO line and press the Enter <←> button.

iv. Scroll down in the DO calibration menu to DO% and press the Enter <←> button.

v. Using the keypad, enter the local barometric pressure obtained from the websites and press the Enter <←> button.

vi. Make sure that the probe is in the calibration cup and is loosely threaded in an upright position; the cup should contain approximately 1/8 in. of water. Also be sure that no water is on the DO membrane from rinsing.

vii. Observe the DO readings and when they show no observable change in 30 seconds, press the Enter <←> button.

viii. Press the Enter <←> button again to return to the DO calibration screen and then the <Esc> button to return to the main calibration screen. The DO sensor is now calibrated.

b. *Conductivity calibration:* To calibrate the conductivity sensor, pour one or more conductivity standards into small specimen cups with lids. To calibrate the probe properly, volumes must be sufficient to cover the opening at the side of the sensor.

i. Remove the storage/calibration cup from the probe and submerge the probe in the lowest conductivity standard, making sure that the opening at the side of the sensor is submerged in the standard

ii. From the main calibration menu, select Conductivity and press the Enter <←> button

iii. From the Conductivity calibration menu, select Specific Conductance and press the Enter <←> button

iv. Enter the specific conductance of the first (lowest) standard to be measured and press the Enter <←> button

v. Observe the conductivity readings and when they show no observable change in 30 seconds, press the Enter <←> button

vi. Press the Enter <←> button again to return to the Conductivity Calibration Menu

vii. If more than one standard is to be measured, repeat the above process for each standard

viii. Be sure to rinse the probe with RO or DI water in between standards and dab dry with a Kimwipe

ix. Once all standards are measured, the conductivity sensor is calibrated. Press the <Esc> button to return to the Main calibration menu.

c. *pH calibration:* To calibrate the pH sensor, one or more pH buffers should be poured into small specimen cups with lids. Volumes must be sufficient to cover the bottom of the pH sensor to calibrate the sensor properly. Three buffer calibrations are typically performed: 4.01, 7.0, and 10.0.

i. From the main calibration menu, use the down arrow button to scroll down to the pH line and press the Enter <←> button.

ii. Place the rinsed and dried probe into the pH 7 buffer and select "3-point calibration" from the menu; press the Enter <←> button.

iii. Using the key pad, enter the pH of the buffer and press the Enter <←> button.

iv. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button to accept the reading.

v. The screen will ask for the pH of the second buffer. Using the key pad, enter 4.01, remove the probe from the pH 7.0 buffer, rinse and dab the probe dry with a Kimwipe, and submerge it in the 4.01 buffer; press the Enter <←> button.

vi. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button again to accept the reading.

vii. The screen will ask for the pH of the third buffer. Using the key pad, enter 10.0, remove the probe from the pH 4.01 buffer, rinse and dab dry the probe with a Kimwipe, and submerge it in the 10.0 buffer; press the Enter <←> button.

viii. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button again to accept the reading.

ix. Once the third buffer has been accepted, press the <Esc> button twice to exit all of the calibration menus and return to the run screen.

3. ***Measurements:*** Following calibration, make the measurements. Make sure the meter is in the run mode by hitting the <Esc> button until all the probes sensor measurements are displayed. Submerge the probe into the solution to be measured. Keep the probe moving slowly to achieve accurate DO readings. When the readings stabilize, record them on a data sheet or press the Enter <←> button to record them into memory.

   a. The readings can be recorded into a list of available files that will appear on the screen or, a new file can be set up using the enter information screen. Refer to the YSI 556 MPS Multi Probe Operations Manual (YSI, 2002) for instruction on setting up the files.

   b. If no files exist, the enter information screen should appear. Using the key pad, enter a file name for the measurement. If you would like to enter optional site or sample descriptions, highlight that field and use the keypad to enter the information. Press Enter <←>, highlight OK, and press Enter <←> again. If logging one sample, the instrument will confirm that the data point was successfully logged.

   c. Remove the probe from the sample, rinse with RO or DI water, briefly shake excess water off the probe, and place it into the next sample to be measured. Repeat the process above to record the data.

4. ***Viewing and recording stored data and calibration information:***

   a. From the run mode, Press the <Esc> button to go to the main menu. Highlight the File line and press the Enter <←> button.

   b. From the File menu, select View File and press Enter <←>.

   c. A list of files will be displayed, including one that ends in .glp. This is the calibration file. Highlight this file and press the Enter <←> button. Scroll down to

the date the calibration was performed and use the right arrow button to scroll the page to the right. Record the information on the DO gain, the DO local gain, conductivity gains from each of the standards measured, as well as the pH gains and offsets that are displayed into the calibration/maintenance log for that instrument. Note: The conductivity gains and the pH gains and offsets are all displayed with the same designation. Be sure to record them as the conductivity standards and pH buffers in the concentration and in the order in which they were measured.

d. Press the <Esc> button to return to the list of files and highlight any files you wish to record on data sheets. Highlight the file name, press the Enter <←> button and scroll to the date of the measurement. The right arrow will take you to other pages with data beyond the date and temperature that is displayed on the first page. When all files are recorded, press the <Esc> button until you return to the Run mode.

Maintenance

1. ***Probe storage:*** Store the probe in its calibration/transportation cup in an upright position in the holding vessels attached to the shelf. A small amount of tap water should be left in the bottom of the calibration/transportation cup to keep the probe environment moist. Do not allow the probe to dry out and do not store the probe with the sensor in water.

2. ***Membrane life:*** According to the instruction manual, the DO membranes will last for weeks but should be changed at least every 30 days, or more frequently if the samples measured have high levels of suspended solids. Follow the instructions above to change out the DO membranes.

If problems occur that this SOP does not address, refer to the YSI 556 MPS Operations Manual (YSI, 2002) or the technical support section at www.YSI.com.

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until the proper technique and accuracy of measurements is ensured.

Safety

1. pH buffers can be acidic or caustic liquids. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with water. A sink is present along the wall of the wet lab in 6009, and an eye flushing station is near the sink. The samples measured may be test waters, effluents, discharges, or other water samples

that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

12.6.3 Procedure for calibration and use of the Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter EERT SOP: I-006

Purpose

- To ensure accurate and reliable water quality measurements (pH) in aqueous solutions.

Materials

- Orion DUAL STAR pH/ISE benchtop meter or similar
- Orion model 9107BN gel-filled low-maintenance pH/ATC Triode
- Electrode holding arm
- pH electrode storage bottle with electrode storage solution
- pH buffers in small scintillation vials (4.01, 7.0, and 10.0)
- Stir plate with small stir bars
- Rinse bottle with RO or DI water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

1. Calibrate the Orion pH probe each day before initial use and log calibrations into the calibration book for that instrument. Recalibration may be necessary throughout the day if measurements against standards deviate more than 5%. This probe and meter are typically used for measuring pH and temperature of samples before ammonia measurements are taken.

Procedure

1. ***Meter setup:*** Consult the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009) for information on setting up the meter's features, including measuring modes, resolution, and units displayed.

   The meter is typically set up for the activation of "hold and ready" function. Once the meter registers a stable reading of the probe, the "hold and ready" function locks in a reading on the display screen.

   a. The display will briefly display RDY and then HLD when the reading is ready to be recorded

b. The probe is kept in the electrode holding arm to keep it upright in the sample vials on the stir plate.

2. ***Calibration:***

a. Turn meter on by pressing the power button.

b. Press the <Channel> button to display the screen for pH measurement.

c. Press the <F2> button at the top to begin the calibration.

d. Rinse the exterior of the probe with DI or RO water with a squirt bottle and dab dry with a Kimwipe.

e. Place the probe into the pH 7.0 buffer on the stir plate and stir at 500 RPM.

f. Press the <F3> button to start and wait for a stable reading. The meter will lock in the reading once it becomes stable. Press <F2> if the reading is the correct concentration; otherwise, enter the correct concentration with the keypad and then select <F2>.

g. Press <F3> to move to the next buffer, remove the probe from the vial, rinse and dab dry the probe, and place the next buffer (4.01) onto the stir plate.

h. Press <F2> to begin measuring the next buffer; wait for a stable reading.

i. Repeat this procedure for the remaining pH 10.01 buffer. When the final buffer is measured and the correct concentration is entered, select <F3> to end the calibration. The meter will display the calibration average slope and all the millivolt readings from each of the standards. If the slope is acceptable (95–105%), then select <F2> to log/print the calibration and return the meter to the measurement mode. Record the slope and time in the instrument calibration log.

j. Allow the last calibration standard measurement to lock into the meter, remove, rinse, and dab dry the probe. The probe is now ready for measurement.

3. ***Adjusting the calibration curve:*** If the calibration slope is not in the acceptable range, the entire calibration curve can be re-measured or individual points can be re-measured or deleted from the curve. Consult the Thermo Scientific Orion DUAL STAR pH/ISE Meter user Guide (Thermo Fisher Scientific, 2009, pp. 40–41) for instructions.

4. ***Measurements:*** Following calibration, measure the concentration of at least one standard buffer solution for verification. If the value is not within 2% of the expected value, repeat the calibration procedure. Once the verification is complete, measure the various samples by placing at least 10 mL of sample into a clean scintillation vial with a magnetic stir bar, place it on the stir plate, place the probe in the sample, and stir at 500 RPM. Press the <1/measure> button to begin measurement of the sample. When the auto read feature senses a stable reading, the measurement will lock in. Record the pH and temperature measurement on the data sheet for the project. Remove the probe from the sample, rinse with DI or RO water, and dab dry before placing it in the next sample.

5. ***Measuring hints:***

    a. Replace buffers at least once a week
    b. Properly seal vial of buffer after each use to prevent evaporation
    c. Do not allow the probe to dry out between measurements
    d. Rinse the probe between each sample and dab dry with a Kimwipe to prevent cross contamination.

Maintenance

1. ***Probe storage:*** Store the pH probe in the electrode storage bottle containing electrode storage solution in an upright position. Perform periodic inspection of the storage solution; replace it if it has evaporated or has become moldy.

2. ***Cleaning:*** Clean the pH probe by soaking in a pH 4.01 buffer or by soaking in an electrode cleaning solution available from multiple vendors. Thoroughly rinse the probe with DI following cleaning, before storing or using. Calibrate the probe after the cleaning procedures but before using them.

For problems encountered that this SOP does not address, refer to the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009) and the Low Maintenance Gel-Filled pH Electrodes User Guide (Thermo Fisher Scientific, 2007b).

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until proper technique and accuracy of measurements is ensured.

Safety

1. pH buffers can be acidic or caustic liquids. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with water. A sink is

present along the wall of the laboratory in building 6000, room 65, and an eye flushing station is near the sink. The samples measured may be pore waters, test waters, effluents, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

12.6.4 Procedure for calibration and use of Orion 9512 HPBNWP Ammonia Probe EERT SOP: I-001

Purpose

- To ensure accurate and reliable ammonia measurements in aqueous solutions.

Materials

- Orion model 9512 HPBNWP high performance ammonia probe
- Orion DUAL STAR pH/ISE benchtop meter or similar
- Magnetic stirrer and small magnetic stir bars
- Bonded membrane caps (Orion #951205) or loose membranes (Orion #951204)
- Electrode internal filling solution (Orion #951202)
- Standard Solution, NH_4Cl, 1,000 mg/L as nitrogen (Orion # 951007) or prepared as described below
- Ionic Strength Adjuster (ISA; Orion #940011)
- Ammonia pH Adjusting ISA (Orion # 951211)
- Rinse bottle with RO or DI water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

1. Calibrate the ammonia probe each day before initial use and every 2 hours if necessary as determined by checks against standards.

Procedure

1. ***Electrode setup:*** When the electrode is first received or after it has been stored dry, soak the inner body in internal filling solution for at least 2 hours before assembling the electrode. For best results, soak the inner body overnight. Then, follow these steps:

    a. Unscrew top cap and remove electrode inner body; drain old filling solution. Set cap with inner body aside carefully.

    b. Remove bottom cap from electrode outer body. If using bonded membrane caps, screw cap in end of electrode until finger tight and proceed to step 1.e. If using loose membranes, proceed with instructions in step 1.c.

    c. Using flat-end tweezers, carefully grasp a white membrane from between paper separators. Hold the membrane at the edge with the tweezers. Hold the electrode outer body in a free hand, loosely stretch the membrane across the opening in the bottom of the probe, holding the ends against the threads with your thumb and forefinger. Avoid excess handling of the membrane, since this may affect its hydrophobic properties and reduce its life.

    d. Replace the cap on the probe and screw it on until it is finger tight. The membrane should be smooth with no wrinkles.

    e. Add internal filling solution up to the embossed line on the outside of the electrode outer body. If measuring low ammonia concentrations (e.g., < 0.06 mg/L ammonia nitrogen), the filling solution can be diluted by 1/10 to increase response time.

    f. Replace inner body into the outer body and screw on upper cap.

    g. Place a finger over the hole in the upper outside of the outer body and shake probe as if it were a clinical thermometer to remove internal bubbles.

    h. Record date of membrane and internal filling solution change in the instrument calibration log.

    i. Soak the assembled probe in a 10 mg/L NH_4Cl standard for at least 2 hours before attempting calibration and making measurements.

2. ***Preparation of standards:*** A 1,000-mg/L ammonia as nitrogen stock standard can be purchased (Orion #951007) or prepared. Prepare by adding 3.82 g of reagent grade NH_4Cl to 500 mL of Milli-Q DI water in a 1,000 mL volumetric flask, stir to dissolve and

dilute to the full 1,000-mL volume with Milli-Q DI water. Make or purchase again before shelf life expiration, approximately six months.

a. Prepare 100-, 10-, 1.0-, and 0.1-mg/L standard solutions by serial dilution from the 1,000-mg/L stock solution using Milli Q water to which 2 mL of ionic strength adjuster (Orion #940011) has been added to each 100 mL. These can be made in scintillation vials:

   1 mL 1,000-mg/L standard + 9 mL ionic adjusted water = 100-mg/L standard
   1 mL 100-mg/L standard + 9 mL ionic adjusted water = 10-mg/L standard
   1 mL 10-mg/L standard + 9 mL ionic adjusted water = 1-mg/L standard
   1 mL 1-mg/L standard + 9 mL ionic adjusted water = 0.1-mg/L standard.

3. ***Perform calibrations using standard solutions:*** For detailed instructions for setting up the Thermo Scientific Orion DUAL STAR pH/ISE Meter consult the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009).

   a. Turn meter on by pressing the power button.

   b. Press the <Channel> button to display the screen for NH_3 measurement.

   c. Press the <F2> button at the top to begin the calibration.

   d. Rinse the exterior of the probe with DI or RO water with a squirt bottle and dab dry with a Kimwipe.

   e. Place five drops of Ammonia adjusting ISA (Orion #951211) and a small stir bar into the scintillation vial containing the lowest concentration standard and place the vial on the stir plate and submerge the tip of the probe into the sample, while stirring the sample at approximately 500 RPM.

   f. Press the <F3> button and wait for a stable reading. The meter will lock in a reading once it becomes stable. Press <F2> if the reading is the correct concentration; otherwise, enter the correct concentration with the keypad and then select <F2>.

   g. Press <F3> to move to the next standard, remove the probe from the vial, rinse, and dab dry the probe and place the next standard onto the stir plate. Be sure to add five drops of Ammonia pH adjusting ISA (Orion # 951211) and a stir bar to the vial before placing it on the stir plate.

   h. Press <F2> to begin measuring the next standard and wait for a stable reading.

i. Repeat this procedure for the remaining standards beginning at the lowest and going to the highest concentration. When the final standard is measured and the correct concentration is entered, select <F3> to end the calibration. The meter will display the calibration average slope and all the millivolt readings from each of the standards. If the slope is acceptable (-54 to -60), then select <F2> to log/print the calibration and return the meter to the measurement mode. Record the slope and time in the instrument calibration log.

j. Allow the last calibration standard measurement to lock into the meter, remove, rinse, and dab dry the probe. The probe is now ready for measurement.

4. ***Adjusting the calibration curve:*** If the calibration slope is not in the acceptable range, the entire calibration curve can be re-measured or individual points can be re-measured or deleted from the curve. Consult the Thermo Scientific Orion DUAL STAR pH/ISE Meter user Guide (Thermo Fisher Scientific, 2009, pp. 40-41) for instructions.

5. ***Measurements:*** Following calibration, measure the concentration of at least one standard solution for verification. If the value is not within 2% of the expected value, repeat the calibration procedure. Once verification is complete, measure the various sample concentrations by placing five drops of the pH ammonia adjusting ISA and a stir bar into each sample in a scintillation vial just before placing the probe in the sample on the stir plate; stir at 500 RPM. Press the <1/measure> button to begin measurement of the sample. When the autoread feature senses a stable reading, the measurement will lock in. Record the measurement on the data sheet for the project. Remove the probe from the sample, rinse with DI or RO water and dab dry before placing it in the next sample.

6. ***Measuring tips:***

a. Rinse the electrode with DI or RO water between measurements.

b. Check the electrode for bubbles on the membrane. If present, bubbles can be removed by shaking the electrode.

c. Magnetically stir all samples during measurement. Magnetic stirrers may generate some heat, and a layer of Styrofoam can be placed below the sample to help limit any change in temperature.

d. The temperature must be consistent for all samples and standards. This ion-selected electrode is not temperature compensated. A 1°C difference will introduce a 2% measurement error.

e. If the electrode response is slow, the membrane may contain a surface layer of contaminants. To restore performance in this case, soak the electrode in DI water for about 5 minutes and then soak in a standard solution for about 1 hour before use or replace the membrane and soak in a 10 mg/L NH_4Cl standard for 2 hours. For large numbers of "dirty" samples, more than one probe may be assembled and used to reduce or eliminate the waiting time for probe equilibration.

f. If samples cannot be measured on the same day, they can be acidified with 1 M HCl to a pH of less than 2 and held refrigerated for up to 28 days without significant loss of ammonia.

Maintenance

1. ***Electrode storage:*** Between measurements, do not allow the electrode tip to dry. For overnight or week-long storage, the electrode tip should be immersed in a 1,000 mg/L standard solution. For storage over 1 week or if the electrode is stored indefinitely, disassemble completely and rinse the inner body, outer body, and bottom cap with DI water. Allow parts to dry, then reassemble the electrode without internal filling solution or a membrane.

2. ***Membrane life:*** According to the instruction manual, membranes will last from 1 week to several months depending on usage. At ERDC, membranes may last less than 1 day when measuring samples with contaminants or high ammonia levels. Membrane failure is characterized by a shift in the electrode potential, drift, or poor response time. Membrane failure may be apparent on visual inspection as dark spots or discoloration of the membrane or carbonate deposits on the membrane and end cap. Follow the above procedure step 1 (*Electrode Setup*) to replace the membrane and add internal filling solution.

For problems encountered that this SOP does not address, refer to the model 9512 HPBNWP high-performance ammonia probe user guide (Thermo Fisher Scientific, 2007a) and the Thermo Scientific Orion DUAL STAR pH/ISE Meter User Guide (Thermo Fisher Scientific, 2009).

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until proper technique and accuracy of measurements is ensured.

Safety

1. Ammonia pH adjusting ISA is a highly caustic liquid. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with

water. A sink is present along the wall of room 65, and an eye flushing station is near the sink. The samples handled may be pore water, effluent, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

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A. Testing Protocol 1: 28-day Chronic Sublethal Sediment Tests Using *Leptocheirus plumulosus*

A.1 Testing Procedures Overview

This protocol provides guidance for conducting 28-day chronic sediment toxicity tests using the estuarine amphipod *Leptocheirus plumulosus*. The protocol is written in basic accordance with the guidance provided in *Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod* Leptocheirus plumulosus (EPA 600-R-01-020) and *Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates* (ASTM 1367-03). The ERDC modified this procedure specifically for evaluating sediments for NOAA. In some instances, reburial was recorded as an endpoint (see test-specific TCTs). In those cases, reburial of surviving adult *Leptocheirus plumulosus* following removal from sediment treatments was assessed. According to ASTM Method E1367-03 (ASTM, 2008) and (U.S. EPA, 1994), this assessment is optional for use in association with the standard 10-day sediment toxicity testing. Use of this approach in association with the standard 28-day protocol is described below.

A.2 Sediment Preparation

A.2.1 Receipt of sediment

Sediments were shipped frozen to the ERDC in a plastic bag. Sediments were kept frozen until testing.

A.2.2 Preparation of sediment

Sediments were prepared for testing following the guidance provided in the latest revision of the sediment preparation SOP entitled *Protocols for Preparation of Spiked Sediment*.

A.3 SOP: Protocol for Preparation of Spiked Sediment

A.3.1 General guidelines

- Prepare all sediments using the *SOP: Protocol for Preparation of Spiked Sediment;* however, do not add oil to the control sediments.

  *Note: Prior to 2014, the ERDC control was not mixed in the stand mixer.

- Prepare each sediment-oil concentration separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

  *Note: Prior to 2014, the mixing paddle was not rinsed with hexane and dichloromethane (DCM); only acetone was used.

- Store unused prepared sediments in a 1-L glass jars and store in the dark at 4°C (short term) or in freezer at -20°C (long term).

A.3.2 Glassware preparation

Prepare all of the equipment in accordance to the *Decontamination SOP* in the QAPP.

A.3.3 Preparation of sediments

- Allow the sediment to thaw
- Remove all debris (grass, shells, etc.) from the thawed sediment and place in a mixer bowl
- Using a Cuisinart SM-70 7-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

A.3.4 Mixing oil into sediment

- Weigh out the appropriate amount of oil as outlined below:

  Weigh slick oil in a pre-cleaned aluminum weigh boat. Tare a weigh boat and a stainless steel spatula on the top loading balance. Using the stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Using the spatula, transfer the oil onto the sediment in the mixing bowl, placing it in several areas around the bowl.

Reweigh the weigh boat and spatula to calculate and record the actual mass of oil transferred.

- Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.
- Mix the oil into the sediment at medium speed (5) with a Cuisinart SM-70 7-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with a stainless steel spoon.
- Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the oiled sediment from the mixing bowl into 4-L glass beakers for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for test initiation.

A.4 Methods

A.4.1 Collection of testing organisms

1. Obtain *Leptocheirus plumulosus* from in-house cultures. Obtain individuals that are in the 250 – 600 µm size class. Be sure to collect plenty of individuals for the test. To ensure a large selection pool of individuals, obtain an additional 1/3 of the total number of organisms that are need for the test from the in-house culture (see test-specific TCTs).

2. Transfer organisms to a holding container and gradually bring them to testing temperature. *Leptocheirus plumulosus* can easily become trapped in the water surface tension and will float. Use a dropping pipette to break surface tension and allow floating amphipods to swim to the bottom of the container. Supply aeration using an air stone or glass pipette. During the acclimation period, hold the animals at 25°C with a photoperiod of 16:8 hours light:dark. Proceed with laboratory acclimation, at testing conditions, without sediment for ~ 24 hours before initiating the test. This period is designated to gradually acclimate organisms to laboratory/testing conditions and ensure that test organisms are healthy before distribution into test chambers.

A.4.2 Test chamber and sediment preparation

1. Use pre-cleaned 1-L beakers (~ 10 cm diameter) during testing. Prepare five beakers per treatment to use as testing chambers and prepare two additional beakers per treatment to use as "dummy beakers." Treat the dummy beakers the same as the testing chambers, but do not collect biological data from these beakers. One dummy beaker is for measuring pore water quality at day 0 and one dummy beaker is for measuring pore water quality and sediment chemistry at day 28. Do not place organisms in the day 0 dummy beaker. Label each beaker with an assigned tank ID designation.

2. Fill beakers with 175 mL (~ 2 cm) of sediment prepared as described in Section A.3, *Protocol for Preparation of Spiked Sediment*. Add the sediment carefully to minimize contact with the sides of the beakers; level the sediment by tapping the sides of the beakers. Fill chemistry sample jars incrementally.

3. Slowly add the overlaying reconstituted seawater (20 ppt), using a turbulence reducer to minimize re-suspension of the sediment. Fill beakers to the 850-mL line. This will allow room for the additional water added with the organisms at test initiation. Once the organisms are added, fill the beakers to the 900-mL mark.

4. Randomly place all treatment replicates in the specified controlled water bath at 25 ± 1°C and cover each beaker with a plastic cover.

5. After the sediment has partially settled, gently aerate ~ 2 to 3 cm from the sediment surface and allow full settling overnight. Provide aeration using plastic tubing connected to an air supply and inserted into a glass pipette; insert the pipette through a hole in the plastic cover. The aeration rate should be adequate to keep DO at ~ 90% saturation but not disturb or re-suspend the test sediment.

6. Measure pore water quality from a dummy beaker for all treatments at day 0 and at day 28.

7. Take pore water quality measurements by obtaining a sediment sample and placing it in a centrifuge tube. Centrifuge the sediment and sample the pore water from the overlying water in the centrifuge tube.

8. Take 10 mL of overlying water (pore water from the centrifuge tube) and place into a 20 mL scintillation vial. See Section 12.4 for the pore water quality guidelines.

A.4.3 Test initiation

1. Remove and count the dead organisms in the holding containers. If the total mortality/inactivity exceeds 5% at day 0, the entire collection is unsuitable for testing.

2. Measure pore water ammonia concentration and other pore water quality parameters (see Section 12.4) in one dummy beaker from each treatment. Testing cannot be initiated unless total ammonia is less than 60 mg/L.

3. Take overlying water quality measurements (temperature, salinity, pH, and DO) in all chambers and record. Measure and record overlying water total ammonia in one composite sample of all replicates within a treatment. Initiate the test if parameters are within the specified ranges according to the U.S. Environmental Protection Agency guidelines.

4. Place *Leptocheirus plumulosus* neonates (250 – 600 µm) in a culture bowl or counting tray. Count ten organisms randomly into high-density polyethylene (HDPE) cups (counting chambers) so that counts and QA/QC are manageable. Transfer organisms using wide-bore pipettes, fully submerging the pipettes to minimize injury; do not use forceps. Add two counting beakers containing 10 organisms each to each test chamber so that the total number of amphipods per replicate is 20. Only select apparently healthy individuals at random for testing; amphipods typically swim or curl up. Do not use any organisms that are dropped or that contact a dry surface in testing. After all the designated counting chambers are filled, verify that each container has 10 individuals. Include an additional six counting chambers for initial weight measurements. During the addition of the two counting chambers to the test replicates, stop aeration to test beakers and gently pour the two counting chambers into a test beaker. Additionally, ensure that a second technician is present to verify that two counting chambers are added to each beaker and that all individuals are removed from the counting chambers. Use wash bottles containing 20 ppt seawater to dislodge amphipods that stick to the sides of the counting chamber. Submerge floating amphipods using a drop of test water from the test beaker using a pipette.

5. Upon the addition of test organisms, record the time of test initiation on the appropriate bench sheet. Re-supply aeration 1 hour after test initiation to allow time for neonates to burrow.

6. Place 20 neonates from two counting chambers on each of three pre-weighed pans for initial weight determination.

7. Approximately 1 hour following the addition of test organisms, observe chambers for injured amphipods and individuals that did not burrow. At this time, replace injured or unhealthy individuals, or individuals that failed to burrow, if the response does not appear to be specific to the particular sediment treatment. Use a dropping pipette to submerge floating amphipods.

A.4.4 Test monitoring and maintenance

1. For the first week of testing, observe each test chamber once daily for floating amphipods. Submerge any amphipods observed floating using water droplets dropped gently from a pipette. Record behavioral observations, floaters, and other notes on appropriate test bench sheets.

2. Water quality: Record the exposure chamber temperature (min/max) daily. Record the overlying water temperature, salinity, pH, and DO three times per week on Mondays, Wednesdays, and Fridays before water exchange in at least one replicate per treatment using the provided bench sheets.

3. Exchange the water (400 mL) in all chambers, including dummy chambers, by siphoning the overlying water using a 1.5-cm ID siphon hose and refilling with 20 ppt seawater using a pitcher and a turbulence reducer to minimize sediment disturbance.

4. Add 20 mg of TetraMin to each test chamber by preparing a seawater TetraMin slurry that contains 20 mg of TetraMin per mL. Deliver 1 mL to each chamber three times per week (Mondays, Wednesdays, and Fridays), following water exchange and water quality monitoring. After 2 weeks, deliver 40 mg of TetraMin by adding 2 mL per chamber.

5. Check light cycle and ensure that each test chamber is adequately aerated daily.

A.4.5 Setup of reburial evaluation chambers

If the test requires the evaluation of the reburial endpoint (see test-specific TCTs), add ERDC control sediment to five replicate pre-cleaned beakers. Add overlying water and place in a temperature controlled chamber or water bath on trickle-flow aeration.

A.4.6 Test termination and breakdown

1. Water quality: Record temperature, salinity, pH, and DO in all replicate exposure chambers for the 28-day test and reburial evaluation beakers. Take total ammonia

measurements of the overlying water total ammonia in one composite sample of all replicates within a treatment and record. Also, take pore water quality measurements from one dummy beaker for each treatment.

2. Survival, reproduction, and growth endpoint determination

   a. Gently pour off all but 200 mL of overlying water through stacked 8- or 12-in. diameter 0.425-mm and 0.25-mm ASTM testing sieves to isolate adults and neonates. Adults are retained on the 0.425-mm sieve and neonates on the 0.25-mm sieve. Swirl and suspend sediment in the remaining overlying water for easier passing of sediment through the sieves. If using an 8-in. sieve, a 12-in. diameter sieve (1-mm mesh) can be placed over the bucket receiving the waste.

   b. Using a squirt bottle with 20 ppt seawater, transfer the material retained on the 0.425-mm sieve to a counting bowl or tray for examination.

   c. Rinse and consolidate the material on the 0.25-mm sieve, containing the neonates, and transfer it to a 1-L wide mouth glass jar. Add 70% ethanol/Rose Bengal stain solution to the jar until the original volume is doubled.

   d. Count recovered *Leptocheirus plumulosus* adults. Remove obviously dead adults and transfer to a labeled vial containing 70% ethanol and retain under chain of custody (COC). If the reburial endpoint is to be evaluated (see test-specific TCTs), transfer living adults to a labeled 1-L beaker and hold for reburial evaluation. If the reburial endpoint is not being evaluated, transfer living adults to a pan for the evaluation of the growth endpoint.

   e. Remove any neonates contained with the adults and place them into the wide mouth glass jar for that treatment/replicate. If the test includes the reburial endpoint (see test-specific TCTs), place surviving adults from each replicate into a corresponding 1-L reburial evaluation beaker and record the number added and the time. After 1 hour, make observations of the beakers and record the number of animals not buried. Remove unburied animals and record number alive and dead. After all reburial data has been collected and recorded, sieve the adults from the reburial evaluation beakers following guidance provided in steps a–d above. Record the number of adults recovered and place all surviving amphipods from each replicate on a pre-dried/pre-tared pan. Dry in oven at 60°C for 24 hours. If there is no evaluation of reburial (see test-specific TCTs), then place all surviving amphipods from each replicate on a pre-dried/pre-tared pan. Dry in oven at 60°C for 24 hours.

A.4.7 Biomass and weight endpoint quantification

Remove pans from oven and allow them to cool for 1 hour in a desiccator. Record the total weight of each pan. Retain pans with dried animals under COC.

A.4.8 Reproduction endpoint quantification

Allow the stain to interact with the organisms for at least 2 days. Rinse the preserved material with tap water through a 0.25-mm sieve and transfer the material placed in the wide mouth glass jars into an 8-in. culture bowl or counting tray and record the number of neonates recovered. Neonates should be pink in color. It may be necessary to transfer small amounts of the preserved material from the 8-in. culture bowl into a 4-in. culture bowl for observation under a dissecting microscope. Transfer preserved neonates to a 20-mL labeled scintillation vial containing 70% ethanol and retain under COC. Record the number of neonates counted on the appropriate bench sheet.

A.4.9 Acceptability

1. The test is not acceptable if any of the following occur.

    a. Less than 80% survival in the ERDC control/reference (less than 60% survival in any individual replicate) or growth not observed in a control replicate

    b. If DO dropped below 40% saturation in any treatment replicate.

A.4.10 Recording data

1. Record all specified data on the appropriate bench sheets

2. If any parameters are not within the specified range, record on the appropriate bench sheet and make a note in the comments.

Appendix References

ASTM. 2008. Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates. ASTM Method E1367-03 (Reapproved 2008).

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods.* EPA/ 600/R-94/025, Office of Research and Development, Washington, DC.

13. Northwest Fisheries Science Center General Laboratory Procedures and Practices

13.1 Introduction

The Northwest Fisheries Science Center (NWFSC) conducted exposure studies with zebrafish, southern bluefin tuna, amberjack, yellowfin tuna, mahi-mahi, and red drum to determine the cardiotoxic effects of *Deepwater Horizon* MC252 crude oil on developing fish embryos [zebrafish were exposed to either Alaska North Slope crude oil (ANSCO) or *Deepwater Horizon* MC252 crude oil]. These tests were often conducted in collaboration with other researchers. The NWFSC General Laboratory Procedures and Practices (GLPP) chapter describes the zebrafish, southern bluefin tuna, and amberjack tests, while other tests are described in the collaborator's chapters of the GLPP (see Section 13.2; *Test Organism Sources and Husbandry*).

13.2 Test Organism Sources and Husbandry

Zebrafish (*Danio rerio*)

Experiments were conducted using embryos from a zebrafish (*Danio rerio*) colony (AB strain) maintained at the NWFSC, in Seattle, Washington. All zebrafish husbandry protocols, including spawning and water quality, are described in Linbo (2009).

Southern bluefin tuna (*Thunnus maccoyii*)

Bluefin tuna embryos were obtained from the Clean Seas hatchery in Arno Bay, Australia. The embryos were collected approximately 4–8 hours after fertilization and then transported to the Lincoln Marine Science Centre in Port Lincoln, Australia, where tests were started immediately upon arrival. The husbandry techniques used by Clean Seas to spawn bluefin tuna are proprietary.

Amberjack (*Seriola lalandi*)

Amberjack embryos were obtained from the Clean Seas hatchery in Arno Bay, Australia. The embryos were then transported to the Lincoln Marine Science Centre in Port Lincoln, Australia, where tests were started immediately upon arrival.

Yellowfin tuna (*Thunnus albacares*)

See *University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS) GLPP* for information on yellowfin tuna husbandry and testing protocols.

Mahi-mahi (*Coryphaena hippurus*)

See RSMAS GLPP for information on mahi-mahi husbandry and testing protocols.

Red drum (*Sciaenops ocellatus*)

See *Stratus Consulting Red Drum and Speckled Seatrout GLPP* for information on red drum husbandry and testing protocols.

13.3 Exposure Media Preparations

For testing protocols included in the NWFSC GLPP, exposure media were prepared according to established protocols with slight modifications. See *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Water accommodated fractions (WAFs) were made using ANSCO and the four *Deepwater Horizon* oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A). The slight modifications included the following:

- Rather than using a gastight syringe, a Rainin positive displacement pipette with positive displacement tips was used to transfer the liquid oil samples.
- For tests that used the Slick A and B oils, a small amount of the viscous oil was transferred to an amber vial that was then heated in a 65°C water bath for approximately 8 minutes, reducing the viscosity to allow the use of the Rainin positive displacement pipette. This oil was pipetted onto the surface of the blade in a three-speed commercial food blender with a 1-gal stainless steel container, before water was added to mix the oil into the water column.
- For tests in which the high-energy WAFs (HEWAFs) were filtered, the exposure media was put through a 2.7-µm glass fiber filter, followed by a 0.7-µm glass fiber filter.
- The water used in all zebrafish exposure studies was prepared with 1-ppt Instant Ocean Salt mixed with reverse-osmosis deionized treated water. The water used in the southern bluefin tuna and amberjack exposure studies was natural seawater obtained from the Clean Seas hatchery; the water matched the water quality parameters of the seawater in

which the embryos were spawned. The techniques used to treat the natural seawater were conducted by Clean Seas and are proprietary.

13.4 Water Quality Monitoring

Water quality was not measured for zebrafish tests. For other tests, water quality measurements were completed following the guideline in the QAPP and the *Hopkins Marine Station of Stanford University and NWFSC GLPP*.

13.5 Analytical Chemistry

For zebrafish tests, analyses of polycyclic aromatic hydrocarbons (PAHs) and other compounds were conducted by NWFSC (Sloan et al., 2014). For all other tests, analyses of PAHs and other compounds were conducted by ALS Environmental, with some samples analyzed at NWFSC. For the collection procedure, see the QAPP.

13.6 Reporting and Testing Documentation

For zebrafish tests, results were recorded in laboratory notebooks and on Excel spreadsheets. Images, videos, and all electronic data were stored on secure hard drives. For all other tests, reporting of the data followed procedures described in the QAPP.

13.7 General Testing Standard Operating Procedures

13.7.1 Preparation of HEWAF for NWFSC GLPP testing protocols

- Glassware/mixer decontamination procedure

Zebrafish tests

Rinse all glassware and HEWAF mixer components with three rinses of acetone, followed by three rinses of dichloromethane (DCM) between each preparation. Allow sufficient time for full evaporation of final solvent rinse. Inspect the inside parts of the blender and wipe down the blades with solvent-soaked Kimwipes®. Use gloved hands throughout all preparation steps, and use common-sense laboratory safety for handling solvents, especially DCM. Refer to appropriate material safety data sheets if necessary.

Other tests

Decontaminate glassware following the *Decontamination SOP* found in the QAPP.

- Preparation of oil HEWAF for Alaska North Slope Crude and *Deepwater Horizon* source and artificially weathered source oils

  1. Measure appropriate volume of water into pre-cleaned Waring blender (1 or 2 L).
  2. Measure desired volume of oil. These oil samples have a low enough viscosity so that a Rainin Displacement Pipette can measure out volumes effectively.
  3. Common dilution of oil: 1:10,000 = 100 µL oil into 1 L of water.
  4. Seal blender with aluminum-wrapped lid.
  5. Blend for 30 seconds on low.
  6. Transfer to glass separatory funnel and allow to settle for 1 hour.

- Preparation of oil HEWAF for *Deepwater Horizon* Slick A and Slick B oils

  1. Warm an aliquot of the oil at 65°C in a water bath for 8 minutes to decrease the viscosity.
  2. Measure the desired volume of oil using the Rainin Displacement Pipette. Dispense oil directly onto blades of pre-cleaned Waring blender.
  3. Add the appropriate volume of water into blender (1 or 2 L).
  4. Seal blender with aluminum-wrapped lid.
  5. Blend for 2 minutes on low.
  6. Check the oil solution; if the oil did not blend well, scrape down the sides of the blender with metal spatula and reblend for 1 or 2 more minutes.
  7. Transfer to glass separatory funnel and allow to settle for 1 hour.

- Filtering of HEWAF

  1. If a filtered HEWAF is required, set up filter apparatus.

  2. While running at low vacuum, drain the bottom layer of the settled HEWAF from the separatory funnel through the filtration apparatus, which should be fitted with a Whatman GF/D filter (0.27 mm). Do not drain the top ~ 100 mL of the HEWAF.

  3. Remove the filter apparatus and replace the dirty filter with a Whatman GF/F filter (0.7 mm). Pour first filtrate through the second filter using the low vacuum.

13.7.2 General morphological analysis of fish embryos and larvae

1. Assess gross morphological defects by inspecting images and videos captured for each animal. Include an overall assessment of all visible organs, including but not limited to, eye, brain, otic capsule, gastrointestinal tract, urinary pore, notochord, myotomes, finfolds, pigment, lateral line neuromasts. Only record abnormalities (i.e., the absence of a score for a particular structure indicates there is no evidence of a defect).

2. Record axial defects (dorsal or upward curvature of the trunk/tail) and tail finfold defects (reduction, irregularities, blisters) based on a comparison to control animals. Only record abnormalities (i.e., the absence of a score for a particular structure indicates there is no deviation from the controls).

13.7.3 Viewing video files for cardiac endpoint measurements

1. Open the ".MOV" or ".JPG" file in ImageJ (download for free at http://rsbweb.nih.gov/ij/download.html; on a Mac computer, drag and drop the filename onto an alias of ImageJ).

2. If it is a ".MOV" file, use "virtual stack" or convert the file to 8-bit grayscale; if it is a ".JPG," proceed to Step 3.

3. Under "Analyze" on the menu bar, select "Set Measurements." In the pop-up box, check the "Display Label" and "Area" box (deselect any other checked boxes). Type "3" into the box following "Decimal Places."

4. Confirm that the resolution of the file located in the upper left corner of the window is 800 x 600 pixels (red drum) or 640 x 480 pixels (mahi-mahi and amberjack); record the resolution of the file located in the upper left corner of the picture. For a ".JPG" file, record the resolution of the file located in the upper left corner of the picture.

5. Continue to the protocol for specific endpoint to be measured.

13.7.4 Heart rate, rhythm, and heart rate variability measurements: Blue fin tuna, yellowfin tuna, and amberjack larvae

1. Open and prepare the video clip according to *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Play the entire video at half-speed, counting the number of heart beats that occur during the video.

3. Calculate the heart rate in beats per minute.

4. For arrhythmia measurements, scroll slowly through the video, counting the number of individual video frames that encompass the onset of contraction (systole phase) and relaxation (diastole phase) for cardiac chambers. Using the number of individual frames, calculate the mean duration of the systole and phase diastole phase for each fish. The duration of the systole and the diastole phase cannot be determined for yellowfin tuna because of their higher heart rates.

5. For heart rate variability (i.e., beat-to-beat variability), count the number of video frames between the initiation of contractions for an entire video clip (Incardona et al., 2009).

13.7.5 Atrial contraction measurements – Red drum

1. Open and prepare video clips according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Scroll through the video. Watch the walls of the ventricle closest to the jaw line (towards where the blood would leave the ventricle) through a couple of heartbeats. Look for indentations or distinguishing characteristics that are visible both during contraction (systolic) and relaxation (diastolic). Pause the video when the atrium is fully contracted and the ventricle is fully dilated/relaxed. Use the line tool to draw a line between the outer edges of the ventricle, trying to keep as perpendicular to the ventricle walls as possible. Keep the line as close to the jaw line as possible (Figure 13.1).

4. Select "Measure" under "Analyze" in the menu bar. This is the diastolic measurement.

5. Continue scrolling through the video until the ventricle fully contracts and the atrium is relaxed. At full contraction, the entire length of the atrium forms a chute, with approximately the same diameter throughout. Using the same distinguishing characteristics of the ventricle walls that were noted in Step 3, draw a line between the outer edges of the ventricle. The resulting line should still be roughly perpendicular to the ventricle walls.

6. Select "Measure" under "Analyze" in the menu bar. This is the systolic measurement.

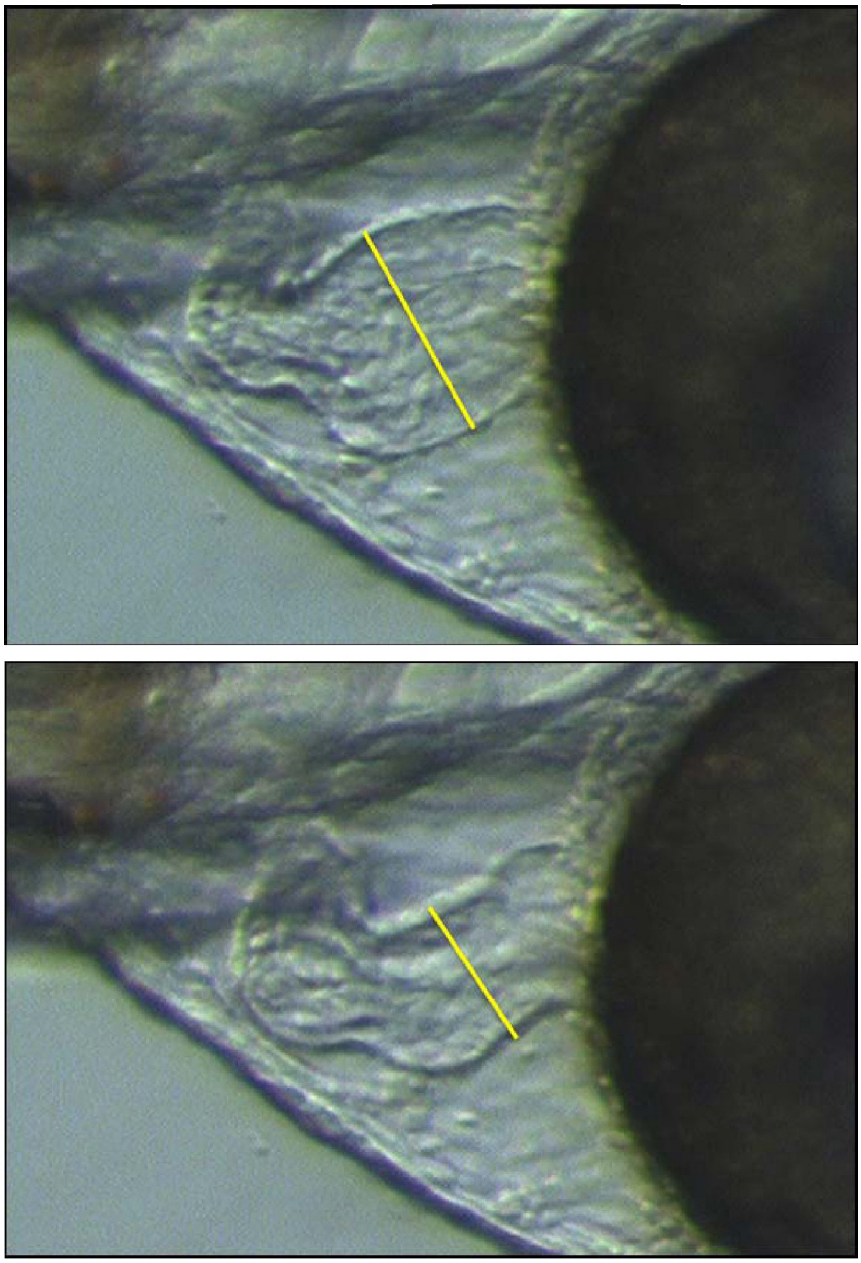

Figure 13.1. Images showing full atrium (top) and contracted atrium (bottom) for diastolic and systolic measurements.

7. Repeat Steps 3 through 6 two more times and save the three sets of measurements for each fish.

   NOTE: Sometimes the atrium is difficult to measure if the orientation of the heart is incorrect or the video is out of focus. In these cases, do not take a measurement.

8. From your three sets of diastolic/systolic measurements, calculate the mean diastolic and the mean systolic measurements for each fish. Then use these means to calculate the percent atrial contractility ((diastolic-systolic)/diastolic * 100) for the fish.

   NOTE: If the first three sets of diastolic and systolic measures differ by greater than 10%, repeat measuring until the values are consistently within 10% of each other. The mean value should be taken from the first three measures within 10%.

9. Take notes throughout the process when necessary.

10. See specific testing protocols and test-specific test conditions tables (TCTs) for number of larvae collected per replicate for each measurement.

13.7.6 Ventricular contraction measurements – Red drum

1. Open and prepare video clip according to *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Scroll through the video. Watch the walls of the ventricle closest to the jaw line (towards where the blood would leave the ventricle) through a couple of heartbeats. Look for indentations or distinguishing characteristics that are visible both during contraction (systolic) and relaxation (diastolic). Pause the video when the atrium is fully contracted and the ventricle is fully dilated/relaxed. See Figures 13.2 and 13.3 for diastolic and systolic measurements, respectively.

4. Use the line tool to draw a line between the outer edges of the ventricle, trying to keep as perpendicular to the ventricle walls as possible. Keep the line as close to the jaw line as possible.

5. Select "Measure" under "Analyze" in the menu bar. This is the diastolic measurement.

6. Continue scrolling through the video until the ventricle fully contracts and the atrium is relaxed.

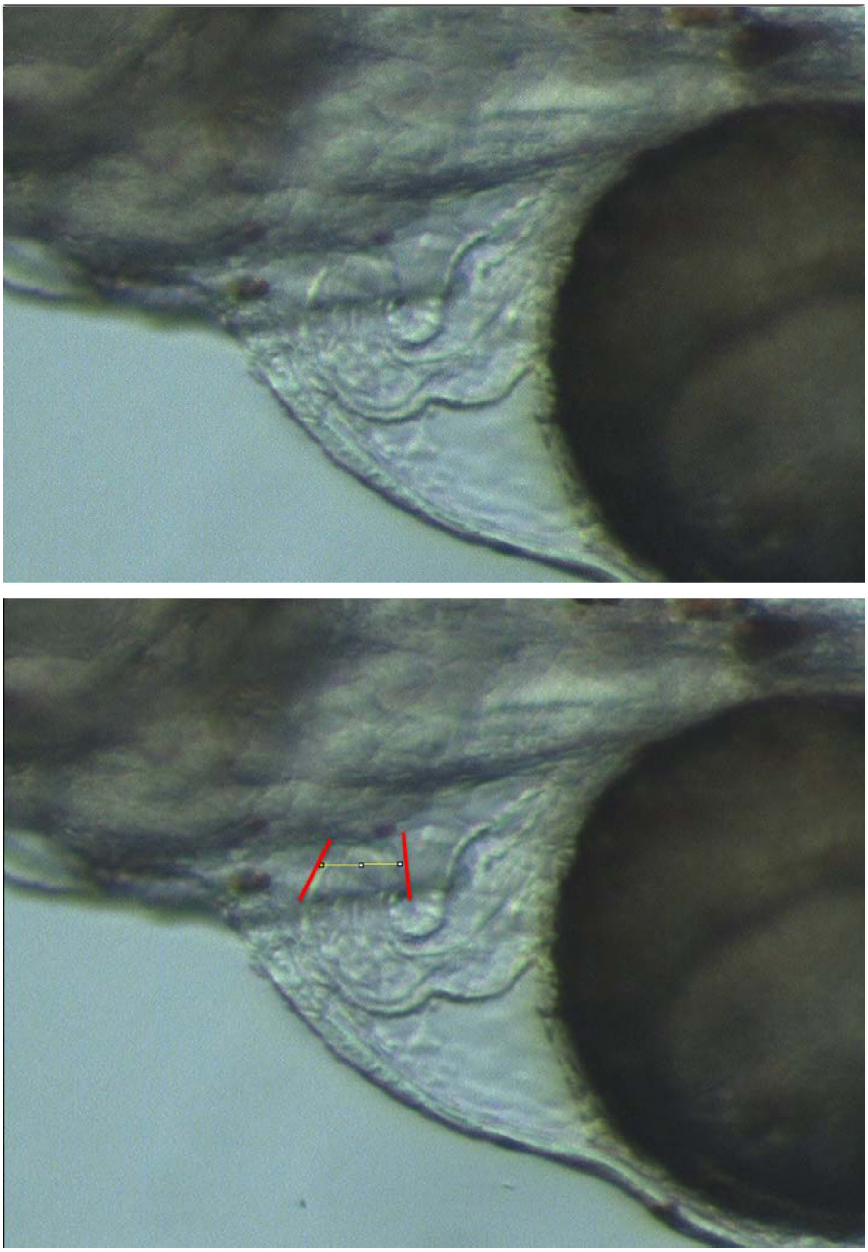

Figure 13.2. Images showing diastolic measurement. Scroll through video until the atrium is fully contracted and the ventricle is relaxed. Draw a line between the outer edges of the ventricle, trying to stay as perpendicular to the ventricle walls as possible. Red lines highlight the angle at which the diastolic measurement line meets ventricle walls.

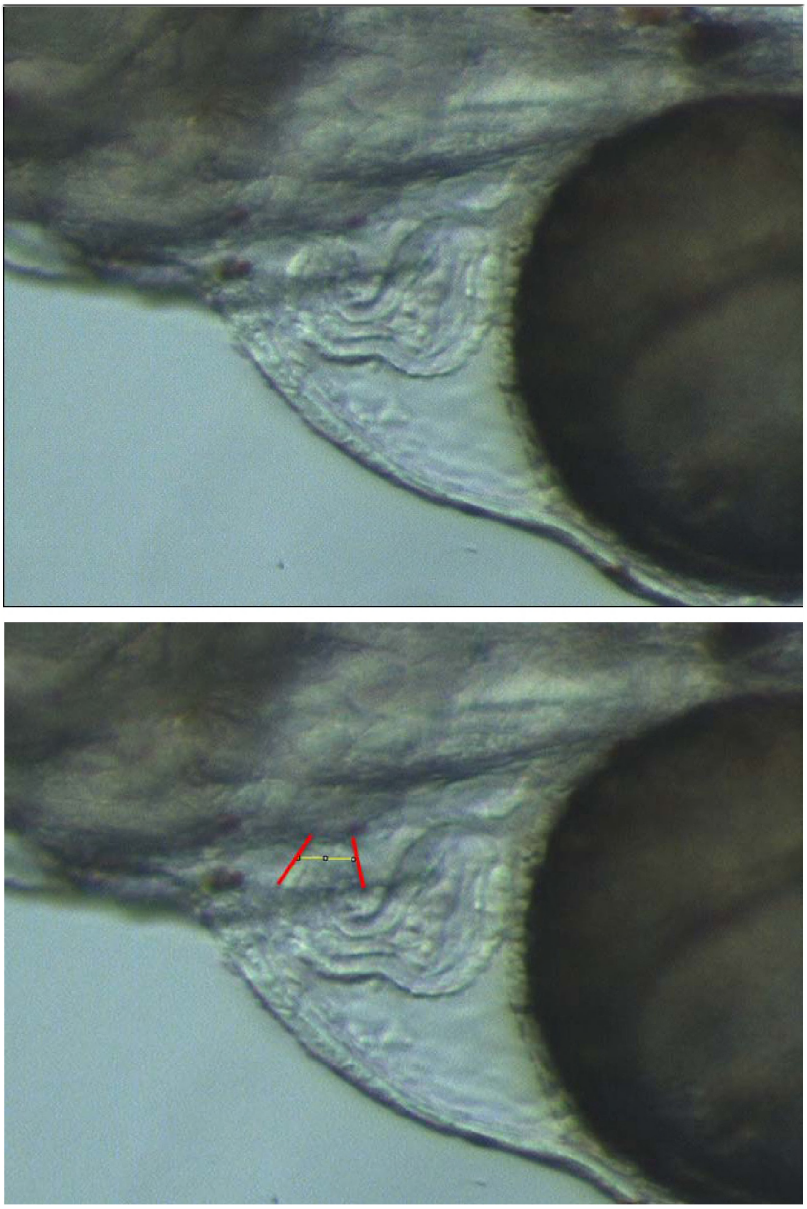

Figure 13.3. Images showing systolic measurement. Scroll through video until the ventricle is fully contracted and the atrium is relaxed. Draw a line between the outer edges of the ventricle, trying to stay as perpendicular to the ventricle walls as possible. Red lines highlight the angle at which the systolic measurement line meets ventricle walls.

7. Using the same distinguishing characteristics of the ventricle walls that were noted in Step 3, draw a line between the outer edges of the ventricle. The resulting line should still be roughly perpendicular to the ventricle walls.

8. Select "Measure" under "Analyze" in the menu bar. This is the systolic measurement.

9. Repeat Steps 3 through 8 two more times and save the three sets of measurements for each fish.

   NOTE: Sometimes pigment or the atrium may obstruct the ventricle wall. In these cases, do not take a measurement for the fish.

10. Transfer measurements (three sets of diastolic and systolic measurements for each fish) to an Excel spreadsheet. For each fish, calculate the percent ventricular contractility using the following equation: (diastolic-systolic)/diastolic * 100).

    NOTE: If the first three sets of diastolic and systolic measurements differ by greater than 10%, repeat the measures until measurements are consistently within 10% of each other. The mean value is taken from the first three measures within 10% of each other.

11. Calculate the means of the diastolic and systolic measurements for each fish. Use the individual fish median to calculate the mean ventricular contraction for a replicate beaker (equation in Step 10).

12. Take notes throughout the process, if necessary.

13. See specific testing protocols or test-specific TCTs for the number of larvae collected per replicate for each measurement.

13.7.7 Pericardial area measurements (as defined in Incardona et al., 2014 and Incardona and Scholz, 2015)

1. Open and prepare video clip or photo according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the free draw tool from the tool bar.

3. Outline the pericardial sac. This area will change, sometimes drastically, depending on the presence or absence of edema and on the severity of edema. For the analyses of red drum images, note that the area of the pericardial sac may exceed the visual field in severely edematous larvae. Make a note of the excluded area with appropriate label

options: "Ventral Cutoff," "Dorsal Cutoff," or "Ventral/Dorsal Cutoff." For mahi-mahi and amberjack analyses, no areas should extend past the oil droplet present at the posterior end of the yolk sac.

a. ***In a fish without edema,*** the outline typically encloses the area anterior to the yolk sac and ventral to the lower jaw (see Figures 13.4 and 13.5).

b. ***In a fish with edema,*** the outline encloses a fluid-filled area that can be seen pushing against the yolk sac. For red drum analyses, sometimes the yolk sac is pushed outside the frame of the picture. Also note that in some red drum, the pericardial sac may curve under the yolk and out of visual range, in which case the label "Ventral Cutoff" should be applied (Figure 13.4). For mahi-mahi and amberjack, only, draw outlines within the boundaries of the yolk sac if a sharp, angular, defined, dark line is present pushing against the yolk sac (Figure 13.5).

4. After the area has been drawn on the image, select "Measure" under "Analyze" in the menu bar.

5. After all measurements have been taken, transfer data to an Excel spreadsheet.

6. Remember to take succinct notes throughout the process, if necessary (e.g., "blurry" for an out-of-focus image).

7. See specific testing protocols or test-specific TCTs for number of larvae collected per replicate for each measurement.

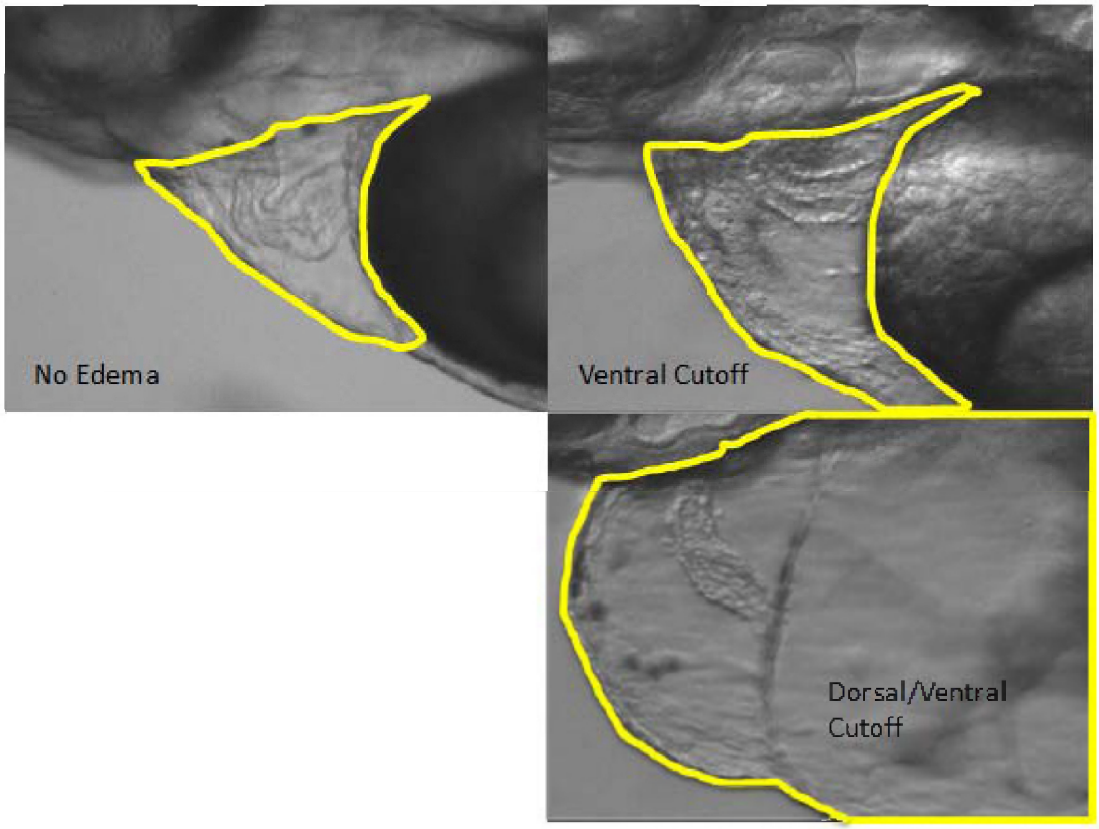

Figure 13.4. Images showing examples the pericardial measurement of red drum with no edema (upper left) and fish with two different severities of edema (upper and lower right).

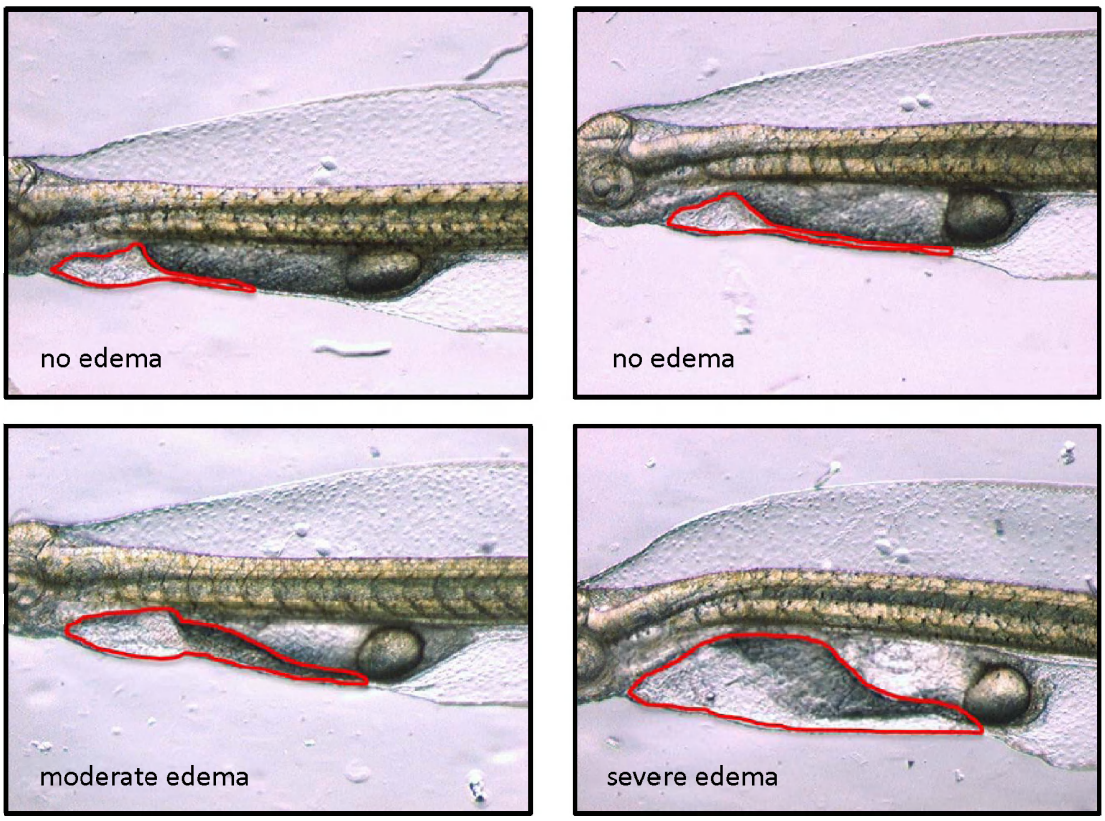

Figure 13.5. Images showing examples the pericardial measurement of mahi-mahi with no edema (upper row) and fish with two different severities of edema (lower row).

13.7.8 Atrioventricular angle measurements of red drum larvae

1. Open and prepare the video clips according to *the Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the angle tool from the tool bar.

3. Scroll through the video and pause the video when the following conditions have been met: the atrium is full, a narrow chute has formed through the ventricle, and the heart has pinched between the atrium and ventricle. For consistency, use the frame where the

atrium is at its fullest right before it starts to contract. Scroll until the atrium just starts to contract and reverse one frame (Figure 13.6).

4. For red drum, start the line by clicking on the outer dorsal edge of the heart, where the ventricle and bulbus arteriosus meet at a pinch. Complete the first segment of line by clicking on the outer dorsal edge of the heart where the AV node is located. Finally, create the second segment of line (and therefore an angle between the two lines) by clicking on the outer dorsal edge of the heart where the atrium and sinus venosus meet (Figures 13.7 and 13.8). For mahi-mahi, draw a line through the center of the ventricle, starting at the anterior end of the ventricle and moving toward the AV node. Stop drawing your line and VISUALIZE an additional line being drawn through the center of the atrium (moving from posterior toward anterior). Now with the visualized atrium line in mind, continue the line that you are drawing through the ventricle by extending it until it would meet the visualized line (even if it extends beyond the wall of the ventricle). At this point, change directions (create the angle) and extend the line through the lumen of the atrium.

   NOTE: If the fish's head is angled downward relative to the viewer, then the measured AV angle will decrease. An indication of this is when the atrium overlaps the ventricle. In Figure 13.8, an example is shown. The yellow outlines the ventricle and the red outlines the anterodorsal edge of the atrium. In this case, the fish cannot be used for the AV angle measurement.

5. Select "Measure" under "Analyze" in the menu bar.

6. Repeat Steps 2 through 4 three times and save the mean value.

   NOTE: If the first three measurements differ by greater than 10%, repeat the measures until the values are consistently within 10% of each other. Take the mean value from the first three measurements that are within 10% of one another.

7. After all measurements have been taken, transfer data to an Excel spreadsheet.

8. Calculate the mean AV angle measurements for each fish.

9. Remember to take notes throughout the process if necessary.

10. See specific testing protocols or test-specific TCTs for number of larvae collected per replicate for each measurement.

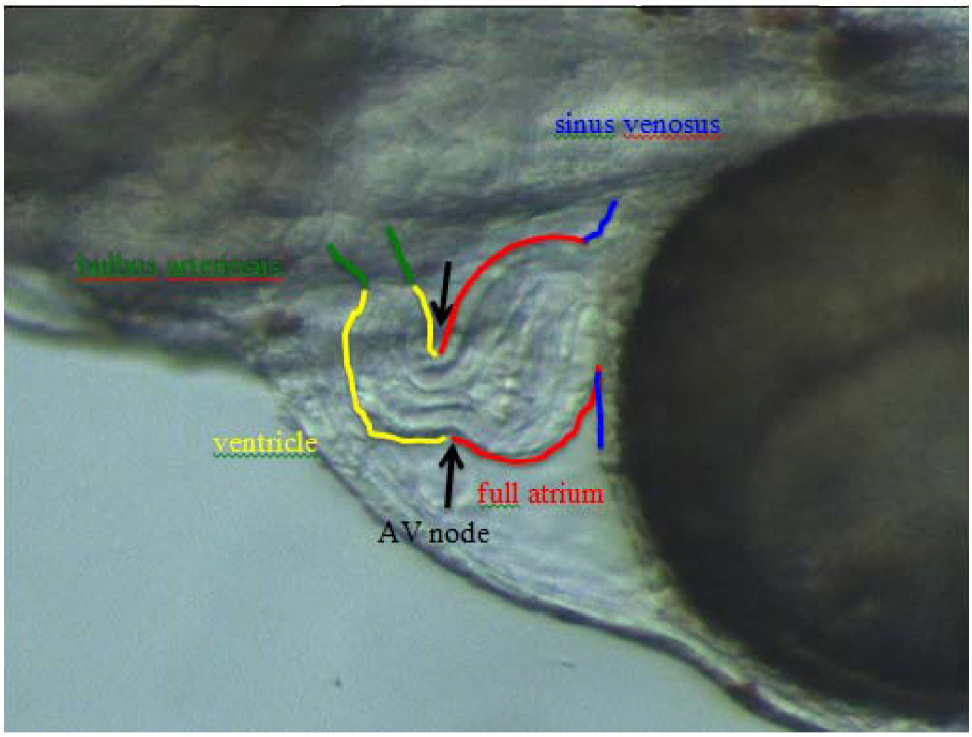

Figure 13.6. Image showing an example frame where the atrium is full, a narrow chute has formed through the ventricle, and the heart has pinched between the atrium and ventricle. This image was obtained by scrolling through the video until the atrium just started to contract and then reversing one frame.

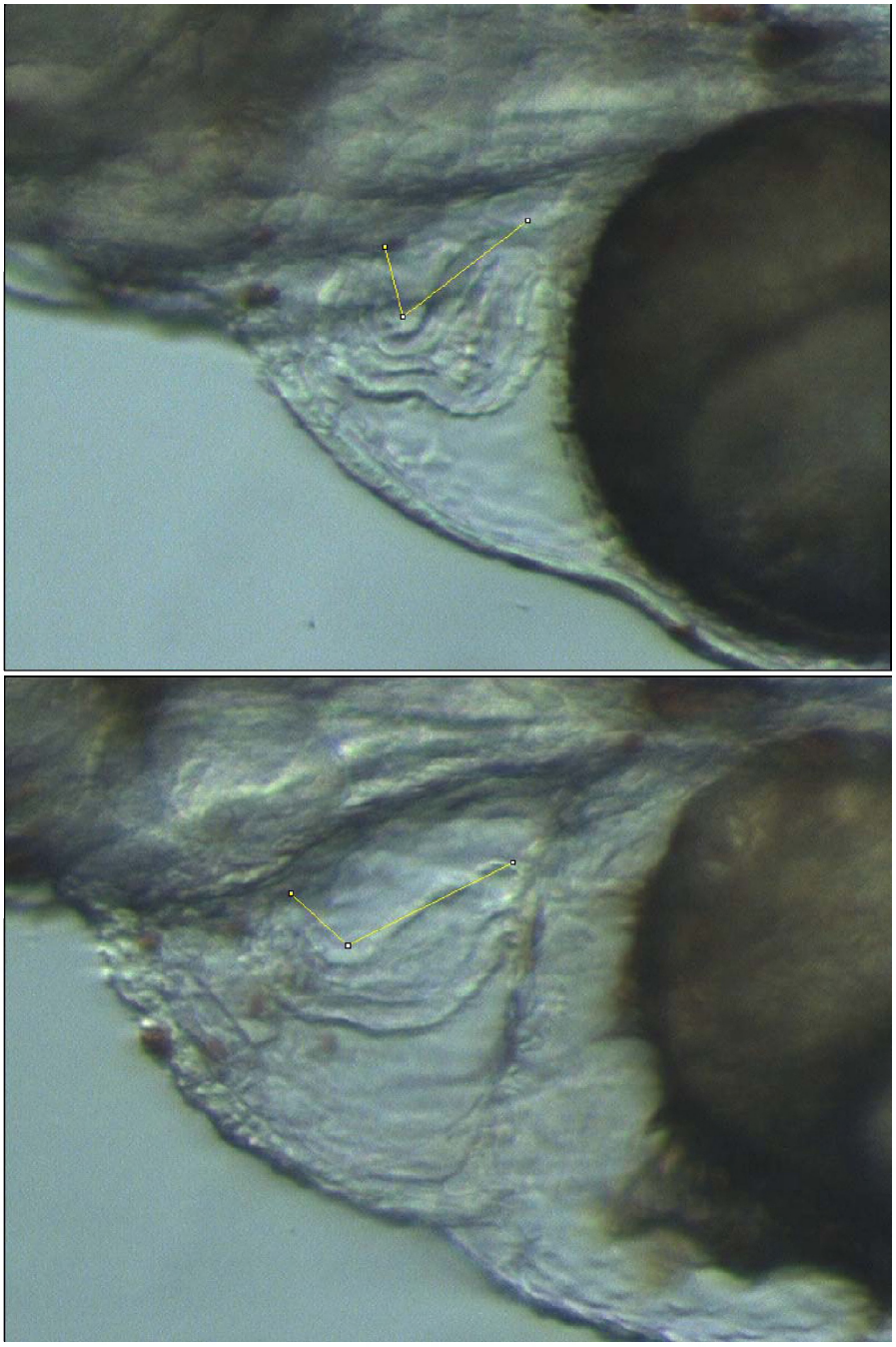

Figure 13.7. Example images of the AV measurement.

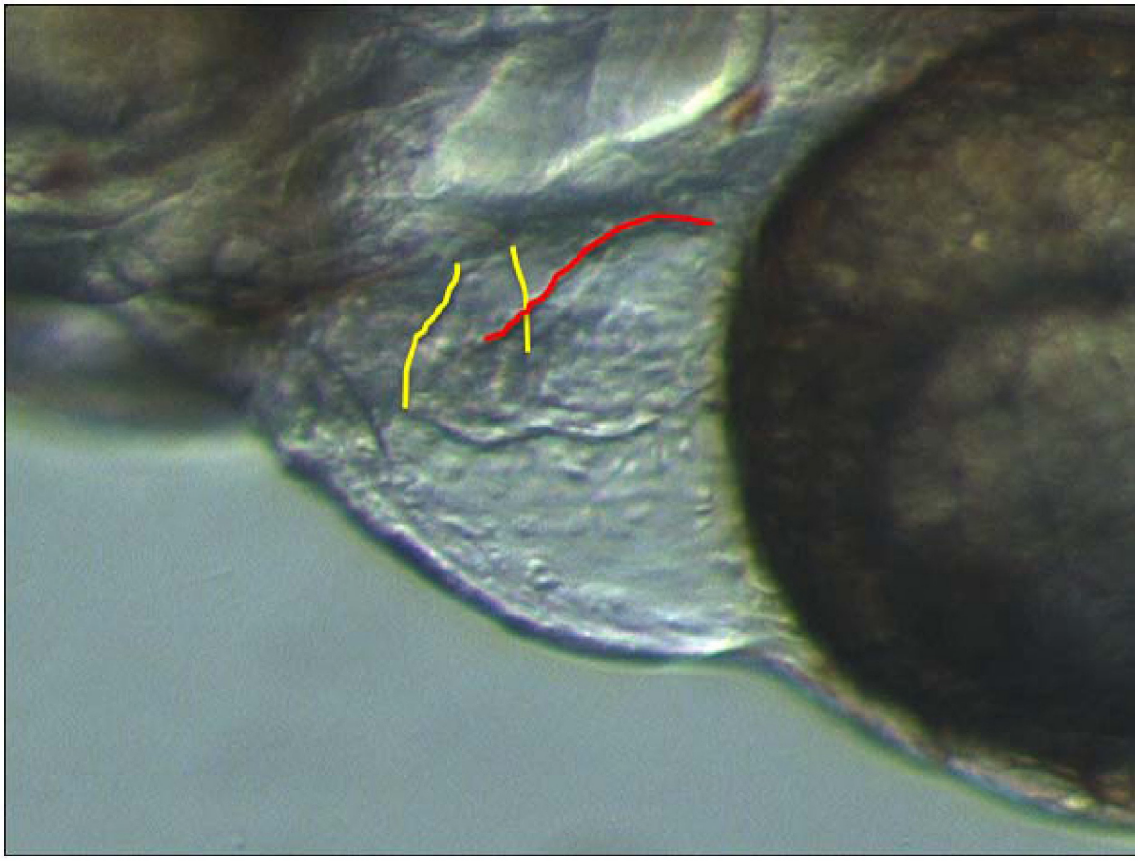

Figure 13.8. Example image where fish is tilted in video and thus AV measurement from the video is not possible because the ventricle and atrium overlap, biasing the measurement. Yellow lines show the outline of the ventricle and the red line outlines the anterodorsal edge of the atrium to show overlap.

13.7.9 Identifying Presence or Absence of Edema (as defined in Incardona et al., 2014 and Incardona and Scholz, 2015)

1. Open and prepare the video clip according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Examine the shape of the yolk sac and pericardial area.

3. Record "edema" or "no edema" based on the definitions below.

Record "no edema" if the anterior portion of the yolk sac is smooth and rounded with a bullet-shaped tip and if there are no obvious indentations on the yolk sac because of pressure from fluid buildup in the pericardial area. See examples below.

Record "edema" if the anterior portion of the yolk sac is concave or pushed to a sharp point or if indentations indicated by dark, angular lines are seen pushing on the yolk sac because of pressure from fluid buildup in the pericardial area (Figure 13.8).

Note: There is a range of yolk sac shapes for normal (non-edema) fish. Sometimes, the yolk sac does not have a perfect rounded, bullet shape, but the fish is still within the range of normal. The rounded, bullet shape can be a bit blunt or can come to a semi-point and still be considered normal (Figure 13.9).

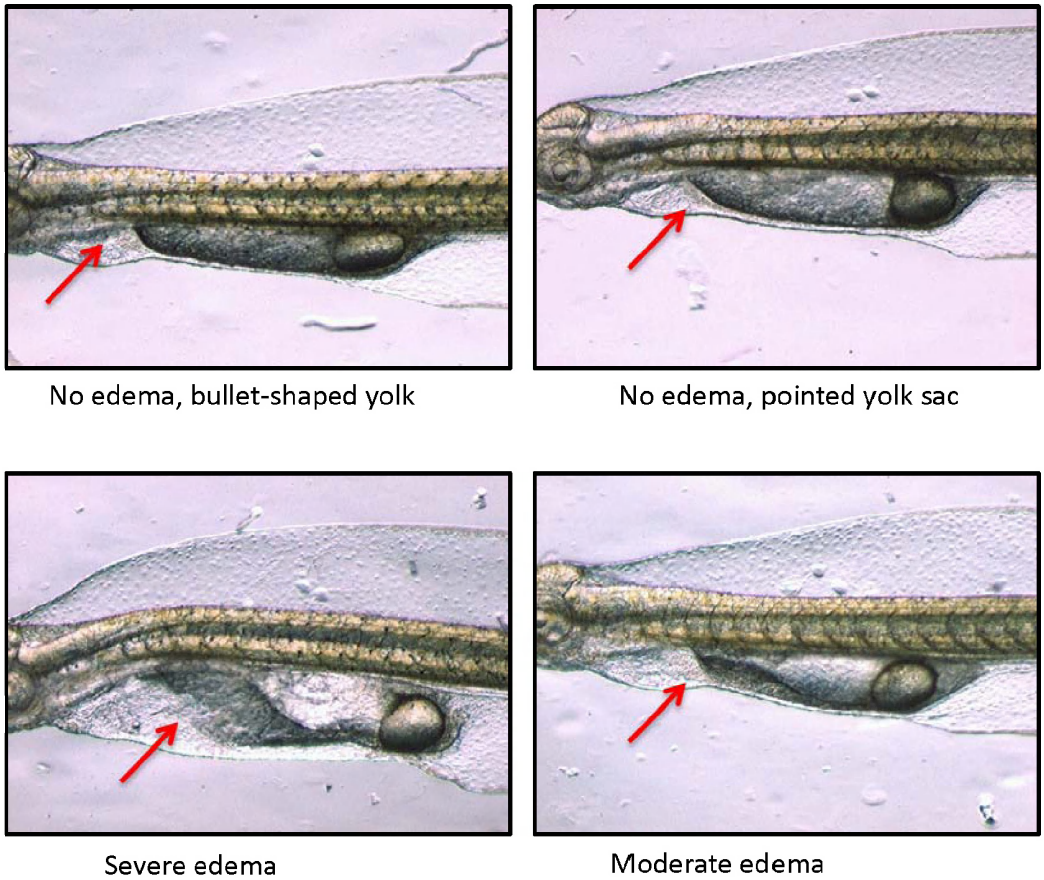

Figure 13.9. Example images of mahi-mahi fish with and without edema.

13.7.10 Atrial yolk mass gap

1. Open and prepare video clip according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Play the video and pause the video when a line becomes visible at the posterior edge of the atrium where it connects to a wall of blood/fluid anterior to the yolk sac. This is typically when the posterior part of the atrium is in contraction.

4. Measure the thickness of this wall of blood/fluid using the line tool. To be consistent, start the line where the atrium meets the wall, centered between the dorsal and ventral sides of the atrium. Draw your line through the wall of blood/fluid until it meets the yolk sac. See examples below (Figure 13.10).

5. Select "Measure" under "Analyze" in the menu bar.

6. Repeat Steps 4 through 8 three times and save the mean value.

   NOTE: If the three measurements differ by greater than 10%, repeat the measures until measurements are consistently within 10% of each other. The mean value is taken from the first three measures within 10% of one another.

7. After all measurements have been taken, transfer data to an Excel spreadsheet.

8. Remember to take notes throughout the process if necessary.

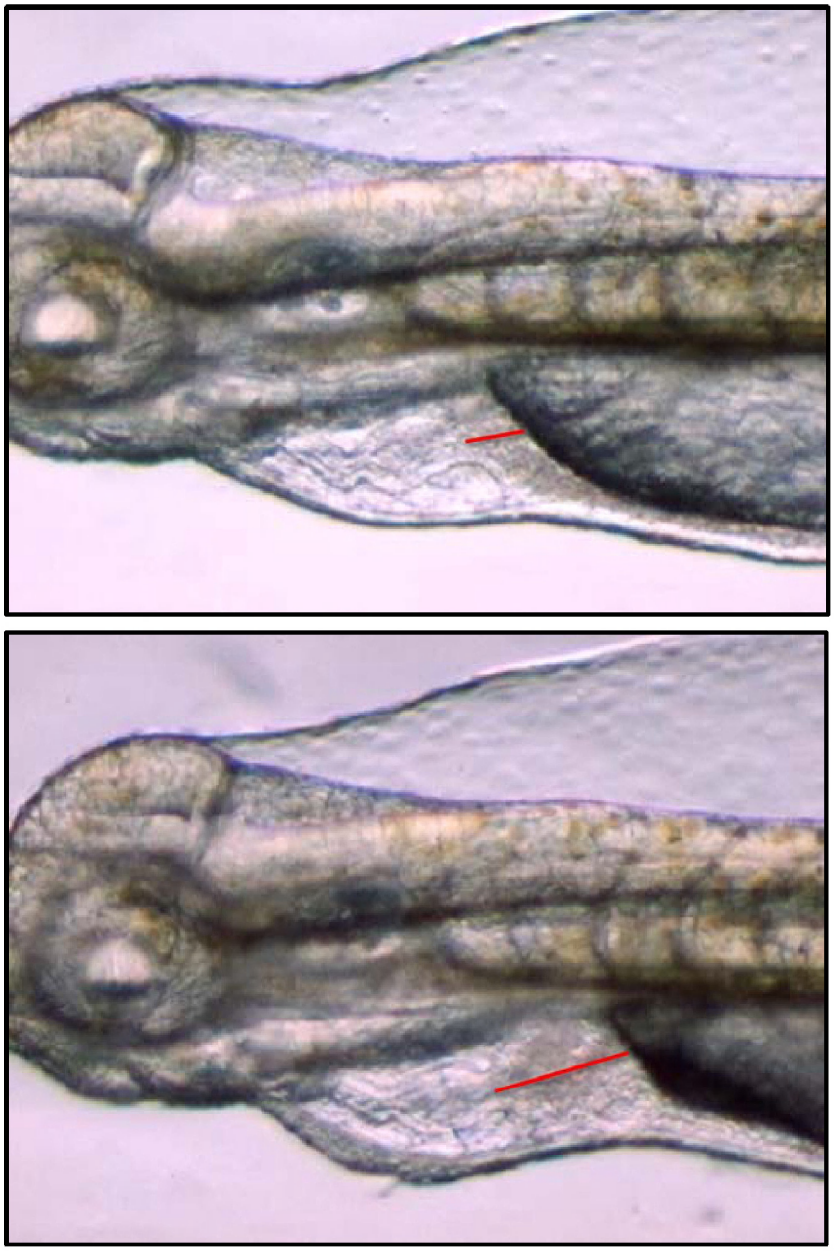

Figure 13.10. Example images of atrial-yolk mass gap measurement. The red lines measure the width of the edema space located posterior to the atrium and anterior to the yolk sac. The video should be paused when the most posterior part of the atrium is contracting.

13.7.11 Molecular assessment of crude oil cardiotoxicity in larval fish

Total RNA extraction

1. To clean homogenizer between samples, prepare three Eppendorf tubes (1.5–2 mL) for each sample, one with 1 mL of RNaseZap® Solution (Ambion, USA) and two with 1 mL of nuclease-free water.

2. Clean homogenizer (e.g., Ultra-Turrax T8; Kia, USA) by running at high speed in RNaseZap® Solution and 2x water washes for 10 seconds each. Dry with a Kimwipe between washes.

3. Remove sample tube from -80°C freezer, weigh, and record weight in grams (e.g., 1.722g).

4. Add 800 µL of TRIzol® (Invitrogen, USA) or TRI-Reagent® (Sigma, USA) to cryovial and homogenize for 10 seconds. Thoroughly clean homogenizer between samples (see Steps 1 and 2 above).

5. For samples snap-frozen in 2-mL Eppendorff SafeLock® tubes: add stainless steel bead (Qiagen) and 800 µL of TRIzol® or TRI-Reagent®. Homogenize using TissueLyser® (Invitrogen).

6. Following homogenization, let the samples stand at room temperature for ≥ 5 minutes (up to 6 hrs).

7. Add 80 µL of BCP (1-Bromo-3-Chloropropane; Sigma-Aldrich, USA) to each sample, shake vigorously for 15 sec, incubate at room temp for 3 min, and then spin: 12,000 g at 4°C for 15 min.

8. During the centrifugation step, label one 1.5-mL Eppendorff tube per sample and place the tube on ice.

9. Remove one sample at a time from chilled 4°C centrifuge, taking care to not disturb phase-separated solution, and transfer the supernatant (~ 500 µL) to a chilled 1.5-mL Eppendorff tube via pipette (e.g., Rainin P200). Avoid touching the interphase; leave a small visible layer of supernatant above the interphase.

10. Return the labeled Eppendorff tube containing the supernatant (RNA) to ice; proceed to the next sample.

11. Purify and on-column the DNAse supernatant (i.e., total RNA) using a Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA), per the manufacturer's instructions, as follows:

    a. Mix an equal volume of supernatant with absolute ethanol and vortex
    b. Load 700 µL of mixture onto column and spin ("soft"): 16,000 g for 1 min
    c. Repeat to finish loading all of the supernatant/ethanol mixture
    d. Add 400 µL of RNA Wash Buffer and spin: 16,000 g for 1 min
    e. Add 80 µL of DNAse master mix directly to the column

        i. DNAse I = 5 µL per sample
        ii. 10x DNAse I Reaction Buffer = 8 µL per sample
        iii. Nuclease-Free Water = 3 µL per sample
        iv. RNA Wash Buffer = 64 µL per sample

    f. Incubate at 37°C on a heat block for 15 min and then spin at 16,000 g for 30 sec
    g. Add 400 µL of RNA PreWash to the column and spin (16,000 g for 1 min); empty the effluent
    h. Repeat: add 400 µL of PreWash and spin (16,000 g for 1 min); empty the effluent
    i. Add 700 µL RNA Wash Buffer and spin (16,000 g for 1 min); empty the effluent
    j. Spin the column for an additional 2 min at 16,000 g to dry
    k. Transfer the column to a new, labeled, RNAse-Free tube
    l. Add 26 µL directly to the column (1 tip per sample)
    m. Incubate at room temperature for 1 min, then spin ("soft") at 16,000 g for 1 min
    n. Remove the column and save until RNA quantity is verified
    o. NOTE: Alternative DNAse treatment is available.

12. Quantify all samples using NanoDrop® 1000 (Invitrogen, USA)

    a. Record quantity (ng/µL) and purity ratios (260/280 nM and 260/230 nM)

13. Begin cDNA synthesis or store RNA (25 µL) on ice overnight in a refrigerator at 4°C.

cDNA Synthesis

Option 1 (High-Capacity RNA-to-cDNA Kit):

1. Prepare cDNA synthesis reaction ON ICE.

2. Calculate the RNA volume need for 2,000 ng per sample (i.e., 2000/[RNA concentration]).

a. NOTE: cDNA synthesis can be performed using lower RNA input (e.g., 200 ng or 50 ng), depending on the amount of RNA available (i.e., individual embryo extractions generally permit 50–100 ng RNA input).

b. All calculations below are based on 2,000 ng per sample input, which results in a final cDNA concentration of [100 ng/µL].

c. All calculations below can be modified according to the RNA input used (experiment specific) by adjusting the stock cDNA concentration accordingly (e.g., if only 50 ng of RNA was used, then the stock cDNA is [2.5 ng/µL].

   i. NOTE: Buffering cDNA with tRNA is not done for individual embryo cDNA because the stock concentration (or 1:2) is required for qPCR.

3. Calculate the water volume needed for a final volume of 10 µL (i.e., 10 µL – (2000/[RNA])); the reaction can be scaled to 2x (e.g., final volume of RNA + water = 20 µL).

4. Calculate the volume of master mix needed for the desired number of reactions as follows:

   a. 10x Buffer: 1x = 2 µL (2x = 4 µL)
   b. 10x Random Primers: 1x = 2 µL (2x = 4 µL)
   c. 25x dNTP Mix: 1x = 0.8 µL (2x = 1.6 µL)
   d. Reverse Transcriptase (RT): 1x = 1 µL (2x = 2 µL)
   e. RNAse-Free Water: 1x = 4.2 µL (2x = 8.4 µL).

5. In strip-tubes or a 96-well plate, combine the RNA and water to a final volume of 10 µL for 1x or 20 µL for 2x reaction size.

6. Add 10 µL of master mix to each well for 1x reaction or 20 µL master mix to each well for 2x reaction.

7. For negativeRT (–RT) control, combine 0.2 µL of each sample and add all master mix constituents, except RT enzyme. NOTE: be sure to add water to make up the appropriate volume.

8. Seal, vortex, and spin down.

9. Run on a standard thermal cycler according to manufacturer's conditions:

    a. 25°C for 10 min
    b. 37°C for 120 min
    c. 85°C for 5 min
    d. 4°C bath.

Option 2 (SuperScriptIII Kit):

1. Use a SuperScriptIII Reverse Transcription® Kit (Invitrogen, USA) with oligo dT_{20} primers to synthesize [100 ng/µL] complementary DNA (cDNA), per manufacturer's instructions.

2. Use post-DNAse quantifications (ng/µL) to calculate the RNA and water volumes required:

    a. For half reaction (10 µL):

        i. RNA volume (µL) = 1000 ng / sample quantity (ng/µL)
        ii. Water volume (µL) = 3 µL – X µL RNA
        iii. 0.5 µL Annealing Buffer + 0.5 µL oligo dT_{20} + 5 µL 2x Buffer + 1 µL SSIII.

    b. For full reaction (20 µL):

        i. RNA volume (µL) = 2000 ng / sample quantity (ng/µL)
        ii. Water volume (µL) = 6 µL – X µL RNA
        iii. 1 µL Annealing Buffer + 1 µL oligo dT_{20} + 10 µL 2x Buffer + 2 µL SSIII.

    c. NOTE: Reaction can be scaled to 2x.

3. For –RT control, combine 0.2 µL of each sample and add all constituents except SSIII.

4. Store strip-tubes or 96-well plate at 4°C overnight (in cycler or on ice in fridge).

Standard curve generation

1. Determine the volume required to pool for "standards" (e.g., 100/10/1/0.1 ng or 20/10/5/2.5 ng):

    a. Calculate the total µL needed to run a total number of target genes (e.g., 15 genes)

        i. 2 µL per replicate x 2 technical replicates = 4 µL of each standard (STD) needed per gene

ii. 4 µL per STD for 15 genes = 60 µL needed for each STD (30 µL per duplicate well)

iii. Add the volume needed for serial dilution, plus ~10% for "slop:"

1. Log_{10} requires 7 µL be taken for each serial dilution, so make 70 µL per STD

2. Log_2 requires 60 µL be taken for each serial dilution, so make 130 µL per STD

iv. Multiply the total volume needed for top STD (e.g., 100 or 20 ng) by the concentration of cDNA required (e.g., [50 ng/µL] or [10 ng/µL]; NOTE: assays use 2 µL of cDNA):

1. For 100 ng top STD (Log_{10}): 3,500 ng of cDNA are needed in 70 µL

2. For 20 ng top STD (Log_2): 1,300 ng of cDNA are needed in 130 µL

v. Divide the total number of nanograms needed by the concentration of stock cDNA ([50 ng/uL]), then divide that number by the total number of samples to be included in the STD curve pool (e.g., 20 samples available across control and treatment replicates):

1. For 100 ng, top STD (Log_{10}): 65 aliquots of 50 ng/µL are needed

   a. Pool 3.5 µL from each of 20 samples into a final volume of 70 µL

2. For 20 ng top STD (Log_2): 26 aliquots of 50 ng/µL are needed

   a. Pool 1.3 µL from each of 20 samples into a final volume of 130 µL

vi. Start with top STD and dilute serially (1:10 or 1:2) into 3–4 subsequent tubes:

1. For 100 ng top STD (Log_{10}): Add 7 µL into 63 µL water for each dilution

2. For 20 ng top STD (Log_2): Add 65 µL into 65 µL water for each dilution

vii. Load duplicates of each STD into wells A1–A12 on **cDNA Plate** (if applicable).

"cDNA Plate" generation

1. Dilute stock tRNA 1:100 in water (to [100 ng/µL]; "tRNA-water")
2. Dilute stock cDNA ([100 ng/µL]) 1:1 with tRNA-water
    a. Add 20 uL tRNA-water into each 20 uL cDNA reaction well = [50 ng/µL]
3. Seal, vortex, and spin down the stock cDNA plate
    a. Each well now contains 40 µL of [50 ng/µL] cDNA
4. Label individual Eppendorff tubes for each cDNA sample (including RT)
5. Dilute cDNA 1:5 (20 ng assay) or 1:50 (2 ng assay) with tRNA-water
    a. 20 µL [50 ng/µL] cDNA into 80 µL tRNA-water for 100 µL cDNA at [10 ng/µL]
    b. 2 µL [50 ng/µL] cDNA into 98 µL tRNA-water for 100 µL cDNA at [1 ng/µL]
6. Vortex and spin down each individual Eppendorff tube containing diluted cDNA
7. Split diluted cDNA volume in Eppendorff tube (100 µL) into 2 wells on 96-well plate
    a. If running an STD curve, load STDs first (A1-A12), then individual assays (B1 onward)
        i. See "Standard Curve Generation," above
    b. If not running STD curve, load individual assays starting at well A1
    c. Load 50 uL per technical replicate into adjacent wells (A1 + 2, A3 + 4, A5 + 6, etc.)
    d. Treat the RT sample the same and load diluted –RT into two wells after last treatment sample
    e. Add 50 µL water into the final two wells after –RT wells for a "no template control" (NTC).

"qPCR Plate" generation

1. Dilute the gene-specific primers (F & R) from 100 µM stock to the desired assay concentration:

   a. For 150 nM: 3 µL of 100 µM primer into 97 µL water for 100 µL at 3.75 µM
   b. For 250 nM: 5 µL of 100 µM primer into 95 µL water for 100 µL at 5 µM

2. Generate master mix based on the number of wells used on the cDNA Plate (see above):

   a. Water = 2 µL each
   b. SYBR® Green = 5 µL each
   c. Forward Primer (3.75 or 5 µM) = 0.5 µL each
   d. Reverse Primer (3.75 or 5 µM) = 0.5 µL each
   e. cDNA (1 ng/µL) = 2 µL each

3. Load 8 µL of gene-specific master mix into the required number of MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, USA) wells using electronic dispensing pipette (e.g., Rainin E4 XLS).

4. Add 2 µL of diluted cDNA from the cDNA Plate (see above) into each corresponding well using a 12-channel pipette, for a 10 µL total reaction volume. Repeat if multiple genes can be run on the same plate (e.g., if total wells on the cDNA Plate is less than 24 or 48, or A1–B12 or A1–D12).

5. Seal the qPCR Plate with MicroAmp™ Optical Adhesive Film (Applied Biosystems, USA).

6. Vortex for 30 seconds (Level 6).

7. Centrifuge at 3,000 g for 30 sec.

8. Load onto a Viia7 Real-Time Detection System (Applied Biosystems, USA).

9. Use manufacturer's pre-set thermal cycling conditions for either fast or slow chemistry.

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A. Testing Protocol 1: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Embryos

A.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared on the morning of the exposure with slight modifications from the QAPP (see Section 13.2.2, *Exposure media preparations*).

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

A.2 Embryo Exposure

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at either 4–6 hours (sphere to shield developmental stage) or 28–30 hours post-fertilization (hpf) in replicate groups of 3 or 6 using either 60- or 100-mm glass Petri dishes (n = 25 or 35 embryos per replicate, respectively).

3. Embryos were cleaned and sorted by stage, then divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL or 40 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of the exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 48 hours.

5. Dead embryos were removed every 24 hours. Mortalities were recorded.

A.3 Morphological Assessment of Embryos

1. Embryos were screened for abnormalities at 48 or 72 hpf based on observations using the stereomicroscope or digital still images and videos.

2. All observations were made under a Nikon SMZ800 stereomicroscope or Nikon Eclipse E600 compound microscope. Images were collected using a SPOT imaging system (Diagnostic Instruments, Inc., Sterling, MI; camera model 2.3.1; software version 4.5.9.9) or Fire-i400 camera (Unibrain, San Ramon, CA).

3. Presence and absence scores for pericardial edema were based on observations in the live embryos or digital video clips. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

4. Intracranial hemorrhage was scored visually in either live embryos or in lateral images and video clips.

5. For quantification of pericardial edema and detailed imaging, embryos were mounted in 2% methylcellulose. Pericardial area was measured in still frames extracted from digital video, as described previously (Incardona et al., 2006).

6. For cardiac dimensional measurements, embryos were mounted without anesthesia in 2% methylcellulose and digital video collected using differential interference contrast optics with a 20x objective lens on the Nikon E600 compound microscope. Diastolic and systolic chamber diameters were measured, as was fractional shortening (contractility) as described in Bendig et al. (2006) and Incardona et al. (2011).

7. For CYP1A expression, approximately 20 embryos from each treatment were dechlorinated and fixed in 4% paraformaldehyde. Standard protocols were used to fluorescently stain the heart for CYP1A and myosin heavy chain protein expression (Incardona et al., 2006).

B. Testing Protocol 2: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Embryos – Phototoxicity

B.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry in between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared the morning of the exposure following the modified QAPP.

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

B.2 Embryo Exposure

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at 4–6 hpf (sphere to shield developmental stage) in replicate groups of 3 using 60-mm glass Petri dishes (n = 25 embryos per replicate).

3. After being cleaned and sorted by stage, embryos were divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 72 hours.

5. Dead embryos were removed every 24 hours and mortalities were recorded.

6. Phototoxicity tests were carried out after exposing embryos to HEWAFs from 4 to 72 hpf as detailed previously (Hatlen et al., 2010).

7. Larvae were transferred into new Petri dishes with clean water, and exposed outdoors to full sunlight for 30 minutes.

8. Larvae were returned to the laboratory for immediate imaging.

B.3 Morphological Assessment of Embryos

1. Embryos were screened for abnormalities after exposure to sunlight based on observations using the stereomicroscope or digital still images and videos.

2. All observations were made under a Nikon SMZ800 stereomicroscope fitted with a Fire-i400 camera (Unibrain, San Ramon, CA).

3. Presence and absence scores for pericardial edema were based on observations in the live embryos or digital video clips. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

4. Intracranial hemorrhage was scored visually in either live embryos or in lateral images and video clips.

5. For caudal finfold measurements, 10 larvae were randomly selected from each replicate, anesthetized with tricaine mesylate and mounted in 2% methylcellulose for imaging. Using ImageJ, area measurements were taken by tracing a roughly half-circular line using the end of the notochord as a landmark along the posterior edge of the caudal finfold.

C. Testing Protocol 3: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Juveniles

C.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry in between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared on the morning of the exposure, following the modified QAPP.

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

C.2 Embryo Exposure and Larval Rearing

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at 4–6 hpf (sphere to shield developmental stage) in replicate groups of 6 using 60-mm glass Petri dishes (n = 30 embryos per replicate).

3. After being cleaned and sorted by stage, embryos were divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 48 hours.

5. Dead embryos were removed every 24 hours and mortalities were recorded.

6. At 48 hpf, embryos were scored for pericardial edema. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

7. HEWAF-exposed embryos were pooled and divided into three replicate groups (n = 30 each) with edema and three replicate groups (n = 30 each) without edema.

8. In control embryos, rates of edema were low enough to provide only a single group with edema (n = 30), while embryos without edema were pooled into five replicate groups (n = 30 each).

9. The new replicate groups were transferred to clean water, incubated at 28.5°C in 100-mm plastic Petri dishes until feeding stage and then raised until juvenile stage (2 weeks post-fertilization) as detailed in Linbo (2009), with mortality assessed daily.

C.3 Morphological Assessment of 2-Week Juveniles

1. At 2 weeks post-fertilization, fish were anesthetized with tricaine mesylate, assessed for morphological defects, and measured for length.

2. Representative individuals were imaged using a Nikon SMZ800 stereomicroscope fitted with a Fire-i400 camera (Unibrain, San Ramon, CA).

3. A subset of individuals from each treatment were randomly selected, fixed, and assessed for craniofacial skeletal defects using an alcian blue and Alizarin Red S stain.

C.4 Alcian Blue and Alizarin Red S Stains

1. Following morphometric data collection, fish were fixed in 4% paraformaldehyde overnight at 4°C on a rocker, dehydrated through a gradient of methanol/phosphate-buffered saline (PBS) (25%/75%, 50%/50%, 75%/25%; 5 minutes each) and stored at -20°C in 100% methanol.

2. For staining, samples were rehydrated through methanol/PBS series (75%/25%, 50%/50%, 25%/75%; 10 minutes each) and washed in water (3 times; 1 hour each).

3. Fish were then stained with alcian blue solution (0.01% in 7:3 ethanol/glacial acetic acid; 24 hours), destained (7:3 ethanol/glacial acetic acid; 30 minutes), transferred into 100% ethanol (30 minutes), rehydrated through ethanol/water series (75%/25%, 50%/50%, 25%/75%; 30 minutes each) and washed in water (2 times; 30 minutes each).

4. Following rehydration, alcian-stained fish were subjected to trypsin digestion (50% saturated aqueous sodium borate, 1.7% trypsin powder; 10 minutes) before 0.5% potassium hydroxide (KOH) enzyme deactivation (5 minutes) and 0.5% KOH washes (2 times; 5 minutes each).

5. Alizarin Red S solution (0.04%) was added to samples (in 0.5% KOH) until solution turned deep purple. Alizarin staining proceeds for 12 hours before clearing through 0.5% KOH/glycerol series (3:1, 1:1, 1:3; 6 hours each) and storage in 100% glycerol.

6. Dissected craniofacial skeletons were mounted in glycerol and imaged using the Nikon Eclipse E600 compound microscope and SPOT imaging system.

7. Multiple images encompassing all focal planes (n = 10) were then aligned and Z-stacked using Hugin version 2011.4.0 (http://hugin.sourceforge.net/) and Enfuse version 4.0 (http://enblend.sourceforge.net/) to generate an extended depth of field.

D. Testing Protocol 4: Assessment of Crude Oil Cardiotoxicity in Southern Bluefin Tuna (*Thunnus maccoyii*) Yolk-sac Larvae – Static Exposure

These tests were performed in collaboration with the NWFSC/University of Miami RSMAS/ Hopkins Marine Station of Stanford University.

Testing protocol of southern bluefin tuna cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were screened at 8–10 hpf and exposed until 8–10 hours post-hatch (~ 36 hours).
- Embryos were kept in a temperature controlled room set at 25°C.
- Exposures were done without any agitation (i.e., without a reciprocating shaker).

E. Testing Protocol 5: Assessment of Crude Oil Cardiotoxicity in Amberjack (*Seriola lalandi*) Larvae – Beaker Exposure

These tests were performed in collaboration with the NWFSC/RSMAS/Hopkins Marine Station of Stanford University.

Testing protocol of amberjack cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were exposed in 1-L beakers at a density of 80 embryos/L.
- Embryos were kept in a temperature controlled room set at 25°C.
- Beakers were agitated on a horizontal shaker water bath.

F. Testing Protocol 6: Assessment of Crude Oil Cardiotoxicity in Amberjack (*Seriola lalandi*) Larvae – Bucket Exposure

These tests were performed in collaboration with the NWFSC/RSMAS/Hopkins Marine Station of Stanford University.

Testing protocol of amberjack cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were exposed in 10-L buckets with an exposure volume of 7 L and stocked at a density of 80 embryos/L.
- Embryos were kept in a temperature-controlled room set at 25°C.
- Buckets were agitated with magnetic stir bars on stir plates.
- Due to the large number of embryos per bucket (> 500), hatching rates were not precisely quantified.
- 10 hatched larvae were randomly captured from each treatment and imaged within 1 hour.

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14. Stratus Consulting/Abt Associates General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp

14.1 Methods

Stratus Consulting/Abt Associates[1] conducted the tests described below in collaboration with the University of North Texas (UNT) and the Northwest Fisheries Science Center (NWFSC) at the Texas Parks and Wildlife Department (TPWD) Sea Center Texas marine hatchery in Lake Jackson, Texas. This chapter describes General Laboratory Procedures and Practices (GLPP) that Stratus Consulting/Abt Associates used at TPWD.

14.1.1 Source water

The natural seawater that was used during toxicity testing was the same as the source water used at TPWD. To obtain source water, TPWD pumps water from Galveston Bay and runs the water through a drum filter. Source water was pulled directly from distribution lines that ran throughout the TPWD hatchery. For static renewal tests, testing staff used source water stored in a 90-gal carboy that was covered in dark plastic and maintained under aeration at ambient temperature (a sample of this 90-gal "batch" was collected and sent to ALS Environmental for chemical analyses). Testing staff used water from the carboy to prepare test exposure solutions. A sample of every batch that was used during testing was sent to ALS Environmental for analyses.

TPWD hatchery source water was also used during flow-through tests. Testing staff plumbed water lines from the TPWD hatchery distribution lines directly to head tanks used for flow-through exposure systems. The amount of source water needed for flow-through tests precluded storing and sampling batches of water. However, a series of water samples were collected from head tanks during each flow-through test and sent to ALS Environmental for chemical analyses.

1. The name of the company changed while tests were ongoing.

14.1.2 Fish culturing

All of the fish used in these studies came from the TPWD hatchery. The TPWD hatchery rears juvenile red drum and speckled seatrout for the purposes of stock enhancement. Mature adults live indoors and spawn in large circular brood stock tanks maintained by a large recirculating filtration system. TPWD hatchery staff regulate spawning by manipulating the water temperature and photoperiod in the brood-stock tanks. During a successful spawning event, female fish release eggs that are externally fertilized by sperm. Fertilized embryos float to the surface of brood-stock tanks. The hatchery staff use skimming devices on the edges of the brood tanks to concentrate embryos in the overflow filter box on the side of each tank. During the spawning season, late each night, early the following morning, or both, TPWD hatchery staff collect embryos from each tank's overflow box to stock into 100-gal circular incubation tanks maintained by a flow-through system. Then they volumetrically enumerate the number of embryos stocked into each incubation tank. The hatchery staff rear embryos in incubation tanks until the embryos hatch and the resulting larvae absorb their yolk sack, which takes approximately 3 to 4 days. After this rearing period, TPWD hatchery staff transfer larvae to one-acre outdoor ponds. Fish at the hatchery live in outdoor ponds for approximately 1 month before being stocked as juveniles (> 15mm) in State waters. Larvae are fed granulated prepared feed (Rangen, Angleton, TX) twice daily and live food (natural, native zooplankton) are available throughout larval and juvenile development.

For early life stage toxicity testing, testing staff collected embryos and larvae directly from the hatchery system described above. For juvenile life stage toxicity testing, test organisms were collected directly from outdoor rearing ponds just before stocking.

Red drum (*Sciaenops ocellatus*) and speckled seatrout (*Cynoscion nebulosus*)

Testing staff obtained red drum and speckled seatrout embryos, larvae, and juveniles from the TPWD hatchery for use in toxicity testing. Embryos were collected directly from the brood stock tank's overflow filter box between 20:00 and 24:00, a few hours after fertilization. Before initiating a test, testing staff examined a sample of embryos in Petri dishes or welled slides using a stereo or compound microscope to determine fertilization status. Spawns with low fertilization rates were not used for definitive testing.

Testing staff collected red drum or speckled seatrout larvae from stocked incubation tanks a few hours after hatching [see test-specific test conditions tables (TCTs)]. Testing staff collected newly hatched larvae by dipping a beaker into incubation tanks. Larval morphology and behavior were grossly assessed by looking through the sides, bottom, and top of the beaker. Only healthy-looking, vigorous larvae were used to stock test exposure vessels.

Testing staff obtained red drum juveniles from outdoor rearing ponds located on the TPWD hatchery property. Juveniles used for testing were collected approximately three weeks post-

hatch, just before being stocked in State waters. Testing staff used a long-handled dip net to capture and transfer fish from rearing ponds to buckets of pond water aerated with pure oxygen. Once enough fish were collected, they were immediately transported to the TPWD hatchery incubation room for acclimation and testing.

Juvenile red drum were held in two 100-gal, circular incubation tanks for approximately 48 hrs before testing. Water quality was maintained via flow-through conditions and monitored throughout the holding period. Red drum juveniles were fed ad libitum with prepared feed (Rangen, Angleton, TX) and newly hatched *Artemia* nauplii until stocked into the testing exposure system. Just before stocking the exposure tanks, testing staff randomly captured juvenile red drum from the incubation tanks using a large net and transferred them to an aerated bucket of clean source water. Fish were taken from this aliquot, inspected, measured, weighed, and stocked into exposure tanks. Only healthy-looking, normal[JH1] fish were used in toxicity tests.

New aliquots of fish were collected from the incubation tanks throughout the stocking procedures. Remaining fish not used to stock exposure tanks were retained and reared in incubation tanks during the course of respective testing. Juvenile red drum tests were conducted under the UNT Institutional Animal Care and Use Committee (IACUC) animal use protocols.

14.1.3 Pacific white shrimp

In addition to red drum and speckled seatrout, Pacific white shrimp (*Litopenaeus vannamei*) were also used during TPWD hatchery toxicity testing. Post-larva (PL) 8 to 10 mm shrimp were obtained from the Shrimp Improvement Systems (SIS) LLC Nucleus Breeding Center in Islamorada, Florida, USA. Although SIS is a specific pathogen-free aquaculture facility, we had an aliquot of SIS shrimp shipped to the University of Arizona Aquaculture Pathology Laboratory for additional diagnostic microbiology and bacteriology screening. Once shrimp were determined to be free of pathogens, SIS sent shrimp to the TPWD hatchery for use in testing. Before importing live shrimp to the TPWD hatchery, we obtained an Exotic Species Permit from the TPWD Exotic Species program (No. RES 07 15-112). Shrimp rearing, testing, and disposal were conducted within permit specifications.

Once shrimp were received, they were slowly acclimated to the TPWD hatchery source water. This was done by slowly dripping source water into the shrimp shipping water until it had the same water quality as the source water. Shrimp were maintained in a 100-gal circular incubation tank under flow-through conditions. Holding/incubation tank outflow water was directed through a series of filters into the flow-through exposure system sump where it was filtered and pumped to a 1,275-gal holding tank. At least once daily, the contents of the shrimp water holding tank were mixed with TPWD hatchery tap water and pumped into a sink drain that ran to the Lake Jackson sanitary sewer system.

Shrimp were fed ad libitum with prepared feed (Raceway Plus from Zeigler, MN) and newly hatched *Artemia* nauplii until stocked into the testing exposure system. Artemia cysts were obtained from INVE Aquaculture (Salt Lake City, UT) and hatched in a series of 10-L hatching cones. Remaining shrimp not used to stock exposure tanks were retained and reared in incubation tanks during the course of respective testing.

14.1.4 Media preparation

The testing media used during toxicity testing included both water accommodated fractions (WAFs), oil slick, and oil spiked sediment exposures. For WAF and slick testing, staff prepared exposure media using source water stored in the 90-gal carboy. The protocols for WAF preparations listed below appear in the "Protocols for Preparing Water Accommodated Fractions" standard operating procedure (SOP) in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The protocols for Slick A and Slick B oils appear in Appendix D *Testing Protocol 4: Red Drum Oil Slick Exposures*. Sediment exposure preparation methods are included in the individual testing protocols listed in the Stratus Consulting GLPP. The types of toxicity testing media preparations were as follows:

- Three different WAF preparations – high-energy, low-energy, and chemically enhanced WAFs (HEWAF, LEWAF, and CEWAF, respectively) – using one of two oil types: Slick A (CTC02404-02) or Slick B (GU2888-A0719-OE701).
- Oil slick exposures using either Slick A (CTC02404-02) or Slick B (GU2888-A0719-OE701) oil.
- Slick B (GU2888-A0719-OE701) oil-spiked sediment exposure. Sediment was obtained from Point aux Pins near Bayou La Batre, Alabama (30.379079, -88.305423). A composite sample of collected sediment was analyzed for a large suite of hydrocarbons, metals, pesticides, and semi-volatile organic compounds, none of which were detected at concentrations that would be deleterious to sensitive aquatic organisms.

14.1.5 Exposure systems

Both static and flow-through exposure systems were used throughout TPWD hatchery testing activities.

For static tests using fish embryos and larvae, staff filled pre-labeled exposure vessels with final-dilution exposure media and placed them into a temperature-controlled water bath. Staff removed the exposure vessels from the water bath, stocked them with fish, and returned them to the water bath one at a time. The water bath temperature was maintained with heaters and flow-

through hatchery water. A small pump was used to recirculate water in the water bath tank, and staff adjusted water depth using standpipes at the water bath outflow.

For flow-through tests, two rack systems were constructed in the TPWD hatchery incubation room. Each rack systems consisted of a 45-gal head tank; 24 individual, covered, 2.5-gal exposure tanks; a 45-gal sump; and a three-stage canister filter system (Figure 14.1). Air lines were run to each exposure tank and air flow was adjustable using gang and inline values. Head tanks were also aerated. The source water distribution system was constructed using 0.25-in. ball valves that adjusted inflow to each tank. Exposure tank effluent flowed through individual, screened overflow bulkheads into an effluent collection pipe. Before entering the sump, effluent was filtered through a 200-micron filter sock at the end of the effluent collection pipe. A float valve was used to turn on the filter pump when the sump was approximately half full of effluent. The filtration system consisted of one 20-micron pleated filter, one 5-micron pleated filter, and an activated carbon filter run in a series. Filter cartridges were changed at least once daily during the experiment. Filtered effluent was either directed to a 1,275-gal holding tank for shrimp or the floor drain for red drum.

Figure 14.1. Shrimp and red drum flow-through exposure systems. Photograph taken by I. Lipton on 7/8/2015.

14.1.6 Water-quality monitoring

Testing staff monitored water quality in exposure vessels at specific time points throughout testing activities. See "Water Quality Protocols" in Section 14.3, below, for detailed water quality methods used throughout testing activities. See the QAPP and TCTs for required monitoring schedule.

In addition to time-point-specific temperature measurements, HOBO data loggers (Onset Computer Corporation, Pocasset, MA) were sometimes used to continuously monitor the temperature in water baths or dummy exposure beakers. When deployed, pendant-type temperature/alarm (UA-001-64) loggers were used in conjunction with the Pendant Base Station (BASE-U-1) and HOBOware Lite version 3 software. Logger data files were named with the test identification (ID), location (water bath or dummy beaker), and date and time deployed, and saved as comma delimited Microsoft Excel files on the project server.

14.1.7 Analytical chemistry sampling

See the QAPP, related testing protocols, and test-specific TCTs for sampling and processing of water and tissue collected for chemical analyses.

14.2 Reporting and Testing Documentation

Reporting and testing documentation was performed as outlined in the QAPP. Samples that we collected were labeled according to the QAPP, using "ST-" for the first sample ID segment.

14.3 Water Quality Protocols – General Laboratory SOPs

Testing staff used commercially available water quality meters, probes, and colorimetric test kits to measure various water quality parameters throughout testing activities. Testing staff measured pH, dissolved oxygen (DO), and conductivity directly in the exposure chambers using meters and probes. Water samples for ammonia measurements were collected for analysis at the beginning and end of the tests. Temperature measurements were taken within the water bath and during the in-chamber pH or DO measurements. Salinity/conductivity was measured by either using a hydrometer or a salinity meter. When using a hydrometer, water samples were collected for measurements at the beginning and end of a test. When using a salinity meter, the measurements that were taken at the beginning and the end of a test were taken directly in the exposure chambers.

To limit the possibility of cross-contamination from the probes, measurements began with the controls and then proceeded in order of treatment concentration. Probes were also rinsed with reverse osmosis (RO) water between exposure chambers. After use, testing staff decontaminated probes and acrylic vials using a non-toxic mild detergent solution and light scrubbing, followed by an RO water rinse.

Prior to use, meters were bench-calibrated for their accuracy and, if possible, the calibrations were verified against standards. Testing staff followed each meter's manufacturer calibration frequency and protocols.

14.3.1 pH and temperature – YSI EcoSense PH100A pH meter/temp probe

1. Calibrate the instrument according to the operations manual using neutral (pH 7) and basic (pH 10) calibration solutions.
2. Rinse the probe with RO water and place the probe into the treatment container to be tested. Confirm that the probe's electrode elements are submerged below the liquid level; continuous movement is not required.
3. Allow the temperature and pH readings to stabilize and record the readings on the "Water Quality Monitoring" datasheet.
4. Repeat steps 2 through 3 as necessary for each treatment container.
5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container in the electrode storage solution.

14.3.2 DO – YSI® ProODO

1. Verify the instrument calibration according to the operations manual.
2. Rinse the probe with RO water and place the probe into the treatment container to be tested. Confirm that the probe's electrode elements are submerged below the liquid level; continuous movement is not required.
3. Allow DO readings to stabilize and record readings on the "Water Quality Monitoring" datasheet.
4. Repeat steps 2 through 3 as necessary for each treatment container.
5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container.

14.3.3 Salinity – Instant Ocean Hydrometer or Pinpoint Salinity Meter

Instant Ocean Hydrometer

1. Ensure that the black plug is inserted into the flow outlet at the bottom of the hydrometer.
2. Add water into the hydrometer until it is full. Remove any air bubbles by lightly tapping the hydrometer until no bubbles are visible. Note that air bubbles on the pointer will cause incorrect readings.
3. Place the hydrometer on a flat surface. Do not tilt the hydrometer as that will cause water to spill from the water inlets.
4. Record the reading for salinity on the "Water Quality Monitoring" datasheet.
5. After using the hydrometer, flush it with warm, fresh tap water to prevent salt deposits from forming on the swing arm, which can distort future readings.

Pinpoint Salinity Meter

1. Verify instrument calibration according to the operations manual.
2. Wash the probe with RO water and place the probe into the treatment container to be tested. Be sure that the probe elements are submerged below the liquid level and gently swirl the probe.
3. Allow salinity readings to stabilize. The salinity meter measures conductivity in millisiemens (mS). Record conductivity results on the "Water Quality Monitoring" datasheet and correct the conductivity units on the datasheet in the respective entry heading. To convert the conductivity measurements to parts per thousand (ppt), use the table provided by the meter manufacturer. Record that value in the datasheet.
4. Repeat steps 2 through 3 as necessary for each treatment container.
5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container.

14.3.4 Total ammonia (NH_3/NH_4^+) – API Ammonia Test Kit

1. Collect 5 mL of test solution and place it into the glass test tube provided in the API test kit. The test tube has a white 5-mL graduation line.

2. Add eight drops of test solution #1 to the test tube.

3. Immediately add eight drops of test solution #2 to the same test tube.

4. Tightly cap the test tube with the provided cap and shake vigorously for 5 seconds. Do not cap the tube with your finger since that might interfere with test results.

5. Set the timer for 5 minutes and wait for the color to develop.

6. After 5 minutes, read the test results by comparing the test solution color to the saltwater ammonia color chart provided in the test kit. View the tube color against a white background in a well-lit area. Determine the closest color match between the test solution and the color chart and record the associated NH_3/NH_4^+ values. Record the value on the "Water Quality Monitoring" datasheet.

7. Pour 5 mL of solution into the appropriate disposal vessel.

8. After using the API kit container, clean it with mild soap and water and store until next use.

Note that samples may also be collected at the end of each test for possible additional ammonia analyses. Log these samples on the "Analytical Sample Inventory Bench Sheet" and archive the samples at -20°C.

A. Testing Protocol 1: Red Drum and Speckled Seatrout Acute Toxicity Procedure

A.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Prepare HEWAFs the evening of the embryo or larval collection. If a single test requires multiple WAF preparations, prepare all WAF solutions at the same time, composite the solutions in a decontaminated stainless steel or glass container, and thoroughly mix before making test treatment dilutions.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary chain-of-custody (COC) documentation, as described in the QAPP.

A.2 Test Media Preparation

Perform static-exposure acute toxicity tests in 250- or 600-mL glass beakers with a total test solution volume of 200 and 400 mL, respectively (see test-specific TCTs). For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add both source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into sample vessels. After filling the sample vessels, collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental as described in the QAPP. Fill each sample jar, provided by ALS Environmental, to capacity. Fill all exposure vessels and sample jars and discard the unused WAF into a lined 55-gal drum for off-site disposal.

A.3 Embryo or Larval Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct temperature. See

test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Before test initiation, measure water-quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) as described in the *Stratus Consulting General Laboratory Procedures and Practices Red Drum, Speckled Seatrout, and Pacific White Shrimp* (Stratus Consulting GLPP) document. Record the water-quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. ***For embryo exposures*** (see test-specific TCTs): Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

   For larval exposures (see test-specific TCTs): Collect the desired aged larvae from incubation tanks. Collect larvae and water by dipping a decontaminated glass beaker into stocked incubation tanks. Please note that hatchery staff will stock incubation tanks with embryos. Embryos hatch into larvae in the early evening approximately 17 to 20 hours post-fertilization.

4. ***For embryo exposures (***see test-specific TCTs): If loading embryos using a compound microscope, use a pipette to transfer the embryos from the collection beaker onto a microscope slide. Use the low-magnification microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Transfer the embryos into test beakers in random order by gently washing them from the surface of the microscope slide. Once the complete transfer is verified, dispose of the slide by inserting it into a hard-sided sharps receptacle. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See the test-specific TCT for the number of organisms required per replicate, as well as the number of replicates required per treatment.

   If loading embryos using a stereo microscope, use a pipette to collect the embryos from the collection beaker into a new Petri dish with source water. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Gently expel the viable embryos into the test beakers. Inspect the pipette to verify complete transfer of the embryos into the test beakers. Record start time for each

replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See test-specific TCTs for the number of organisms required per replicate, as well as the number of replicates required per treatment.

For larval exposures (see test-specific TCTs): Transfer larvae from the collection beaker to exposure vessels using a pipette. Before dispensing them into exposure vessels, count the larvae by looking through a pipette cylinder that is backlit with a desk lamp. After dispensing, reexamine the pipette to ensure all larvae were successfully transferred. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain larvae. See test-specific TCTs for the number of organisms required per replicate, as well as the number of replicates required per treatment.

5. Keep the beakers in the water bath for the duration of the test, except when you are inspecting them. The light cycle may vary during test maintenance because of routine hatchery operations.

6. After 24 hours of exposure, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record the organism as dead if you can fully identify intact carcasses or dead eggs. Do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole dead organisms and carcass or embryo remnants from the exposure chamber into a freezer-safe sample container using a treatment-dedicated pipette and archive at -20°C according to the QAPP.

7. Repeat step 6 for each 24-hour time period prior to test termination.

8. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 6. Pipettes may also be used to inspect and count larvae, as described above. Note that immobile or morbid organisms may be removed from the exposure chamber by using a pipette and then inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. The number of morbid organisms may be enumerated using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of test. Test organisms may be fixed for potential future analyses. To preserve samples, collect live and morbid organisms and fix them in 10% buffered formalin. Archive all remaining live and dead organisms that are not fixed in formalin at -20°C according to the QAPP. Record test end times on the "Tank ID,

Dilution, or Stock Code Definitions" data entry bench sheet. See test-specific TCTs for exposure duration and test length.

9. If needed, collected end-of-test water chemistry samples and discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

B. Testing Protocol 2: Red Drum and Speckled Seatrout Acute Differential Exposure Toxicity Testing

B.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Prepare HEWAFs the evening of the embryo or larval collection.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

B.2 Test Media Preparation

Perform acute differential static-exposure toxicity tests in 250- or 600-mL glass beakers with a total test solution volume of 200 and 400 mL, respectively (see test-specific TCTs). For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger-volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into sample vessels. After filling sample vessels, collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental, as described in the QAPP. Fill each sample jar, provided by ALS Environmental, to capacity. After all exposure vessels and sample jars are filled, discard unused WAF into a lined 55-gal drum for off-site disposal.

B.3 Embryo Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature that is indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct

temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop times and dates.

2. Before test initiation, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) as described in the Stratus Consulting GLPP document. Record water quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. Collect embryos from the egg-collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

4. Use a pipette to transfer the embryos from the collection beaker onto a microscope slide. Use a low-magnification compound microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Transfer the embryos into test beakers in random order by gently washing them from the surface of the microscope slide. Once the complete transfer is verified, dispose of the slide by inserting it into a hard-sided sharps receptacle. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See the test-specific TCT for the number of organisms required per replicate, as well as the number of replicates required per treatment.

5. Keep the beakers in the water bath for the duration of the test, except when you are inspecting them. The light cycle may vary during test maintenance because of routine hatchery operations.

6. After the appropriate exposure duration time, transfer embryos/larvae from the treatment solutions into clean source water. Perform all of the transfers using pipettes. Use a new pipette for each treatment to avoid cross-contamination. See test-specific TCTs for exposure durations and test lengths.

7. During transfer and at 24 hours, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record an organism as dead if you can fully identify intact carcasses or dead eggs. As such, do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole, dead organisms, and remnants into a freezer-safe

sample container using a treatment-dedicated pipette and archive at -20°C, according to the QAPP.

8. Repeat step 7 for each 24-hour time period prior to test termination.

9. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 7. Pipettes may also be used to inspect and count larvae, as described above. Note that immobile or morbid organisms may be removed from the exposure chamber by using a pipette and then inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. The number of morbid organisms may be enumerated using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of test. Test organisms may be fixed for potential future analyses. To preserve samples, collect live and morbid organisms and fix them in 10% buffered formalin. Archive all the remaining live and dead organisms that are not fixed in formalin at -20°C. Collect and archive them according to the QAPP. Record test end times on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet. See test-specific TCTs for exposure duration and test length.

10. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

C. Testing Protocol 3: Morphological Assessment of Oil Cardiotoxicity in Red Drum Embryos

These tests were conducted in in collaboration with NWFSC.

C.1 General Considerations

Decontaminate all glassware, spatulas, and materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see TCTs) according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A. Prepare HEWAFs the evening of the embryo or larval collection. If a single test requires multiple WAF preparations, all WAF solutions will be prepared at the same time, composited in a decontaminated stainless steel or glass container, and thoroughly mixed before making test treatment dilutions.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless noted otherwise; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

C.2 Test Media Preparation

Perform acute differential static exposures toxicity tests in 600-mL glass beakers with a total test solution volume of 400 mL. For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add both source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger-volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into replicate/sample vessels. After filling the sample vessels, collect 250 mL of each bulk solution and ship it overnight on ice to ALS Environmental, as described in the QAPP. Each sample jar, provided by ALS Environmental, should be filled to capacity. After all exposure vessels and sample jars are filled, discard unused WAF into a lined 55-gal drum for off-site disposal.

C.3 Embryo Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, as indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Before starting the test, measure the water-quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH), as described in the Stratus Consulting GLPP document. Record water-quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

4. Use a pipette to collect the embryos from the collection beaker. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Gently expel the embryos into the test beakers. Load approximately 200 organisms per replicate.

5. Keep the beakers in the water bath, except when being inspected. The light cycle may vary during test maintenance because of routine hatchery operations.

6. At roughly 24 hours post-fertilization, harvest 100 mL of test solution containing the newly hatched larvae. Pour the solution through a small straining basket to collect the larvae, placing approximately 20 larvae into a labeled microcentrifuge tube containing RNAlater for possible genetic analyses. Immediately place the tube in liquid nitrogen. Repeat for each replicate.

7. At approximately 12 hours post-hatch or approximately 36 hours post-fertilization, observe and record mortality. Count organisms by gross observation. Enumerate dead organisms only if you can fully identify intact carcasses or dead eggs. Decomposed carcass remnants may not be counted as dead organisms. Remove all whole dead organisms and remnants from the exposure chamber using a treatment-dedicated pipette and archive them at -20°C, according to the QAPP.

8. Also at approximately 12 hours post-hatch or approximately 36 hours post-fertilization, harvest 100 mL of test solution containing fish. Pour the solution through a small straining basket to collect the fish. Place approximately 20 individuals into a labeled microcentrifuge tube containing RNAlater® for possible genetic analyses. Immediately place the tube into liquid nitrogen. Repeat for each replicate.

9. Divide the remaining 200 mL of test solution with larvae into two equal 100-mL volumes. Try to divide the samples so that an equal number of larvae remain in each aliquot. Set one 100-mL aliquot aside for harvesting at the end of imaging. Repeat for each replicate.

10. Use the remaining 100 mL aliquot from each replicate for imaging. To capture images of individuals, follow the protocols described in Section C.3 (Digital Videomicroscopy of Hatched Larvae). Repeat for each replicate.

11. Collect any remaining live organisms and fix them in paraformaldehyde.

12. Archive all of the imaged larvae, together with the dead larvae collected in step 7, at -20°C according to the QAPP.

13. After imaging is complete, harvest larvae from the 100-mL aliquot that was set aside in step 9. To harvest fish, pour the solution through a small straining basket and place approximately 20 individuals into a labeled microcentrifuge tube containing RNAlater® for possible genetic analyses. Immediately place the tube into liquid nitrogen. Repeat for each replicate.

14. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

C.4 Digital Videomicroscopy of Hatched Larvae

1. Randomly select a replicate from one of the treatment groups.

2. Capture two or three larvae at a time using a wide-bore glass pipette.

3. Mount larvae in 2% methyl cellulose in source water.

4. Image all the larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

5. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereo microscope fitted with a phototube and Unibrain Fire-i400 1394 camera, connected via

firewire to a laptop with BTV Pro. Keep the magnifications for imaging identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

a. Video capture. Take a 10-second video at the highest magnification (6.3x). Focus on the cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

b. Image capture. Capture composite images of the entire larvae for three fish per beaker at 5x magnification.

6. After imaging, use a wide-bore glass pipette to transfer larvae to an appropriate container for archiving. Repeat the process of capturing and mounting two or three larvae at a time until 20 larvae from the treatment vessel have been imaged, then move on to the next beaker. Refresh the methyl cellulose as needed.

7. Save and copy files to two back-up hard drives.

8. Repeat steps 1–7, imaging all of the exposure vessels.

9. Process images according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP in the NWFSC GLPP.

D. Testing Protocol 4: Red Drum Oil Slick Exposures

D.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

D.2 Preparation of Polyvinyl Chloride Coupling

To prepare the polyvinyl chloride (PVC) coupling for slick exposures, gather the required number of 2-inch PVC couplings. Use one coupling for each replicate (see test-protocol TCTs). Drill a hole through both sides of the top half of the PVC coupling so that a small wooden dowel can slide through the holes. Thoroughly wash and rinse the PVC coupling with Simple Green and tap water, followed by an RO rinse. Soak the PVC coupling in RO water for a minimum of two hours. Dry before using.

D.3 Slick Preparation

1. Fill the labeled exposure chamber with 450 mL of source water. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, as indicated in the test-specific TCT. Place labeled test beakers in the water bath to acclimate the test solutions to the correct temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Weigh 2 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 2 g of oil as possible around the inside of the bottom half of the PVC coupling. Keep the oil ring approximately 1 cm away from the edge of the PVC coupling.

6. Weigh the PVC coupling with the oil to determine the total amount of oil that was added to the PVC coupling. Record the results.

7. Place a dowel through the holes drilled in the top portion of the PVC coupling.

8. Repeat steps 1–7 for each replicate.

9. Rest the ends of the wooden dowel on the top of the exposure beaker so that the oil from the PVC coupling is in contact with source water (Figure D.1). Repeat for each replicate.

10. If a PVC coupling control is required (see test-specific TCTs), set up one treatment that includes the PVC coupling but excludes the oil.

11. Place the PVC coupling in the water and allow the PVC coupling to soak in the exposure beakers for approximately 5.5 hours while in the water bath.

Figure D.1. Top view of the slick exposure set-up. Photo: Abt Associates.

D.4 Embryo Exposure

1. Before starting the test, measure water-quality parameters in the control beakers without PVC couplers (temperature, conductivity/salinity, DO, ammonia, and pH), as described in the Stratus Consulting GLPP document. Record water quality results on standard "Water Quality Monitoring" data entry bench sheets.

2. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the

egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm. Collect the embryos from the collection beaker into a new Petri dish with source water.

3. Use a pipette to collect embryos from the Petri dish. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Collect a total of 20 embryos.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously, expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into test beakers. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. During testing, keep the beakers in the water bath except when being inspected. The light cycle may vary during test maintenance because of routine hatchery operations.

7. After 24 hours of exposure, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record the organism as dead if you can fully identify intact carcasses or dead eggs. Do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole dead organisms and remnants from the exposure chamber using a treatment-dedicated pipette; archive them at -20°C, according to the QAPP.

8. Repeat step 7 for each 24-hour time period prior to test termination.

9. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 6. Immobile or morbid organisms may be removed from the exposure chamber using a pipette and inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. Enumerate the number of morbid organisms using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of the test. Collect live and morbid organisms and fix them in 10% buffered formalin for potential analysis, and archive all remaining live and dead organisms that are not fixed in formalin at -20°C, according to the QAPP. See test-specific TCTs for exposure duration and test length.

10. Use a siphon to composite the water from the four replicates of each treatment into a clean beaker. Avoid collecting the top 50 mL of water.

11. Swirl the composite water beaker and pour 250 mL of the solution into the analytical chemistry bottle. Ship it on ice overnight to ALS Environmental for analysis.

12. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

E. Testing Protocol 5: Red Drum Chronic Spiked Sediment Exposures

E.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

Place any unused prepared sediment into a zip-lock bag and store in the dark at 4°C (short-term) or in a freezer at -20°C (long-term). Store large quantities (> 5 kg) of prepared sediment in metal pans, covered with aluminum foil and stored in the dark at 4°C.

E.2 Test Organisms

Obtain red drum juveniles at approximately three weeks post-hatch, from one of the one-acre outdoor ponds located at the TPWD hatchery in Lake Jackson, Texas. Capture juvenile red drum from the deepest section of the pond, near the weir, using a long-handled dip net. Transfer fish from the net to buckets filled with pond water aerated with oxygen. Immediately transport fish to the TPWD hatchery incubation room for acclimation and testing.

Acclimate juvenile fish to 100% dilution/culture (source) water and hold for 2 days before test initiation to fully acclimate fish to testing water characteristics. Keep fish within 2°C of the test temperature for the 48 hours immediately before initiation of the study (see test-specific TCTs). Feed fish commercial fish food at least twice daily during the holding and test periods. See Source water and Fish culturing sections of the *Stratus Consulting General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp* for additional information. Only healthy looking, vigorous juveniles should be used to stock test exposure tanks.

E.3 Test Media Preparation

Mix oil into uncontaminated sediments (ALAJ46-C1127-SB701B) to prepare test media; use a KitchenAid stand mixer using the following procedure:

1. For each treatment, weigh and thaw 4 kg of sediments overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs.

2. Starting with the control treatment, weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, turn mixer on for 30 minutes at moderate speed (4.5 on mixer), and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

3. Transfer control sediment to a clean, decontaminated mixing bowl and cover with aluminum foil.

4. Working in order from low to high concentration treatments, measure out required mass of slick oil in a pre-cleaned aluminum weigh boat or glass beaker. Tare a weigh boat or beaker and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat.

5. Weigh approximately 4 kg of sediment, dispense into the KitchenAid mixing bowl, and transfer the oil from the measuring vessel into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

6. Mix the oil into the sediment for 30 minutes at moderate speed (4.5 on mixer) and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

7. Once the mixing is complete, transfer sediment oil mixture to a clean, decontaminated stainless steel mixing bowl and cover with aluminum foil. Be sure to thoroughly scrape the mixer bowl and paddle with a stainless steel putty knife or spoon to remove all of the excess oiled sediment.

8. Repeat steps 4 through 7 until all of the sediments in each treatment are prepared.

9. For each treatment, load four pre-cleaned exposure tanks and an 8-oz glass analytical chemistry sample sediment jar with prepared sediment. To load, add scoops of approximately 200 g sediment to each tank followed by a smaller scoop of approximately 10 g sediment to the sample jar. Repeat 5 times so that all sediment is used and each tank contains approximately 1–1.2 kg of wet sediment with the sample jar containing approximately 50 g of sediment (i.e., 3/4 full). To ensure sediment is equally divided among four replicate tanks, weigh each tank with sediment, and adjust volumes as needed.

10. Randomly place tanks with sediment back onto the rack system and replace tank lids, airlines, waterlines, and effluent lines.

11. Store labeled samples at 4°C until shipment to ALS Environmental using guidelines described in the project QAPP.

12. After all tanks are loaded with their appropriate sediment mixtures, open up the water inflow values and adjust to a slow and steady flow until each tank is filled to just under the overflow.

13. Turn off the water inflow and airlines to all tanks and allow the sediment to settle in the tank overnight under static conditions.

14. Several hours before beginning the test, turn on the flow-through water and aeration. Do this by opening up water inflow values and use ball valves to adjust inflow to a slow and steady flow. Flow rates should be set by observing the stream of water flowing into each tank. A slow flow rate is set at the point where the incoming water is a series of individual drops that are just less than a steady solid stream. This steady series of drops equates to approximately 3 L per hour flowing into each exposure tank. The air lines should then be turned on and adjusted to a slow inflow at one bubble per second.

15. After a few hours of running clean source water through the exposure tanks, take water quality measurements near the sediment/water interface. If the water quality is similar to the source water, then it is acceptable to start loading test organisms into exposure tanks. If the water quality is not similar to the source water, check water quality at the water surface. If the water surface water quality is better than at the sediment/water interface, increase the air and allow more time to equilibrate. Recheck the water quality at the sediment-water interface and near the surface, as needed, until it is similar to the source water.

E.4 Test Initiation

Tests will be initiated when juvenile red drum are transferred to each exposure tank following the procedure described below.

1. Set up a digital balance, a fish measuring board with millimeter increments, and a few 1-L plastic pour beakers for transferring fish. Also obtain enough *Tank ID Dilution or Stock Code Definitions* bench sheets to document start times for each treatment and tank and enough *Organism Length and Weight* bench sheets to record individual fish lengths and weights for each treatment and tank.

2. Obtain a random grab of juvenile red drum from the holding/incubation tank and transfer them into a clean tub of source water. Aerate the staging tub with an air stone while working on fish.

3. Randomly capture one fish from the staging tub by hand and inspect for gross abnormalities. Body condition should also be assessed. Abnormal or emaciated individuals will not be used for testing. Do not use MS-222 or any other method to anesthetize fish.

4. Place normal-looking juvenile fish on a damp measuring board and determine standard length to the whole millimeter. Standard length is measured from the tip of the upper jaw to the end of the hypural plate where the vertebral elements support the rays of the caudal fin.

5. After measuring standard length, quickly and carefully transfer the fish to a small weigh boat with water on a tared digital balance. Record the weight of the fish to the nearest hundredth of a gram.

6. Pour the fish from the weigh boat to a 1-L plastic pour beaker filled with fresh source water.

7. Repeat steps 2 to 6 until 10 individual red drum are measured and weighed.

8. Transfer all 10 fish to a randomly selected treatment exposure tank by lifting the tank lid and slowly pouring fish and water to avoid disturbing sediments. Record the time when transferred on the *Tank ID Dilution or Stock Code Definitions* data entry bench sheet.

9. Repeat steps 2 through 8 until all tanks have been stocked with 10 measured and weighed fish. Note that after every 4 to 5 tanks, return the batch of fish in the staging tub to the holding tank, and net a new random grab of fish and transfer them into the staging tub filled with fresh source water.

10. Collect a composite Day 0, unfiltered water sample from the control and each oil treatment for polycyclic aromatic hydrocarbon (PAH) analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface so sediments are not disturbed.

E.5 Test Maintenance

Tests will be monitored and maintenance performed at least twice daily for the entire test duration. Monitoring and maintenance will include assessing test performance, checking water quality, feeding test organisms, and verifying that the exposure system is working as designed by following the procedure described below.

1. Each morning, before doing any maintenance procedures that would potentially disturb the fish and cloud the water with suspended sediment, slowly and methodically search each tank for dead fish.

2. If a dead fish is observed, remove it using long-handled forceps or your gloved hand, and take and record standard length and weight measurements on a new *Organism Length and Weight* bench sheet. If the dead fish is too decomposed to be handled, forgo this step. Archive each dead fish or remnants thereof according to the project QAPP. If the tank is too cloudy to be thoroughly inspected, note it on the *Test Performance Monitoring* bench sheet.

3. After inspecting tanks and removing any dead fish, take water quality measurements. At a minimum, water quality measurements (temperature, pH, DO, conductivity/salinity, and total ammonia) will be taken from one tank from each treatment per day, so that after four days all tanks will be sampled. If warranted (i.e., aeration system stops working, all fish from a tank die suddenly), take water quality measurements from more tanks.

4. After recording water quality measurements, assess pH, DO, and total ammonia water quality results to determine if the tank inflow and aeration are sufficient for maintaining good water quality. Target water quality parameters are: pH > 7.50, DO > 4 mg/L, and total ammonia < 1 mg/L. If tanks are not meeting target water quality parameters, increase the inflow and/or increase aeration.

5. After taking water quality measurements and adjusting inflows as needed, feed each tank with the standard ration of prepared fed (Salmon Starter #2, Brand). The standard ration is 0.25 g of feed per feeding, with two feedings per day.

6. If *Artemia* are available, squirt approximately 1 mL of concentrated nauplii into each tank using a graduated, disposable pipette.

7. On day 1, 2, 4, 7, or 10, take water chemistry samples. Collect one unfiltered composite water sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each

bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface so sediments are not disturbed. Store labeled samples at 4°C until shipment to ALS Environmental, using guidelines described in the project QAPP.

8. Next, inspect the exposure system and, if necessary, replace the filter cartridges. Cartridges will be replaced at least once daily, but may need to be replaced more often when water is backing up in the sump while the pump is running, the pump housing is hot to the touch, or the pump is constantly running with water above the sump float switch. Also check that the water supply in the head tank is maintaining a consistent head, water lines are flowing, airlines are bubbling, and drain lines inserted into the drain pipe manifold are not clogged. Tank overflow bulkhead screens may also need to be periodically cleaned. Do this by having a spare, clean screen assembly on hand; remove the old screen and immediately replace with the clean screen. Do not leave the overflow bulkhead unscreened for more than a second or two while it is replaced with a clean screen.

9. Return in the late afternoon/early evening to feed fish. Perform the second daily feeding by adding 0.25 gm of feed into each tank.

10. Repeat steps 1 through 9 each day of the test. On the day the test is terminated, assess each tank for dead fish, conduct water quality measurements, and feed fish. In addition, collect end-of-test water chemistry samples. Water chemistry samples will be taken as described in step 7 above. Additional test termination procedures are described in the next section.

E.6 Test Termination

Upon test termination, testing staff should have all or most of the sample containers pre-labeled and water quality, first feeding, and water chemistry sampling described in Section E.5 above completed. All assessment and tissue sampling equipment and reagents should also be prepared and ready for use before the following test termination protocols are followed:

1. Pull the first randomly selected exposure tank from the system. Disconnect and turn off the inflow and airline tubing. The outflow tubing line may also need to be disconnected from the outside hose barb on the exposure tank overflow bulkhead.

2. Net all of the fish from the tank and transfer into a 2-L plastic pour beaker with fresh source water, aerated with an air stone. You may have to carefully pour the tank overlying water through a net over a bucket to capture all the fish in the tank and ensure that no fish remain in the tank. Decant as much overlying water as possible into a bucket.

Using a clean decontaminated stainless steel scoop, thoroughly mix and scoop out approximately 2 ounces of sediment into a treatment-specific 8-oz composite sediment chemistry sampling jar. This process will be repeated with each tank until each jar is filled with sediment from all remaining replicates for that treatment. Keep jars capped and cool between tanks. Dump the remaining sediment into a waste bucket for offsite disposal. Note that most of step 2 can be done while other testing staff are working on fish from the previous tank; however, to limit the length of time fish are held in the 2-L pour beaker, do not break down and net fish out of more than one tank while fish from the previous tank are being worked on.

3. Label the 2-L pour beaker containing the fish and bring into the wet laboratory for weight/length measurements and dissection.

4. Once fish are delivered to the wet laboratory, testing staff will immediately start to work on the fish.

5. First, randomly grab one to two fish from the 2-L pour beaker and place into a weight boat containing a 400-mg/L solution of MS-222 in source water. Wait approximately 30 sec. until all opercular movement has stopped.

6. Next, transfer one of the fish from the MS-222 bath to the measuring board and record standard length to the nearest millimeter. Record results on the *Organism Length and Weight* bench sheet.

7. Next, transfer the fish from the measuring board to a new, clean dry weigh boat on a tared balance and weigh to the nearest hundredth of a gram. Record results on the *Organism Length and Weight* bench sheet.

8. After recording weight, transfer the weigh boat and fish to the microbiome sampling station.

9. Using clean, ethanol dipped, and flamed micro scissors, carefully cut and peel back the right gill operculum to expose the gills. Detach one to three gill arches by cutting the dorsal and ventral ends of the gill arches. Using the same micro scissors, transfer the gill tissue to a 1.5-mL certified DNA/RNA free micro centrifuge tube. Fill micro centrifuge tube with at least 1 mL of RNAlater® RNA Stabilization Reagent (Qiagen, Duesseldorf, Germany) to fully submerse the tissue.

10. Using a second set of clean, ethanol dipped, and flamed micro scissors, carefully slice open the abdominal cavity from the anus to the head and tease out the gastrointestinal tract (GI). If possible, pull back the GI to expose its attachment to the stomach. Try not to disturb any other organs or tissue. Detach the GI from the stomach and anus so that the

entire GI is sampled. Using the same micro scissors, transfer the GI tissue to a 1.5-mL certified DNA/RNA-free micro centrifuge tube. Fill the micro centrifuge tube with 1 mL of RNAlater® RNA Stabilization Reagent (Qiagen, Duesseldorf, Germany) to fully submerse the tissue.

11. Between each use, clean dissection tools with soap and hot water, dip into 90% ethanol, flame off remaining ethanol, and let air dry on a new, clean paper towel before using to collect microbiome samples.

12. Place fish and remaining tissues into a 50-mL conical Falcon tube with 10% buffered formalin. All post-dissection fish from one exposure tank/replicate will be stored in the same conical Falcon tube.

13. Repeat steps 5 through 12 until the first four fish in each tank are sampled for microbiome and histological analyses. If needed, top off the 50-mL Falcon tube with more 10% buffered formalin and store microbiome samples at 4°C for the first 24 hrs, then transfer to -20°C.

14. Measure and weigh each remaining fish as described in steps 5 through 7. After weighing each fish, use a scalpel or micro scissors to carefully cut open the abdominal cavity laterally from the anus to the isthmus and place into a 50-mL Falcon tube with 10% buffered formalin. All remaining fish from one exposure tank/replicate not sampled for potential microbiome analysis will be stored in the same conical Falcon tube.

15. If needed, top off the 50-mL Falcon tube with more 10% buffered formalin and store at room temperature.

16. Repeat steps 1 through 14 until all exposure tanks have been taken down and fish and sediments have been sampled.

17. Clean exposure tanks and system. All remaining sediments from a single test should fit into one 5-gallon bucket. Oil-contaminated overlying water generated during tank breakdown and cleaning should be decanted into the sump of the exposure system with the filtration system running. Any solids and oily water remaining in the sump after disconnecting the filter system will be collected in 5-gallon buckets. Buckets containing oily water and sediments will be capped and transported to the nearest offsite oil waste recycling center for disposal. Any oil water generated during testing activates will also be containerized and either dropped off or be picked up by an offsite oil waste recycling company.

F. Testing Protocol 6: Pacific White Shrimp Chronic Spiked Sediment Exposures

F.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

Place any unused prepared sediment into a zip-lock bag and store in the dark at 4°C (short-term) or in a freezer at -20°C (long-term). Store large quantities (> 5 kg) of prepared sediment in large metal pans, covered with aluminum foil, and stored in the dark at 4°C.

F.2 Test Organisms

Obtain PL Pacific white shrimp, 8 to 10 mm in total length, from SIS. Transfer all of the shrimp and the water they were shipped in to an empty, clean incubation tank. Turn on the inflow so that a small trickle of source water begins to fill the incubation tank. After approximately 12 hrs trickling water into the incubation tank, measure incubation tank water quality and compare to raw source water quality. If incubation water quality is similar to source water quality, open the incubation tank inflow to a steady stream. Inflow should be set at a rate that is sufficient to cause some circular current around the tank, but not overwhelm the incubation tank overflow screens or rack system sump and filter. Refer to the *Stratus Consulting General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp* for a detailed description of the shrimp incubation and rack system. Hold PL shrimp in 100% dilution/culture (source) water for 2 days before test initiation to fully acclimate fish to testing water characteristics. Keep shrimp within 2°C of the test temperature for the 48 hrs immediately before initiation of the study (see test-specific TCTs).

F.3 Test Media Preparation

Mix oil into uncontaminated sediments (ALAJ46-C1127-SB701B) using a KitchenAid stand mixer, following the procedure described below.

1. For each treatment, weigh and thaw 4 kg of sediments overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs.

2. Starting with the control treatment, weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, turn mixer on for 30 minutes at moderate speed (4.5 on mixer), and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

3. Transfer control sediment to a clean, decontaminated mixing bowl and cover with aluminum foil.

4. Working in order from low to high concentration treatments, measure out required mass of slick oil in a pre-cleaned aluminum weigh boat or glass beaker. Tare a weigh boat or beaker and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat.

5. Weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, and transfer the oil from the measuring vessel into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

6. Mix oil into the sediment for 30 minutes at moderate speed (4.5 on mixer) and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

7. Once the mixing is complete, transfer the sediment oil mixture to a clean, decontaminated stainless steel mixing bowl and cover with aluminum foil. Be sure to thoroughly scrape the mixer bowl and paddle with a stainless steel putty knife or spoon to remove all of the excess oiled sediment.

8. Repeat steps 4 through 7 until all of the sediments in each treatment are prepared.

9. For each treatment, load four pre-cleaned exposure tanks and an 8-oz glass, analytical chemistry sample sediment jar with prepared sediment. To load, add scoops of approximately 200 g sediment to each tank followed by a smaller scoop of approximately 10 g sediment to the sample jar. Repeat 5 times so that all sediment is used and each tank contains approximately 1–1.2 kg of wet sediment with the sample jar containing approximately 50 g of sediment (i.e., 3/4 full). To ensure the sediment is equally divided among four replicate tanks, weigh each tank with sediment, and adjust volumes as needed.

10. Randomly place sediment-loaded tanks onto the rack system and replace tank lids, airlines, waterlines, and effluent lines.

11. Store labeled samples at 4°C until shipment to ALS Environmental using guidelines described in the project QAPP.

12. After all tanks are loaded with sediment mixtures, open up water inflow values and adjust to a slow and steady flow until each tank is filled to just under the overflow.

13. Turn off the water inflow and airlines to all tanks and allow the sediment to settle in the tank overnight under static conditions.

14. Several hours before beginning the test, turn on the flow-through water and aeration. Do this by opening up water inflow values and use ball valves to adjust inflow to a slow and steady flow. Flow rates should be set by observing the stream of water flowing into each tank. A slow flow rate is set at the point where the incoming water is a series of individual drops, which is less than a steady solid stream. This steady series of drops equates to approximately 3 L per hour flowing into each exposure tank. Airlines should then be turned on and adjusted to a slow inflow at one bubble per second.

15. After a few hours of running clean source water through sediment loaded and settled exposure tanks, take water quality measurements near the sediment water interface. If the water quality is similar to source water, then it is acceptable to start loading test organisms into exposure tanks. If the water quality is not similar to the source water, check the water quality at the water surface. If the water surface water quality is better than at the sediment/water interface, increase the air and allow more time to equilibrate. Recheck the water quality at the sediment/water interface and near the surface, and increase the air and/or water inflow as needed until the exposure tank water quality is similar to the source water.

F.4 Test Initiation

Tests will be initiated when PL shrimp are transferred to each exposure tank following the procedure described below.

1. Obtain a few 2-L plastic pour beakers and enough *Tank ID Dilution or Stock Code Definitions* bench sheets to document start times for each treatment and tank.

2. Dip the plastic pour beaker into the shrimp incubation/holding tank to obtain a random grab of shrimp. Obtain a new random grab of shrimp for the incubation/holding tank after stocking approximately every four to five exposure tanks.

3. Using a disposable pipette with the tip cut off, aspirate a random grab of a few shrimp and dispense into a plastic Petri dish. Inspect each shrimp for gross abnormalities and presence of food in gut (PL shrimp are transparent). Dispose of any abnormal-looking shrimp and shrimp that do not contain food in their digestive system.

4. Repeat steps 2 and 3 until 20 individual, healthy-looking shrimp are in the plastic Petri dish.

5. Randomly select an exposure tank and gently pour shrimp from the Petri dish into the tank. Thoroughly inspect the Petri dish to ensure that all 20 shrimp were transferred. If needed, gently wash any remaining shrimp from the Petri dish into the exposure tank using a disposable pipette and source water. Record start time on the *Tank ID Dilution or Stock Code Definitions* bench sheet.

6. Repeat steps 2 through 5 until all exposure tanks have been stocked.

7. Collect a composite Day 0, unfiltered water chemistry sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface while not disturbing the sediment. Confirm that shrimp are not aspirated into the pipette each time it is used to collect exposure tank water by thoroughly inspecting pipette contents behind a dark-colored background.

F.5 Initial Shrimp Length Measurements

Measure total shrimp length to the nearest millimeter for 40 individual shrimp and calculate an average length for the shrimp at the start of the test. Given that shrimp length measurements can cause damage to the shrimp, do not use any of these shrimp in test exposures. Follow the steps below to measure total shrimp length.

1. Using a 2-L plastic pour beaker, collect a sub-sample of shrimp from the stock shrimp tank.

2. Pour a sample with shrimp through a nylon screen to concentrate shrimp on the screen.

3. Using clean, metal forceps, carefully transfer an individual shrimp from the screen to a black, plastic metric ruler.

4. Align shrimp so that its rostrum is facing left and the body of the shrimp is fully stretched out.

5. Measure to the nearest millimeter the length of the shrimp starting from the rostrum to the tip of the uropod (i.e., tail).

6. Archive all in shrimp in a 15-mL conical tube filled with 10% neutral buffered formalin.

F.6 Test Maintenance

Tests will be monitored and maintained at least twice daily for the entire duration of the test. Monitoring and maintenance will include assessing test performance, checking water quality, feeding test organisms, and verifying that the exposure system is working as designed by following the procedure described below.

1. Take water quality measurements. At a minimum, water quality measurements (temperature, pH, DO, conductivity/salinity, and total ammonia) will be taken from one tank from each treatment per day, so that after four days all tanks will be sampled. If warranted, take water quality measurements from more tanks.

2. After recording water quality measurements, assess pH, DO, and total ammonia water quality results to determine if tank inflow and aeration are sufficient in maintaining good water quality. Target water quality parameters are: pH > 7.50, DO > 4 mg/L, and total ammonia < 1 mg/L. If the tanks are not meeting target water quality, turn up the inflow and/or increase aeration.

3. After taking water quality measurements and adjusting inflows as needed, feed each tank with the standard ration of *Artemia* nauplii. Fed each tank by aspirating 200 µL of nauplii feeding stock solution into a micro pipette and dispense into each tank. Keep the nauplii feeding stock solution well mixed when drawing standard rations. Refer to the next procedure in Section F.7 for a description of how to prepare the nauplii feeding stock solution.

4. After all shrimp exposure tanks have received food, pour remaining nauplii feeding stock into the incubation/holding tank.

5. On test Day 4, take water chemistry samples. Collect one unfiltered composite water sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under

the water surface while not disturbing the sediment or aspirating shrimp. Store labeled samples at 4°C until shipment to ALS Environmental using the guidelines described in the project QAPP.

6. Next, inspect the exposure system and, if necessary, replace the filter cartridges. Cartridges will be replaced at least once daily, but may need to be replaced more often when water is backing up in the sump while the pump is running, the pump housing is hot to the touch, or the pump is constantly running with water above the sump float switch. Also check that the water supply in the head tank is maintaining a consistent head, water lines are flowing, airlines are bubbling, and drain lines inserted into the drain pipe manifold are not clogged. Tank overflow bulkhead screens may also need to be periodically cleaned. Do this by having a spare, clean screen assembly on hand; remove the old screen and immediately replace with a clean screen. Do not leave the overflow bulkhead unscreened for more than a second or two while it is replaced with a clean screen.

7. Return in the late afternoon or early evening to conduct the second daily feeding. Perform the second daily feeding by adding 200 µL of nauplii feeding stock solution into each tank using a micro pipette. Pour all remaining nauplii feeding stock solution into the incubation/holding tank after all exposure tanks have received food.

8. Repeat steps 1 through 7 each day of the test. On the day the test is terminated, conduct water quality measurements and collect end-of-test water chemistry samples; however, do not feed the shrimp. Collect water chemistry samples following procedures described in step 5 above. Additional test termination procedures are described in the Test Termination (Section F.8) procedures section.

F.7 Preparation of Nauplii Feeding Stock Solution

Nauplii feeding stock solution is used to feed experimental shrimp and maintain shrimp held in the incubation/holding tank throughout testing activities. Two batches of 24-hrs-old *Artemia* nauplii cultures will be cultured, but staggered in age by approximately 12 hrs so that each culture is used to make a fresh feeding stock during each feeding. Stocks are prepared by testing staff daily following the procedure described below.

1. Thoroughly clean one 10-L *Artemia* hatching cone using clean source water and a soft bristled brush.

2. Set hatching cone into the cone rack and ensure that the bottom petcock is closed.

3. Fill hatching cone with 10 L of clean, UV-sterilized, 5-µm, filtered TPWD hatchery source water. The UV sterilized filtered source water can be obtained from the source water hose attached to the filter system located at the back of the incubation room, near the shrimp holding tank. Ensure that the 250-gal holding tank that is attached to the filter system does not run dry. Fill the holding tank with raw source water as needed.

4. Insert a hard plastic air line into the hatching cone and adjust airflow so that water becomes turbulent, but does not bubble over the top.

5. Check the salinity of hatching cone water and add commercial sea salt mix or RO water to adjust salinity to approximately 32 ppt.

6. Obtain *Artemia* cysts from the refrigerator, measure out 40 grams of cysts, and pour into one hatching cone.

7. After approximately 12 hrs, turn off or remove airline and let settle for approximately 1 hr. Turn on the spotlight and aim it at the bottom of the hatching cone to aid settlement. Settlement is complete when empty and unhatched cysts are floating, the center section is clear, and the bottom contains a thick layer of reddish-orange, live nauplii.

8. Harvest all of the bottom layer of nauplii by opening up the bottom petcock over an empty, clean 2-L plastic pour beaker. Close the petcock when almost all of the bottom layer has drained.

9. Add clean source water to the 2-L plastic pour beaker to bring the total volume of nauplii stock to 1 L.

10. Use stock solution to feed shrimp exposure tanks and holding tank. Stock solution should contain approximately 3,000 nauplii per millimeter.

11. Repeat steps 1 through 10 after each feeding.

F.8 Test Termination

Upon test termination, testing staff should have all or most of the sample containers pre-labeled and water quality, first feeding, and water chemistry sampling (described in Section E.6) completed. All equipment and reagents should also be prepared and ready for use before the following test termination protocols are performed:

1. Pull the first randomly selected exposure tank from the system. Disconnect and turn off the inflow and airline tubing. The outflow tubing line may also need to be disconnected from the outside hose barb on the exposure tank overflow bulkhead.

2. Carefully pour the tank overlying water and shrimp through a 200-µm Nitex screen positioned over a bucket to capture water as it is poured through the screen. Decant as much overlying water as possible, without disturbing sediment, through the screen. Visually inspect to ensure no shrimp are still in the tank.

3. Pour clean source water through the screen to rinse as much sediment off the screen as possible, and place the screen with shrimp into the tray. Rinse shrimp from the screen into a staging tub filled with clean source water. Ensure that no shrimp remain on the screen. Thoroughly clean or replace the screen between tanks.

4. Transfer shrimp from the screen to an aluminum weigh boat using metal forceps. Blot dry, count, and weigh. Record counts on the *Test Performance Monitoring Bench Sheet* and weights on the *Weight and Length Bench Sheet*.

5. Transfer all shrimp from the weigh boat to a 15-mL conical Falcon tube containing 10% neutral buffered formalin. It may be necessary to decant most of the formalin and refill with fresh formalin after all shrimp have been added.

6. Repeat steps 1 through 5 until all exposure tanks have been taken down and shrimp and sediments have been sampled.

7. Using a clean decontaminated stainless steel scoop, thoroughly mix and scoop out approximately 2 oz of sediment into a treatment-specific 8-oz composite sediment chemistry sampling jar. This process will be repeated with each tank until each jar is filled with sediment from each replicate tank for each treatment. Cap jars and store at 4°C until shipped to ALS for analysis. Discard remaining sediment into a waste bucket for offsite disposal.

8. Clean exposure tanks and system. All remaining sediments from a single test should fit into one 5-gal bucket. Oil-contaminated overlying water generated during tank breakdown and cleaning should be decanted into the sump of the exposure system with the filtration system running. Any solids and oily water remaining in the sump after disconnecting the filter system will be collected in 5-gal buckets. Buckets containing oily water and sediments will be capped and transported to the nearest offsite oil waste recycling center for disposal. Any oil water generated during testing activates will also be containerized and either dropped off or picked up by an offsite oil waste recycling company.

9. The following day, transfer shrimp from the 15-mL sample tube with 10% neutral buffered formalin to a small holding vessel (e.g., weigh boat). Pull off excess formalin with a transfer pipette and return to the 15-mL tube.

10. Measure final shrimp length for each individual in the sample according to steps 3–5 in Section F.5. Once the length has been measured, place individual shrimp back in the appropriate sample tube.

11. Repeat steps 9 and 10 for each shrimp sample so that the total length of all individual shrimp collected during test termination has been measured.

15. Marin Biologic Laboratories, Inc. General Laboratory Procedures and Practices

15.1 Introduction

Marin Biologic Laboratories, Inc. (Marin), conducted human adrenal cell line toxicity tests to identify toxicological effects of the 2010 *Deepwater Horizon* (DWH) oil spill. This chapter describes general laboratory practices and procedures used at Marin. Marin provided Stratus Consulting with work plans and data reports describing the procedures used to conduct each test. Stratus Consulting used information from work plans and reports to prepare this General Laboratory Procedures and Practices (GLPP) chapter.

15.2 Testing Methods

Marin tested the effects of DWH oil exposure on cortisol, aldosterone, and progesterone production, as well as steroidogenesis genes in human adrenal cells. Additional adrenal cell line exposures were conducted to characterize adrenocorticotropic hormone (ACTH) and foskolin activity in stimulating cortisol and aldosterone production.

15.2.1 Test organisms

Experiments conducted by Marin used the human adrenal cell line H295R obtained from the American Type Culture Collection (ATCC) and grown in culture according to the ATCC standard protocol (available at http://www.atcc.org/products/all/CRL-10296.aspx#culturemethod).

The base-growth medium for this cell line is ATCC-formulated DMEM:F12 Medium (ATCC Catalog No. 30-2006). The base-growth medium was amended to include the following components; 0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, and 0.00535 mg/ml linoleic acid. After these additions, the amended base-growth medium was adjust to a final concentration of 2.5% Nu-Serum IV (Corning #355104) and 1% penicillin/streptomycin antibiotic (Penn Strep). Additives [insulin, transferrin, selenium, bovine serum albumin (BSA), and linoleic acid] were obtained from ITS + Premix (Corning #354352).

Adherent adrenal cells were grown at 37°C in a 5% CO_2-air incubator. Cells were sub-cultured by removing the culture medium and briefly rinsing with TryplExpress (trypsin-EDTA solution). After 5 to 15 min, the adherent cell layer dispersed. The cells were then diluted with the appropriate volume of fresh growth medium. The target density when sub-culturing cells was 4-8 x 10^4 cells/cm^2.

A cell culture was established by conducting an initial freeze of six cell vials in liquid nitrogen. To determine the proper conditions of maintenance for the cell line and for initiating a cell assay, a seven-day grow-out study was performed and growth curve established.

15.2.2 Exposure media preparation

Human adrenal cells were exposed to different concentrations of high-energy water accommodated fractions (HEWAFs) and low-energy water accommodated fractions (LEWAFs) that were prepared according to the *Quality Assurance Project Plan:* Deepwater Horizon *Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Standard water accommodated fraction (WAF) preparation bench sheets used to document the analyst, method steps, volumes/weights, and times when preparing HEWAFs and LEWAFs.

Slick A (DWH4748co or DWH4748cp), Slick B (DWH7616), and artificially weathered source oil (DWH7977) HEWAFs and Slick A (DWH4748co or DWH4748cp) LEWAFs were tested. WAFs were prepared using cell-line growth medium without fetal bovine serum or other protein supplements, to avoid their inactivation during sonication. These protein supplements were added at the appropriate concentrations for testing.

Aliquots (250 mL) of each undiluted HEWAF or LEWAF were sampled and sent for chemical analysis to ALS Environmental as described in the project QAPP.

15.3 Reporting and Testing Documentation

A workbook was created to document the analyst, method steps, volumes/weights, and times used. This workbook was used for all assays. In addition, data reports were prepared for each experiment. Raw data files were also submitted to Stratus Consulting.

A. Testing Protocol 1: Assessing HEWAFs for Potential Microbial Contamination

HEWAFs prepared with Slick A, Slick B, and artificially weathered source oil were tested for microbial contamination before initiation of definitive tests.

A.1 Test Design

For microbial contamination assessment, each test included four different HEWAF concentrations prepared in the cell culture media prepared as described in the Marin GLPP, but with the penicillin/streptomycin antibiotic added. Tests were carried out in 96-well flat bottom plates and 5-mL T-25 culture flasks. Cultures were maintained at 37°C in a humidified incubator for up to seven days and monitored microscopically for microbial outgrowth. All treatments were run in triplicate.

B. Testing Protocol 2: Assessing Cell Growth Following Exposure to HEWAF

B.1 Test Design

To establish sub-lethal effect concentrations for subsequent HEWAF tests, three dilutions of HEWAF were assessed for their effect on H295R cell line growth; these were the same three HEWAF concentrations tested in Testing Protocol 1. Cell growth was assessed daily for seven days by fluorescence using Alamar blue dye. At each time point, 20 µL of 0.6 M Alamar blue was mixed with 200 µL of cell cultures in a white, 96-well plate. After 1–4 hrs of incubation, staff recorded the fluorescence resulting from the intracellular reducing conditions of live cells. Cell growth in each treatment was reported as growth relative to a control cell culture. All treatments were run in triplicate.

C. Testing Protocol 3: Assessing Effects on Steroid Production and Gene Expression of Adrenal Cells Following Exposure to HEWAF

HEWAF made from two oil samples was tested for effects on cortisol, aldosterone, and progesterone production; and STARD1, CYP11A1, and HSD3B2 gene expression in the human adrenal H295R cell line.

Each test included five HEWAF concentrations, prepared using 1:2 serial dilutions starting at 1:10 stock HEWAF to growth medium, and two sets of control treatments. Additionally, 20 µL of 10^{-6} M ACTH was added to one set of controls to serve as a positive control. All treatments were run in triplicate.

C.1 Cell Culture Exposure

1. Plate 2 mL H295R cells in wells of a 12-well plate at 4 x 10^4 cells/cm^2 and allow adherence for 24 hrs

2. Remove the supernatant and add 2 mL of appropriate WAF treatment, or ACTH agonist

3. Incubate cultures at 37°C in a humidified CO_2 incubator for 48 h.

C.2 Steroid Assays (Cortisol, Aldosterone, and Progesterone)

1. After 48 hrs of exposure, collect culture medium from 96-well plate and freeze the sample at -80°C until analysis.

2. For progesterone and aldosterone, thaw samples on ice and assayed directly, using Cayman Enzyme Immunoassay (EIA) kit #582601 at a 1:20 dilution of samples, or using IBL International kit #RE52301 with undiluted samples, respectively.

3. For cortisol, extract samples before analysis. To extract, thaw medium on ice and acidify 500-µL samples to ~ pH 2 with three drops of 3M HCl.

4. Extract two times with 2–5 mL diethyl ether.

5. Evaporate under a gentle stream of nitrogen to remove all solvent.

6. Reconstitute the remaining residue in 500 µL enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA) and vortex several times to mix. Freeze extract and store at -80°C until further analysis.

7. Measure steroids by competitive ELISA, following the manufacturer's procedures for cortisol kit #500360, aldosterone kit #10004377, progesterone kit #582601 (Cayman Chemical, Ann Arbor, MI).

C.3 Quantitative PCR Assay for Gene Expression

For this procedure, gene expression was measured by quantitative polymerase chain reaction (qPCR) and expressed relative to a housekeeping gene. In addition, transcription of three steroidogenic genes, STARD1, CYP11A1, and HSD3B2, was quantified in H295R cells.

1. Expose cells according to procedures in Section C.1.

2. After 48 hrs, extract and purify RNA from cells using RNeasy Mini Spin Columns (Qiagen #74104), QIA Shredder (Qiagen #79656), and RNase-Free DNase Set (Qiagen #79254).

3. Prepare complementary DNA (cDNA) from each RNA sample. For reverse transcription, use the iScriptTM cDNA Synthesis Kit (BioRad, Hercules, CA, USA).

4. Perform real-time qPCR on the resulting cDNA by mixing 10 µL TaqMan Gene Expression Master Mix, 1 µL TaqMan gene expression assay, 5 µL RNase-free water, and 4 µL cDNA template. Set the thermal cycle profile to the following: 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 s; and 60°C for 1 min.

5. Quantify expression of mRNA using the Ct method. Ct is the PCR cycle at which the signal crosses the amplification threshold value set in the linear phase of the reaction. Normalize the Ct values for each gene of interest to the "housekeeping" gene β-actin. Use normalized values to calculate the degree of induction or inhibition and express as a "fold difference," compared to normalized control values. Report all data as the statistical difference in "fold induction" between the exposed and control cultures.

D. Testing Protocol 4: Assessing Steroidogenesis Effects on Adrenal Cells Following Exposure to HEWAF and LEWAF

Adrenal cells were exposed to HEWAF and LEWAF made with Slick A oil to determine their effect on cortisol and aldosterone production to the supernatant by the human adrenal cell line H295R after 2 and 7 days of incubation and to determine their CYP11A1 and CYP11B2 gene expression. All treatments were run in triplicate.

D.1 Cell Culture Exposure

Adrenal cells were assessed for steroidogensis effects following two and seven days of exposure. For each test, a matrix of six WAF concentrations (including control) and up to three agonist conditions (ACTH induction, forskolin induction, or no agonist) were assessed. All treatments were run in triplicate. Different initial cell concentrations are necessary to achieve approximately 50–90% confluence at the Day 2 and Day 7 time points.

1. For the two-day exposures, plate 2 mL H295R cells at 4 x 10^4 cells/cm^2 in a 12-well plate and allow adherence for 24 hrs. For the seven day exposures, plate 2 mL of H295R cells at 1 x 10^4 cells/cm^2 in a 12-well plate and allow adherence for 24 hrs.

2. After adherence, aspirate off the cell culture medium and replace with 2 mL of the appropriate treatment medium. For the two-day exposures, also add appropriate agonist to wells: use a 1-µM solution for ACTH and a 5-µM solution for forskolin. For the seven-day exposures, add agonist to wells on the fifth day.

3. Incubate cultures at 37°C in a humidified CO_2 incubator for 2 or 7 days based on the treatment (see test-specific TCT for exposure durations).

D.2 Viable Cell Measurement

After appropriate incubation time (2 or 7 days), quantify viable cells to normalize assay results.

1. To quantify viable cells, remove 1 mL of cell culture supernatant from each well and set aside for assays

2. Add 100 µL of Alamar blue to the remaining cell culture in each well and incubate for 2 to 4 hrs

3. Measure fluorescence at 560 nm/590 nm.

D.3 Steroid Assays

1. For aldosterone quantification, pipette 100 µL of the cell culture supernatant that was set aside, above, into a 96-well plate from Cayman EIA kit #582601 or an IBL International kit #RE52301, and follow instructions. For cortisol, acidify 100 µL of cell culture supernatant to ~ pH 2 using three drops of 3M HCl.

2. Extract two times with 2–5 mL diethyl ether solvent.

3. Evaporate under a gentle stream of nitrogen to remove all solvent.

4. Reconstitute the remaining residue in 500-µL EIA buffer provided with the kit (Cayman Chemical, Ann Arbor, MI, USA, Kit #500360) and vortex several times to mix.

5. Dilute samples 1:10 in EIA buffer and then analyze according to instructions for Cayman EIA kit #500360.

6. If not analyzed immediately, freeze extract and store at -80°C until analysis.

D.4 Gene Expression

This protocol describes the quantification of CYP11A1 and CYP11B2 gene expressions measured by QPCR and expressed relative to a housekeeping gene.

1. On Day 2 and Day 7, collect cell culture supernatant from each well, and spin to create cell pellet.

2. Freeze and store at -80°C until extraction. Extract RNA from cell pellets and purify using RNeasy Mini Spin Columns (Qiagen #74104), QIA Shredder (Qiagen #79656), and RNase-Free DNase Set (Qiagen #79254).

3. Prepare cDNA from each RNA sample. For reverse transcription, use the iScriptTM cDNA Synthesis Kit (BioRad, Hercules, CA, USA).

4. Perform real-time qPCR on the resulting cDNA by mixing 10 µL TaqMan Gene Expression Master Mix, 1 µL TaqMan gene expression assay, 5 µL RNase-free water, and 4 µL cDNA template. Set the thermal cycle profile to the following: 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 s; and 60°C for 1 min.

5. Analyze cDNA for expression of the genes CYP11A, CYP11B2, β-actin "housekeeping gene."

6. Quantify expression of mRNA using the Ct method. Ct is the PCR cycle at which the signal crosses the amplification threshold value set in the linear phase of the reaction. Normalize the Ct values for each gene of interest to the "housekeeping" gene β-actin. Use normalized values to calculate the degree of induction or inhibition and express as a "fold difference," compared to normalized control values. Report all data as the statistical difference in "fold induction" between the exposed and control cultures.

16. Louisiana Universities Marine Consortium General Laboratory Procedures and Practices

16.1 Introduction

The goal of this project is to determine the effects of *Deepwater Horizon* (DWH) oil on mortality rates, hatch rates, and the development of four resident fish species in the Gulf of Mexico. These endpoints were assessed in a series of toxicity tests conducted on Gulf menhaden (*Brevoortia tyrannus*) embryos, larvae, and juveniles; Bay anchovy (*Anchoa mitchilli*) embryos and larvae; red snapper (*Brevoortia patronus*) embryos; and sand seatrout (*Cynoscion arenarius*) embryos.

16.2 Methods

16.2.1 Test organisms

Menhaden and sand seatrout embryos: Wild Gulf menhaden and sand seatrout eggs were collected at sea approximately 25–30 miles offshore into the Gulf of Mexico from Cocodrie, LA. Eggs were collected with a neuston net (0.8-mm mesh) that was towed at the water surface. Unsorted net collections were transported back to the Louisiana Universities Marine Consortium (LUMCON) Marine Center in large coolers. At the laboratory, menhaden eggs were sorted from other plankton by allowing them to float and skimming them with 500-µm netting or a small beaker. The skimmed embryos were transferred into clean, filtered seawater with the same temperature and salinity as the seawater at the collection site; the embryos were kept in this water until the test began. Collections occurred in February and March, as weather allowed.

Surface temperatures and salinity measurements were collected during egg collection to help determine an appropriate temperature during housing, exposures, and post-exposure incubations.

Menhaden juveniles: Wild Gulf menhaden juveniles were collected by cast net in the LUMCON boat basin near Cocodrie, LA and held in a recirculating seawater system overnight before oil exposures. A pilot test was conducted to monitor ammonia and oxygen and to establish test loading rates that did not stress the fish under the expected conditions of the planned test.

Bay anchovy: Bay anchovy embryos and larvae were obtained from broodstock maintained at LUMCON. Broodstock were established by collecting juvenile anchovy off the LUMCON dock (29°15’14.42”N, 90°39’49.64”W) using neuston net (0.8-mm mesh), fitted with a solid glass

codend to reduce the chance of damage to the juveniles; laboratory staff reared the juveniles to adult stage. The adult anchovy population was maintained in a recirculating seawater tank (575-gallon, Red Ewald, Inc., Karnes City, TX, USA) with continuous aeration at 26–27°C, 25 ppt, and a 15:9 light:dark photoperiod. Fish were fed dry commercial fish feed (size 0.3, Cargill®, Minneapolis, MN, USA) and a liquid mixture of live-hatched Artemia (Brine Shrimp Direct, Ogden, UT, USA), cultured rotifers, and *Nannochloropsis oculata* phytoplankton. Both were delivered by automatic feeders set to distribute feed at eight-minute intervals throughout daylight hours. A total of 4 L of liquid feed and 10–15 g dry feed were distributed daily to approximately 35 fish. Spawned embryos were caught in an egg collection device within the same system as adult fish and held overnight until testing began the following morning.

Red snapper: Mature red snapper (*Lutjanus campechanus*) were collected ~ 36 miles offshore of Louisiana in June and July 2015 by hook and line and transported back to LUMCON in a transport tank fitted with oxygen aeration. Amquel™ was added to reduce ammonia stress during transport (~ 1 g per 4 L). At the time of collection all mature fish (> 43 cm total length) were inspected on the boat to tentatively identify sex. Suspected males were immediately sacrificed by placing them under ice, while females were held in a live tank fitted with aeration. At the laboratory, all iced males were dissected to remove their gonad and the gonads cut into 2-cm sections. The sperm from the 2-cm sections were then stripped into a solution of cold Hank's balanced salt solution (202 mM) and stored at 4°C until use. All living female snapper were anesthetized using clove oil (3.3 mg:20 L clove oil:seawater) and sexed by catheterization with Teflon™ tubing attached to a 10 ml syringe. A small sample of gonad was removed from each female to confirm sex and to evaluate the size and condition of the oocytes for spawning. Suitable females were then injected with a priming dose (250 IU per kg) of Ovaprim® (Western Chemical, Inc., Ferndale, WA, USA) on the day of collection and held in a recirculating seawater system overnight at 30 ppt and 27–28°C on a 14:10 light:dark photoperiod. The next morning they were given a resolving dose (500 IU per kg) of Ovaprim®. That evening each female was inspected for properly developed, hydrated oocytes. Females with properly developed hydrated oocytes were stripped spawned and their oocytes placed into a glass bowl with sperm solution warmed to 27°C for fertilization. Fertilized embryos were water hardened and monitored for proper development. Embryos of females with properly developing embryos and a high fertilization rate were selected for use in oil exposures.

16.2.2 Source water

The source water used to maintain all fish embryos and larvae, and that was used in all oil exposures, was raw seawater collected regularly by boat from offshore Louisiana and stored in a holding system at LUMCON. Before use, the source water was UV sterilized and diluted down to appropriate salinity using 5-µm filters (Whirlpool®, Benton Charter Township, MI, USA).

16.2.3 Exposure media preparations

Gulf menhaden toxicity tests were conducted using two different water accommodated fraction (WAF) preparations: high-energy and low-energy WAFs (HEWAFs and LEWAFs, respectively). Both HEWAFs and LEWAFs were prepared as described in the standard operating procedure (SOP) *Protocols for Preparing Water Accommodated Fractions* described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. WAFs were prepared using Slick B (GU2888-A0719-OE701) or Slick A oil (CTC02404-02). See test-specific test condition tables (TCTs) for details.

Tests were also conducted using the slick preparation method. Generally, slick preparation consisted of placing a polyvinyl chloride (PVC) coupler with a 1-cm-thick strip of oil into the exposure chamber and allowing the PVC to soak for a given length of time before initiating the exposure. A more detailed discussion of this preparation can be found in test-specific procedures in the LUMCON General Laboratory Procedures and Practices (GLPP).

16.2.4 Water quality

Ammonia, dissolved oxygen, water temperature, and pH were monitored daily in all exposures and controls. For some tests, water temperature was also monitored continuously using a HOBO™ data logger (see test-specific TCTs). The exposure chambers were maintained at the appropriate temperature with the water batch connected to a heater/chiller. Salinity was measured in controls at the beginning and end of each experiment with a refractometer. See Section 16.4 for details on water quality protocols.

16.2.5 Analytical chemistry and archive sampling

To sample water for polycyclic aromatic hydrocarbons (PAHs), each 250-mL sample jar (provided by ALS Environmental) was filled to capacity. Sample collection, labeling, and handling were conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Sample storage and documentation [e.g., preparing required chain-of-custody (COC) forms] was conducted as described in the QAPP. Analytical chemistry samples were shipped on ice to ALS Environmental as soon as possible after collection

Analysis of PAHs in the water of each treatment was conducted by ALS Environmental Laboratories. Filtered and unfiltered water samples were collected. Methods for filtration can be found in the project QAPP, Appendix G. See test-specific TCTs for details on water sampling plans.

All unused or remaining WAF was disposed of according to LUMCON's waste disposal protocols.

16.3 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the QAPP. Photographs taken during planned tests were handled and documented as described in the QAPP.

16.4 General Testing Protocols

16.4.1 Water Quality SOPs

Temperature measurement SOP

1. Temperature measurement is performed using the Atkins AquaTuff™ 351 temperature probe.
2. Place thermometer in solution and wait for 1 minute. Record value.
3. Make sure to clean thermometer with absolute ethanol and distilled water between measurements.

Measurement of salinity SOP

1. Salinity is measured using a refractometer (Vital Sine SR-6 Refractometer). Using a glass Pasteur pipette, place 1–2 drops of test solution on the measurement window and close the lid. Point toward the light, making sure to keep the refractometer level.
2. Record value.
3. Decontaminate after each use by gently washing with warm soapy water, rinsing with deionized (DI) water, and carefully drying with Kimwipes.

Measurement of dissolved oxygen SOP

1. Dissolved oxygen is measured using a handheld digital YSI ProODO dissolved oxygen meter and probe (#626279).
2. The oxygen meter should be calibrated daily and the calibration logs should be filled out.

3. Calibration:

   a. *Salinity*: Because the solubility of oxygen in water decreases as salinity increases, it is important to input the test salinity into the meter before calibration and use. To input the salinity value into the meter, push the probe button and select *salinity* from the menu. Enter the appropriate value. Input the mean value of the measured salinity for the test replicates; salinity should not vary much between replicates. Record the calibration salinity used on the calibration log.

   b. *Temperature*: This meter does not need temperature calibration, although the meter reading should be recorded on the calibration log.

   c. *One-point calibration in water-saturated air*: This procedure is as described in the manufacturer manual. Moisten the calibration sponge with a small amount of water and ensure no water droplets are present on the sensor/temperature caps; do not immerse the sensors in water. Place sensors in calibration sleeve and allow to equilibrate for 5 minutes. Press *calibration* button, highlight *DO %* and press *enter*. Highlight the barometer value and press *enter*. Although the value can be changed, the value measured by the meter should be used. If the barometer value is off, then a barometric pressure calibration should be performed. Record the barometric pressure value on the calibration log. Wait for temperature and DO % values to stabilize and highlight *accept calibration* and press *enter*. No calibration efficiency rating is provided, but the user should check off on the calibration log that the meter was calibrated.

   d. *Barometric Pressure*: This value does not need to be routinely calibrated. If the value appears to be inaccurate, press the calibrate button and select *barometer*. Simply input an acceptable value and select *calibration*.

4. To test the dissolved oxygen in the test replicates, first set the meter to auto-stabilize by pressing probe and selecting *auto-stable*; the sensitivity should be set to the middle value, which allows a data variance of 1.275%. Place probe in the sample and briefly move to release air bubbles from the sensor. Continued movement is not necessary because of the optical luminescent measurement. Allow the value to stabilize and record it on the data sheet.

5. Between replicates, the probe should cleaned using Simple Green Cleaning Solution followed by distilled water.

Total ammonia (NH_3/NH_4^+) – API ammonia test kit SOP

1. Collect 5 mL of test solution and place it into the glass test tube provided in the API test kit. The test tube has a white 5-mL graduation line.

2. Add eight drops of test solution #1 to the test tube.

3. Immediately add eight drops of test solution #2 to the same test tube.

4. Tightly cap the test tube with the provided cap and shake vigorously for 5 seconds. Do not cap the tube with your finger because that might interfere with the test results.

5. Set the timer for 5 minutes and wait for the color to develop.

6. After 5 minutes, read the test results by comparing the test solution color to the saltwater ammonia color chart provided in the test kit. View the tube color against a white background in a well-lit area. Determine the closest color match between the test solution and the color chart and record the associated NH_3/NH_4^+ values. Record the value on the "Water Quality Monitoring" datasheet.

7. Pour the 5-mL solution into the appropriate disposal vessel.

8. After using the API kit container, clean it with mild soap and water and store until next use.

Measurement and maintenance of copper in broodstock tanks SOP

1. Measure copper levels 2 to 3 times per week in broodstock tanks using the HACH DR/890 Portable Colorimeter (Free Copper kit) Method 8506, briefly discussed below.

2. Fill one sample vial provided by HACH with 10 mL of DI water (the blank).

3. Select the appropriate program (Program 20) and press *enter* on the HACH portable colorimeter.

4. Place the blank into the colorimeter and cover with the instrument cap and press *zero*; the display should show 0.00 mg/L.

5. Fill another sample vial with 10 mL of the water sample and add the contents of one CuVer 1 Copper reagent powder pillow to the sample. Swirl to mix.

6. Press *timer* and *enter* and begin the 2-minute reaction period.

7. After the timer beeps, place the prepared sample into the colorimeter and cover with the instrument cap. Press the *read* button.

8. Record the value in the laboratory notebook.

9. Add copper to tanks where measured values are below 0.25 mg/L.

A. Testing Protocol 1: Gulf Menhaden/Sand Seatrout Embryo and Larval Acute Toxicity WAF Exposures

A.1 Test Solutions

HEWAF preparations followed the SOP provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated (acetone/hexane/DCM rinse) stainless steel commercial Waring™ CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. WAF was transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before using it in the exposure.

The appropriate amount of 100% stock WAF was added to each test beaker and two dummy beakers for each treatment (treatment-dependent), followed by clean seawater to bring the volume up to 1 L for embryo exposures and 2 L for larval exposures. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

A.2 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. Embryos were loaded into each test beaker, once the exposure solution reached desired temperature; exposures were allowed to run without renewals for 48–96 hours. All test beakers were covered with food-grade plastic wrap for the duration of the exposure and were maintained at 18 ± 1°C on a 8 L:16 D light cycle. See test-specific TCTs for number of treatments, number of replicates per treatment, number of organisms per replicate, and number of hours per test. See LUMCON GLPP for source and maintenance of test organisms.

4. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

5. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding. For some tests, live larvae were transferred to beakers with 2 L of clean water; latent mortality was assessed for an additional five days (see test-specific TCT).

6. All embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term archive storage at LUMCON.

A.3 Water Sample Analysis

1. Unfiltered water samples (250 mL) were collected from replicate "dummy" beakers prepared for each treatment at test initiation and from one test beaker per treatment at test termination.

2. Samples were analyzed for PAH analytes by gas chromatography/mass spectrometry (GC/MS) in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

B. Testing Protocol 2: Bay Anchovy Embryo Acute Toxicity WAF Exposures

B.1 Bay Anchovy Embryos

1. Bay anchovy are evening spawners; spawned embryos were caught in an egg collection device within the same system as adult fish and held there overnight until use in exposure testing the following morning. See LUMCON GLPP for the sourcing and maintenance of test organisms.

B.2 Source of Crude Oil and Test Solutions

1. HEWAF and LEWAF preparations followed the SOPs provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated [acetone/hexane/dichloromethane (DCM) rinse] stainless steel commercial Waring™ CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. WAF was then transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before the WAF was used in exposure. Stocks of LEWAF were prepared one day before exposure in a decontaminated aspirator bottle (Kimax®, Elmsford, NY, USA) at an oil:seawater loading rate of 1 g:1 L. LEWAF was stirred on a stir plate for 18–24 hours before use in exposure. For both oil preparation methods, the appropriate amount of 100% stock WAF was added to each test beaker and one dummy beaker for each treatment (treatment-dependent), followed by clean seawater to bring the volume up to 1 L. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

B.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. Embryos (n = 25) were then loaded into each test beaker and exposures allowed to run without renewals for 48 hours or 72 hours (see test-specific TCT).

4. Tests consisted of 4–6 oil treatments, plus a control using clean source water, and 3–4 replicates, depending on the availability of embryos (see test-specific TCT).

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 26–27°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

7. At test conclusion, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

B.4 Water Sample Analysis

1. 250 mL were collected from filtered samples (HEWAF exposures only) and unfiltered samples from replicate dummy beakers prepared for each treatment at test initiation, and from one test beaker per treatment at test termination.

2. The samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

C. Testing Protocol 3: Bay Anchovy Embryo Acute Toxicity Slick Exposures

C.1 Bay Anchovy Embryo

1. Bay anchovy are evening spawners; spawned embryos were caught in an egg collection device within the same system as adult fish and held there overnight until they were used in exposure testing the following morning. See LUMCON GLPP for source and maintenance of test organisms.

C.2 Test Solutions

1. Surface slicks were prepared by applying an approximately 1-cm-thick strip of oil (2 g) to the inner side of a 3-inch PVC coupler fitting. The PVC fitting was then placed into a 1-L beaker filled with seawater and suspended by a wooden dowel such that the oil ring aligned with the surface of the water. The oiled fitting was allowed to soak for 4–6 hours immediately before exposure so the oil could migrate off the PVC and onto the water's surface. Two dummy beakers for each treatment were prepared using the same methods for test beakers. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

C.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. During the surface slick soak, test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. At the conclusion of the slick preparation steps, embryos (n = 25) were carefully loaded beneath the slick by pipetting them between the PVC ring and the edge of the beaker at the same time the PVC ring was removed. Exposures were allowed to run without renewals for 48 hours.

4. Tests consisted of 4–6 oil treatments, plus a control using clean source water, and 3–4 replicates, depending on the availability of embryos (see test-specific TCT).

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 26–27°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in dummy beakers to avoid disturbing the surface slick, and in actual test beakers for the control treatment.

7. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

C.4 Water Sample Analysis

1. Unfiltered water samples (250 mL) were collected from replicate dummy beakers prepared for each treatment at test initiation and termination. To capture PAHs on the surface slick, a decontaminated 10-cm-diameter absorbent pad was placed on the slick for 3 minutes; upon removal, the pad was transferred into a wide-mouth jar for chemical analysis.

2. Water and pad samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

D. Testing Protocol 4: Red Snapper Embryo Acute Toxicity WAF Testing

D.1 Fish Collection and Spawning

1. Mature red snapper (*Lutjanus campechianus*) were collected from offshore Louisiana by hook and line and transported back to LUMCON in a live tank fitted with aeration. See LUMCON GLPP for source and spawning procedures for red snapper test organisms.

D.2 Test Solutions

1. HEWAF preparations followed the SOP provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated (acetone/hexane/DCM rinse) stainless steel commercial Waring™ CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. The WAF was then transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before use in exposure testing. The appropriate amount of 100% stock WAF was added to each test beaker (treatment-dependent), followed by clean seawater to bring the volume up to 1 L.

D.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at desired temperature.

3. Embryos (n = 25) were then loaded into each test beaker and exposures allowed to run without renewals for 48 hours.

4. Tests consisted of five oil treatments, plus a control that used clean source water, and relied on four replicates per treatment.

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 27–28°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

7. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

D.4 Water Sample Analyses

1. Unfiltered water samples (250 mL) were collected from replicate dummy beakers prepared for each treatment at test initiation and from one test beaker per treatment at test termination.

2. Samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

2. Analytical Methods Used in the *Deepwater Horizon* Natural Resource Damage Assessment Toxicity Testing

2.1 Toxicity Testing Analyses

In analyzing toxicity test results, we primarily modeled dose-response curves, from which we calculated effect and lethal concentrations (EC_x/LC_x). We based these models on the measured concentrations of polycyclic aromatic hydrocarbons (PAHs) or the known loading rates of Corexit. For experiments with ultraviolet (UV) treatments, we also performed analyses based on measured UV and phototoxic PAH concentrations. In some cases, we supplemented or replaced dose-response models with additional analyses, such as time-to-event plots or analyses of variance, to determine treatment levels. The following sections describe how we processed the data and performed the analyses.

2.1.1 Types of effects: Endpoints

While performing toxicity tests, scientists in testing laboratories measured many different toxic effects, or endpoints, of *Deepwater Horizon* (DWH) oil and dispersant. These endpoints included mortality, growth, reproduction, development, and others. In general, the testing laboratories measured most endpoints directly (e.g., organism weight) or provided calculated endpoint data (e.g., calculated oxygen use in swim-performance tests based on machine data). In some cases, however, the testing laboratories provided us with their original data, and we used the data to calculate response values. For example, if a testing laboratory recorded the number of dead organisms at multiple time points, we summed the number of dead organisms at each time point for each experimental replicate to produce records for cumulative mortality. In these cases, we analyzed the cumulative values. For continuous-response variables, we performed dose-response analyses on the average response of all measured individuals within a treatment replicate. The following sections contain descriptions for response values with complex or variable calculation procedures – procedures that have not been described in published literature or other technical reports.

2.1.1.1 Cumulative mortality

When toxicity laboratories collected information on mortality at multiple time points, we calculated cumulative mortality for each test replicate at each nominal time point. For larger organisms – those classified as juveniles or adults – we calculated cumulative mortality using the

number of dead organisms observed at each time point. For smaller organisms – those classified as embryos or larvae – or in cases where testing laboratories only counted the number of living organisms, we calculated cumulative mortality using the number observed alive at each time point. We corrected the latter type of cumulative mortality for non-treatment mortalities, such as mortality from sampling.

2.1.1.2 Estimation of oyster mortality

Dead oyster embryos and veligers before the pediveliger stage disintegrate quickly and are therefore hard to count. Oyster tests on these tiny life stages were performed with thousands of individuals per testing chamber, which made it impractical to count all living oysters. Therefore, rather than relying on observed mortality or survival, we estimated mortality from the stocking density (i.e., the number of individuals added to testing chambers), fertilization rates (when necessary), and the number of oysters still alive at the end of each test. A description of these calculations follows.

We estimated the number of oysters alive in each testing chamber by using the average number of oysters found alive in each subsample and the volume of the total test solution.

To estimate the number of dead oysters, we subtracted the estimated number of oysters still alive from the number of oysters expected to be alive in each tank. We calculated the number of oysters expected to be alive in each tank based on the initial stocking density [(number of oysters/mL) × (tank volume at the end of the test)]. This method accounted for any organisms removed during subsampling for other endpoints during the tests.

Some tests began with the exposure of gametes and continued through multiple life stages. For these tests, we corrected the mortality estimates for fertilization rates. To do this, we calculated the percentage of fertilized eggs and multiplied by the stocking density to estimate the maximum number of embryos that could be alive in each tank.

2.1.1.3 Estimating oyster settlement

Unlike oysters at earlier life stages (Section 2.1.1.2), the oysters in settlement tests do not decay readily; this allowed us to estimate stocking density from subsamples of oysters from stocked chambers, rather than from the subsamples of the stocking solution before placing the oysters in chambers. To determine the number of settled oysters in each test chamber, researchers counted all of the oysters that had settled on cement board tiles and on the chamber walls (Morris et al., 2015).

Oysters loaded into test chambers with sediment were difficult to count because some of them became intermixed with sediment. To address this problem, the researchers stocked some control chambers without sediment to determine the stocking densities for actual sediment test chambers. To estimate the number of stocked oysters in each test chamber, we scaled the average number of oysters that the researchers counted in subsamples to the test chamber volume. To calculate the total number of unsettled oysters in each chamber, we subtracted the total number of oysters that had settled from the estimated number of stocked oysters.

To estimate the number of oysters per sample for tests run without sediment, we summed the number of settled oysters and the estimate of unsettled oysters. To estimate the number of unsettled oysters, researchers identified and counted dead, living, and metamorphosed oysters that had not attached to the substrate in subsamples. We scaled average counts of these unsettled oysters to the test chamber volume to estimate the total number of unsettled oysters for each test chamber. To determine the total number of oysters in each test chamber, we added the number of settled oysters to the number of unsettled oysters.

2.1.1.4 Hatching

The testing laboratories collected slightly different data on some species or endpoints for hatching. Some tests included only one measurement of hatched versus unhatched eggs for each replicate. Other tests included measurements at additional time points, additional counts of deformed larvae, or distinctions between dead and living, but unhatched, eggs. In cases where records of dead or deformed organisms existed, we added these to the number of unhatched eggs to produce a measurement of "unviable" organisms. Because eggs may continue to hatch throughout a test, we did not provide analyses of unviable organism measurements for time periods before the mean hatching time in the control treatment for an experiment.

2.1.1.5 Growth

For juvenile fish in tests with initial measurements of each fish, we calculated growth using initial and final measurements of standard length or total weight [(final measurement – initial measurement)/#days]. For tests where it was not possible to follow individuals from beginning to end, we estimated growth using initial measurements of groups of organisms and comparing those to final measurements.

2.1.1.6 Heart rate

We presented heart rates in beats per minute. In some cases, testing laboratories measured heart rate over periods shorter than one minute; we converted these measurements to beats per minute.

2.1.2 Additional Stressors

For tests that included multiple exposures to additional stressors, such as UV light, salinity, temperature, and so forth, we fitted curves separately for each treatment. We recommend that users consider all treatments collectively when examining the data.

2.2 Dose Interpretations

For initial estimates of EC_x and LC_x, we based exposure concentrations on measured concentrations of unfiltered samples taken at test initiation (time zero) whenever available. When time-zero measurements were unavailable because of sample breakage or other issues, we estimated initial exposure concentrations from available samples and reported dilutions.

Validated chemistry data contained measurements for many analytes. For tests including oil, we used the sum of the concentrations of 50 PAH analytes (TPAH50) as our measurement of oil exposure (Forth et al., 2015).

2.2.1 Initial measurements of the TPAH50 analytes and dispersant concentrations

We calculated TPAH50 concentrations at the initial time point for tests that included oil. We used validation qualifiers to determine whether to include analytical measurements in summations. We removed all records with validation qualifiers for "remove" or "do-not-report," and set all records qualified as "non-detects" to zero before summation.

For tests of dispersant toxicity, we also analyzed the data based on reported loading rates of the dispersant.

For most tests, we based exposure concentrations for each treatment level on direct measurements of samples. In some cases, samples were unavailable for some or all treatment levels; this occasionally happened when samples broke or the sampling scheme included only samples for a subset of the treatment levels. In cases with no sample measurement for a treatment level, we inferred the initial exposure concentrations using available information. For example, if the sample for treatment level A for an experiment was broken, but the sample for treatment level B was not, we used a dilution coefficient and the direct measurement of treatment level B to determine the exposure concentration for treatment level A, rather than simply using the nominal exposure concentration for treatment level A.

2.3 UV380 Energy Measurements

Testing laboratories carried out UV experiments indoors under artificial light and outdoors under natural light. During outdoor tests, the researchers collected measurements of UV light at the 380 nm wavelength (UV_{380}) throughout each day of the experiments (Morris et al., 2015; Lay et al., 2015). We calculated the integrated UV_{380} dose based on UV_{380} measurements collected over the course of each day. To do this, we approximated the curve that connected the UV_{380} measurements from each day with *approxfun* (R Development Core Team, 2015), and then integrated under that curve using the quadrature method in the *pracma* package (Borchers, 2013).

2.4 Time Points: Nominal Hour Estimation

We used categories of time points (nominal hours) to compare measurements taken at different times. For instance, if testing laboratory staff began collecting mortality measurements 22.5 hrs after a test began and continued collecting them until 25.1 hrs after the test began, we treated all the measurements as being done at nominal hour 24. Nominal hours provide a reasonable way to compare exposure duration across different tests and species. To find nominal hours, we used several methods.

In some cases, laboratory personnel recorded the nominal hour they felt best described each time point for a test. In other cases, we derived the number from a set of typical nominal hour categories (24, 48, 72, 96, etc.) and the time elapsed between the recorded start time for each tank at the beginning of the test and each recorded measurement at the end of a test. For extended-duration tests, we generally included analyses for a subset of the time points recorded over the course of a test.

2.5 Methods for Modeling Dose-Response Relationships

2.5.1 Fitting dose-response curves

To fit dose-response curves, we used the *drc* and *bbmle* packages in R (Ritz and Streibig, 2005, Bolker and R Development Core Team, 2015). For each binomial endpoint (such as mortality) of each test, we fit a three-parameter log-logistic model (Ritz, 2010). This produced estimates for the inflection point, a parameter proportional to the steepness of the line tangent to the curve at the inflection point, and, in most cases, the lower limit of the curve (Figure 2.1). This lower limit can be thought of as the modeled mortality when the dose is equal to zero. If the initial optimization (using maximum likelihood estimation, or MLE) produced a negative

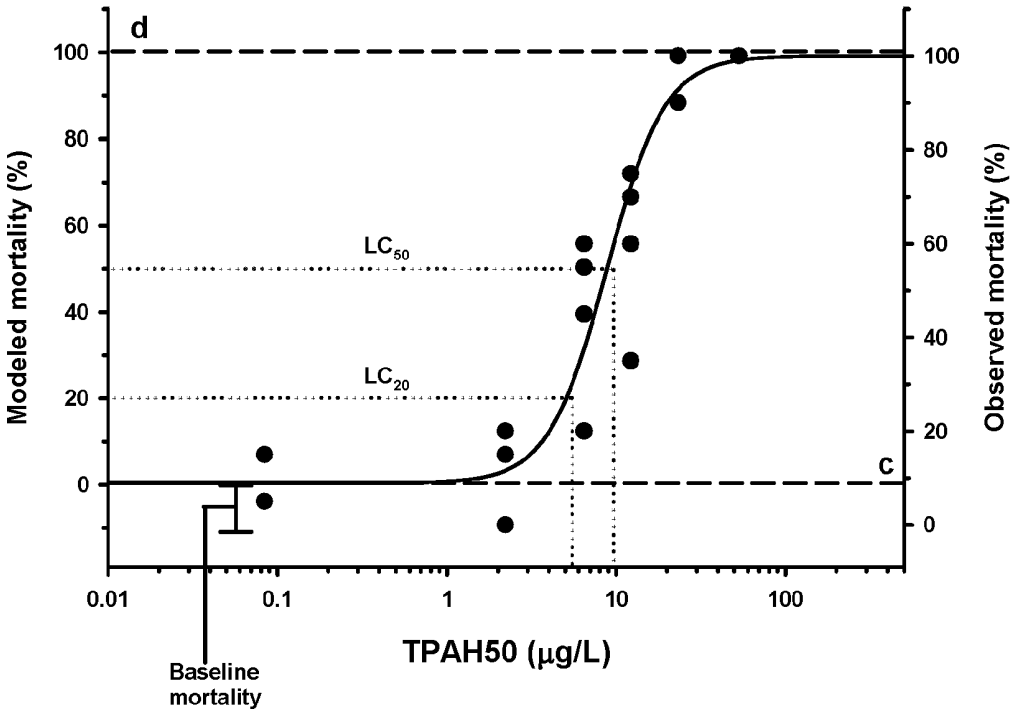

Figure 2.1. Example of fitted, three-parameter log-logistic curve for mortality showing (c) modeled lower limit of the curve, (d) upper limit of the curve, and modeled 50% effect level (LC_{50}) at the inflection point of the curve. The parameter (b), not shown here, is proportional to the steepness of the curve at the inflection point. This plot also shows how we found the LC_{20} for each test and how the lower limit c describes baseline mortality.

estimate for the lower limit of the curve, we set this limit to zero and fit a two-parameter log-logistic model. For each continuous endpoint (such as size) of each test, we fit a four-parameter log-logistic model. This model is similar to the three-parameter log-logistic model, but also includes an estimate for the upper limit of the curve.

2.5.2 Identification of effect quantiles of interest

For binomial endpoints, such as mortality and abnormality, a 50%-effect level represented the point along the dose-response curve that was halfway between the control response and a full response, where all individuals were affected (Figure 2.1). For continuous endpoints with *decreasing* dose-response curves, such as size, the 50%-effect level represented the point along

the curve where the response differed from the control response by 50%. For continuous endpoints with *increasing* dose-response curves, such as gill telangiectasia, the 50%-effect level represented the point along the curve that was halfway between the lower and upper limits.

2.5.3 Estimating effect concentrations and confidence intervals

To obtain effect concentrations and confidence intervals, we used the *bbmle* and *boot* packages in R (Bolker and R Development Core Team, 2015; Canty and Ripley, 2015). We reparameterized the log-logistic model for each desired effect level (U.S. EPA, 2013) and optimized the model using the *bbmle* package, based on the profile likelihood method; and the *boot* package, based on the bootstrap resampling method (Venzon and Moolgavkar, 1988; Faraggi et al., 2003; Bolker and R Development Core Team, 2015; Canty and Ripley, 2015). In most cases, we reported confidence intervals based on the profile likelihood method. If any of the model parameters estimated through the *bbmle* package differed from the original fitted parameters through the *drc* package by more than 1%, we used the original model to estimate effect concentrations using the *drc* package. In some cases, we reported confidence intervals based on the bootstrap resampling method using the *boot* package (Canty and Ripley, 2015). We did not report confidence intervals in cases where a visual inspection of diagnostic plots suggested that the data did not support confidence intervals. We adjusted effect concentrations for modeled control response in all methods.

2.5.4 Reporting criteria

Two readers evaluated the fitted models to determine whether they met reporting criteria. If both readers determined that a particular value did not meet reporting criteria, we did not report the value. For experiments with treatments other than oil or dispersant (e.g., UV, temperature, etc.), we did not provide independent EC_x values for each treatment.

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A. Analytical Results Lethal and Effects Concentrations from the Deepwater Horizon Natural Resource Damage Assessment Toxicity Testing Program

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
198	NWFSC	Amberjack	Embryo	WS HEWAF	Arrhythmia	–	48	EC20	2.8	2.2	3.6	15.0
198	NWFSC	Amberjack	Embryo	WS HEWAF	Arrhythmia	–	48	EC50	9.2	7.4	11.8	15.0
198	NWFSC	Amberjack	Embryo	WS HEWAF	Edema	–	48	EC20	8.3	7.0	9.7	15.0
198	NWFSC	Amberjack	Embryo	WS HEWAF	Edema	–	48	EC50	13.7	12.0	15.9	15.0
198	NWFSC	Amberjack	Embryo	WS HEWAF	Heart rate (bpm)	–	48	EC10	3.6	0.8	5.7	15.0
198	NWFSC	Amberjack	Embryo	WS HEWAF	Heart rate (bpm)	–	48	EC20	5.6	2.9	9.2	15.0
929	LUMCON	Bay anchovy	Embryo	A HEWAF	Mortality	–	48	LC20	3.2	2.7	n.s.	19.5
929	LUMCON	Bay anchovy	Embryo	A HEWAF	Mortality	–	48	LC50	3.9	3.3	n.s.	19.5
936	LUMCON	Bay anchovy	Embryo	A LEWAF	Mortality	–	48	LC20	3.3	2.8	4.0	19.2
936	LUMCON	Bay anchovy	Embryo	A LEWAF	Mortality	–	48	LC50	4.3	3.8	4.9	19.2
925	LUMCON	Bay anchovy	Embryo	B HEWAF	Mortality	–	48	LC20	6.6	n.s.	n.s.	68.3
925	LUMCON	Bay anchovy	Embryo	B HEWAF	Mortality	–	48	LC50	7.2	n.s.	n.s.	68.3
926	LUMCON	Bay anchovy	Embryo	B HEWAF	Mortality	–	48	LC20	1.3	0.9	2.8	15.9
926	LUMCON	Bay anchovy	Embryo	B HEWAF	Mortality	–	48	LC50	1.4	1.3	3.5	15.9
931	LUMCON	Bay anchovy	Embryo	B LEWAF	Mortality	–	48	LC20	1.5	1.1	n.s.	4.7
931	LUMCON	Bay anchovy	Embryo	B LEWAF	Mortality	–	48	LC50	1.6	n.s.	n.s.	4.7
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	48	LC20	27.9	21.1	36.7	46.1
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	48	LC50	55.4	41.8	91.9	46.1
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	72	LC20	16.3	12.6	20.4	46.1
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	72	LC50	26.7	22.1	32.9	46.1
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	96	LC20	12.7	9.5	16.3	46.1
148	GCRL	Blue crab	Zoea	A CEWAF	Mortality	–	96	LC50	20.8	17.0	26.1	46.1
118	UNT	Blue crab	Zoea	A HEWAF	Mortality	50	24	LC20	20.3	16.4	23.9	127.9
118	UNT	Blue crab	Zoea	A HEWAF	Mortality	50	24	LC50	25.7	22.5	28.7	127.9
118	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	48	LC20	56.8	40.2	83.5	127.9
118	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	48	LC50	93.4	75.8	116.1	127.9

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	24	LC20	OOR	n.s.	n.s.	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	24	LC50	OOR	n.s.	n.s.	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	24	LC20	19.3	7.8	41.3	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	24	LC50	94.6	47.2	219.6	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	36	LC20	OOR	n.s.	n.s.	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	36	LC50	OOR	n.s.	n.s.	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	36	LC20	8.0	3.5	16.2	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	36	LC50	34.8	18.3	70.6	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	48	LC20	105.1	31.6	245.3	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	10	48	LC50	293.8	166.9	1,431.0	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	48	LC20	5.3	2.8	8.9	209.0
322	UNT	Blue crab	Zoea	A HEWAF	Mortality	100	48	LC50	14.8	9.1	27.0	209.0
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	24	LC20	50.2	22.5	78.7	59.4
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	48	LC20	22.6	8.4	52.2	59.4
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	72	LC20	26.5	13.6	37.5	59.4
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	72	LC50	49.2	38.7	71.2	59.4
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	96	LC20	12.9	7.6	17.4	59.4
144	GCRL	Blue crab	Zoea	B CEWAF	Mortality	–	96	LC50	19.7	15.2	23.9	59.4
117	UNT	Blue crab	Zoea	B HEWAF	Mortality	50	24	LC20	10.0	7.3	12.6	71.8
117	UNT	Blue crab	Zoea	B HEWAF	Mortality	50	24	LC50	15.0	12.4	18.2	71.8
117	UNT	Blue crab	Zoea	B HEWAF	Mortality	100	24	LC20	1.1	0.0	4.3	71.8
117	UNT	Blue crab	Zoea	B HEWAF	Mortality	100	24	LC50	2.2	0.2	6.0	71.8
117	UNT	Blue crab	Zoea	B HEWAF	Mortality	10	48	LC50	79.0	n.s.	n.s.	71.8
320	RSMAS	Cobia	Embryo	S HEWAF	Mortality	–	60	LC20	60.8	46.2	76.2	123.8
320	RSMAS	Cobia	Embryo	S HEWAF	Mortality	–	60	LC50	92.2	79.8	106.5	123.8
320	RSMAS	Cobia	Embryo	S HEWAF	Mortality	–	96	LC20	27.5	n.s.	n.s.	123.8

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
320	RSMAS	Cobia	Embryo	S HEWAF	Mortality	–	96	LC50	29.7	25.0	30.8	123.8
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	36	LC20	72.2	n.s.	n.s.	77.3
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	36	LC50	83.8	n.s.	n.s.	77.3
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	60	LC20	65.3	n.s.	n.s.	77.3
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	60	LC50	72.4	56.7	76.1	77.3
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	96	LC20	17.3	n.s.	n.s.	77.3
339	RSMAS	Cobia	Embryo	WS HEWAF	Mortality	–	96	LC50	18.7	11.9	29.3	77.3
923	RSMAS-UNT	Copepod	Adult	A HEWAF	Mortality	10	96	LC20	56.4	30.0	84.0	264.4
923	RSMAS-UNT	Copepod	Adult	A HEWAF	Mortality	10	96	LC50	87.0	60.1	110.0	264.4
923	RSMAS-UNT	Copepod	Adult	A HEWAF	Mortality	100	96	LC20	3.7	2.5	5.1	264.4
923	RSMAS-UNT	Copepod	Adult	A HEWAF	Mortality	100	96	LC50	5.4	4.3	6.8	264.4
910	RSMAS	Copepod	Adult	A HEWAF	Mortality	–	96	LC20	33.0	17.2	51.4	207.8
910	RSMAS	Copepod	Adult	A HEWAF	Mortality	–	96	LC50	64.4	46.7	83.4	207.8
109	FGCU	Eastern oyster	Early spat	A CEWAF	Mortality	–	168	LC20	68.3	49.2	95.3	182.7
109	FGCU	Eastern oyster	Early spat	A CEWAF	Mortality	–	168	LC50	150.8	124.9	190.9	182.7
103	FGCU	Eastern oyster	Embryo	A CEWAF	Abnormality	–	24	EC20	12.2	11.6	12.8	26.1
103	FGCU	Eastern oyster	Embryo	A CEWAF	Abnormality	–	24	EC50	15.6	14.9	16.5	26.1
106	FGCU	Eastern oyster	Veliger	A CEWAF	Mortality	–	96	LC20	24.3	23.8	24.8	213.0
106	FGCU	Eastern oyster	Veliger	A CEWAF	Mortality	–	96	LC50	34.3	33.9	34.8	213.0
175	FGCU	Eastern oyster	Veliger	A CEWAF	Development	–	48	EC20	42.6	37.3	49.0	178.5
175	FGCU	Eastern oyster	Veliger	A CEWAF	Development	–	48	EC50	102.5	84.4	136.8	178.5
175	FGCU	Eastern oyster	Veliger	A CEWAF	Mortality	–	96	LC20	26.0	25.4	26.5	178.5
175	FGCU	Eastern oyster	Veliger	A CEWAF	Mortality	–	96	LC50	41.8	41.2	42.4	178.5

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
175	FGCU	Eastern oyster	Veliger	A CEWAF	Shell length	–	48	EC10	7.0	n.s.	n.s.	178.5
107	FGCU	Eastern oyster	Early spat	A HEWAF	Mortality	–	336	LC20	32.5	n.s.	n.s.	878.8
107	FGCU	Eastern oyster	Early spat	A HEWAF	Mortality	–	336	LC50	OOR	n.s.	n.s.	878.8
263	FGCU	Eastern oyster	Embryo	A HEWAF	Mortality	–	96	LC20	118.3	115.2	121.5	3,208.5
263	FGCU	Eastern oyster	Embryo	A HEWAF	Mortality	–	96	LC50	219.9	216.0	223.8	3,208.5
263	FGCU	Eastern oyster	Embryo	A HEWAF	Shell length	–	96	EC10	138.2	n.s.	n.s.	3,208.5
263	FGCU	Eastern oyster	Embryo	A HEWAF	Shell length	–	96	EC20	937.1	n.s.	n.s.	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Fertilization	–	1	EC20	1,649.5	n.s.	n.s.	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Fertilization	–	1	EC50	2,253.2	n.s.	n.s.	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Mortality	–	96	LC20	234.2	n.s.	n.s.	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Mortality	–	96	LC50	307.3	n.s.	n.s.	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Shell length	–	24	EC10	374.0	309.1	450.7	3,208.5
264	FGCU	Eastern oyster	Gamete	A HEWAF	Shell length	–	96	EC10	260.4	n.s.	n.s.	3,208.5
564	UNT	Eastern oyster	Gamete	A HEWAF	Fertilization	10	1	EC20	18.6	14.4	23.3	106.4
564	UNT	Eastern oyster	Gamete	A HEWAF	Fertilization	10	1	EC50	115.6	93.4	148.7	106.4
564	UNT	Eastern oyster	Gamete	A HEWAF	Fertilization	100	1	EC20	5.7	4.5	7.2	106.4
564	UNT	Eastern oyster	Gamete	A HEWAF	Fertilization	100	1	EC50	12.8	10.9	15.0	106.4
507	FGCU	Eastern oyster	Veliger	A HEWAF	Abnormality	–	48	EC20	645.4	387.5	3,180.0	473.7
507	FGCU	Eastern oyster	Veliger	A HEWAF	Abnormality	–	48	EC50	OOR	n.s.	n.s.	473.7
256	FGCU	Eastern oyster	Pediveliger	B SPIKED SED	Settlement	–	72	EC20	6.5	5.0	8.4	113.7
256	FGCU	Eastern oyster	Pediveliger	B SPIKED SED	Settlement	–	72	EC50	18.8	16.1	21.9	113.7
576	FGCU	Eastern oyster	Embryo	FIELD SED ELUTRIATE	Abnormality	–	24	EC20	77.7	62.8	96.9	989
576	FGCU	Eastern oyster	Embryo	FIELD SED ELUTRIATE	Abnormality	–	24	EC50	151.2	133.8	172.2	989

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
609	FGCU	Eastern oyster	Gamete	FIELD SED ELUTRIATE	Abnormality	–	24	EC20	1.1	0.2	2.4	152.8
609	FGCU	Eastern oyster	Gamete	FIELD SED ELUTRIATE	Abnormality	–	24	EC50	11.0	6.1	15.3	152.8
609	FGCU	Eastern oyster	Gamete	FIELD SED ELUTRIATE	Fertilization	–	1	EC20	40.6	29.8	54.1	152.8
609	FGCU	Eastern oyster	Gamete	FIELD SED ELUTRIATE	Fertilization	–	1	EC50	173.2	148.1	209.6	152.8
610	FGCU	Eastern oyster	Veliger	FIELD SED ELUTRIATE	Abnormality	–	48	EC20	95.9	42.3	174.2	563.2
610	FGCU	Eastern oyster	Veliger	FIELD SED ELUTRIATE	Abnormality	–	48	EC50	OOR	n.s.	n.s.	563.2
579	Auburn-UNT	Fiddler crab	Zoea	A SED CLEAN + Surface Oiling	Mortality	10	12	LC20	OOR	n.s.	n.s.	25.7
579	Auburn-UNT	Fiddler crab	Zoea	A SED CLEAN + Surface Oiling	Mortality	10	12	LC50	OOR	n.s.	n.s.	25.7
579	Auburn-UNT	Fiddler crab	Zoea	A SED CLEAN + Surface Oiling	Mortality	100	12	LC20	0.6	0.5	0.8	25.7
579	Auburn-UNT	Fiddler crab	Zoea	A SED CLEAN + Surface Oiling	Mortality	100	12	LC50	2.6	2.2	3.1	25.7
131	UNT	Fiddler crab	Zoea	B HEWAF	Mortality	50	24	LC20	11.0	n.s.	n.s.	9.3
131	UNT	Fiddler crab	Zoea	B HEWAF	Mortality	100	24	LC20	4.1	3.6	4.6	9.3
131	UNT	Fiddler crab	Zoea	B HEWAF	Mortality	100	24	LC50	6.7	6.0	7.6	9.3
131	UNT	Fiddler crab	Zoea	B HEWAF	Mortality	50	48	LC20	2.5	2.0	3.1	9.3
131	UNT	Fiddler crab	Zoea	B HEWAF	Mortality	50	48	LC50	4.0	3.5	4.5	9.3

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	24	LC20	107.1	91.2	122.5	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	24	LC50	129.6	116.4	144.7	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	48	LC20	96.3	80.8	109.3	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	48	LC50	118.7	106.8	133.0	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	72	LC20	93.1	74.4	107.3	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	72	LC50	116.3	103.4	131.2	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	96	LC20	73.0	56.5	86.8	182.1
283	GCRL	Grass shrimp	Adult	A CEWAF	Mortality	–	96	LC50	94.8	82.0	107.4	182.1
195	LSU	Gulf killifish	Embryo	B SPIKED SED	Hatching failure and mortality	–	480	EC10	11.7	6.1	18.6	402.0
195	LSU	Gulf killifish	Embryo	B SPIKED SED	Hatching failure and mortality	–	480	EC20	15.5	9.3	22.1	402.0
195	LSU	Gulf killifish	Embryo	B SPIKED SED	Hatching failure and mortality	–	480	EC50	24.8	18.5	30.4	402.0
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	24	LC20	78.4	67.8	90.9	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	24	LC50	99.6	89.5	113.3	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	48	LC20	73.6	63.0	85.5	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	48	LC50	94.0	84.8	106.2	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	72	LC20	80.6	70.3	89.3	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	72	LC50	90.3	83.6	104.4	165.8
211	Mote	Inland silverside	Larvae	A CEWAF	Mortality	–	96	LC50	86.5	n.s.	n.s.	165.8

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
114	PER	Leptocheirus	Juvenile	A SPIKED SED	Mortality	–	240	LC20	15.5	12.1	21.7	215.2
114	PER	Leptocheirus	Juvenile	A SPIKED SED	Mortality	–	240	LC50	24.5	20.3	30.5	215.2
342	PER	Leptocheirus	Juvenile	A SPIKED SED	Mortality	–	240	LC20	2.9	2.1	3.8	108.9
342	PER	Leptocheirus	Juvenile	A SPIKED SED	Mortality	–	240	LC50	5.1	4.2	6.0	108.9
115	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	234	LC20	3.3	2.4	4.4	127.3
115	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	234	LC50	10.8	8.6	13.4	127.3
262	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	240	LC20	49.9	41.7	56.3	422.3
262	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	240	LC50	69.8	63.2	77.2	422.3
340	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	240	LC20	15.4	11.9	19.0	43.1
340	PER	Leptocheirus	Juvenile	B SPIKED SED	Mortality	–	240	LC50	32.3	27.8	38.8	43.1
272	USACE	Leptocheirus	Neonates	B SPIKED SED	Estimated average weight gain (µg/day)	–	672	EC10	0.6	n.s.	n.s.	24.2
272	USACE	Leptocheirus	Neonates	B SPIKED SED	Estimated average weight gain (µg/day)	–	672	EC20	1.1	n.s.	n.s.	24.2
272	USACE	Leptocheirus	Neonates	B SPIKED SED	Neonates per adult	–	672	EC10	0.5	0.0	2.3	24.2
272	USACE	Leptocheirus	Neonates	B SPIKED SED	Neonates per adult	–	672	EC20	0.6	0.1	2.1	24.2

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
630	PER	Leptocheirus	Juvenile	FIELD SED	Mortality	–	240	LC20	5.4	3.2	8.9	601.7
630	PER	Leptocheirus	Juvenile	FIELD SED	Mortality	–	240	LC50	12.8	9.0	17.7	601.7
631	PER	Leptocheirus	Juvenile	FIELD SED	Mortality	–	240	LC20	5.4	3.2	8.9	1,532.4
631	PER	Leptocheirus	Juvenile	FIELD SED	Mortality	–	240	LC50	12.8	9.0	17.7	1,532.4
288	RSMAS-NWFSC	Mahi-mahi	Embryo	A CEWAF	Edema	–	48	EC20	8.7	4.8	14.8	16.37
288	RSMAS-NWFSC	Mahi-mahi	Embryo	A CEWAF	Edema	–	48	EC50	13.7	9.4	19.7	16.37
288	RSMAS-NWFSC	Mahi-mahi	Embryo	A CEWAF	Mortality	–	24	LC20	16.1	n.s.	n.s.	16.4
288	RSMAS-NWFSC	Mahi-mahi	Embryo	A CEWAF	Mortality	–	48	LC50	15.9	12.0	18.3	16.4
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	24	LC20	15.5	11.5	21.3	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	48	LC20	10.4	7.7	13.7	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	48	LC50	18.2	15.5	21.4	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	72	LC20	9.5	7.5	11.9	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	72	LC50	13.9	12.0	16.0	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	96	LC20	8.9	n.s.	n.s.	33.2
316	RSMAS	Mahi-mahi	Embryo	A CEWAF	Mortality	–	96	LC50	9.6	8.7	10.1	33.2
278	RSMAS-NWFSC	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC20	OOR	n.s.	n.s.	5.1
278	RSMAS-NWFSC	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC20	OOR	n.s.	n.s.	5.1
278	RSMAS-NWFSC	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC50	OOR	n.s.	n.s.	5.1
278	RSMAS-NWFSC	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC50	OOR	n.s.	n.s.	5.1
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	24	LC20	54.9	n.s.	n.s.	53.2

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC20	19.4	15.0	24.6	53.2
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	48	LC50	41.8	34.7	53.1	53.2
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	72	LC20	15.0	12.4	17.8	53.2
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	72	LC50	25.1	22.1	28.8	53.2
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	96	LC20	5.1	3.7	6.6	53.2
279	RSMAS	Mahi-mahi	Embryo	A HEWAF	Mortality	–	96	LC50	8.8	7.4	10.4	53.2
328	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC20	2.4	n.s.	n.s.	22.5
328	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC50	12.8	8.6	21.0	22.5
330	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC20	0.947	0.529	1.48	18.3
330	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC50	3.28	2.45	4.41	18.3
663	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC20	1.74	1.22	2.29	21.5
663	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC50	6.84	5.57	8.57	21.5
667	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC20	6.5	4.3	8.6	20.3
667	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC20	7.8	5.44	10.2	20.3
667	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC50	10.1	8.2	12.0	20.3
667	RSMAS	Mahi-mahi	Embryo	A LEWAF	Mortality	–	96	LC50	12	10	14.4	20.3
285	RSMAS	Mahi-mahi	Embryo	S CEWAF	Mortality	–	72	LC20	29.7	n.s.	n.s.	41.6
285	RSMAS	Mahi-mahi	Embryo	S CEWAF	Mortality	–	72	LC50	45.1	n.s.	n.s.	41.6
285	RSMAS	Mahi-mahi	Embryo	S CEWAF	Mortality	–	96	LC20	20.6	18.0	23.2	41.6
285	RSMAS	Mahi-mahi	Embryo	S CEWAF	Mortality	–	96	LC50	25.4	23.4	27.4	41.6
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	48	LC20	132.2	89.1	175.7	213.1
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	48	LC50	201.8	174.0	252.6	213.1
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	72	LC20	79.4	51.9	103.5	213.1
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	72	LC50	124.8	100.9	146.7	213.1
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	96	LC20	40.2	n.s.	n.s.	213.1
299	RSMAS	Mahi-mahi	Embryo	S HEWAF	Mortality	–	96	LC50	43.5	n.s.	n.s.	213.1

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
512	RSMAS-NWFSC	Mahi-mahi	Embryo	S HEWAF	Mortality	–	48	LC20	OOR	OOR	n.s.	21.6
512	RSMAS-NWFSC	Mahi-mahi	Embryo	S HEWAF	Mortality	–	48	LC50	OOR	OOR	n.s.	21.6
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	24	LC20	442.7	283.8	849.1	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	48	LC20	46.2	29.0	n.s.	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	48	LC50	75.5	55.1	134.1	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	72	LC20	25.5	n.s.	n.s.	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	72	LC50	28.8	n.s.	n.s.	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	96	LC20	6.6	n.s.	n.s.	596.0
291	RSMAS	Mahi-mahi	Embryo	WS CEWAF	Mortality	–	96	LC50	7.4	n.s.	n.s.	596.0
290	RSMAS-NWFSC	Mahi-mahi	Embryo	WS HEWAF	Edema	–	48	EC20	1.3	0.2	3	15.86
290	RSMAS-NWFSC	Mahi-mahi	Embryo	WS HEWAF	Edema	–	48	EC50	5.2	2.1	8.5	15.86
290	RSMAS-NWFSC	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	48	LC20	OOR	n.s.	n.s.	15.9
290	RSMAS-NWFSC	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	48	LC50	OOR	n.s.	n.s.	15.9
295	UNT	Mahi-mahi	Embryo	WS HEWAF	Hatching failure	10	48	EC20	10.6	6.7	16.8	67.9
295	UNT	Mahi-mahi	Embryo	WS HEWAF	Hatching failure	10	48	EC50	40.1	27.9	65.6	67.9
295	UNT	Mahi-mahi	Embryo	WS HEWAF	Hatching failure	100	48	EC20	3.8	1.6	n.s.	67.9
295	UNT	Mahi-mahi	Embryo	WS HEWAF	Hatching failure	100	48	EC50	5.2	3.5	6.3	67.9
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	48	LC20	24.5	15.7	36.1	393.7
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	48	LC50	47.6	35.9	63.1	393.7
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	72	LC20	30.8	n.s.	n.s.	393.7
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	72	LC50	34.5	n.s.	n.s.	393.7

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	96	LC20	11.6	n.s.	n.s.	393.7
298	RSMAS	Mahi-mahi	Embryo	WS HEWAF	Mortality	–	96	LC50	12.7	n.s.	n.s.	393.7
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	0	120	LC20	81.7	45.0	143.9	138.6
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	0	120	LC50	198.9	136.2	467.4	138.6
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	50	120	LC20	5.3	3.7	7.3	138.6
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	50	120	LC50	8.3	6.8	10.3	138.6
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	100	120	LC20	4.3	3.0	5.5	138.6
517[a]	UNT	Mysid shrimp	Juvenile	A HEWAF	Mortality	100	120	LC50	6.2	5.1	7.6	138.6
655	Stratus	Pacific white shrimp	Post-larvae	B SPIKED SED	Growth (total mm)	–	168	EC20	4.3	0.0	35.8	538.0
655	Stratus	Pacific white shrimp	Post-larvae	B SPIKED SED	Growth (total mm)	–	168	EC50	62.7	0.0	208.0	538.0
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	24	LC20	22.6	15.7	32.1	72.1
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	24	LC50	52.0	42.9	66.0	72.1
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	48	LC20	33.8	28.6	37.9	72.1
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	48	LC50	42.0	38.3	45.9	72.1
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	72	LC20	21.9	18.4	24.5	72.1
381	Stratus	Red drum	Embryo	A HEWAF	Mortality	–	72	LC50	26.3	24.0	28.8	72.1
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Atrial arrhythmia	–	48	EC10	9.7	n.s.	n.s.	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Atrial arrhythmia	–	48	EC20	11.8	n.s.	n.s.	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Atrioventricular angle	–	48	EC10	5.9	3.0	8.3	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Atrioventricular angle	–	48	EC20	9.7	7.0	13.7	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Mortality	–	36	LC20	45.6	36.4	65.4	31.5

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Mortality	–	36	LC50	OOR	93.2	360.3	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Ventricular arrhythmia	–	48	EC10	6.5	1.1	9.6	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Ventricular arrhythmia	–	48	EC20	8.3	4.3	14.3	31.5
636	Stratus-NWFSC	Red drum	Embryo	A HEWAF	Ventricular arrhythmia	–	48	EC50	18.1	n.s.	n.s.	31.5
391	Stratus-UNT	Red drum	Larvae	A HEWAF	Mortality	50	24	LC20	6.6	4.2	10.0	11.8
391	Stratus-UNT	Red drum	Larvae	A HEWAF	Mortality	50	24	LC50	12.1	9.5	18.9	11.8
391	Stratus-UNT	Red drum	Larvae	A HEWAF	Mortality	100	24	LC20	2.4	1.5	3.3	11.8
391	Stratus-UNT	Red drum	Larvae	A HEWAF	Mortality	100	24	LC50	3.5	2.7	4.3	11.8
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Atrial arrhythmia	–	48	EC10	4.8	2.9	5.9	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Atrial arrhythmia	–	48	EC20	6.8	5.8	7.9	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Atrioventricular angle	–	48	EC10	3.5	2.0	4.9	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Atrioventricular angle	–	48	EC20	5.3	3.8	6.6	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Atrioventricular angle	–	48	EC50	10.0	8.7	11.4	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Mortality	–	36	LC20	n.s.	n.s.	n.s.	17.9

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Mortality	–	36	LC50	n.s.	n.s.	n.s.	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Ventricular arrhythmia	–	48	EC10	1.6	0.5	2.9	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Ventricular arrhythmia	–	48	EC20	2.9	1.6	4.2	17.9
638	Stratus-NWFSC	Red drum	Embryo	A LEWAF	Ventricular arrhythmia	–	48	EC50	7.9	6.6	9.5	17.9
640	Stratus	Red drum	Embryo	A LEWAF	Mortality	–	60	LC20	14.5	10.9	18.5	17.7
640	Stratus	Red drum	Embryo	A LEWAF	Mortality	–	60	LC50	16.2	13.6	18.7	17.7
X17	Stratus	Red drum	Embryo	B CEWAF	Mortality	–	60	LC20	7.0	6.0	8.1	19.2
X17	Stratus	Red drum	Embryo	B CEWAF	Mortality	–	60	LC50	8.6	7.8	9.4	19.2
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	24	LC20	21.912	0.468	75.516	34.1
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	24	LC50	OOR	n.s.	n.s.	34.1
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	48	LC20	9.201	6.521	12.26	34.1
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	48	LC50	15.885	13.143	18.993	34.1
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	72	LC20	10.82	7.841	14.167	34.1
384	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	72	LC50	14.812	12.261	17.636	34.1
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Atrial arrhythmia	–	48	EC10	8.8	6.1	11.5	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Atrial arrhythmia	–	48	EC20	15.7	12.6	19.6	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Atrioventricular angle	–	48	EC10	5.5	1.9	10.3	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Atrioventricular angle	–	48	EC20	11.2	5.8	16.5	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Atrioventricular angle	–	48	EC50	32.8	24.7	41.0	51.8

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Mortality	–	36	LC20	35.0	29.5	41.4	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Mortality	–	36	LC50	OOR	101.6	203.0	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Ventricular arrhythmia	–	48	EC10	2.3	1.0	4.2	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Ventricular arrhythmia	–	48	EC20	4.5	2.8	6.8	51.8
637	Stratus-NWFSC	Red drum	Embryo	B HEWAF	Ventricular arrhythmia	–	48	EC50	17.4	13.4	22.8	51.8
653	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	48	LC20	28.2	18.2	40.5	56.8
653	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	48	LC50	71.1	53.3	127.5	56.8
653	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	60	LC20	21.7	19.0	24.3	56.8
653	Stratus	Red drum	Embryo	B HEWAF	Mortality	–	60	LC50	30.9	28.2	34.0	56.8
392	Stratus-UNT	Red drum	Larvae	B HEWAF	Mortality	50	24	LC20	4.6	4.0	5.2	8.3
392	Stratus-UNT	Red drum	Larvae	B HEWAF	Mortality	50	24	LC50	5.8	5.3	6.3	8.3
392	Stratus-UNT	Red drum	Larvae	B HEWAF	Mortality	100	24	LC20	1.4	1.0	1.8	8.3
392	Stratus-UNT	Red drum	Larvae	B HEWAF	Mortality	100	24	LC50	2.0	1.7	2.3	8.3
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Atrial arrhythmia	–	48	EC10	1.0	0.4	2.2	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Atrial arrhythmia	–	48	EC20	4.5	2.6	6.7	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Atrial arrhythmia	–	48	EC50	54.7	38.3	88.2	14.8

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Atrioventricular angle	–	48	EC10	1.1	0.7	12.7	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Atrioventricular angle	–	48	EC20	1.3	n.s.	n.s.	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Ventricular arrhythmia	–	48	EC10	0.8	n.s.	n.s.	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Ventricular arrhythmia	–	48	EC20	1.0	n.s.	n.s.	14.8
639	Stratus-NWFSC	Red drum	Embryo	B LEWAF	Ventricular arrhythmia	–	48	EC50	1.7	n.s.	n.s.	14.8
654	Stratus	Red drum	Juvenile	B SPIKED SED	Growth (SL, mm)	–	312	EC10	17.5	7.6	32.2	538.0
654	Stratus	Red drum	Juvenile	B SPIKED SED	Growth (SL, mm)	–	312	EC20	37.1	20.9	56.8	538.0
654	Stratus	Red drum	Juvenile	B SPIKED SED	Growth (SL, mm)	–	312	EC50	134.0	103.0	178.0	538.0
477	Hopkins-NWFSC	Southern bluefin tuna	Embryo	WS HEWAF	Cardiac edema	–	36	EC20	0.6	0.1	1.4	9.4
477	Hopkins-NWFSC	Southern bluefin tuna	Embryo	WS HEWAF	Cardiac edema	–	36	EC50	1.6	0.7	2.6	9.4
477	Hopkins-NWFSC	Southern bluefin tuna	Embryo	WS HEWAF	Heart rate (bpm)	–	36	EC10	1.9	n.s.	n.s.	9.4
477	Hopkins-NWFSC	Southern bluefin tuna	Embryo	WS HEWAF	Heart rate (bpm)	–	36	EC20	3.3	n.s.	n.s.	9.4
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Gill epithelial proliferation	–	762	EC10	0.1	0.0	0.5	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Gill epithelial proliferation	–	762	EC20	0.3	0.0	7.2	394.9

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Gill telangiectasis	–	762	EC10	0.5	n.s.	10.1	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Gill telangiectasis	–	762	EC20	1.3	0.2	6.4	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Growth (SL, mm)	–	762	EC10	8.4	5.0	18.5	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Growth (SL, mm)	–	762	EC20	12.8	8.8	23.7	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Growth (SL, mm)	–	762	EC50	26.7	17.6	37.4	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Growth (total weight, g)	–	762	EC10	8.1	2.6	5.9	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Growth (total weight, g)	–	762	EC20	12.0	55.5	44.3	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatic vacuolization	–	762	EC10	9.5	n.s.	n.s.	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatic vacuolization	–	762	EC20	10.4	n.s.	n.s.	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatic vascular congestion	–	762	EC10	14.2	4.5	46.1	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatic vascular congestion	–	762	EC20	23.8	12.8	48.7	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatosomatic index	–	762	EC10	8.1	n.s.	51.4	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Hepatosomatic index	–	762	EC20	8.9	n.s.	50.6	394.9
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Mortality	–	762	LC20	36.3	20.2	54.7	394.9

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
113	GCRL	Southern flounder	Juvenile	B SPIKED SED	Mortality	–	762	LC50	78.1	56.6	104.2	394.9
540	GCRL	Southern flounder	Juvenile	FIELD SED	Mortality	–	336	LC20	432.2	263.6	701.0	3,940.4
540	GCRL	Southern flounder	Juvenile	FIELD SED	Mortality	–	336	LC50	1,489.9	1,052.8	2,170.0	3,940.4
540	GCRL	Southern flounder	Juvenile	FIELD SED	Mortality	–	672	LC20	434.9	268.5	730.6	3,940.4
540	GCRL	Southern flounder	Juvenile	FIELD SED	Mortality	–	672	LC50	1,203.1	873.8	1,664.3	3,940.4
394	Stratus	Speckled sea trout	Embryo	A HEWAF	Mortality	–	48	LC20	49.1	39.9	65.4	68.2
394	Stratus	Speckled sea trout	Embryo	A HEWAF	Mortality	–	48	LC50	68.9	62.0	81.2	68.2
394	Stratus	Speckled sea trout	Embryo	A HEWAF	Mortality	–	72	LC20	25.5	23.1	29.0	68.2
394	Stratus	Speckled sea trout	Embryo	A HEWAF	Mortality	–	72	LC50	30.3	28.1	33.1	68.2
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	10	24	LC20	OOR	n.s.	n.s.	2.4
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	10	24	LC50	OOR	n.s.	n.s.	2.4
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	50	24	LC20	2.3	1.6	n.s.	2.4
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	50	24	LC50	2.4	2.2	2.8	2.4
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	100	24	LC20	0.5	n.s.	n.s.	2.4

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
393	Stratus-UNT	Speckled sea trout	Larvae	A HEWAF	Mortality	100	24	LC50	0.8	n.s.	n.s.	2.4
647	Stratus	Speckled sea trout	Embryo	B CEWAF	Mortality	–	48	LC20	12.2	9.2	15.0	30.0
647	Stratus	Speckled sea trout	Embryo	B CEWAF	Mortality	–	48	LC50	19.1	16.6	21.6	30.0
647	Stratus	Speckled sea trout	Embryo	B CEWAF	Mortality	–	72	LC20	6.2	5.5	7.6	30.0
647	Stratus	Speckled sea trout	Embryo	B CEWAF	Mortality	–	72	LC50	7.6	7.0	8.4	30.0
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	24	LC20	OOR	n.s.	n.s.	69.6
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	24	LC50	OOR	n.s.	n.s.	69.6
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	48	LC20	35.0	29.0	41.0	69.6
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	48	LC50	54.8	48.9	62.6	69.6
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	72	LC20	23.1	n.s.	n.s.	69.6
388	Stratus	Speckled sea trout	Embryo	B HEWAF	Mortality	–	72	LC50	24.7	23.2	25.5	69.6
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	10	24	LC20	378.3	n.s.	n.s.	1.6
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	10	24	LC50	OOR	n.s.	n.s.	1.6
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	50	24	LC20	0.7	0.6	1.5	1.6

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	50	24	LC50	1.0	0.8	1.5	1.6
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	100	24	LC20	0.1	n.s.	n.s.	1.6
395	Stratus-UNT	Speckled sea trout	Larvae	B HEWAF	Mortality	100	24	LC50	0.2	n.s.	n.s.	1.6
268	GCRL	White shrimp	Juvenile	A CEWAF	Mortality	–	72	LC20	66.2	41.6	97.8	126.3
268	GCRL	White shrimp	Juvenile	A CEWAF	Mortality	–	72	LC50	138.6	96.0	352.7	126.3
268	GCRL	White shrimp	Juvenile	A CEWAF	Mortality	–	96	LC20	50.9	34.4	67.1	126.3
268	GCRL	White shrimp	Juvenile	A CEWAF	Mortality	–	96	LC50	83.9	65.7	112.0	126.3
261	GCRL	White shrimp	Juvenile	A HEWAF	Mortality	–	24	LC20	114.7	70.5	189.5	304.5
261	GCRL	White shrimp	Juvenile	A HEWAF	Mortality	–	48	LC20	114.7	70.5	189.5	304.5
261	GCRL	White shrimp	Juvenile	A HEWAF	Mortality	–	72	LC20	114.7	70.5	189.5	304.5
261	GCRL	White shrimp	Juvenile	A HEWAF	Mortality	–	96	LC20	80.4	48.5	121.9	304.5
261	GCRL	White shrimp	Juvenile	A HEWAF	Mortality	–	96	LC50	333.4	208.3	823.2	304.5
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Arrhythmia	–	48	EC20	0.5	n.s.	n.s.	3.9
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Arrhythmia	–	48	EC50	4.0	n.s.	n.s.	3.9
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Edema	–	48	EC20	1.7	1.3	3.9	3.9
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Edema	–	48	EC50	2.6	2.2	3.8	3.9
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Heart rate (bpm)	–	48	EC10	2.0	n.s.	n.s.	3.9
537	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Heart rate (bpm)	–	48	EC20	3.1	n.s.	n.s.	3.9

Table A.1. Results for waterborne and sediment PAH exposures. Waterborne values in µg/L; sediment values in mg/kg.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint(s)	UV (%)	Duration hour	EC/ LC	Value	Lower 95% CI	Upper 95% CI	High concentration
543	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Heart rate (bpm)	–	48	EC10	2.9	n.s.	n.s.	5.0
543	RSMAS-NWFSC	Yellowfin tuna	Embryo	A HEWAF	Heart rate (bpm)	–	48	EC20	4.1	n.s.	n.s.	5.0
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	24	LC20	0.7	0.3	1.4	56.1
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	24	LC50	24.7	13.9	54.9	56.1
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	48	LC20	5.3	n.s.	n.s.	56.1
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	48	LC50	5.9	5.0	7.0	56.1
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	60	LC20	1.2	n.s.	n.s.	56.1
539	RSMAS-NWFSC	Yellowfin tuna	Embryo	WS HEWAF	Mortality	–	60	LC50	2.8	1.6	5.2	56.1

bpm: beats per minute; n.s.: no solution; OOR: out of range.

a. Calculations for test 517 were performed on data prior to validation. Validated data will be provided on DIVER.

Table A.2. Results for waterborne Corexit exposures. Values in mg/L Corexit.

Test ID	Laboratory	Species	Life stage	Contaminant and exposure	Endpoint	UV (%)	Duration hour	EC	Value	Lower 95% CI	Upper 95% CI	High concentration
110	FGCU	Eastern oyster	Embryo	COR DISP	Development	–	24	EC20	5.3	5.2	5.5	182.7
110	FGCU	Eastern oyster	Embryo	COR DISP	Development	–	24	EC50	5.7	5.6	6.3	182.7
267	GCRL	White shrimp	Juvenile	COR DISP	Mortality	–	24	EC20	43.1	31.3	54.7	0.0
267	GCRL	White shrimp	Juvenile	COR DISP	Mortality	–	24	EC50	62.6	50.4	79.7	0.0
304	RSMAS	Mahi-mahi	Embryo	COR DISP	Mortality	–	72	EC20	25.1	22.1	28.2	50.0
304	RSMAS	Mahi-mahi	Embryo	COR DISP	Mortality	–	72	EC50	30.6	28.4	33.1	50.0
304	RSMAS	Mahi-mahi	Embryo	COR DISP	Mortality	–	96	EC20	25.1	22.1	28.2	50.0
304	RSMAS	Mahi-mahi	Embryo	COR DISP	Mortality	–	96	EC50	30.6	28.4	33.1	50.0

3. Quality Assurance Project Plan: *Deepwater Horizon* Laboratory Toxicity Testing (Version DARP)

SC11571

Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing

Version DARP

Prepared for:

U.S. Department of Commerce
National Oceanic and Atmospheric Administration
The State of Louisiana

Prepared by:

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SC11571

Contents

Appendices

Acronyms and Abbreviations

BTEX	benzene, toluene, ethylbenzene, and xylenes
CEWAF	chemically-enhanced water accommodated fraction
COC	chain-of-custody
CV	curricula vitae
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOSS	dioctyl sulfosuccinate
DOT	Department of Transportation
DWH	*Deepwater Horizon*
EDD	electronic data deliverable
EPA	U.S. Environmental Protection Agency
ERDC	Engineering Research and Development Center
FBS	fetal bovine serum
FGCU	Florida Gulf Coast University
GLP	Good Laboratory Practice
GLPP	General Laboratory Procedures and Practices
HDPE	high-density polyethylene
HEWAF	high-energy water accommodated fraction
IACUC	Institutional Animal Care and Use Committee
IATA	International Air Transport Association
ID	identification
LEWAF	low-energy water accommodated fraction
NOAA	National Oceanic and Atmospheric Administration
NRDA	Natural Resource Damage Assessment

PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PI	principal investigator
PPE	Personal Protective Equipment
QA	quality assurance
QAPP	Quality Assurance Project Plan
QC	quality control
RFU	relative fluorescence unit
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SOP	standard operating procedure
TCT	test conditions table
TPAH	total polycyclic aromatic hydrocarbon
TPH	total petroleum hydrocarbon
TSA	Transportation Security Administration
VOA	volatile organic analysis
VOC	volatile organic compound
WAF	water accommodated fraction

1. Introduction

This Quality Assurance Project Plan (QAPP) was developed to provide data collection guidance for *Deepwater Horizon* (DWH) laboratory toxicity testing project activities. Stratus Consulting,[1] now Abt Associates, is conducting this work on behalf of the National Oceanic and Atmospheric Administration (NOAA) and the State of Louisiana Trustees to support the DWH oil spill Natural Resource Damage Assessment (NRDA) activities. Guidance and requirements provided herein are based on U.S. Environmental Protection Agency's (EPA's) *Guidance for QAPPs* (U.S. EPA, 2002) for the purpose of data collection and analysis, with modifications to reflect project goals. This QAPP, which outlines the procedures that will be used to ensure that data are collected and analyzed to meet project requirements, contains the following information:

- Project management procedures, objectives, and approaches
- Procedures and guidelines for generating the data, including methods for documenting test results, collecting samples for laboratory analysis, and submitting results to Stratus Consulting
- Project assessment and oversight
- Data validation and assessment of data usability.

2. Project Management

This section presents the project administrative functions and approaches that will be followed.

2.1 Project Organization

Stratus Consulting is performing the work described herein on behalf of NOAA and the State of Louisiana. The laboratories that conduct toxicity testing are subcontractors under contract to Stratus Consulting or, in certain cases, to one of its subcontractors. The exceptions are the Northwest Fisheries Science Center, which is a NOAA laboratory and not under contract to Stratus Consulting; and the U.S. Army Corps of Engineers Engineering Research and Development Center (ERDC), which is under contract directly with NOAA. Stratus Consulting is the primary manager for all laboratories and provides oversight for work conducted at all

1. Stratus Consulting merged with Abt Associates in 2015; since much of this work was conducted by Stratus Consulting prior to the merger, both firm names will appear.

testing laboratories. In addition, Stratus Consulting conducts toxicity testing activities at independent or subcontractor laboratories. At least one principal investigator (PI) has been designated at each laboratory. The PI is responsible for work conducted at the laboratory and for ensuring that work is conducted as described in this QAPP. Subcontractors will document deviations from project planning objectives and will provide this documentation to Stratus Consulting as soon as possible following a change so that we can implement adaptive management if needed. This QAPP will be distributed to each PI for review when finalized. Definitive toxicity tests conducted after finalization of this QAPP will be subject to requirements set forth herein.

2.2 Project Timing

The data collection and analysis methods described in this QAPP will be followed for the duration of the DWH laboratory toxicity testing project.

2.3 Special Training/Certification

All PIs and supporting staff will have experience in conducting toxicity tests, documenting test results, and collecting analytical samples. Training and certification requirements related to testing and health and safety are detailed in each testing laboratory's standard operating procedures (SOPs) and/or health and safety plans. Laboratory-specific testing methods and operating procedures are provided in their respective laboratory protocols. Any collection permits, Institutional Animal Care and Use Committee (IACUC) approvals, and respective requirements that are related to project toxicity testing activities will be the responsibility of the laboratories; Stratus Consulting may request such documentation as appropriate.

PIs and supporting staff will participate in a presentation and discussion about the NRDA process, which Stratus Consulting will host. Participants will either attend a presentation in person or join remotely via a conference call and computer with an internet connection. Testing laboratory staff must schedule a time when they can attend the presentation. Contact Stratus Consulting for scheduling information.

PIs and all supporting staff will sign a project-specific confidentiality agreement before conducting any work on this project. All project staff will submit their signed confidentiality agreements, together with their current curricula vitae (CVs) to Stratus Consulting.

2.4 Documents and Records

The laboratory toxicity test documentation may generate the following records:

- Laboratory notebooks in which testing activities are documented
- Data entry bench sheets and electronic files in which testing data and sample inventories are documented
- Electronic data files
- Photographs documenting testing activities, observations, and events
- ALS Environmental analytical chemistry laboratory documents
- Chain-of-custody (COC) and shipping forms.

Each record type is described in detail below.

2.4.1 Laboratory notebook

Laboratory notebooks will record the details of all testing activities. The notebooks will:

- Document and describe testing methods
- Record observations made in the laboratory
- Identify, locate, and track samples
- Document any deviations from the project approach, QAPP, and changes in project personnel
- Record any project-related information that would not be appropriately documented elsewhere.

2.4.2 General notebook requirements

- Notebooks will be bound. Notebooks with water-resistant covers and pages are recommended. Every notebook page will be sequentially numbered starting with the first page of the notebook. If pages are not pre-printed with page numbers, the numbers will be handwritten at the top of each page. Loose-leaf sheets, other than dedicated sampling

forms, should not be used to record notes. If notes must be taken on loose sheets, the same person who took the notes will place the loose sheets securely into a notebook, and then sign and date them. The notebook and all loose-leaf notes will be retained under strict custody. Pages will not be removed from the notebook.

- All entries will be written legibly using indelible ink pen or pencil. Writing will be dark enough to allow legible photocopies to be made and should not bleed through the paper, which would cause any notes on the backside of the page to become illegible.

- The following information should be written on the front cover of the notebook; some information may need to be entered after the project has ended:

  - Notebook user name, affiliation, address, and phone number
  - Notebook number (1 for the first notebook, 2 for the second, etc.)
  - Name of the site, city, and state
  - Project name and type of activity
  - Beginning and ending dates of activities entered into the notebook
  - "Property of [*testing laboratory*]"
  - "DWH ATTORNEY WORK PRODUCT / ATTORNEY-CLIENT COMMUNICATIONS."

- Only information that is related to the project should be entered into the notebook. All notebook entries should remain factual and objective.

- A new page should be started for each day.

- The following information should be entered at the beginning of each day:

  - Date
  - Starting time (all time entries should be recorded in military or 24-hour time format)
  - Specific location
  - General description of anticipated activities.

- A diagonal line should be drawn across any blank space of more than one line to prevent unauthorized entries.

- An approximate scale for all diagrams should be provided. If this is not feasible, write "not to scale." Orientation relative to north should be indicated with an arrow and diagram features should be labeled.

- The number, file name, orientation, and subject of all photographs should be recorded.

- Corrections should be made by drawing a single line through the corrected entry, leaving the information legible, and initialing and dating the correction.

- The following information should be entered into the notebook when collecting samples:

  - Sample information, including description/reason for sampling, stock solution preparation method [e.g., high-energy water accommodated fraction (HEWAF), species/weight/length for tissue samples, and similar information]
  - Names of sampling personnel present
  - Sample collection time (military or 24-hour time format) and time zone
  - Sample collection method (e.g., filtered/unfiltered, grab, composite)
  - Sample identification (ID) number (see naming convention in Section 4.6.1)
  - Designation of sample type (e.g., definitive sample, duplicate sample)
  - Sample media (water/sediment/tissue)
  - Sample observations (e.g., color, odor, consistency)
  - COC page and form number in which samples will be relinquished to ALS Environmental (top right-hand corner of COC sheet) – to be filled in by sampler
  - Source water preparations or origin (date, time, and storage location)
  - Field collections, shipments, and husbandry performance of test organisms.

- Custody of laboratory notebooks should be maintained at all times, and notebooks should be stored in a safe, secure place at all times when not in the possession of personnel.

2.4.3 Data entry bench sheet requirements

Data entry bench sheets are provided in Appendix B of this QAPP. Laboratories will print and use hard copy bench sheets to facilitate entering data into the data files (electronic versions of the bench sheets) and perform a quality assurance/quality control (QA/QC) check of 100% of the test results electronically entered into the data files. Water-resistant paper should be used for hardcopy testing bench sheets.

The following are data entry bench sheet requirements for recording information generated during toxicity testing:

- There will be one set of bench sheets for each test.

- Prior to beginning a definitive test, the PI or appropriate test manager should confirm that all required types and number of blank bench sheets are available.

- All staff conducting the test and test information recorders should be identified on each sheet. Fill out time using military or 24-hour time format.

- All bench sheet entry fields will be filled out. "NA" should be recorded in entry fields that are not applicable. Do not leave any blanks.
- All entries will be made using indelible ink.
- All mistakes should be corrected by drawing a single line through the incorrect entry and entering the correct entry in an understandable manner. The initials of the recorder should be entered next to any corrected entries.
- At the end of each day, completed bench sheets will be organized, reviewed, and, if needed, corrected by the PI or appropriate test manager. Corrections should be made by following the aforementioned method and initialing the corrected entry.
- Photocopies should be made of each day's completed bench sheets. Copied bench sheets will be separated from originals and stored in a secure place.

Toxicity testing data bench sheets and files are described in Section 4.1 and reporting requirements in Section 4.11.2.

2.4.4 Electronic data files

Electronic data files will be generated throughout the course of this project. These files may include but are not limited to data entry files, emails, photographs, scanned documents, and work plans. Electronic data files should be named with the date when the file was created. Where possible, a ReadMe should be included that describes when the file was created and by whom. If a data file is revised, a new copy should be made and the original should be preserved. Files should be securely stored in password-protected folders. Only people that are working on this project with completed confidentiality agreements on file should have access to saved files. Files should be routinely backed up during the course of this project. If the backup files are stored on an external device, this device needs to be stored in a secure location. All data files will be retained under custody procedures until notified otherwise by Stratus Consulting.

"DWH ATTORNEY WORK PRODUCT / ATTORNEY-CLIENT COMMUNICATIONS" should be included in all electronic files. This statement should be entered into the subject line when sending project-related emails. Alternatively, the confidentially statement may be written on the top line of the email.

2.4.5 Photographic log requirements

Photographs and digital videos may be taken during testing to record activities, observations, and events. Before being used to take any photographs, cameras will be set to the local time (in

military or 24-hour time format) and date. Personal cameras, including those on cell phones, smartphones, and other mobile devices, are not to be used for project-related work. Photographs and video data will be collected and stored in a legally defensible fashion using requirements described in this section. There are two major elements to satisfying these requirements: preserving original files and maintaining a complete photographic record.

Photographs and digital videos will be retained as original files on the removable memory card in the camera. Once a file is created (i.e., picture taken), it will not be deleted from the microSD card and will be kept under strict COC procedures. At no time will information stored on a digital memory card or a camera's internal memory be erased or overwritten, even if the photograph is out of focus or was taken accidentally. Additionally, digital photograph files must be stored sequentially on the microSD card and not renamed.

When saving and viewing photographs and videos on a computer, each digital media file must first be downloaded to an archive file that cannot be opened, deleted, or renamed. Prior to viewing pictures, staff will upload photograph files directly from the camera to an archive file location that is secured and routinely backed up. Photos will be maintained as unopened files until notified by Stratus Consulting. After uploading unopened files, digital media files may then be copied or downloaded to a local working folder where they can be accessed. Note that files in the working folder will not be deleted. After copying files into working folders, the media storage device will be removed from the camera and sent to Stratus Consulting under COC procedures using the project-specific COC form.

A photograph/digital video log will be created for all photographs taken for this project. Microsoft Excel should be used to create the photograph/video log. Each camera used to take photographs during each test will have a unique photograph or video log. Entries in the photograph log should be made at the time each photograph is taken. The log will contain the camera ID, memory card ID, original file name, date, time, time zone, photographer name/affiliation, location where photograph was taken, and a description of the subject. The camera ID is the serial number that is clearly stamped on the camera body or other ID that is unique to the camera and can be used to identify which camera was used. If the original file name is not apparent when taking photographs (i.e., it does not display on the camera screen), the original file name can be filled out after files have been downloaded to a working folder. All other information in the photograph log should be filled out while photographs are being taken. A ReadMe worksheet should also be provided with each photograph log file, and describe when the photograph log was created and by whom, as well as any pertinent information not already provided within the log itself. Once complete, photograph logs will be checked for completeness and accuracy. The photograph log will then be provided to Stratus Consulting.

2.4.6 ALS Environmental laboratory documents

The following types of documents will be generated by analytical laboratories for this project

- A description of analytical methods and measurements performed on the collected samples and on QA/QC samples (e.g., blanks, calibration standards)
- Supporting documentation, including copies of laboratory notebooks, sample tracking forms, raw outputs from instruments, chromatograms, run logs, and similar documentation that is sufficient to conduct full data validation, if needed
- Project narrative reports.

The analytical laboratory will provide the data to Stratus Consulting in an electronic data deliverable (EDD) format.

In addition to ALS Environmental, other laboratories may be used throughout the course of this project. These laboratories will generate their own types of documents that are specific to the analyses being conducted. When used, documentation requirements will be specified in the PI SOPs prior to testing.

2.4.7 COC and shipping forms

A blank COC form is provided in Appendix C. This form will document sample retention and storage; relinquish samples; relinquish any test materials such as data sheets, microSD cards, and similar materials; and request analyses when relinquishing samples to an analytical laboratory. A description of COC requirements and procedures is provided in Section 4.8.

3. Testing Method Documentation and Review Process

This section describes the method documentation and review process for the definitive toxicity tests. Testing method documents include work plans, SOPs, and test conditions tables (TCTs). Testing laboratories will draft these test method documents and provide them to Stratus Consulting for review and approval prior to conducting definitive tests. Stratus Consulting will use finalized test method documents to draft the *General Laboratory Procedures and Practices: Deepwater Horizon Laboratory Toxicity Testing* (see Attachment 1). General Laboratory

Procedures and Practices (GLPP) will document finalized methods that laboratory personnel used during definitive testing.[2]

Descriptions of each of the test methods documents follows:

- ***Work plans:*** These plans will document general testing methods and information. These work plans document elements or testing procedures that are common to all or a subset of similarly related tests being conducted by a specific laboratory. These elements may include, but are not limited to, sources of test organisms and water, test endpoint descriptions and how they will be assessed, and analytical sampling requirements. These work plan elements should be written to a level of specificity such that someone trained in the scientific discipline being utilized to conduct the test could accurately repeat it. Where applicable, work plans may reference methods described in this QAPP or published journal articles; for example, personnel may reference this QAPP when describing methods for labeling and shipping analytical samples, or when reporting test results. Work plans may be updated as needed to reflect changes in general testing methods. New version numbers will be assigned to updated work plans.

- ***SOPs:*** These documents will detail how toxicity tests or elements of tests will be performed. SOPs should be written as a step-by-step process in chronological order. SOPs should be written to a level of specificity such that someone trained in the scientific discipline could use them to accurately repeat procedures. If appropriate, one SOP can be used to document multiple tests. For example, one SOP could detail how to perform water quality analyses, directions for calibrating diluter systems, and the process for conducting an acute toxicity exposure for multiple species or life stages. Work plans may reference laboratory-specific SOPs and project-wide SOPs provided in appendices in this QAPP when describing testing procedures. SOPs for HEWAF, chemically-enhanced water accommodated fraction (CEWAF), and low-energy water accommodated fraction (LEWAF) preparations, the most common water accommodated fraction (WAF) preparations used during definitive testing, are provided in Appendix A.1. Additional test procedures, such as spiking sediment with oil and preparing oil slick exposures for use in toxicity testing, can be found in the most recent version of the GLPP. GLPP SOPs may be referenced in laboratory method documentation materials and used and when finalized. If applicable, standard method and test-specific QA/QC limits and test performance criteria will be specified in testing laboratory SOPs or work plans if applicable to multiple testing procedures.

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2. The project GLPP acronym should not be confused with Good Laboratory Practice (GLP) regulations that specify the management QC system for clinical and nonclinical research laboratories. Although the project GLPP provides GLP-like guidance and requirements, it should not be confused with a document that strictly adheres to U.S. Food and Drug Association regulations or Organization for Economic Cooperation and Development guidelines for testing of chemicals.

- ***TCTs:*** These tables will document experimental conditions for each definitive toxicity test. TCTs contain specific test information such as the number of treatments, nominal dilution series concentrations, and environmental conditions such as salinity, temperature, and feeding schedule. As such, TCTs are the final test method documents generated prior to definitive testing. Each test will have a single TCT, even if the test is repeated using the same experimental conditions. If a test is repeated, a new TCT will be drafted that clearly documents which test is being repeated in addition to any test condition changes. TCT templates are available from Stratus Consulting.

- ***GLPP:*** Work plans and SOPs will be used to create the GLPP for each laboratory conducting toxicity tests. During the course of a test, minor deviations may be made to the SOPs and work plans. The GLPP will be finalized after the completion of a definitive test and will therefore reflect the confirmed testing actions. This document will contain the final/modified versions of protocols for each definitive test. The GLPP will be regularly updated throughout the DWH laboratory toxicity testing projects lifespan.

The documents described above are required to facilitate efficient timely reviews of test methods generated by testing laboratories. Once established, work plans and SOPs may be used for multiple tests. These method documents may not have to be resubmitted to Stratus Consulting when seeking approval for each new test. In practice, TCTs may be the final method documents that are reviewed by Stratus Consulting. Stratus Consulting will give approval to start each test after review and acceptance of TCTs. To facilitate this review process, TCTs should be sent to Stratus Consulting as standalone Microsoft Word documents, so that each test will be described and provided in a separate file. Additionally, TCTs should clearly reference work plans or SOPs where appropriate. When approved, Stratus Consulting will notify the testing laboratories via email. If an approved test is rerun using the same test conditions, Stratus Consulting will assign a new test ID. If not provided, Stratus Consulting will create a new TCT file documenting the repeated test and new test ID information.

It is the responsibility of each testing laboratory to have final approved versions of work plans, SOPs, and TCTs available for reference when conducting definitive toxicity tests. If requested, these documents should be readily available for review. We recommend that testing laboratories create a hardcopy binder that contains these documents and routinely refresh it with updated work plans, SOPs, and new TCTs. Stratus Consulting will also document and inventory draft and currently approved test method documents.

4. Data Generation and Acquisition

This section describes definitive toxicity testing results and sample handling, QC, and data management for the project. A definitive test is a multi-concentration exposure that consists of

control and exposure treatments that provide dose-response information within a prescribed period of time. With respect to this QAPP, range-finding or pilot tests are not considered definitive tests. The methodology described in this QAPP only pertains to work performed during definitive testing. However, general document and file COC and retention requirements described in Section 2.4 (*Documents and Records*) and the sample retention policy described in Stratus Consulting (2011) applies to all tests, including definitive, pilot, and range-finding tests.

4.1 Toxicity Testing Data Recording

As stated in Section 2.4, toxicity testing information will be documented in notebooks, bench sheets, data entry files, photograph files and logs, COC forms, and shipping forms. These data will be provided to Stratus Consulting as hard copies and/or electronic files. Electronically scanned files of hard-copy and handwritten documents are acceptable and preferred.

When a test is complete, testing laboratory staff will enter all data from notebooks and/or hardcopy bench sheets into electronic Microsoft Excel files (Appendix B). All file entries will be checked against the original notebook and/or hardcopy entry bench sheets where data were originally recorded. All hand-written information recorded on the toxicity testing results reporting bench sheets will be clearly legible. Testing laboratories will complete the transcription of test results as soon as possible after a test is ended.

Stratus Consulting will provide each laboratory with blank templates of the toxicity testing data files prior to beginning toxicity testing. Testing laboratories will use the most recent versions of these files to record test information. Data files will be printed and used as bench sheets to record test results while conducting tests. Data entry files will be in Microsoft Excel format and have separate worksheets for documenting the following items:

- ***Experimental design:*** The "Tank ID, Dilution, or Stock Code Definitions" and "Test Conditions Table" bench sheets document general testing conditions (e.g., start and end times, species and life stage tested, feeding regime) and serve as lookup tables for tracking test IDs, tank IDs, and defining stock and dilution series codes that are used in subsequent sample inventory bench sheets. These bench sheets will be printed and filled out prior to conducting tests. Test and tank ID codes are described in Section 4.3.

- ***Preparing test solutions:*** Three bench sheets document test solution preparation: "Water Accommodated Fraction Preparation and Sampling Table," "Fluorescence Analysis of Test Solutions," and "Development of Fluorescence Analysis Standard Curve." WAF documentation includes preparation start and stop times, experimental procedures, and analytical sample collection for each test and test renewal. When used, fluorescence data entry bench sheets document the preparation and analysis of solutions used to generate a

standard curve, associated standard curve equation parameters, and preparation and analysis of all samples from each WAF dilution series.

- ***Daily and periodic water quality analyses:*** The "Water Quality Monitoring" bench sheet documents water quality analyses and results during the course of each test.

- ***Daily inspections:*** The "Test Performance Monitoring Bench Sheet" documents daily inspections of each tank during the course of each test. All tanks inspected will be recorded on this bench sheet. Inspectors are required to record the number of test organisms in each tank at the start of the test and to make note of any organisms found dead. Only organisms that can be observed as dead should be counted in the "Number observed treatment mortalities" entries. These organisms should then be removed and archived/sampled. There is a separate column for treatment and non-treatment mortalities. Examples of non-treatment mortalities include accidental spilling, loss of a tank, or losing fish that jump out of the tanks. If a non-treatment mortality occurs, briefly describe the event in the "Notes" column. Each inspection will also provide the number of organisms remaining in each tank, which will be recorded in the "Number of observed alive" entry.

- ***Sample collections:*** The "Analytical Sample Inventory Bench Sheet" documents all sample collection events that occur during the course of the definitive tests. Sampling events include, but are not limited to, WAF stock, archive, fluorescence, and archive tissue samples collected during or at the end of the test. WAF stock and archive sample entries will reference codes defined by each laboratory in the "Tank ID, Dilution, or Stock Code Definitions" bench sheet. Sample IDs are also documented along with a short description of the sample event, contents, and use. Storage information should also be recorded for each sample.

In addition to the test results reporting bench sheets described above, Appendix B contains a field entry "Data Dictionary" table that defines all entry field headings found on each bench sheet.

If the data entry bench sheets that Stratus Consulting provides to the laboratories are insufficient for documenting testing results, testing laboratories will draft revised or test-specific bench sheets. Testing laboratories will draft test-specific bench sheets during the test method work plan development and provide the draft data files to Stratus Consulting for review and approval. Testing laboratories should consult with Stratus Consulting during this process to determine if a needed bench sheet has already been created and to ensure that the format is consistent with other data files. In general, bench sheets should include columns for replicate ID (tank ID), date, time of measurement, and initials of recording staff. Revised and approved data entry files should be drafted in Microsoft Excel and used as data entry bench sheets as described above.

4.2 Sample Retention Requirements

Sample retention is required for specific samples and solutions generated during project activities as stipulated in the June 24, 2011 U.S. District Court, Eastern District of Louisiana *Pretrial Order No. 37 Relating to the United States' and Natural Resource Trustees' Testing of Samples*. Project sample retention policies will apply to samples and solutions generated during all pilot, range-finding, and definitive testing and analytical activities. The following sample and solution retention requirements are summarized from the June 2011 Pretrial Order No. 37:

- All glassware, containers, and equipment used to mix or administer oil and exposure solutions [e.g., needles, syringes, gavage tubing, gas-tight syringes, exposure chambers, and sampling equipment (including analytical sample containers)] *may be washed or discarded after use.*
- Exposure solutions, including unused oil-sediment slurries, materials removed from exposure chambers as part of regular maintenance, and oil remaining in exposure chambers at the conclusion of toxicity tests, *may be discarded after any necessary samples have been collected for chemical analysis or archiving.*
- All analytical chemistry extracts of oil, water, sediment, or tissues remaining after analysis *must be retained.*
- Requirements for retention of organisms or parts of organisms used in toxicity testing vary depending upon where they were obtained or if their tissues or blood were destroyed during analysis, as follows:
    - For organisms collected in the field for this project, any organisms or parts of organisms remaining after the testing *must be retained* regardless of whether they were used for toxicity testing
    - For organisms collected in the field for this project, any organisms or parts of organisms that died or were removed from the testing program for any reason before being used in toxicity tests *must be retained*
    - For organisms purchased or otherwise obtained from third parties and available to all parties for purchase or acquisition and not used in exposure tests or for controls *do not need to be retained*
    - Organisms that were used in exposure tests, including controls, *must be retained*
    - Organisms collected in the field or from which eggs were collected *must be retained.*

- If analysis of the organism and/or its tissues or blood results in the destruction of the tissues or blood, then any extracts or preparations (e.g., histology slides and uncut paraffin-embedded tissue) *must be retained*, regardless of effective shelf lives or hold times.
- All unused oil, dispersant, and sediment *must be retained*. Under no circumstances will oil, dispersant, or sediment be used or distributed outside of tests or analyses approved by Stratus Consulting.
- If it is unclear at any time as to whether a sample of any kind (chemical or biological) should be retained, contact Stratus Consulting before attempting disposal.

4.3 Test and Tank ID Codes

Test IDs, consisting of a three-digit number, track results for each definitive test. Unique test IDs will be assigned to each proposed test for each laboratory. Upon approval of testing methods documentation and before tests begin, Stratus Consulting will assign unique test IDs to each proposed test for each laboratory. When a test is rerun or new tests are added, they will receive a new test ID. Stratus Consulting will provide additional test IDs if needed. These test IDs will be part of the sample label, which is described in Section 4.6.

Prior to starting a test, each testing laboratory will establish tank ID, WAF stock sample, and dilution series codes in the "Tank ID, Dilution, or Stock Code Table" results reporting data bench sheet. These codes will be used in subsequent data reporting bench sheets and files for each test. Codes must be specific enough to avoid duplication and facilitate easy tracking between sampling from individual tanks and samples from WAF stocks or archive/dilution series. Note that these codes are not part of sample IDs.

Tank ID codes will track data generated for individual tanks during a test. WAF stock codes will designate WAF stock water samples from other sample types in the "Analytical Sample Inventory" bench sheet. The WAF stock water sample code will be entered into the "Tank ID Dilution or Stock Code" bench sheet when WAF stock samples are inventoried. Archive samples taken from each dilution series also need to be referenced to a specific dilution series. Therefore, unique dilution series codes will be generated, defined on the "Tank ID, Dilution, or Stock Code Table," bench sheet and used when inventorying archive series samples. Given the relationship between dilution series and tank IDs, we recommend that dilution series codes also be associated with tank ID codes. For example, when using dilution code "A" for a given dilution series; tank IDs would be A-1, A-2, and so on for each replicate. Refer to the "example" test results in the reporting data entry Excel file provided to each laboratory for more examples of coding tank IDs, WAF stock samples, and dilution series.

4.4 Sampling and Chemical Analysis

Types of sample matrices that may be collected during toxicity testing include water, sediment, and biological samples such as tissue. The types of samples collected and respective analyses conducted will depend on the particular test, but will follow the basic methodology described in this section. Non-analytical samples will also be collected by testing laboratories throughout the course of this project. These samples may include, but are not limited to, bacterial cultures, molecular biology samples, test organism whole-body tissue samples for use in histological analyses, or archives of any media type. Non-analytical samples may be analyzed in the future. Therefore, they should be collected, handled, and stored so that they do not reasonably jeopardize potential future analyses. If guidance on collection, handling, and storage of these samples is lacking herein, then testing laboratories should document collection, handling, and storage requirements in respective work plans or SOPs.

Please refer to the *Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment Analytical Quality Assurance Plan* (analytical QAPP; Appendix D) for details on analytical methods, reporting and detection limits, and the relevant laboratory parameters for the various analytical samples collected during definitive toxicity testing.

The majority of samples that testing laboratories collect may be water samples; these include samples from each newly prepared WAF stock solution, dilution series samples from the different treatments including control treatments, and source water samples. In most cases, water samples will be collected for one of four types of analyses: (1) polycyclic aromatic hydrocarbon (PAH) with alkylated homologues; (2) benzene, toluene, ethylbenzene, and xylenes (BTEX); (3) dioctyl sulfosuccinate (DOSS); and (4) fluorescence. Section A.4 in Appendix A provides a summary guide for water sampling, including information on when to sample and what analyses are needed for different sample types, what bottles/volumes are required, basic sampling instructions, and storage information.

Note that guidance provided in the following sections may not be applicable to all tests or testing laboratories. Testing laboratories will provide sampling and analysis information to Stratus Consulting when drafting work plans, SOPs, and TCTs. Upon review of draft testing methods documents, Stratus Consulting will specify any additional or modified sampling requirements. Sampling requirements may be modified according to the capabilities of the testing laboratory and the type of test.

Additional details on the types of samples that may be collected are described in the following sections.

4.4.1 WAF stock and dilution series samples

WAF stock or dilution series sampling may be conducted for oil exposure toxicity tests. This sampling guidance does not apply to dispersant-only tests, which are described in Section 4.4.3. Water samples may be collected from each toxicity test WAF stock solution prepared during the course of each test (Section A.1, Appendix A). Specifically, for PAH analysis, WAF stock water samples will be collected if an undiluted WAF stock will be used as the highest concentration exposure treatment. If the WAF stock is not the highest exposure concentration, then the highest exposure concentration should be sampled in lieu of sampling the WAF stock. Dilution series water samples may also be collected for analysis or extract and archive purposes only (Section 4.4.2). Dilution series water samples may be collected and analyzed for PAHs as explained in this section. For CEWAF tests, DOSS samples may also be taken from the highest exposure concentration for each new CEWAF stock prepared. A DOSS sample may also be collected from the control water. DOSS water samples may not be needed for the other exposure concentrations in a CEWAF test. For tests using source oil, BTEX samples may be collected for the stock WAF/highest exposure concentration and control water only. See Table 1 for the stock WAF/highest exposure concentration sampling scheme. In some exposure setups such as flow-through tests, the analytical sampling may differ from what we describe above. For these tests, the analytical sampling plan will be specified in test method documents prior to conducting definitive tests.

Table 1. Analyses designations for WAF stock and dilution series solution water samples

Stock preparation method	Oil type	Analyses requested[a]
HEWAF/LEWAF	Slick A	PAH/Alk (8270C SIM/PAH)
	Slick B	PAH/Alk (8270C SIM/PAH)
	Source	PAH/Alk (8270C SIM/PAH) BTEX (8260C VOCs)
	Weathered source	PAH/Alk (8270C SIM/PAH)
CEWAF	Slick A	PAH/Alk (8270C SIM/PAH) DOSS (ALS Environmental SOP)
	Slick B	PAH/Alk (8270C SIM/PAH) DOSS (ALS Environmental SOP)
	Source	PAH/Alk (8270C SIM/PAH) BTEX (8260C VOCs) DOSS (ALS Environmental SOP)
	Weathered source	PAH/Alk (8270C SIM/PAH) DOSS (ALS Environmental SOP)

a. Testing laboratories will also check the "Archive extract?" box for each sample on the COC forms when requesting analyses.

One set of WAF stock/highest exposure concentration water samples may be collected for each new WAF stock solution prepared, according to the guidelines listed below. This will include the collection of WAF stock/highest exposure concentration water samples at each renewal for the duration of the test. WAF stocks that are used for toxicity tests can only be stored for 24 hours and need to be kept in a cool, dry, and dark location. Stock solutions stored for more than 24 hours will not be used or sampled. If the renewal occurs within 24 hours and the same stock solution from which samples were previously drawn is used to renew test media, another sample from the WAF stock/highest exposure concentration should be collected at each renewal time point and sent in for analysis. If filtered WAFs are to be tested, then sampling should be conducted on the filtered WAF stock, unless directed otherwise. WAF stock samples should be taken by filling sample bottles directly from the stock solution aspirator bottle. Dilution series water samples should be taken by filling sample bottles as surrogate exposure vessels. Note that:

- WAF stock samples should be collected prior to diluting the stock solution to make subsequent test treatment solution
- WAF stock preparations should be sufficient to accommodate volumes needed for water samples and subsequent dilutions
- If more than one WAF preparation needs to be mixed to achieve a sufficient volume to conduct a test, all WAF solutions should be composited into one solution prior to collecting water samples for analysis and making test treatment dilutions
- The composited WAF stock solutions should be well mixed prior to taking water samples and making test treatment dilutions.

Dilution series samples may also be collected for PAH analysis from each new WAF preparation used throughout the duration of the test. These samples may be collected for extract and archive purposes. Similar to WAF stock samples, dilution series samples may be filtered or not filtered. Dilution series samples should be taken by filling sample bottles directly from the vessel in which treatment dilutions are made. As such, dilutions will be well mixed and be of sufficient volume to fill required sample bottles and dilution series test chambers. Reduced or additional dilution series sampling will be specified in test work plans, SOPs, and/or TCTs. As mentioned above, for CEWAF tests, only the highest exposure concentration and the control water are sampled for DOSS. Likewise, for source oil tests, only the highest exposure concentration and the control water are sampled for BTEX.

Table 1 provides the types of chemical analyses that may be requested for each stock preparation method and oil type. Note that in addition to analyses summarized in Table 1, the analytical laboratory will archive all WAF stock and dilution series solution sample extracts. Therefore, testing laboratories will check the "Archive extract?" box in addition to analyses requested

(Table 1) for each sample on COC forms when relinquishing samples to and requesting analyses from ALS Environmental.

WAF stock and dilution series water samples will be sent to ALS Environmental for chemical analysis. Until being packaged and sent to ALS Environmental for analysis, water samples should be stored according to the requirements listed in Tables 2 and 3. Sample handling and packaging should follow methods provided in Section A.2 in Appendix A (*Analytical Sample Shipping and COC SOP*). Section A.5 in Appendix A provides a sample shipping checklist that can be used when shipping analytical samples to ALS Environmental (see Table A.4).

Table 2. Sample bottles, preservation techniques, and holding times for each type of WAF stock and dilution series water sample/analysis

Analysis requested	Sample container	Bottle volume (mL or oz); sample volume	Preservation technique	Maximum holding time before extraction or analysis
PAH/Alk (8270C SIM/PAH)	Amber glass, wide mouth (n = 1)	250 mL; with minimal headspace	Store at 4°C in darkness	7 days (14 days if acid preserved)
BTEX (8260C VOCs)	VOA bottle set with HCl preservative (n = 3)	40 mL each; with zero headspace	Store at 4°C in darkness	14 days (7 days if not acid preserved)
DOSS (ALS Environmental SOP)	Plastic centrifuge tube set (n = 4)	15 mL each, but only add 10 mL of sample	Freeze at -20°C ± 10°C in darkness	Not established

VOA = volatile organic analysis.

Note that the maximum holding times listed in Tables 2 and 3 reflect the time from sample collection to the time when sample extraction occurs. ALS Environmental must receive samples such that at least one full business day (Monday–Friday) is allowed for conducting extractions within the listed holding times. Additional information on hold times for sample types not listed here can be found in the analytical QAPP (Appendix D). Ideally all samples should be shipped to the analytical laboratory the day when sampled or as soon as possible thereafter so that the sample holding times are not exceeded. Care should be taken to avoid sending samples over weekends or holidays. If unavoidable circumstances warrant sending samples to ALS Environmental over weekends or holidays, contact Stratus Consulting so that proper arrangements can be made with ALS Environmental sample intake personnel.

Table 3. Sample bottles, preservation techniques, and holding times for each type of sample/analysis

Analysis	Sample container	Bottle volume (mL or oz); sample volume	Preservation technique	Maximum holding time
Archive samples (water)	Amber glass, wide mouth	250 mL; with minimal headspace	Store at 4°C in darkness	7 days (14 days if acid preserved)
DOSS samples (water)	Plastic centrifuge tube set (n = 4 vials)	15 mL each, but only add 10 mL of sample	Freeze at -20°C ± 10°C in darkness	Not established
VOCs (water)	VOA bottle set with HCl preservative (n = 3)	40 mL each; with zero headspace	Store at 4°C in darkness	14 days (7 days if not acid preserved)
Fluorescence samples (water)	Borosilicate scintillation vial (n = 1 vial)	7 mL; with zero headspace	Add equal volume of 100% ethanol; store at 4°C in darkness	48 hours for freshwater; 1 week for saline
Source sample (water)	See Section 4.4.5 text		Store at 4°C in darkness	7 days
PAHs (sediment)	Glass jar	8 oz; load at least ¾ full	Store at 4°C in darkness	14 days[a]
VOCs (sediment)	Glass vial set with MeOH or $NaHSO_4$ preservative (n = 3 vials)	40 mL each; load 5 g sediment each and mix gently	Store at 4°C in darkness	14 days
Archive samples (tissue)	Wrapped in foil and bagged[b]	Whole organism tissue samples	Freeze at -20°C or colder	2 years[c]
Blood cell counts (blood smear)	Two glass microscope slides; air dry and preserve within 5 hours		Dry at room temperature in a slide box	None
Blood chemistry (plasma)	Cryovials	1.5 mL	Freeze (-20°C)	None
Blood protein levels (serum)	Cryovials	1.5 mL	Freeze (-80°C)	None
Blood endocrine markers (plasma)	Cryovials	1.5 mL	Freeze (-80°C)	None
Blood immunology (plasma, serum whole blood)	Cryovials	1.5 mL	Freeze (-20°C)	None
Microbiomics (lesions and skin secretions)	Swab tip in dry Eppy tube	2.0 mL	Freeze (-20°C)	None

Table 3. Sample bottles, preservation techniques, and holding times for each type of sample/analysis (cont.)

Analysis	Sample container	Bottle volume (mL or oz); sample volume	Preservation technique	Maximum holding time
Genetics (tissue)	DNA/RNA free microcentrifuge (snap-cap) tube	1.5 mL	RNALater at 4°C for 12 hrs then freeze at -20°C	None
DNA damage (tissue)	Glass scintillation vial	6 oz.	RPMI + 10%FBS + DMSO; freeze at -80°C	Two years, -80°C
Histology (tissue)	One HDPE screw-cap wide-mouth jar	Any	10% neutral buffered formalin, store at room temperature	None
Disease screen (tissue)	Microcentrifuge (snap-cap) tube	1.5 mL	Glycerol; freeze at -20°C	None
Dendrochronology and microchemistry (sagittae otoliths)	Manila paper scale envelope		Dry at room temperature	None
PAH metabolites (bile)	Clear glass vial with foil-lined cap; wrapped with foil	20 mL	Freeze (-80°C)	4+ years, -80°C
Chemical extracts (liquid solvent)	Depends on type of extract; contact Stratus Consulting for additional guidance[c]			

a. Maximum holding time may be extended to four years or longer if samples are stored at -20°C (Appendix D).
b. New, certified pre-cleaned borosilicate glass or polytetrafluoroethylene bottles may also be used to store archive tissue samples.
c. Archive tissue and chemical extract samples will be retained until notified otherwise regardless of holding time.

DMSO = dimethyl sulfoxide; DNA = deoxyribonucleic acid; DOSS = dioctyl sulfosuccinate; FBS = fetal bovine serum; HDPE = high-density polyethylene; RNA = ribonucleic acid; RPMI = Roswell Park Memorial Institute; VOCs = volatile organic compounds.

4.4.2 Archived water samples for PAH analysis

A single archive sample may be taken from each dilution series created from each WAF stock made at the beginning of the test and subsequent renewals for the duration of the test. Archive samples may also be collected from control treatments. Archive samples will be processed into sample extracts which will then be archived at the analytical laboratory. Archive-only samples

will not be required if dilution series samples are collected for chemical analysis; extracts from these samples will be archived.

Archive samples should be taken by filling sample bottles directly from the graduated cylinder or mixing vessel in which treatment dilutions are made. Archive samples do not need to be collected for dispersant only tests.

All archive water samples will be collected in 250-mL amber glass bottles and stored at 4°C (Table 3) until sent to ALS Environmental. Archive water samples will also be collected after dilutions are made and prior to being divided into testing chambers. COC forms should indicate that these samples are to be extracted and archived but not analyzed at this time by marking the "Extract and archive only" box for each sample.

4.4.3 Dispersant-only test DOSS water samples

When conducting dispersant-only and variable dispersant tests DOSS sampling and analysis may be performed on all dilutions made from dispersant-only stocks. DOSS water samples may also be collected from control treatments used in dispersant-only tests. Samples should be taken by filling sample bottles directly from the graduated cylinder or mixing vessel in which treatment dilutions are made, just prior to filling test chambers.

For each DOSS sample, four 15-mL plastic centrifuge tubes will be filled to approximately two-thirds full (Table 3). The four sample bottles will have the same sample ID. Respective COC forms will specify that the sample is contained in four sample containers and each container with the same sample ID should be labeled "1 of 4," "2 of 4," and so on. DOSS samples will be stored at or below 4°C and shipped to ALS Environmental. If stored for more than ten days before sending to ALS Environmental, DOSS samples will be frozen (Tables 2 and 3). If not already frozen, ALS Environmental will freeze all DOSS samples when received and store frozen until analyzed.

4.4.4 Fluorescence water samples

Fluorescence analysis may be used to check accuracy of making dilution series from WAF stock solutions. The relative total PAH concentrations in WAF stock solutions and dilution series solutions may be analyzed using a fluorometer. Fluorescence analysis requires that water samples be collected from the WAF stock (or 100% WAF treatment) and from each dilution series prior to being divided into testing chambers. Fluorescence samples may also be collected from control treatments. Fluorescence samples from the WAF stock solution may be used to generate standard curves for analyzing samples. A standard curve compares the relative

difference in fluorescence (e.g., total PAHs) between the WAF stock and all the dilution treatments in order to quantify the actual dilution of each treatment relative to the stock WAF.

Specifically, one fluorescence water sample may be collected from each WAF stock and each dilution series at the beginning of the test and subsequent renewals for the duration of the test. Samples should be taken by filling sample bottles directly from the aspirator bottle, graduated cylinder, or mixing vessel in which stocks and/or treatment dilutions are made. A single sample will consist of 3.5 mL of test solution added to 3.5 mL of ethanol in a 7-mL borosilicate scintillation vial (i.e., 1:1 solution-to-ethanol ratio). Vials should have either a foil-lined or Teflon cap. After filling and capping the vial, mark the liquid level with a permanent marker, weigh the vial, add a Parafilm wrap outside of the cap, and store upright at 4°C. Rather than sending them to ALS Environmental, each testing laboratory will analyze and securely store fluorescence samples. Freshwater samples should be analyzed within 48 hours of sampling, while saline water samples should be analyzed within one week, although it is recommended that all samples be analyzed as soon as possible after collection. Fluorescence sampling and analyses should be conducted according to the *Standard Operating Procedure – Fluorescence Spectroscopy to Verify Dilutions of Water Accommodated Fraction for Toxicity Testing* (Appendix F).

4.4.5 Source water samples

Water used to make exposure solutions is referred to as source water. Source water sources vary among testing laboratories and may come from natural sources such as filtered seawater or filtered saline groundwater, or may be prepared using filtered municipal water and commercial sea salt mixes. Source water samples will be sampled periodically during the course of this project. Specifically, one unfiltered source water sample will be taken from the dilution water used for making control treatment exposure and dilution treatment solutions. The source water sample will be shipped to ALS Environmental and analyzed for PAHs, metals, polychlorindated biphenyls (PCBs), pesticides, and BTEX. Source water samples will be collected in two 1-L amber glass bottles, three VOA bottles, one 500-mL plastic bottle (1.5 L for saline waters), one 250-mL plastic bottle without acid preservative, and one 250-mL plastic bottle with acid preservative. Sample bottles will be provided to laboratories by ALS Environmental. Samples will be stored at 4°C in darkness prior to sending to ALS Environmental. Maximum hold time for all sample bottles is seven days. Source water sample IDs will be generated by each laboratory and are not described in this QAPP. COC forms will specify that all samples will be analyzed for the full contaminants scan. Stratus Consulting will be contacted prior to conducting source water sampling to set up bottle delivery and analysis requests.

4.4.6 Sediment samples

Sediment sampling may be conducted during the course of this project. Sediment samples will be collected according to laboratory-specific protocols for conducting sediment toxicity tests. Required sediment analytical sample bottles are specified in Table 3. Sample labeling and shipping methods will follow requirements outlined in Section 4.6 of this QAPP. Archive sediment samples may also be collected.

4.4.7 Tissue samples

Tissue samples will be collected during the course of this project. Tissue samples may consist of, but are not limited to, whole test organisms, blood, and excised organs (Table 3). Tissue samples not designated for specific chemical, histological, or genomic analyses will be collected as archive tissue samples. Tissue samples that are designed for specific analyses will be collected according to laboratory-specific protocols required for each analysis. Some tissue samples may be subject to special biological substance containerization and shipping requirements. More information on shipping tissue samples that are considered biological substances is provided in Section 4.9.3, *Shipping biological samples*.

Archive tissue samples

All organisms used in toxicity tests but not designated for specific analysis will be retained as archive tissue samples. Remaining tissues from samples not consumed by designated test-specific analyses will be archived. Types of tissue samples that are subject to retention under a project-wide court order are described in the *Retention of Samples and Solutions Generated during Toxicity Testing* internal confidential memorandum, dated July 14, 2011 (Stratus Consulting, 2011) and Section 4.2.

Archive tissue samples and samples subject to the organism retention order should be sampled and preserved according to established methods for conducting organic contaminant analyses on tissue samples (U.S. EPA, 2000). Specifically, tissue samples should be placed in sample containers with as little water as possible. Equipment used to process tissue samples should be made of stainless steel, anodized aluminum, borosilicate glass, polytetrafluoroethylene, ceramic, or quartz. Tissue samples should be wrapped in aluminum foil and placed into a properly labeled sample container or plastic bag. New, certified pre-cleaned borosilicate glass or polytetrafluoroethylene bottles or vials may also be used to store tissue. Archive tissue sample labeling requirements are provided in Section 4.6.1.

Organisms removed from the same exposure chamber at the same time may be included in a single archive tissue sample, unless analysis of individually identified organisms is required. It is possible that test organisms may be too small, too fragile, or decomposed to a state such that sampling is not feasible. If an archive tissue sample cannot be made, Stratus Consulting will be

notified and it will be noted in a laboratory notebook. The notebook entry will provide sufficient detail to identify which test and treatment tissue samples could not be made and why the tissue sample could not be collected. If testing laboratories know that tissue sampling will not be feasible, they will contact Stratus Consulting/NOAA for approval to not collect tissue samples for that test.

Archive samples may be securely stored at -20°C at each laboratory for short-term storage or sent to ALS Environmental for long-term storage. If sent to ALS Environmental, sample shipping requirements provided in Section 4.9 will be followed.

4.4.8 Analytical chemistry sample extracts

If testing laboratories conduct chemical analyses, any remaining sample extracts will be retained (Section 4.2). This requirement pertains to pilot, range-finding, and definitive toxicity tests (i.e., all tests).

When generated, remaining chemical extracts may be securely stored under appropriate conditions at each laboratory for short-term storage or sent to ALS Environmental for long-term storage. If sent to ALS Environmental, sample shipping requirements provided in Section 4.9 will be followed.

4.5 Sample Containers, Preservation, and Holding Times

The analytical laboratory (ALS Environmental) will provide the PIs with appropriate containers for PAH, BTEX, and DOSS analysis of the WAF stock, archive water, source water, and sediment samples. Information on sample bottle type, preservation method, and holding times before extraction or analysis for each combination of matrix and analysis is provided in Tables 2 and 3 and in Appendix A, Section A.4. More details on sample containers, preservation, and holding times can be found in the *Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment Analytical Quality Assurance Plan* (Appendix D).

4.6 Analytical Sample Labeling Procedures and Designations

All sample containers will be labeled legibly in permanent ink with the following information:

- Sample ID (details provided below)
- Time of sample collection (military or 24-hour time format)
- Sample preservative (if applicable)
- Sample collector's name.

All sample labels will be covered with clear packing tape that completely encircles the sample bottle to prevent smearing or physical damage to the label. See Section A.2 in Appendix A for more details.

Note that:

- In some cases separate tests may be run using the same WAF stock and dilution series solutions. Therefore, a single WAF stock sample and a subsequent set of archive samples could be used to characterize water chemistry for more than one test. When practiced, laboratories will generate a single sample ID for each sample type using the methodology described below and record that same sample ID for all tests in which the same WAF stock and dilution series solutions are used. Sample IDs will be documented in each test's "Sample Inventory Table" bench sheet. This methodology facilitates tracking all tests in which the same WAF stock and dilution series solutions are used, regardless of sample ID coding.

- For analysis of volatiles (BTEX), three VOA vials per sample are needed. All three vials will receive the same sample ID. This sample ID will be entered once (i.e., on only one line) on the COC forms when requesting analyses and sending samples to ALS Environmental. COC forms have an entry, "# of containers;" for volatile analysis, enter "3."

- For analysis of DOSS, four DOSS centrifuge tubes per sample are needed. All four tubes will receive the same sample ID. This sample ID will be entered once (i.e., on only one line) on the COC forms when requesting analyses and sending samples to ALS Environmental. COC forms have an entry, "# of containers;" for DOSS analysis, enter "4."

- Cryos™ Cryomarkers should be used for labeling cryogenic vials that are subject to extreme cold temperatures.

- If the sample bottle is too small to contain all the label information above, the sample ID will be the only information written on the bottle. Do not try to fit all the information on a small bottle because the information may be illegible. Record the remaining sample label information in a laboratory notebook and/or in the appropriate test results entry bench sheet.

4.6.1 Sample designations

Each sample will receive a unique alphanumeric designation to identify the sampling location, date, sample type, and sample number. This methodology is similar to NOAA field sample labeling guidance. The following format will be used:

XX-Y####-ZZ-###-###

Samples will be identified as follows:

First segment (XX): laboratory-specific two-letter designations. Two-letter designations are as follows:

- Florida Gulf Coast University (FGCU)/University of North Carolina Wilmington = FG
- Hopkins Marine Station (Stanford University) = HS
- Miami University (Ohio) = MU
- Mote Marine Laboratory =MM
- Northwest Fisheries Science Center (NOAA) = NF
- Queen's University = QU
- University of Maryland = UM
- University of Miami, the Rosenstiel School of Marine and Atmospheric Science = RS
- University of North Texas = NT
- University of Southern Mississippi, Gulf Coast Research Laboratory = GR
- Auburn University Department of Fisheries = AB
- U.S. Army Corps of Engineers/ERDC = CE
- Louisiana State University = LS
- Pacific EcoRisk = PE
- University of South Florida = SF
- Marin Biologic Laboratories = MB
- Stratus Consulting = ST
- Louisiana Universities Marine Consortium = LM
- Florida Atlantic University = FA.

Second segment (Y####): sampling date. This five-digit date code includes a letter to represent the year, with 2012 = C, 2013 = D, 2014 = E, and 2015 = F. Following the year letter code are four digits for the month and day, including zeroes. Do not use slashes or dashes between digits. For example, the date code for May 3, 2015, would be F0503.

Third segment (ZZ): two-letter sample type and matrix designations that correspond to respective sample analyses. See Table 4 for a summary of sample type/matrix two-letter designations.

Table 4. Sample type/matrix two-letter designations

Matrix	Analysis	Type code
Water – filtered	PAH	FP
Water – unfiltered	PAH	UP
Water – filtered	VOC	FV
Water – unfiltered	VOC	UV
Water – filtered	DOSS	FD
Water – unfiltered	DOSS	UD
Water – filtered	Archive	FA
Water – unfiltered	Archive	UA
Water – filtered	Fluorescence	FF
Water – unfiltered	Fluorescence	UF
Sediment	Any	SE
Sediment	Archive	SA
Tissue	Any	TS
Tissue	Archive	TA
Extracts	Archive	AX
Bacterial culture	Any	BC
Molecular biology	Any	MO

Fourth segment (###): test ID. This is a unique three-digit number that identifies the toxicity test ID number that the sample is associated with. Each test will have a unique number that Stratus Consulting will assign prior to testing.

Fifth segment (###): sequential three-digit sample number for each sample. The sample number sequence will begin with 101 for each test and be sequential regardless of the sample type or matrix. Replicate samples will receive different sequential sample numbers.

Example sample IDs: the sample ID for Mote Marine Laboratory's fourteenth sample during test number 104, a filtered water sample for PAH analysis collected on July 23, 2015, would be MM-F0723-FP-104-114. A replicate filtered sample from the same WAF would be designated as MM-F0723-FP-104-115.

4.7 Equipment Decontamination

Care should be taken to avoid any cross-contamination of testing equipment, including but not limited to test exposure chambers, laboratory glassware, water quality meter probes, and analytical samples. To the extent possible, new, certified, clean materials should be used to conduct testing and sampling activities. When new testing materials are unavailable, all equipment used during any aspect of testing will be decontaminated before and after use following one of the three protocols outlined in Section A.3 (*Decontamination SOP*) of Appendix A. Equipment or testing materials that cannot withstand any of the decontamination procedures cannot be reused.

4.8 Sample COC

All samples collected during this project will be maintained under strict COC, which is the documentation of a sample's history from the time of collection through sample analysis to final disposal or complete consumption. Sample COC forms will be used to document sample COC, request chemical analyses, transfer samples between laboratories, and transfer samples to long-term storage. A blank COC form is provided in Appendix C of this QAPP. A printable, electronic COC form will also be provided to each testing laboratory.

The individual who prepares and labels a sample is responsible for the care and custody of all samples in his/her possession. A sample is considered to be appropriately in the custody of the sampler only in the following situations:

- The sample is in the individual's possession and no one else has access to the sample
- The sample is in a sealed container that cannot be tampered with or opened without breaking a tamper-proof seal
- The sample is in a designated secure area, cold storage room, locked refrigerator, or similar storage area to which only the person with custody has access
- The sample is in a shipping cooler, envelope, or box that is tamper resistant, properly prepared for shipping, and secured using custody seal tape.

A COC transfer occurs when custody of the samples is transferred from one individual to another (e.g., from the sample collector to the sample packing/shipment individual) or when the samples are shipped to and received by the laboratory. All COC transfers that occur during the course of this project will be documented on the COC form, which will indicate the individual who is relinquishing custody of the sample and who is receiving custody. The date and time of transfer

will also be recorded on the COC form. When samples are in the custody of the receiver, the person accepting the sample will sign and date the COC form.

When the samples are packed in coolers or other containers for shipment to the laboratory, the samples will be accompanied by completed original COC records (see Section A.2 in Appendix A, *Analytical Sample Shipping and COC SOP*). Each cooler will only contain the specific samples that are listed on the accompanying COC form(s). The COC record will contain the following information:

- Project name
- Sample shipper contact information
- Stratus Consulting contact information
- Any special instructions
- Sample ID (unique for each sample collected during a test)
- Date and time of sample collection
- Sample matrix (e.g., sediment)
- Analysis required for each sample (see Table 1) for definitive samples
- Name and signature of individual relinquishing custody
- Inclusive dates and times of possession for each person
- Sample shipping date and mode.

Custody seals will be used on shipping containers. These seals detect unauthorized tampering with the sample shipping container from the time the laboratories relinquish samples until they are received. Signed and dated gummed paper seals may be used for this purpose. ALS Environmental will provide the seals with the sample bottles. Seals will be attached so that the next individual to open the shipping container cannot do so without disturbing the seals, as detailed in Section A.2 of Appendix A. Custody seals will also be used when shipping any test materials and can be affixed to envelopes or boxes prior to shipment. Evidence tape may also be used as a custody seal if it is signed and dated by the person relinquishing test materials.

Only individuals authorized to receive the samples at the analytical laboratory can open coolers or other containers containing samples. The containers will first be inspected for integrity of the custody seals or other signs of tampering. The receipt of each sample in a cooler or container will be verified on the COC form. After verification, the signed COC form will be photocopied or scanned, and the copy will be mailed or emailed to the sending party. Samples will be stored in a secure area according to procedures documented for each analytical facility.

Note that these same guidelines apply for the physical transfer of written materials and file storage devices from one individual to another. For these transfers, the box on the COC labeled "Data" is to be checked and the contents being transferred (hardcopies, electronic, SD cards, etc.) are to be checked in the rows provided under the analytical sample information entry fields. Samples subject to long-term storage will be documented using the "Samples stored on-site"

checkbox on respective COC forms (Appendix C). Storage location information should also be provided on the COC form.

4.9 Sample Shipping

All samples will be packed in such a manner that they are not compromised during shipment and are received in good condition by the recipient laboratory. All sample containers will be shipped in accordance with all applicable shipping regulations. If shipping frozen samples using dry ice as a refrigerant, refer to Section 4.9.2. If shipping biological samples, refer to Section 4.9.3.

All analytical samples will be packed with enough ice to maintain an ambient temperature of approximately 4°C until the laboratory receives them. Ice will be in gel packs, water frozen in excess sample bottles, and/or ice cubes within multiple resealable plastic bags to prevent water from touching sample bottles and damaging labels. Bubble wrap or a similar packing material should be used to protect glass bottles during shipment. Glass bottles should not contact other glass bottles or hard objects in the shipping container. All shipping containers that contain analytical and archive samples being sent to ALS Environmental should include a clearly labeled temperature blank (plastic bottle containing cold water used by the analytical laboratory to verify sample temperature upon receipt). All sample coolers will be sealed using custody seals and shipped to the laboratory analyzing the samples via overnight delivery as soon as practical after collection. All sample coolers will be shipped in accordance with all applicable regulations. An original COC record, placed in a sealed plastic bag and taped to the inside of the cooler lid, will accompany each shipping container containing samples. An appropriate carbon- or photocopy of the COC record page must be retained prior to sealing the cooler. Appendix A, Section A.5, provides a sample shipping checklist that can be used as a reference guide when shipping samples to ALS Environmental.

4.9.1 Shipping archive samples

Each toxicity test may generate samples that need to be archived, including tissue samples and solvent extracts from in-house analyses, which must be retained as described in the sample retention order memorandum (Stratus Consulting, 2011). Archive samples requiring long-term cold storage can be sent to ALS Environmental. When shipping archived samples to ALS Environmental for long-term storage, samples will be shipped in separate coolers from chemical analysis samples being sent to ALS Environmental. Each individual cooler should contain no more than 100 individual archive samples. Each sample in the cooler must be properly documented on the designated DWH toxicity testing COC form (Appendix C), including sample ID and date and time of sample collection. The samples should be organized in such a way as to allow for easy ID of the samples once they arrive at ALS Environmental. For example, if 100 samples are going to be shipped, the samples will be divided by putting 10 samples in

labeled zip-top plastic bags, and then indicating on the COC form which samples are in which bag. In some cases, only a handful of samples will fit in each cooler (e.g., when shipping large brood stock fish). Thus, for any cooler containing 10 or fewer samples, the sample ID for each individual sample is sufficient, and the samples do not need to be further divided using the above approach. If shipping multiple coolers, then each cooler needs a separate COC form that lists only the samples included in the corresponding cooler. In the special instructions section on the top of the COC form, include the phrase "all samples are for long-term storage only, no analysis required" to indicate that these samples are for storage only. Since ALS Environmental does not need to perform extractions or analyses on these samples, leave the "Analyses Requested" boxes unchecked. Also, the storage temperature for the samples will be indicated on the COC form (e.g., store at -20°C); samples with different storage temperature requirements must be shipped separately from each other. Note that ALS Environmental is only accepting archive samples that require storage at 4°C or -20°C. For archive samples that have different storage requirements, please contact Stratus Consulting for further instructions.

If samples are shipped to ALS Environmental in Kelso, Washington, the shipping address is:

ALS Environmental
1317 South 13th Avenue
Kelso, WA 98626
800-695-7222 (telephone)

4.9.2 Shipping frozen samples using dry ice

Samples may be kept frozen during shipping using dry ice. When dry ice is used as a refrigerant, special shipping regulations must be followed and special packaging materials must be used. Standard coolers are not acceptable shipping containers. Acceptable shipping containers are two-piece units made of a polystyrene (Styrofoam®) inner container that is placed inside an outer corrugated cardboard box. Polystyrene cannot be used as outer packaging. The outer packaging carrying the shipment and the dry ice must be able to withstand the loading and unloading process. Note that the outer package must allow for release of CO_2 gas and should not be completely sealed with packaging tape. Dry ice should not contact or have potential for contacting sample containers. Dry ice will freeze plastic containers or bags, causing them to break apart.

Transportation Security Administration (TSA), International Air Transport Association (IATA), and Department of Transportation (DOT) requirements for the labeling the shipping container will be followed. The maximum amount of dry ice that can be shipped is 200 kg. It is recommended that shipments contain 5–10 pounds (2.3–4.5 kg) of dry ice per 24 hours of transit time. See Appendix A, Section A.6, for detailed dry ice shipping procedures.

4.9.3 Shipping biological samples

Only Biological Substance Category B, Exempt Animal Specimen tissue samples will be shipped. Examples of Category B, Exempt Animal Specimen samples that may be generated and shipped during project activities include, but are not limited to, turtle blood and its components; fresh (not frozen) tissue samples; bacteria cultures; and molecular biology samples not related to exposure and diagnosis of an infectious disease. Exempt Animal Specimen tissue samples will be shipped according to TSA, IATA, and DOT shipping package construction and labeling requirements. See Appendix A, Section A.7, for detailed Category B, Exempt Animal Specimen shipping procedures.

4.10 Sample Storage

Samples for chemical analysis will be sent to the analytical laboratory as soon as possible but may require short-term storage at the testing laboratory prior to shipment. Individual laboratories will store fluorescence samples. While stored at testing laboratories, samples will be kept under strict custody in a lockable refrigerator or freezer. Alternatively, sample custody can also be maintained if the refrigerator or freezer is in a locked room.

Archive tissue samples and remaining chemical extracts generated by testing laboratories may be sent to ALS Environmental for long-term storage as described in Section 4.9. ALS Environmental will store sample extracts, long-term archive samples, and unused portions of all samples under proper COC, temperature, and lighting conditions for at least 24 months. The analytical laboratory will contact Stratus Consulting before disposing of any samples.

4.11 Quality Control

QC is the system established for the project to assess the variability in data that arises from the sampling and analysis procedures used.

4.11.1 Laboratory QC measures

Quantitative analytical data, such as QC limits on precision, accuracy, bias, and detection limits, as well as QC samples prepared and analyzed by testing laboratories, will be specified in the laboratory work plans when applicable. For ALS Environmental QC measures refer to the *Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment Analytical Quality Assurance Plan* (Appendix D) for details.

Non-analytical QC measures will be the responsibility of testing laboratories. Details will be provided in laboratory work plans and SOPs.

4.11.2 Terminating tests

Toxicity tests may be terminated at any time before, during, or after testing. The decision to terminate a test will be made at the discretion of the testing laboratory and/or Stratus Consulting staff. Tests may be terminated due to poor control performance, failure to maintain specified exposure, husbandry issues, COC issues, or any other circumstance that would jeopardize validity or reliability of a test. Laboratory PIs will immediately notify Stratus Consulting when it is determined that a test should be terminated. Alternatively, Stratus Consulting staff will notify PIs if Stratus Consulting staff determines that a test should be terminated.

Stratus Consulting staff will provide guidance on how to handle analytical chemistry samples taken before a test is terminated. If not already sent to ALS Environmental, Stratus Consulting may require that analytical chemistry samples be submitted for analysis. Alternatively, samples already sent to ALS Environmental prior to test termination may subsequently have the requested analyses canceled. Samples collected prior to test termination and not sent for analysis will be discarded, but only after Stratus Consulting approval. If discarded samples were documented on the *Water Accommodated Fraction Preparation and Sampling Table* or *Analytical Sample Inventory Bench Sheet*, the testing laboratory will clearly note on the bench sheets that respective samples have been discarded. As previously mentioned, the sample retention policy described in this QAPP (Section 4.2) applies to all tests, including terminated tests. Therefore, all test organisms used in a terminated test will be archived according to procedures described in Sections 4.4.7 and 4.9.1. Archive tissue samples will be documented using an *Analytical Sample Inventory Bench Sheet* and sample IDs will reference the terminated test ID.

Testing laboratories will retain all toxicity testing data entry bench sheets and any other related files or documents that were used prior to test termination, under the general retention and COC guidelines provided in Section 2.4. Stratus Consulting may request these documents at any time.

Stratus Consulting may choose, on a case-by-case basis, to salvage tests performance and/or analytical chemistry results generated during terminated tests. If this occurs, terminated test results will be subject to data entry and QA/QC (Section 4.11.3) and validation process (Section 6) described in this QAPP. If standard processes described herein are not amenable, then Stratus Consulting will develop, implement, and document an alternative data entry, QA/QC, and validation process.

4.11.3 Toxicity testing data entry

Each test laboratory will check all data entered into the Excel-based toxicity testing data entry files against the original hard-copy bench sheets before sending to Stratus Consulting. A person other than the individual who originally entered information into the data entry files should check transcription accuracy from hard-copy bench sheets. QC checks will be performed on 100% of the entries. Reviewers will correct and note transcription errors using the comment call-out boxes in affected data entry cells. When completed, the reviewer's name and date when checked will be recorded on each test's toxicity testing results reporting data entry file.

After test information is entered into the respective toxicity testing results reporting data entry files and entries are checked against original hardcopy bench sheets, a copy of the workbook will be sent to Stratus Consulting. Testing laboratories will also provide electronic scans or copies of original hard-copy bench sheets to Stratus Consulting. Copies of laboratory notebooks and other electronic data files, photographs, and other records in which testing results are documented will also be provided to Stratus Consulting.

Please note that the toxicity testing data entry file table structure should not be edited. The files are arranged so that they are standardized among testing laboratories and can be efficiently read and analyzed. If the toxicity testing results reporting data entry files are not suitable for a given test, contact Stratus Consulting for guidance. In some cases, testing laboratories may need to use their own data entry files. Stratus Consulting will review and accept these files prior to beginning tests.

4.11.4 Data management

The laboratory will maintain original copies of all records, data, electronic files, backup files, and study documentation and will not purge the records without permission from Stratus Consulting. Laboratory analytical data will be provided to Stratus Consulting in electronic and hard-copy format. Stratus Consulting will maintain copies of laboratory notebooks, COC forms, data entry files, photographs, and photographic logs.

5. Project Assessment and Oversight

Assessment and oversight includes those actions taken to ensure that this QAPP is implemented properly (U.S. EPA, 2002).

5.1 Assessments and Response Actions

Laboratory PIs working together with Stratus Consulting will direct all testing activities. PIs are responsible for:

- Ensuring that their staff have read and understood all related protocols and this QAPP prior to conducting any definitive toxicity tests
- Directing all sampling activities, including sample collection, handling, and labeling, as well as equipment decontamination
- Reviewing test documents.

PIs will communicate directly with their staff to ensure that appropriate response actions, as well as related efforts to document them, are taken to address any problems or issues.

The analytical laboratory staff will follow their internal procedures, as well as those specified in the analytical methodologies, when performing project oversight and instituting appropriate response actions as outlined in the *Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment Analytical Quality Assurance Plan* (Appendix D).

5.2 Reports to Stratus Consulting

PIs will communicate testing activities regularly to Stratus Consulting. The analytical laboratory will also report to Stratus Consulting, who in turn will communicate with laboratory PIs and other project staff, as appropriate.

Each testing laboratory will submit scanned test-specific toxicity testing results bench sheets as PDFs and data entry files in Microsoft Excel after each test has been completed and QC checks have been completed on all data entry. These test results should be provided to Stratus Consulting no later than one week following the conclusion of a test.

6. Data Validation and Assessment of Data Usability

Data validation and assessments will be conducted on the laboratory and analytical data to review laboratory documentation regarding the stated methods and acceptability of results.

Testing laboratories will conduct internal validation assessments on the toxicity testing data that they submit to Stratus Consulting. Testing laboratories will assess test validation according to laboratory acceptability criteria and adherence to work plans. Invalid tests will be identified and

reported to Stratus Consulting as soon as possible so that appropriate measures can be implemented. These measures may include, but are not limited to test termination, stopping analysis of invalid test chemistry samples, and assigning new test IDs for make-up tests.

Once a test is complete and test and chemistry data have been provided to Stratus Consulting, Stratus Consulting will perform a data validation assessment of testing materials. Any issues will be brought up with testing laboratories on a case-by-case basis and appropriate actions will be taken to validate the usability of a test. If completed tests cannot be validated, testing laboratories may repeat invalid tests using new test IDs.

The analytical data review will be conducted according to the standards and criteria set forth in the standard methods and protocols being used by the analytical laboratory. The review process will evaluate the degree to which the analytical laboratory followed the prescribed methods, the results of internal QC sample analyses, and the implications for data usability or any deviations from the prescribed methods.

References

Stratus Consulting. 2011. Memorandum Re: Retention of Samples and Solutions Generated during Toxicity Testing. Prepared by Stratus Consulting, Boulder, CO. Confidential. July 14.

U.S. EPA. 2000. *Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories. Volume 1 Fish Sampling and Analysis*. Third Edition. EPA 823-B-00-007. U.S. Environmental Protection Agency. November. Available: http://water.epa.gov/scitech/swguidance/fishshellfish/techguidance/risk/upload/2009_04_23_fish_advice_volume1_v1cover.pdf. Accessed July 2011.

U.S. EPA. 2002. *EPA Guidance for Quality Assurance Project Plans*. EPA QA/G-5. EPA/240/R-02/009. U.S. Environmental Protection Agency. December. Available: http://www.epa.gov/quality/qs-docs/g5-final.pdf. Accessed April 2011.

A. Protocols and Standard Operating Procedures

A.1 Protocols for Preparing Water Accommodated Fractions

General guidelines:

- Controls should be prepared for all WAF types. Controls should be prepared using the same WAF technique, with the exception of the addition of oil. CEWAFs may require additional control preparations with dispersant only.
- For instructions on cleaning and preparing equipment, refer to the *Decontamination SOP* (see Section A.3 in this appendix).
- If filtered WAF is required, see the *Water Accommodated Fraction Filtration Standard Operating Procedure* provided in Appendix G.
- For each step during sample preparation and collection, all appropriate information must be entered into the bench sheets and data entry files provided by Stratus Consulting (see Section B.4 in Appendix B). Prior to starting an experiment, staff should acquaint themselves with the information that they are required to record.
- All unused WAF solutions left over after analytical sampling and preparing test exposure water dilution series will be disposed of according to the testing laboratory waste management procedures. All test exposure water should also be disposed of according to the testing laboratory waste management procedures when tests are concluded and any samples are taken.
- After it is prepared, WAF solution may be used to make dilution series exposure water for up to 24 hours. Similarly, dilution water may also be used for up to 24 hours after being prepared. These guidelines would only apply for tests that have a renewal within a 24-hour timeframe. WAF and dilution series will be securely stored in a cool, dry, and dark location.

SOP for HEWAF

Materials:

- Waring™ CB15 commercial food blender
- Source water (e.g., seawater or embryo culture solution)

- 1-L or larger graduated cylinder
- 1-L or larger separatory funnels and ring stand
- Heavy-duty aluminum foil, cut into 30 by 30 cm squares, rinsed with acetone/hexane or DCM
- Kimwipes
- Nitrile gloves
- Aluminum weigh boats
- Glass gastight syringes with Teflon plunger (appropriate volumes)
- Stainless steel spatulas
- Miscellaneous glassware to transfer solutions (Erlenmeyer flasks, carboys, beakers, etc.).

Procedure:

A. Preparation of blender lid (this is repeated for each WAF preparation; Figure A.1):

1. Invert blender lid on bench top
2. Center foil square over inside of lid and carefully push down into the lid; push and fold inward to avoid tearing and to keep foil centered
3. Fold excess out over the lip so it can be trimmed with scissors
4. Trim around the edge, leaving ~ 1 cm to fold over the first sealing ridge
5. Press around the edges to make the foil as flat as possible over the sealing ridge
6. Discard and replace foil for each preparation.

Figure A.1. Steps for lining the blender lid with solvent-rinsed foil.

B. Prepare oil HEWAF:

1. Obtain source water from a clean source (e.g., sand filtered, ozonated) and record temperature and salinity. Fill the pre-cleaned blender pitcher with source water; note that the blender pitcher should always be filled to capacity (blender capacity is 3.75 liters) even if the entire volume of WAF is not required. Record start time and source water volume on the "Water Accommodated Fraction Preparation and Sampling Table" bench sheet.

2. Add desired amount of oil to the blender pitcher.

    a. *Source oil and weathered source oil* should be added using a pre-cleaned gastight syringe. It is best to fill the syringe with oil and dispense it prior to taring it on a balance. This will fill the needle and any voids, allowing for a more accurate dispensing weight. Fill the syringe with the desired weight and record the initial weight. After dispensing, record the final weight and determine the actual amount added by mass difference. Note: one gram of oil is equivalent to about 1.2 mL of oil. It is best to have a syringe dedicated to source oil to avoid contamination.

b. *Slick A and B oil* should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. With the weigh boat over the blender pitcher, slightly bend the weigh boat to create a narrower spout. Carefully transfer the oil using a spatula to scrape the oil into the pitcher. Wipe any oil remaining on the spatula using the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass transferred (Figure A.2).

Figure A.2. Taring Kimwipes and aluminum weigh boat and reweighing scraped weigh boat and Kimwipe used to clean spatula.

3. Close the blender lid.

4. Blend 30 seconds on low.

5. Transfer contents to a pre-cleaned, decontaminated separatory funnel.

6. Note time of transfer.

C. Separation:

1. Transfer separatory funnel to a ring stand, preferably in a hood

2. Allow to separate for 1 hour; be sure not to use the top layer (~ 100 mL) for downstream applications

3. Collect the bottom layer of the unfiltered HEWAF in an intermediate container (e.g., an Erlenmeyer flask); any aliquots for definitive analytical chemistry samples should be gently transferred from the intermediate container to the appropriate sample container (provided by ALS Environmental) immediately prior to the next downstream application (e.g., filtration, serial dilution, or direct-exposure testing).

SOP for LEWAF and CEWAF

Materials:

- Source water (e.g., seawater or embryo culture solution)
- 1-L or larger graduated cylinder
- Aluminum foil
- Stir plate
- Stir bars, Teflon coated
- Aspirator bottles (at least 1 liter or larger capacity)
- Tygon tubing with hose clamps
- Syringes, glass/Teflon gastight
- Container for source water, 20-L Nalgene carboy with spigot
- Top-loading bench scale (should have ≥ 300 g limit)
- Aluminum weigh boats
- Stainless steel spatula
- Kimwipes
- Nitrile gloves.

Procedure:

A. Preparing LEWAF:

1. Obtain source water from a clean source (e.g., sand filtered, ozonated) and record temperature and salinity.

2. Place clean, decontaminated aspirator bottles on stir plates.

3. Secure Tygon tubing and clamps onto bottom outlet.

4. Place stir bar in bottom of aspirator bottle, 1 in. for 1-L, 2 in. for 2-L; do not forget this step as it cannot be done after oil has been added.

5. Add desired amount of source water to each aspirator bottle.

6. Begin to stir with no vortex (180–240 rpm for 2-L with 2-in. stir bar).

7. Add desired amount of oil to aspirator bottle.

    a. *Source and weathered source oil* should be added using a pre-cleaned gastight syringe (Figure A.3). It is best to fill the syringe with oil and dispense it prior to taring it on a balance. This will fill the needle and any voids, allowing for a more accurate dispensing weight. Fill the syringe with the desired weight and record the initial weight. After dispensing, record the final weight and determine the actual amount added by mass difference. Note: one gram of oil is equivalent to about 1.2 mL of oil. It is best to have a syringe dedicated to source oil to avoid contamination.

    b. *Slick A and B oil* should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. With the weigh boat over the aspirator bottle, slightly bend the weigh boat to create a narrower spout (Figure A.4). Gently transfer the oil using a spatula so that oil does not drop to the bottom and come into contact with the stir bar. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass transferred (Figure A.2).

8. Cover with aluminum foil and stir for 18–24 hours. No settling time is required for LEWAF. It is best to use it immediately to avoid VOC loss, but it may sit for up to 24 hours if necessary.

Figure A.3. Transferring source oil to aspirator bottle.

Figure A.4. Transferring slick oil into aspirator bottle.

9. Prior to use, allow 20–40 mL to drain to waste container. This will clear any water that has been sitting in the Tygon tubing.

10. Unfiltered LEWAF may be used directly for making definitive analytical chemistry samples and dilution series for exposure assays, or may be filtered and then used for making definitive analytical chemistry samples and dilution series for exposure assays; do not use the top layer (~ 100 mL).

B. Preparing CEWAF:

1. Follow steps 1–5 as for LEWAF.

2. Begin to stir with minimal vortex (less than 25% of solution height).

3. Add oil to the center of the vortex.

4. Increase the mixing speed if the vortex decreases to less than 25% of the solution height.

5. Add dispersant to center of the vortex using a gastight syringe, and again calculate the delivery mass by difference; prior to taring the syringe, prefill and dispense the syringe in order to achieve a more accurate weight, as was done with the source oil (use a syringe dedicated to Corexit to avoid contamination).

6. Adjust vortex to 25%.

7. Stir for 18–24 hours. Turn stirrer off. Let settle for 3–6 hours.

8. Prior to use, allow 20–40 mL to drain to waste container. This will clear any water that has been sitting in the Tygon tubing.

9. Unfiltered CEWAF may be used directly for making definitive analytical chemistry samples and dilution series for exposure assays, or it may be filtered and then used for making definitive analytical chemistry samples and dilution series for exposure assays; be sure not to use the top layer (~ 100 mL).

Refer to the figures for photographs of the following subjects:

- Preparing blender lid (Figure A.1)
- Reweighing scraped weigh boat and Kimwipe used to clean spatula taring Kimwipes and aluminum weigh boat (Figure A.2)

- Transferring source oil to aspirator bottle (Figure A.3)
- Transferring slick oil to aspirator bottle (Figure A.4).

A.2 Analytical Sample Shipping and COC SOP

This SOP describes how to properly ship samples for chemical analysis while maintaining established COC requirements.

As described in the project QAPP, COC forms are used to relinquish custody of samples when sent to ALS Environmental or placed in locked storage. When the samples are sent to an analytical laboratory, the COC is used to request which analyses will be conducted for each sample. After all samples have been aliquoted into appropriate bottles and labeled correctly (with clear packing tape to protect the label), each sample collected must be stored and shipped following these COC standards:

1. Wrap labeled sample bottles in bubble wrap bags or similar packing material and place into coolers with blue ice. All samples will be packed with enough ice to keep the samples cooled to approximately 4°C until received by the laboratory. Ice will be in gel packs, water frozen in excess sample bottles, and/or ice cubes in multiple resealable plastic bags to prevent water from touching sample bottles.

    a. Samples should be packed so that they are not directly touching each other or hard objects such as ice packs.

    b. A temperature blank bottle should be included in each cooler. This bottle should be in the empty cooler when it is received. The analytical laboratory will use this bottle to document the temperature of the samples when they are received. If a temperature blank bottle was not provided by ALS Environmental, make one by adding cold water to a capped 250-mL plastic bottle clearly labeled "Temperature Blank."

2. Record the ID for each sample that is placed into the cooler on the COC form(s).

    a. Note that the COC forms are used to request the chemical analyses to be performed for each sample. See the project QAPP for details on the specific analyses required for each type of sample.

3. Sign and date the COC form(s).

4. Retain one photocopy of the COC form(s) for your records in a secure location.

   a. Document shipment details and COC information in your project notebook.

5. Seal the remaining original copies of the COC form(s) in a plastic resealable bag and tape the bag to the underside of the cooler lid (or to the inside of the cooler).

6. Sign and date at least two COC seals (small stickers provided by ALS Environmental with a line for a signature) for each shipping container.

7. Place signed COC seals on opposite corners of the cooler across the seam between the cooler lid and the main body of the cooler (Figure A.5). COC seals must be arranged so that the cooler cannot be opened without disturbing the seals.

8. Place clear packing tape over the COC seals.

9. Seal the cooler by taping around the seam between the lid and body of the cooler and around the entire cooler (Figure A.6).

10. Deliver cooler(s) to a FedEx location or have FedEx pick up the cooler(s). **Do not** leave the cooler(s) at an unattended FedEx drop-off location. Samples will be shipped using overnight delivery as soon as possible following collection in order to provide ample time to perform extraction/analysis within the appropriate holding time. Avoid shipping samples over weekends. If samples are shipped to ALS Environmental in Kelso, Washington, the shipping address is:

    ALS Environmental
    1317 South 13th Avenue
    Kelso, WA 98626
    800-695-7222 (telephone)

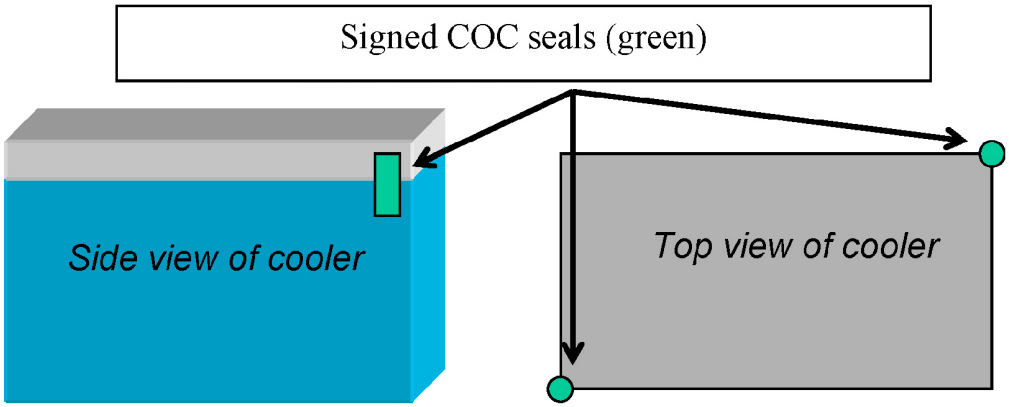

Figure A.5. Placement of signed COC seals (green) on the outside of shipping coolers.

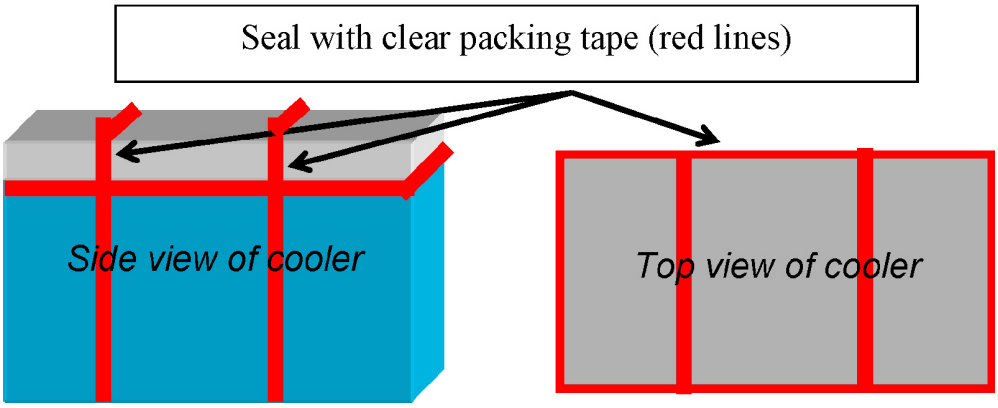

Figure A.6. Placement of clear packing tape (red lines) on shipping coolers.

A.3 Decontamination SOP

These decontamination protocols describe the procedures for preparing all non-disposable equipment prior to experimental analysis. Decontamination should be performed 24 hours in advance of experimental analysis to allow for evaporation of solvents. Three methods are presented below. The first is a solvent-based method and the second is a NaOH-based method used for glassware, stainless steel, or other materials that are inert to solvent and/or caustic chemical exposure. Either of these two methods may be used, depending on resources available to each laboratory. The third method (detergent only) should be used to decontaminate non-disposable test materials that could be destroyed by exposure to solvents and/or caustic

chemicals. Examples of such test materials may include, but are not limited to, water quality meter probes, plastic water holding tanks, and flow-through exposure tank pluming.

General considerations:

- Use gloved hands throughout all preparation steps and use commonsense laboratory safety and Personal Protective Equipment (PPE) when handling solvents.
- Hexane is helpful in removing visible residue from heavily oiled equipment. When cleaning blenders, the underside of blender prongs may need to be carefully scrubbed by hand with hexane-soaked Kimwipes, particularly after use with weathered surface oil samples; note that it is easy to puncture a glove on the blender blades.
- If glassware is too large or bulky (e.g., carboys, aquaria) to submerse into detergent or NaOH solutions, it is acceptable to rinse and scrub it three times, with a 5-minute wait between each rinse/scrub.

Acetone/Hexane/DCM method

Materials:

- Laboratory-grade soap such as Sparkleen or Liqui-Nox; Simple Green® All-Purpose Cleaner
- Reagent-grade acetone and Teflon wash bottle
- Reagent-grade hexane and Teflon wash bottle
- Reagent-grade DCM and Teflon wash bottle (optional)
- Kimwipes
- Nitrile gloves (thicker reusable gloves recommended for extended contact use)
- Aluminum foil.

Procedure:

1. Wash all equipment that will come in contact with the sample (glassware, spatulas, stir bars, etc.) with laboratory-grade soap or Simple Green® and hot water. When cleaning blenders, soap and hot water can be blended on low for 1 minute.

2. Rinse 3 times with reverse osmosis (RO) or organic-free de-ionized (DI) water to remove any soap residue.

3. Rinse all equipment 3 times with acetone. Allow sufficient time for full evaporation of solvent.

4. Rinse all equipment 3 times with hexane. Allow sufficient time for full evaporation of solvent.

5. If available, rinse all equipment 3 times with DCM. If DCM is not used, perform another triplicate hexane rinse. Allow sufficient time for full evaporation of solvent.

6. Cap glassware with aluminum foil and store in a closed dust-free cabinet.

7. Rinse with source water before using.

NaOH method

Materials:

- Phosphate-free laboratory detergent such as Alconox or Liqui-Nox; Simple Green® All-Purpose Cleaner may also be used
- Cleaning brushes
- Reagent-grade NaOH
- Kimwipes
- Nitrile gloves
- Aluminum foil.

Procedure:

1. Dispose of any remaining waste material left in used glassware to be decontaminated in an approved manner.

2. Soak glassware in a solution of phosphate-free laboratory detergent or Simple Green® using the manufacturer's suggested dilution for more than 1 hour, preferably 24 hours.

3. Thoroughly brush-wash the glassware and then rinse 6 times with tap water.

4. Soak glassware in a solution of 0.25 M NaOH (10 g NaOH/L DI water) for more than 1 hour, preferably 24 hours.

5. Rinse 6 times with RO or DI water.

6. Rinse 6 times with reagent-grade Acetone. Allow sufficient time for full evaporation of solvent.

7. Rinse 6 times with organic-free DI water.

8. Drain well and air or oven dry.

9. Cap glassware with aluminum foil and store in a closed dust-free cabinet.

10. Rinse with source water before using.

Detergent only method

Materials:

- Laboratory-grade soap such as Sparkleen or Liqui-Nox; Simple Green® All-Purpose Cleaner
- Cleaning brushes
- Nitrile gloves (thicker reusable gloves recommended for extended contact use).

Procedure:

1. Wash all equipment that has and will come in contact with the exposure solutions with laboratory-grade soap or Simple Green® and hot water.

2. When cleaning water quality meter probes, use a small diameter, soft bristle brush to carefully scrub inside of any orifices or seams. You may have to remove protective caps to thoroughly clean the probe. Be careful not to scratch or damage any exposed sensors.

3. Flush with ample amounts of tap water until all traces of detergent are gone.

4. Rinse 3 times with RO or DI water.

A.4 Water Sampling Matrix/Reference Guide

Table A.1. Sampling matrix for aqueous samples when conducting final/definitive DWH toxicity tests

Analyses requested	Bottles	Filling instructions	Holding time	Storage / handling	When to sample
WAF stock solution sampling: Prior to making dilution series					
PAH/Alk + archive extract	250 mL glass amber (1 per stock)	Fill to top; no headspace	7 days; 14 days if acid preserved[a]	Store at 4°C until sent to ALS Environmental	> For **all** WAF types and **all** oil types
BTEX + archive extract	40 mL glass amber VOA (3 per stock)	Fill until positive meniscus is formed, then cap vial; any bubble in vial must be < 4 mm wide	14 days; 7-days if not acid preserved[a]	Store at 4°C until sent to ALS Environmental	> **Only** WAFs made using **Source** oil type > PAH analyses will also be conducted in addition to BTEX for tests using source oil
DOSS + archive extract	15 mL plastic (4 per treatment)	Fill to ~10 mL	14 days; Indefinite when frozen at -20°C[a]	Store at 4°C until sent to ALS Environmental	> For **all CEWAF** tests > PAH analyses will also be conducted in addition to DOSS for all CEWAF tests
Dilution series – exposure solution sampling: Prior to pouring into exposure chambers; includes control treatments					
Extraction, archive only and PAHs	250 mL glass amber (1 per treatment)	Fill to top; no headspace	7 days; 14 days if acid preserved[a]	Store at 4°C until sent to ALS Environmental	> For **all** WAF types and **all** oil types
DOSS + archive extract	15 mL plastic (4 per treatment)	Fill to ~10 mL	14 days; Indefinite when frozen at -20°C[a]	Store at 4°C until sent to ALS Environmental	> For **dispersant-only** and **variable-dispersant** tests > No PAH samples are needed for **dispersant-only** tests
Fluorescence (PAH)	7 mL borosilicate[b] (1 per treatment)	3.5 mL sample/ 3.5 mL ethanol; no headspace in vial	Freshwater: 48 hours Saline: 1 week	Store at 4°C onsite prior to analysis	> For **all** WAF types and **all** oil types > If not using a 100% WAF treatment, then also need to sample the WAF stock to generate a standard curve

a. ALS Environmental must receive samples with at least 1 full business day (Monday–Friday) to conduct solvent extraction within holding time.
b. ALS Environmental will provide all aqueous sample bottles *except* for 7-mL bottles.

A.5 Sample Shipping Checklist/Reference Guide

Use when sending PAH, BTEX, DOSS, and Extraction/Archive samples to ALS Environmental when conducting final/definitive DWH toxicity tests.

- ❑ Check that sample hold times are not expired (see QAPP).
  Note: ALS Environmental needs at least one business day (Monday–Friday) before hold times expire to process samples. When ALS Environmental receives samples on Saturday, they do not process them until the following Monday.
- ❑ Place clear packaging tape over each sample label to prevent loss or damage to label and check that bottle lids are secure.
  Tape should cover the entire label and circle the bottle at least one time; dry any surface water condensation prior to affixing label and tape.
- ❑ Each sample bottle should be wrapped in bubble wrap, taped again to secure the wrapping, and placed standing up in the bottom of the cooler.
  Do not over-pack coolers – can use multiple coolers; samples should not directly touch each other.
- ❑ Include one temperature blank in each individual cooler.
  Temperature blank should be clearly labeled and inventoried on COC form; temperature blank sample bottles should accompany coolers sent from ALS Environmental; if needed, make a temperature blank using a 250-mL plastic bottle filled with tap water.
- ❑ Fill remaining open cooler space with ice to keep sample temperature 4°C for 24 hours.
  Best practice is to put ice in doubled zip-top baggies placed directly on top of sample bottles until cooler is full; ice is preferred over gel-ice packs.
- ❑ Fill out a COC form (Appendix C). Make a photocopy of the completed form to keep in your records and seal the original form in a clear plastic bag taped to the inside lid of the cooler.
  Note: See QAPP for Analyses Requested.
- ❑ Place custody seals across cooler seam on opposite corners of the lid and wrap the cooler multiple times with clear packaging tape, ensuring that custody seals are taped over but still visible.
- ❑ Ship coolers using FedEx Standard Overnight Express Package Service. Hand coolers over directly to a person. Do not leave them at a pick-up location, where they will be unattended. Retain the shipping receipt. Instructions for filling out the shipping label are below.
  - ▫ 3. Recipient: ALS Environmental, 1317 South 13th Avenue, Kelso, WA 98626
    - — Telephone: 800-695-7222
  - ▫ 4a. Express Package Service: check "FedEx Standard Overnight" – do not fill out Section 4b
  - ▫ 5. Packaging: check "Other"
  - ▫ 6. Special Handling: leave blank, but check "No" for *Does this shipment contain dangerous goods?*
  - ▫ 8. Residential Delivery Signature Options: check "No Signature Required"

A.6 Shipping Samples Using Dry Ice

The following procedures must be followed when shipments contain dry ice. These procedures will be followed in addition to the archive sample requirements or any other applicable sample shipping requirements described in this QAPP.

Acceptable dry ice shipping containers are two piece units made with a polystyrene (Styrofoam) inner container that is placed inside an outer corrugated cardboard box. Styrofoam cannot be used as an outer packaging. The packaging carrying the shipment and the dry ice must be able to withstand the loading and unloading process. The outer package must allow for release of CO_2 gas and should not be completely sealed with packaging tape. The maximum amount of dry ice that can be shipped is 200 kg. It is recommended that shipments contain 5–10 pounds (2.3–4.5 kg) of dry ice per 24 hours of transit time.

The following shipping container labeling and airbill guidelines are required when using dry ice as a refrigerant:

1. Obtain a "Dry Ice – Class 9" placard and write the shipper's and consignee's name and address and total weight of dry ice placed in the shipping container on the face of the placard using a permanent marker (fine-tip Sharpie).

   Note that the "Dry Ice – Class 9" placard was modified in October 2014 (Figure A.7). Use the updated placard for all dry ice shipments. If you need "Dry Ice – UN1845" placards, contact Stratus Consulting.

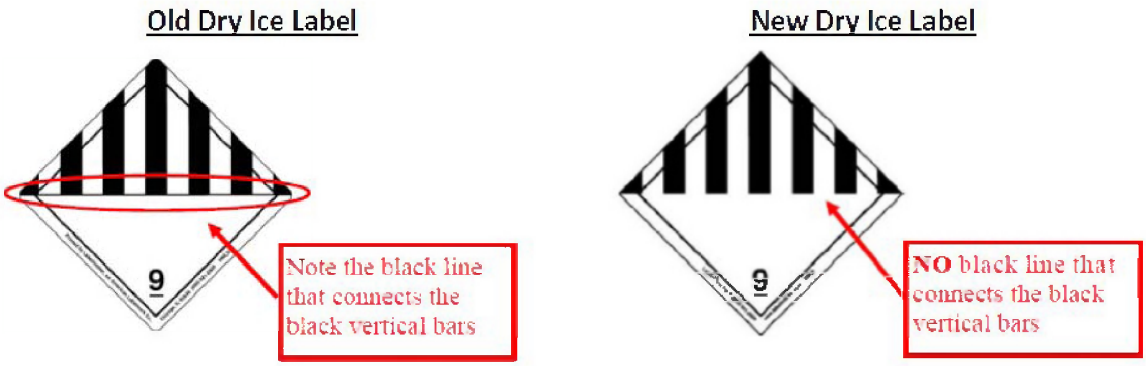

Figure A.7 "Dry Ice – Class 9" placards. Be sure to use the newer placard without the horizontal black line.

2. Affix both a "Dry Ice – Class 9" placard in the center and a "Dry Ice – UN1845" statement sticker in the upper left-hand corner of the largest side of the outer shipping container. The "Dry Ice – UN1845" statement sticker size must be 12 mm or larger. Alternatively, "Dry Ice – UN1845" can be handwritten (printed) in the upper left-hand corner if it is clearly legible and text size is 12 mm or larger.

   Note that "Dry Ice – Class 9" placards often have "Dry Ice – UN1845" printed on them. This is not sufficient to replace the 12 mm or larger "Dry Ice – UN1845" statement sticker labeling requirement described in this step.

3. Write name, address, and phone number of both the shipper and recipient on the outer shipping container on the same side as the "Dry Ice – Class 9" placard.

4. Fill out a FedEx airbill. Retain the sender's copy for your records and record the FedEx Tracking Number (located at the top of the airbill) in the laboratory notebook with respective sample information.

5. Deliver package(s) to a FedEx location or have FedEx pick up the package(s). Do not leave the cooler(s) at an unattended FedEx drop-off location. Turn coolers over directly to a person and retain shipping receipt.

Instructions for filling out the shipping label for containers containing dry ice are provided below:

- Recipient: provide recipient's name, phone number, company, and address; do not check "HOLD" Weekday or Saturday location information
- Express Package Service: check Next Business Day "FedEx Priority Overnight"
- Packaging: check "Other"
- Special Handling and Delivery Signature Options: check "No Signature Required"
- Check "Yes" for *Does this shipment contain dangerous goods?* Check "Dry Ice" and record net weight of dry ice in the shipping container

A.7 Shipping Biological Samples

This SOP describes how to properly ship biological samples while maintaining established COC requirements. Biological samples will be shipped using FedEx, and may be shipped cold or frozen using liquid or dry ice. If shipping biological materials with dry ice, both dry ice and biological shipping requirements should be followed. Biological samples that could be generated

and shipped for this project will fall into the following categories: Category B biological substances and exempt diagnostic samples:

- ***Category B biological samples*** include materials known or reasonably expected to contain a pathogen or infectious substance (e.g., bacterial, viruses, parasites, fungi, prion) that is not generally capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals when exposure occurs. This includes infectious substances transported for research, diagnosis, or investigational activities. Category B biological samples will be shipped according to UN3373 shipping requirements. Note that Category B shipments do not require completing a "Shipper's Declaration for Dangerous Goods" form.

- ***Exempt diagnostic samples*** include materials that are unlikely to cause disease in humans or animals or for which there is only a minimal likelihood that pathogens are present. Examples may include excreta, secreta, blood and its components, tissue and tissue swabs, and body parts. Exempt diagnostic samples will be shipped using similar shipping guidelines as Category B biological samples, with the exception of outer packaging labeling and airbill requirements.

Note that project staff will not ship Biological Substance Category A materials. Category A materials are infectious substances that are capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. If you have any questions regarding biological substance category classification, contact Stratus Consulting for assistance.

Both Category B and exempt diagnostic biological samples will be shipped using the triple packaging system:

1. Confirm that the primary sample container(s) is watertight and has a positive closure cap, and that it contains no more than 1 L of liquid in aggregate; if a solid, it must not exceed the outer packaging weight. Acceptable primary sample containers include metal, plastic, or glass canisters; jars; or vials with screw-on, snap-on, or push-on lids.

   Note that selection of sample container will also consider requirements outlined in *Archive tissue samples* in Section 4.4.7 and/or work plans for test-specific analyses.

2. Wrap the primary sample container(s) lid with packaging tape for extra closure reinforcement.

3. Wrap the primary sample container(s) with cushioning material so that the samples do not contact each other or the secondary receptacle.

4. Place the prepared primary sample container(s) into a watertight secondary receptacle. Acceptable secondary receptacles include sealed plastic bags, plastic canisters, or screw-cap cans.

5. Stuff absorbent material between the primary and secondary receptacles; use enough absorbent material to absorb the entire contents of the sample(s); paper towels are acceptable absorbent material.

6. Place the sealed secondary container into a sturdy outer package. The outer packaging must be rigid and constructed of corrugated fiberboard, wood, metal, or rigid plastic (use of Styrofoam outer packaging is not permitted); must be capable of withstanding a 4-ft drop; and the package size must be at least 4”×4” on each side to accommodate placards and stickers. Note that more than one secondary container may be placed into the outer package as long as the total volume of liquid samples is less than 4 L or 4 kg of solids.

7. Stuff the outer package with absorbent or other cushioning material to prevent the secondary container from being jostled during shipping.

8. If using dry ice as a refrigerant, place it between the secondary container and outer packaging. For best results, place dry ice above and below the secondary container and place additional absorbent below the dry ice. Use cushioning material to prevent the secondary container from being jostled during shipping when the dry ice sublimates.

   Packages shipped with dry ice must comply with the dry ice shipping protocol (Section A.6).

9. Place a completed COC form sealed in a plastic bag between the secondary receptacle and the outer packaging. The COC form can be used as an itemized list of package contents (a requirement for all Category B biological shipments).

10. Once packaged, tape up the outer packaging container with packaging tape and label the largest side of the package using the following guidelines

    a. Labeling packages containing Category B biological samples

       i. Name and address of the shipper and the consignee.

       ii. Name and telephone number of the responsible person.

       iii. UN3373 placard, with each side being at least 50 mm in length; see example UN3373 placard below (Figure A.8).

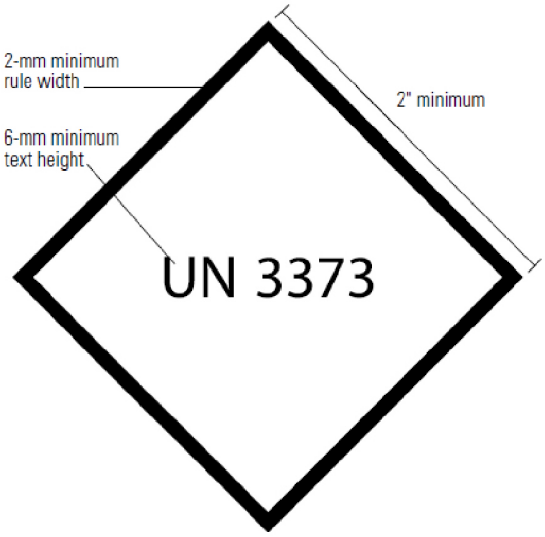

Figure A.8. Example Biological Substance, Category B "UN3373" placard.

iv. If not included on the UN3373 placard, the statement "BIOLOGICAL SUBSTANCE CATEGORY B," in capital letters at least 12 mm high, must be placed on the outer packaging adjacent to the UN3373 placard.

v. If using dry-ice as a refrigerant, affix the "Dry Ice – UN1845" placard and "Dry Ice – UN1845" statement sticker (or handwrite statement) to the outer package. Refer to A.6 SOP above for detailed package labeling procedures.

b. Labeling packages containing exempt diagnostic samples

i. Name and address of the shipper and the consignee

ii. The statement "EXEMPT ANIMAL SPECIMENS," in capital letters at least 6 mm high, must be placed on the outer packaging.

11. Use the following information when filling out biological shipment airbills.

a. In the Internal Billing Reference entry, write:

For Category B shipments – "UN 3373 Biological Substances Category B"

For exempt diagnostic shipments – "Exempt Human/Animal Specimen"

b. Recipient: provide recipient's name, phone number, company, and address; do not check "HOLD" Weekday or Saturday location information

c. Express Package Service: check Next Business Day "FedEx Priority Overnight"

d. Packaging: check "Other"

e. Special Handling and Delivery Signature Options: check "No Signature Required" and the following:

For Category B shipments – Check "Yes" for *Does this shipment contain dangerous goods?*

For exempt diagnostic shipments without dry ice – Check "No" for *Does this shipment contain dangerous goods?*

For any shipments containing dry ice – Check "Yes" for *Does this shipment contain dangerous goods?* Check "Dry Ice" and record net weight.

A.8 Shipping Histology Samples

This SOP will be used when shipping or transporting histology samples that have been fixed in solutions containing formalin. Specimens in formalin can be shipped via air cargo or land freight as "Dangerous Goods in Excepted Quantities" as long as the amount of pure formaldehyde is 30 mL or less in each individual container and less than 500 mL in the shipping container. Unless specified otherwise, a 10% buffered formalin solution will be used for fixing project-specific histology samples. Therefore, individual sample containers will not contain more than 300 mL and the shipping container will not contain more than 5 L of 10% buffered formalin. Histology samples will not be shipped in the same package as other sample types.

1. Once samples have been placed in fixative for at least 24 hrs, they can be prepared for shipment.

2. Obtain two, 1-quart Ziploc freezer bags for each sample, a few rolls of non-dyed paper towels, plain paper index cards, and a pencil.

3. Put on a new pair of nitrile gloves.

4. Check that the sample bottle contains no more than 300 mL of 10% buffered formalin and the cap is tight and does not leak. If not already done, wrap the sample container with clear packaging tape and/or Para-film™ so that the label and cap are completely covered.

5. Place each sample bottle into a Ziploc bag with enough paper towels or similar absorbent material so that they would absorb the entire contents of the sample bottle.

6. Partially seal the bag and then carefully expel as much air as possible without crushing or damaging the sample. After removing the air, completely seal the bag.

7. Write the sample ID on the outside of the sealed Ziploc bag.

8. Repeat steps 5 through 7 until all of the samples are processed.

9. Determine the total volume of 10% buffered formalin of all samples being shipped. Obtain enough shipping containers to ship all samples without going over the maximum 5 L of 10% buffered formalin per shipping container.

10. Pack prepared samples into a cooler or similar rigid shipping container that is lined with the larger plastic bag. The best way to do this is to open up and place the large plastic bag into the shipping container before filling it with samples. Fill the remaining space between the samples and the large plastic bag with more paper towels so that they are not jostled during shipping.

11. Record the ID for each sample that is placed in the cooler on the COC form(s).

12. Sign and date the COC form(s).

13. Retain one photocopy of the COC form(s) for your records in a secure location; document shipment details and COC information in your project notebook.

14. Seal the remaining copies of the COC form(s) in a plastic resealable bag and tape the bag to the inside of the shipping container.

15. Sign and date at least two COC seals (small stickers with a line for a signature) for each shipping container.

16. Place signed COC seals on opposite corners of the shipping container across the seam between the lid and the main body of the shipping container (see Figure A.1). COC seals must be arranged so that the shipping container cannot be opened without disturbing the seals.

17. Place clear packing tape over the COC seals.

18. Seal the shipping container by taping around the seam between the lid and body of the shipping container and around the entire container (see Figure A.2).

19. Label the outer shipping container with the following:

    A properly filled out "Dangerous Goods in Excepted Quantities" label with shipper signature, title, name, address, and date affixed to a vertical side of the outer container. Check "Class 9 material" and enter "UN 3334" under the "Applicable UN Number" field. These labels can be obtained from FedEx. Alternatively labels can be printed in color with overall dimensions of at least 4 in. by 4 in.

    Clearly write the shipper (From:) and consignee (To:) names, addresses, and phone numbers next to the "Dangerous Goods in Excepted Quantities" in letters at least 6-mm tall. The shipper contact information should match that on the "Dangerous Goods in Excepted Quantities" label.

20. Fill out a FedEx US Airbill using the information provided below. Retain the sender's copy for your records and record the FedEx Tracking Number (located at the top of the airbill) in the laboratory notebook with respective sample information.

21. Deliver cooler(s) to a FedEx location or have FedEx pick up the cooler(s). **Do not** leave the cooler(s) at an unattended FedEx drop-off location. Turn coolers over directly to a person and retain shipping receipt.

Histology sample shipment airbill information

Histology samples cannot be shipped using the online airbill option. Therefore, shippers will obtain and fill out a hard copy airbill as described below.

Note that only one airbill may be required when sending multiple packages to the same recipient. In this case, the FedEx agent will affix a matched barcode sticker to all other packages going to the same recipient.

1. Internal Billing Reference: DWH Task Order #X (where X is the Task Order #).

2. Recipient: provide recipient's name, phone number, company, and address; do not check "HOLD" Weekday or Saturday location information.

3. Express Package Service: check Next Business Day "FedEx Standard Overnight."

4. Packaging: check "Other."

5. Special Handling and Delivery Signature Options: check "No Signature Required" and check "Yes" for "Does this shipment contain dangerous goods?"

6. Clearly write "Dangerous Goods in Excepted Quantities" on the top of air bill above the Tracking Number.

7. Payment: check "Sender"; write $100.00 in the "Total Declared Value" entry.

B. Sample ID Look Up Tables and Toxicity Testing Results Reporting Data Entry Bench Sheets

B.1 Sample ID Look Up Tables

Sample ID laboratory codes

Laboratory name	Laboratory code
FGCU/University of North Carolina Wilmington	FG
Hopkins Marine Station (Stanford University)	HS
Miami University (Ohio)	MU
Mote Marine Laboratory	MM
Northwest Fisheries Science Center (NOAA)	NF
Queen's University	QU
University of Maryland	UM
University of Miami, the Rosenstiel School of Marine and Atmospheric Science	RS
University of North Texas	NT
University of Southern Mississippi, Gulf Coast Research Laboratory	GR
Auburn University Department of Fisheries	AB
U.S. Army Corps of Engineers/ERDC	CE
Louisiana State University	LS
Pacific EcoRisk	PE
University of South Florida	SF
Marin Biologic Laboratories	MB
Stratus Consulting	ST
Louisiana Universities Marine Consortium	LU
Florida Atlantic University	FA

Sample ID sample type codes

Matrix	Analysis	Type code
Water – filtered	PAH	FP
Water – unfiltered	PAH	UP
Water – filtered	VOC	FV
Water – unfiltered	VOC	UV
Water – filtered	DOSS	FD
Water – unfiltered	DOSS	UD
Water – filtered	Archive	FA
Water – unfiltered	Archive	UA
Water – filtered	Fluorescence	FF
Water – unfiltered	Fluorescence	UF
Sediment	All analyses	SE
Sediment	Archive	SA
Tissue	All analyses	TS
Tissue	Archive	TA
Extracts	Archive	AX
Bacterial culture	Any	BC
Molecular biology	Any	MO

B.2 Field Entry Dictionary

Data Dictionary

Worksheet name (data table title)	Column Header (data field name)	Column Contents (data field definition)
Analytical Sample Inventory Bench Sheet	Notes	Used to record additional information about each sample; Note for archive tissue samples, record the number of test organisms in sample.
Analytical Sample Inventory Bench Sheet	Number organisms per sample	Documents how many organisms are in a single archive tissue sample
Analytical Sample Inventory Bench Sheet	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Analytical Sample Inventory Bench Sheet	Sample ID	Sample ID is the concatenation of the following elements: Lab code-Date code-Sample type code-Test ID-Unique sample ID (XX-Y####-ZZ -xxx-xxx)
Analytical Sample Inventory Bench Sheet	Sampling date	Date when the given sample was collected; Use Day-Month-Year format
Analytical Sample Inventory Bench Sheet	Tank ID Dilution or Stock Code	Water samples may be taken from each tank, stocks, and/or dilution series. If samples were made using tank water then the tank ID will be recorded. If samples were made from stock and dilution water then the respective codes will be entered
Analytical Sample Inventory Bench Sheet	Sampling time	Time of day when the given sample was collected; Record in 24-hr format
Analytical Sample Inventory Bench Sheet	Storage location	Record where the given sample will be stored; If not being stored on-site, state where it has been sent
Analytical Sample Inventory Bench Sheet	Description of Sample	Short description of the sample; examples include dilution series, WAF-stock, etc.
Analytical Sample Inventory Bench Sheet	Unique ID Number	Sequential three-digit sample number for each test; First number in the sequence shall be 101. Sequential regardless of the sample type or matrix; Used to provide a unique ID number for each
Tank ID Dilution or Stock Code Definitions	Tank ID Dilution or Stock Code	Tank ID will be determined by each lab when conducting tests; Make sure that it is unique for each test; generate a stock and dilution series codes and record in this cell
Tank ID Dilution or Stock Code Definitions	Start date	Date in which test was initiated - MM/DD/YEAR format
Tank ID Dilution or Stock Code Definitions	Start time	Time of day when the given test was initiated - test organisms added to exposure solution; Record in 24-hr format
Tank ID Dilution or Stock Code Definitions	End date	Date in which test was stopped - MM/DD/YEAR format
Tank ID Dilution or Stock Code Definitions	End time	Time of day when the given test was ended; Record in 24-hr format
Tank ID Dilution or Stock Code Definitions	Nominal Treatment Concentration	Record the treatment or dilution series for the given tank
Tank ID Dilution or Stock Code Definitions	Treatment units	Record the treatment or dilution series concentration units
Tank ID Dilution or Stock Code Definitions	Replicate number	Sequential replicate number, starting from zero for controls
Tank ID Dilution or Stock Code Definitions	Notes	Used to record additional information on how test was set-up
Tank ID Dilution or Stock Code Definitions	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Test Conditions Table	Aeration (y/n)	Was aeration used to improve dissolved oxygen concentrations during the test
Test Conditions Table	Fed during test (y/n)	Documents if test organisms be fed during exposures.
Test Conditions Table	Feeding regime	If fed during the test, record the feeding schedule; Example entries could include: Daily, twice per day, or prior to renewal
Test Conditions Table	Food source	If test organisms are fed, what dietary items were they given; Example entries could include: artemia nauplii, Iso.algae, or pellets
Test Conditions Table	Life-stage/ age	Documents what life stage and age test organisms were at the beginning of the test
Test Conditions Table	Test duration	Total duration of test in hours
Test Conditions Table	Test type	Static renewal, static, or flow-through
Test Conditions Table	Oil type	Type of oil being tested - slick A, slick B, weathered source, or source
Test Conditions Table	Oil loading rate	Nominal WAF stock oil to water ratio or concentration
Test Conditions Table	Notes	Used to record additional information on how test was set-up
Test Conditions Table	Organisms per tank	Documents how many test organisms will be placed into each replicate tank
Test Conditions Table	Photoperiod (hrs light/drk)	Records how many hours of light and dark a tank will receive in a 24-hr day; Use a #/# format
Test Conditions Table	Endpoints	General description of what endpoints will be assessed
Test Conditions Table	Temperature monitoring schedule	How and when will temperature be monitored
Test Conditions Table	pH monitoring schedule	How and when will pH be monitored
Test Conditions Table	Diss. oxygen monitoring schedule	How and when will oxygen be monitored
Test Conditions Table	Conductivity monitoring schedule	How and when will conductivity be monitored
Test Conditions Table	Salinity monitoring schedule	How and when will salinity be monitored
Test Conditions Table	Alkalinity monitoring schedule	How and when will alkalinity be monitored
Test Conditions Table	Hardness monitoring schedule	How and when will hardness be monitored
Test Conditions Table	Total ammonia monitoring schedule	How and when will ammonia be monitored
Test Conditions Table	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Test Conditions Table	Test chamber cleaning	If exposure chambers are cleaned during the test, describe when and how
Test Conditions Table	Renewal frequency	Documents the schedule for renewing test solutions; Record as total number of hours between renewals
Test Conditions Table	Temperature	Optimal testing temperature
Test Conditions Table	Salinity	Optimal testing salinity
Test Conditions Table	Light source	What kind of lights are used during test; important for UV exposures
Test Conditions Table	Light intensity	General description for most tests; more details can be provided for UV testing
Test Conditions Table	Replicate number	A sequential count of the number of replicate within each treatment (must be >= 1)
Test Conditions Table	Species tested	Record the common or scientific Name (or initials) of the test organism that is being used for the given test
Test Conditions Table	Dilution/control water	Type of water used for making test solutions; identify commercial salt mixes when used
Test Conditions Table	Test chamber volume (ml)	Document the size of the exposure chamber or tank in milliliters; This is different then the volume of exposure water that is recorded in the "Test solution volume (ml)" field
Test Conditions Table	Test ID	Three digit test identification code; See Stratus Consulting Test ID assignments workbook/table
Test Conditions Table	Test solution volume (ml)	Record the approximate volume of exposure media in each replicate tank; Does not refer to the test chamber size, which is recorded in the "Test chamber volume (ml)" field
Test Conditions Table	Treatment (nominal % WAF)	Record the treatment or dilution series for the given test
Test Conditions Table	WAF prep. method	Record the method that is used for preparing the WAF stock (HEWAF or CEWAF)
Test Performance Monitoring Bench Sheet	Date	Record the date when test performance observations were made; Use Day-Month-Year format
Test Performance Monitoring Bench Sheet	Notes	Used to record additional information on test performance observations
Test Performance Monitoring Bench Sheet	Number at test start	Records the number of test organisms that were added to the tank at the beginning of the exposure/test
Test Performance Monitoring Bench Sheet	Number observed treatment mortalities	Keeps track of how many test organisms died from the listed tank for the observation date and time (not cumulative)

Test Performance Monitoring Bench Sheet	Number observed alive	Used to keep track of the number of organisms in each tank at each time point.
Test Performance Monitoring Bench Sheet	Number from nontreatment mortality	Records any mortalities that accidently occurred from the given tank for the given observation date. Examples include spilling a tank and it's contents or fish that jump out
Test Performance Monitoring Bench Sheet	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Test Performance Monitoring Bench Sheet	Tank ID	Tank ID will be determined by each lab when conducting tests; Make sure that it is unique for each test
Test Performance Monitoring Bench Sheet	Time	Record time when test performance/organisms health inspections were made for each tank; Use 24-hr format
Water Accommodated Fraction Preparation and Sampling Table	Calculated nominal concentration	Target nominal dilution series concentrations (dispersant tests) or WAF compositions (% WAF)
Water Accommodated Fraction Preparation and Sampling Table	CEWAF settle end time	Time when CEWAF was used after being stirred and left to settle; See the WAF Preparation SOP for required settle times
Water Accommodated Fraction Preparation and Sampling Table	CEWAF settle start time	Time when CEWAF was left to settle after being stirred; See the WAF Preparation SOP for required settle times
Water Accommodated Fraction Preparation and Sampling Table	CEWAF stir end date	Date when CEWAF solution mixing ended; Might be the same time as the CEWAF settle start time; See the WAF Preparation SOP for required mixing times
Water Accommodated Fraction Preparation and Sampling Table	CEWAF stir end time	Time when CEWAF solution mixing ended; Might be the same time as the CEWAF settle start time; See the WAF Preparation SOP for required mixing times
Water Accommodated Fraction Preparation and Sampling Table	CEWAF stir start time	Time when CEWAF solution began to be mixed/stirred; See the WAF Preparation SOP for required mixing times
Water Accommodated Fraction Preparation and Sampling Table	Control (y/n)	Note that control treatment test solutions should be handled similarly as WAF treatment solutions, as shown in this example.
Water Accommodated Fraction Preparation and Sampling Table	Est. total volume needed for test/renewal and samples (L)	Record the estimated total volume of WAF stock needed to fill analytical sample bottles and make treatment dilution series; Includes making each treatment-dilution series, definitive test samples
Water Accommodated Fraction Preparation and Sampling Table	Filtered before use (y/n)	Records whether or not the WAF was filtered prior to being used to make treatment dilution series
Water Accommodated Fraction Preparation and Sampling Table	Filtered DOSS sample ID	If a filtered stock sample was taken for DOSS analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	Filtered PAH sample ID	If a filtered stock sample was taken for PAH analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	Filtered VOC sample ID	If a filtered stock sample was taken for VOC analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	Mass of dispersant added (mg)	Records the weight of dispersant added to seawater when making CEWAF and Corexit only stock solutions
Water Accommodated Fraction Preparation and Sampling Table	Mass of oil added (mg)	Records the weight of oil added to seawater when making the WAF stock solution
Water Accommodated Fraction Preparation and Sampling Table	Notes	Used to record additional information on how the WAF was prepared; If WAFs were composited, this information would be recorded in this field
Water Accommodated Fraction Preparation and Sampling Table	Oil type	Documents the type of oil that was used to prepare the given WAF
Water Accommodated Fraction Preparation and Sampling Table	Prep end date	Documents the day when a WAF stock preparation ended and is ready for use
Water Accommodated Fraction Preparation and Sampling Table	Prep end time	Documents the time when a WAF stock preparation ended and is ready for use; Record in 24-hr format
Water Accommodated Fraction Preparation and Sampling Table	Prep start date	Documents the day when a WAF stock preparation started
Water Accommodated Fraction Preparation and Sampling Table	Prep start time	Documents the time when a WAF stock preparation started; Record in 24-hr format
Water Accommodated Fraction Preparation and Sampling Table	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Water Accommodated Fraction Preparation and Sampling Table	Time when drain sep. funnel	Record time when the WAF solution being prepared was transferred from the separatory funnel to being used to make samples and treatment dilutions; Note that at least 1-hr settling time is
Water Accommodated Fraction Preparation and Sampling Table	Time when HEWAF X-fer to sep. funnel	Record time when the WAF solution being prepared was transferred from the aspirator bottle to the separatory funnel; Use 24-hr format
Water Accommodated Fraction Preparation and Sampling Table	Unfiltered DOSS sample ID	If a filtered stock sample was taken for DOSS analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	Unfiltered PAH sample ID	If a filtered stock sample was taken for PAH analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	Unfiltered VOC sample ID	If a filtered stock sample was taken for VOC analysis, then record it's sample ID
Water Accommodated Fraction Preparation and Sampling Table	WAF prep. method	Record the method that is used for preparing the WAF stock (HEWAF or CEWAF)
Water Accommodated Fraction Preparation and Sampling Table	Water/diluent volume (L)	Record the volume of seawater added to the blender or aspirator bottle when making the given WAF stock
Water Quality Monitoring	Cond (µS/cm)	Record specific conductance measurement
Water Quality Monitoring	D.O. (mg/L)	Record dissolved oxygen measurement
Water Quality Monitoring	Date	Record the date when WQ measurements were made; Use Day-Month-Year format
Water Quality Monitoring	Period	Indicates sampling period type category as "initial", "daily", "per renewal" or "final".
Water Quality Monitoring	Notes	Used to record additional information on how WQ was measured
Water Quality Monitoring	pH (S.U.)	Record pH measurement
Water Quality Monitoring	Recorded by	Name (or initials) of the individual that is recording test information in the respective form
Water Quality Monitoring	Salinity (ppt)	Record the salinity measurement in parts per thousand
Water Quality Monitoring	Tank ID Dilution or Stock Code	Water quality parameters may be measured in each tank and/or dilution water after filling all of the tanks. If measurements were made using tank water then the tank ID will be recorded. If
Water Quality Monitoring	Test ID	Three digit test identification code; See Stratus Consulting Test ID assignments workbook/table
Water Quality Monitoring	Time	Record time when water quality measurements were made; Use 24-hr format
Water Quality Monitoring	Total ammonia (mg/L)	Record total ammonia measurement
Water Quality Monitoring	Water temp. (C)	Record temperature measurement

B.3 Test Conditions Bench Sheet

Test Conditions Table

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Data entered by _____ Data QC _____ page_____ of _____

Test Condition Parameter	Value
Test ID number	
Species tested	
Life-stage/ age	
Test duration	
Test type	
Renewal frequency	
Oil type	
WAF preparation method	
Oil Loading rate	
Units of measure for nominal treatment levels	
Nominal Treatment 1 (control)	
Nominal Treatment 2	
Nominal Treatment 3	
Nominal Treatment 4	
Nominal Treatment 5	
Nominal Treatment 6	
Nominal Treatment 7	
Nominal Treatment 8	
Nominal Treatment 9	
Nominal Treatment 10	
Test chamber volume (ml)	
Test solution volume (ml)	
Number of replicates per treatment	
Organisms per tank	
Temperature (°C)	
Salinity	
Light source	
Light intensity	
Photoperiod (hours light/drk)	
Fed during test (y/n)	
Feeding regime	
Food source	
Test chamber cleaning	
Aeration (y/n)	
Endpoints	
Temperature monitoring schedule	
pH monitoring schedule	
Dissolved oxygen monitoring schedule	
Conductivity monitoring schedule	
Salinity monitoring schedule	
Alkalinity monitoring schedule	
Hardness monitoring schedule	
Total ammonia monitoring schedule	
Notes	
Recorded by	
Test Participants	

B.4 WAF Preparation Bench Sheet

Water Accommodated Fraction Preparation and Sampling Table

CONFIDENTIAL ATTORNEY/CONSULTANT WORK PRODUCT

TEST ID _____ Data entered by _____ Data QC_____ page_1____ of ___1___

	WAF Prep 1	WAF Prep 2	WAF Prep 3	WAF Prep 4
Prep start date				
Prep start time				
Prep end date				
Prep end time				
Control (y/n)				
WAF prep. method				
Oil type				
Est. total volume needed for test/renewal and samples (L)				
Water/diluent volume (L)				
Mass of dispersant added (mg)				
Mass of oil added (mg)				
Calculated nominal concentration				
Units of nominal concentration				
CEWAF stir start time				
CEWAF stir end date				
CEWAF stir end time				
CEWAF settle start time				
CEWAF settle end time				
Time when HEWAF X-fer to sep. funnel				
Time when drain sep. funnel				
Filtered before use (y/n)				
Unfiltered PAH sample ID				
Filtered PAH sample ID				
Unfiltered VOC sample ID				
Filtered VOC sample ID				
Unfiltered DOSS sample ID				
Filtered DOSS sample ID				
Notes				
Recorded by				

B.5 Fluorescence Analysis Standard Curve Bench Sheet

Development of Fluorescence Analysis Standard Curve

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TEST ID _____ Data entered Data QC Page of

Item	Value	Dilution (% v/v)	Stock added (uL)	Ethanol Added (mL)	Water Added (mL)	Salt Pellet Visable (y/n)	Total Peak Area	Notes
Test ID		-------	-------	-------	-------	-------	-------	
Date		-------	-------	-------	-------	-------	-------	
Data Entered By		-------	-------	-------	-------	-------	-------	
Oil Type		-------	-------	-------	-------	-------	-------	
Dispersant Type		-------	-------	-------	-------	-------	-------	
WAF Type		-------	-------	-------	-------	-------	-------	
Salinity (ppt)		-------	-------	-------	-------	-------	-------	
Oil:Water Ratio		-------	-------	-------	-------	-------	-------	
Dispersant:Oil Ratio		-------	-------	-------	-------	-------	-------	
Centrifugation Time (min)		-------	-------	-------	-------	-------	-------	
Centrifugation Speed (x g)		-------	-------	-------	-------	-------	-------	
Volume of Sample (mL)		-------	-------	-------	-------	-------	-------	
Volume of Ethanol (mL)		-------	-------	-------	-------	-------	-------	
Wavelength Optimization Standard Used (%v/v)		-------	-------	-------	-------	-------	-------	
Wavelength OptimizationExcitation (nm)		-------	-------	-------	-------	-------	-------	
Wavelength Optimization Emission (nm)		-------	-------	-------	-------	-------	-------	
Ethanol:Water Blank	-------							
Dilution 1	-------							
Dilution 2	-------							
Dilution 3	-------							
Dilution 4	-------							
Dilution 5	-------							
Dilution 6	-------							
Dilution 7	-------							
Dilution 8	-------							
Dilution 9	-------							
Dilution 10	-------							

B.6 Fluorescence Analysis Test Solution Bench Sheet

Fluorescence Analysis of Test Solutions

CONFIDENTIAL ATTORNEY/CONSULTANT WORK PRODUCT

TEST ID _____ Data entered _____ Data QC _____ Page _____ of _____

Test ID	Sample ID	Sample Date	Expected Dilution (%v/v)	Salt Pellet Visable (y/n)	Total Peak Area	Comments

B.7 Water Quality Monitoring Bench Sheet

Water Quality Monitoring
CONFIDENTIAL ATTORNEY/CONSULTANT WORK PRODUCT

TEST ID _____ Data entered by _____ Data QC_____ page_____ of _____

Tank ID Dilution or Stock Code	Date	Time	Period	Water temp. (C)	pH (S.U.)	D.O. (mg/L)	Cond (µS/cm)	Salinity (ppt)	Total ammonia (mg/L)	Notes	Recorded by

B.8 Test Performance Monitoring Bench Sheet

Test Performance Monitoring Bench Sheet - each tank to be inspected daily

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TEST ID _____ Data entered by _____ Data QC_____ **page**_____ **of** _____

Tank ID	Date	Time	Number at test start	Number observed treatment mortalities	Number from non-treatment mortality	Number observed alive	Notes	Recorded by

B.9 Tank Dilution or Stock Codes Bench Sheet

Tank ID Dilution or Stock Code Definitions - *For keeping track of Tank IDs and experimental conditions*.
CONFIDENTIAL ATTORNEY/CONSULTANT WORK PRODUCT

TEST ID _____ Data entered _____ Data QC _____ page___1_ of ___1__

Tank ID Dilution or Stock Code	Start date	Start time	End date	End time	Nominal Treatment Concentration	Treatment Units	Replicate number	Notes	Recorded by

B.10 Sample Inventory Bench Sheet

Analytical Sample Inventory Bench Sheet - Document all samples taken during test

CONFIDENTIAL ATTORNEY/CONSULTANT WORK PRODUCT

TEST ID _____ Data entered by _____ Data QC_____ page_____ of _____

Sampling date	Sampling time	Tank ID Dilution or Stock Code	Number organisms per sample	Unique ID Number	Sample ID	Description of Sample	Storage location	Notes	Recorded by

C. Chain-of-Custody Form

NRDA Chain of Custody Form: DWH Stratus Consulting

COC Form _____ of _____

Check a box: Samples to ALS ☐ Samples to other lab _____ ☐ Samples analyzed on-site ☐ Samples stored on-site ☐ Data

Sampler/Shipper Information	Stratus Contact Information
Contact/Phone/Email:	Contact/Phone/Email:
Affiliation:	

Incident Name: Mississippi Canyon MC252 Deepwater Horizon

☐ These data require validation

Special Instructions:

ALS Contact Information	
Contact	Greg Salata
Address	1317 S. 13th Ave Kelso WA 98626
Phone	360-501-3376
ALS SR#:	

Sample ID	Sample collection Date	Sample collection Time	Matrix	PAH/Alk (8270C SIM/PAH)	Total Extractable Hydrocarbons							# of containers	Comments
	mm/ddyyyy	(24-hr local)		Enter x's in boxes below.									

Relinquished by					Received by				
Date	Time	Signature	Printed Name/Org.		Date	Time		Signature	Printed Name/Org.

D. Mississippi Canyon 252 (*Deepwater Horizon*) Natural Resource Damage Assessment Analytical Quality Assurance Plan

SC11571

ANALYTICAL QUALITY ASSURANCE PLAN

MISSISSIPPI CANYON 252 (DEEPWATER HORIZON) NATURAL RESOURCE DAMAGE ASSESSMENT

Version 4.0 (draft)

Prepared for:

U.S. Department of Commerce
National Oceanic and Atmospheric Administration

March 7, 2014

TABLE OF CONTENTS

VERSION 4.0 CHANGES FROM VERSION 3.1:

Version 3.1 Page No.	Change
Cover	Updated version # & date
ii	Added PCB definition
1	Added PCB congeners to list of potential analytes of concern
5	Added a brief description of the analytical methods for PCB congeners as the last bulleted item and added a column for PCBs in the table at the end of the section
None (Table 1.1i)	Added Table 1.1i PCB Congeners Target Analyte List
17	Added PCB Congeners to Table 3-1 (Sample Holding Times)
17	Corrected footnote references in 'Holding Time to Analysis' column (from 12 to 14)
22	Added Table 1.1i to list of tables in text
23	Added Table 1.1i to list of tables in text
23	Added Table 6.1i to list of tables in text
24	Corrected MQO verbiage for MS/MSD (from ">5x" to "<5x"). Changed "SRM 1974b" to "SRM 1974c"
25, 26, & 30	Corrected MQO verbiage for MS/MSD (from ">5x" to "<5x").
None (Table 6.1i)	Added Table 6.1i Measurement Quality Objectives for PCB Congeners
33	Added Table 6.1i to list of tables in text (3 places)
33	Added 'and PCB congeners' to Section 6.3.5 (2 places)
34	Added Table 6.1i to list of tables in text (4 places)
34	Added discussion of PCB congeners to Section 6.3.8
38	Added Reference for naming PCB Congeners (Ballschmiter & Zell)

Acronyms and Abbreviations

%D	Percent difference
%R	Percent recovery
ASTM	American Society for Testing and Materials
BS/BSD	Blank spike/blank spike duplicate
CCV	Continuing calibration verification
CRM	Certified reference material
DISP	Dispersant
DOSS	Dioctylsulfosuccinate salt
DOT	U.S. Department of Transportation
DQO	Data quality objectives
EDD	Electronic data deliverable
EIP	Extracted ion Profile
EPA	U.S. Environmental Protection Agency
GC/MS-SIM	Gas chromatography with low resolution mass spectrometry using selected ion monitoring
GC/FID	Gas chromatography with flame ionization detection
LC	Liquid chromatography
MC 252	Mississippi Canyon 252 (Deepwater Horizon)
MDL	Method detection limit
MQO	Measurement quality objectives
MS/MSD	Matrix spike/matrix spike duplicate
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NRDA	Natural resource damage assessment
OPA	Oil Pollution Act
OSHA	Occupational Safety and Health Administration
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
PIANO	Paraffins, isoparafins, aromatics, naphthenes, olefins
QA	Quality assurance
QAP	Quality assurance plan
QC	Quality control
QL	Quantitation Limit
RM	Reference material
RPD	Relative percent difference
RSD	Relative standard deviation
SHC	Saturated hydrocarbons
SOP	Standard Operating Procedures
SRM	Standard Reference Material
TEH	Total extractable hydrocarbons
TEM	Total extractable matter
TEO	Total extractable organics
TOC	Total organic carbon
USEPA	U.S. Environmental Protection Agency
VOC	Volatile organic compounds

INTRODUCTION

On April 20, 2010, a fatal explosion struck the Deepwater Horizon offshore oil platform approximately 50 miles off the Louisiana coast in the Gulf of Mexico, ultimately leading to the destruction of the platform and the connecting riser pipe to the seafloor a mile below the water surface, and the ongoing release of thousands of barrels of crude oil from the seafloor per day. The incident has been declared a Spill of National Significance by the U.S. Secretary of Homeland Security and a major spill response effort is in progress. The spill threatens a broad expanse of the U.S. Gulf Coast in addition to the natural resources in the path of the oil slick which has spread across thousands of square miles at sea. Federal and state natural resource trustees have begun collecting ephemeral data to support a natural resource damage assessment (NRDA). Currently, NOAA is the lead administrative trustee. Although a formal agreement has not yet been reached, BP America has indicated an interest in cooperating with the natural resource trustees in the damage assessment.

This Analytical Quality Assurance (QA) Plan describes the minimum requirements for the chemical analysis of the environmental samples that are collected in support of this NRDA. This plan does not address the actual field collection or generation of these samples. The scope of the laboratory work is twofold: (1) generate concentrations for key chemicals used in injury determinations for crude oil releases, and (2) produce more extensive chemical data to use in fingerprinting for source identification. The applicable chemicals, need and frequency of environmental sample analyses, quality control requirements, and data usage vary for these two purposes, although implementation of this plan enables both to be achieved. In recognition of these differences, sampling plans may reference the Analytical QA Plan and cite to specific tables of chemical analyses that are appropriate to the needs of the particular sampling effort.

The requirements specified in this plan are designed to: (1) monitor the performance of the measurement systems to maintain statistical control over the reported concentrations of target analytes and provide rapid feedback so that corrective measures can be taken before data quality is compromised and; (2) verify that reported data are sufficiently complete, comparable, representative, unbiased and precise so as to be suitable for their intended use.

The analytes of concern addressed in this QA Plan are polycyclic aromatic hydrocarbons (PAHs) including alkyl homologues, saturated hydrocarbons (SHC), total extractable hydrocarbons (TEH)[1], polychlorinated biphenyl (PCB) congeners, volatile organic compounds (VOCs), and petroleum biomarkers. Additional analytes of concern are potentially toxic polar and non-polar components found within or formed from the dispersant agents utilized during the response to the incident, although the appropriate target analytes and methods are not yet established. A variety of matrices may be analyzed including water, filters, sediment/soil, tissues, vegetation, absorbent materials (e.g. Teflon nets, etc.), oils and oil debris. In addition to the primary analytes of concern, ancillary tests may include: percent moisture, total organic carbon (TOC) and grain size for sediment samples, and

[1] TEH is the total aromatic and aliphatic content as determined by GC/FID. If the sample extract is not "cleaned up" to remove biogenic material prior to the GC/FID analysis, then the result from the GC/FID analysis is termed Total Extractable Matter (TEM).

total extractable organics (TEO) for tissues. Additional tests not currently addressed in the QAP but may be of interest are: SARA (%Saturate, %Aromatic, %Resin, % Asphaltene) content in oil[2]; carbon, hydrogen, and nitrogen (CHN)[3] for sediments and particulate material in water. Performance criteria will be added to the QAP for additional tests when requested under the NRDA program.

The work plans and associated QA plans under which these samples were generated or collected are independent documents and not included or considered herein. This Analytical QA Plan describes the minimum requirements to be taken to provide for the chemical analyses (and associated physical normalizing parameters) of the previously generated or collected samples in a technically sound and legally defensible manner.

This Analytical QA Plan is consistent with the intent of NRDA regulations under OPA (33 U.S.C. §§ 2701 *et seq.*) and satisfies the requirements listed in the relevant EPA guidance for QA plans (USEPA 2002 and USEPA 2001) as far as the documents relate to analytical testing services. This QA plan will be revised as appropriate, as changes are made to the NRDA and the QA program.

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[2] SARA according to method published by Zumberge et al (2005) or equivalent. [Zumberge, J., J.A. Russell, and S.A. Reid. 2005. Charging of Elk Hills reservoirs as determined by oil geochemistry AAPG Bull. v. 89, pp. 1347-1371]

[3] CHN by micro elemental analyzer using the Dumas method of complete and instantaneous oxidation (flash dynamic combustion) at >1,000 °C following exposure of the sample to HCl fumes to remove inorganic carbon.

1.0 PROJECT DESCRIPTION

A number of laboratories will be analyzing samples associated with this NRDA. The intent of this plan is to present the minimum requirements for the performance criteria for the laboratories providing data in support of this investigation. The analytes of specific interest and brief descriptions of the analytical methods are as follows:

- PAHs including alkyl homologues by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM). The analytical procedure is based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the target analytes. Alkyl PAH homologues are quantified using a response factor assigned from the parent PAH compound. Analytes, associated response factors and target detection limits are listed in **Table 1.1a.** The following references discuss the method options in further detail:

  Federal Register 40CFR300, Subchapter J, Part 300, Appendix C, 4-6-3 to 4-6-5 pp. 234-237.

  Murphy, Brian L. and Robert D. Morrison (Editors). 2007. *Introduction to Environmental Forensics*, 2nd Edition. Chapter 9, p. 389 – 402.

  Page, D.S., P.D. Boehm, G.S. Douglas, and A.E. Bence. 1995. Identification of hydrocarbon sources in the benthic sediments of Prince William Sound and the Gulf of Alaska following the *Exxon Valdez* oil spill. *In*: *Exxon Valdez Oil Spill: Fate and Effects in Alaskan Waters, ASTM STP 1219*, P.G. Wells, J.N. Bulter, and J.S. Hughes, Eds, American Society for Testing and Materials, Philadelphia. pp 44-83.

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- Saturated hydrocarbons by gas chromatography with flame ionization detection (GC/FID) based on EPA Method 8015. Analytes and target detection limits are listed in **Table 1.1b**.

- Total Extractable Hydrocarbons (TEH[4]) representing the total aromatic and aliphatic hydrocarbon content of sample extracts after silica gel clean-up and analysis by GC/FID (**Table 1.1b**). The result is reported based on integration of the FID signal over the entire hydrocarbon range from *n*-C9 to n-C44 and calibrated against the average alkane hydrocarbon response factor.

  If the sample extract does not receive any clean-up then the result will be reported as Total Extractable Matter (TEM) because the extract may contain non-hydrocarbon compounds. Either TEH or TEM may reported by the laboratory depending on the handling of the extract.

- Standard volatile organic compounds (VOC) by GC/MS based on EPA Method 8260B but for aromatics hydrocarbons only. Analytes and target detection limits are listed in **Table 1.1c.**

- Extended list of VOCs for a specialized fingerprinting analysis of paraffins, isoparaffins, aromatics, naphthenes, and olefins (PIANO) by GC/MS. Analytes and target detection limits are provided in **Table 1.1d** for this source identification list.

- Petroleum biomarkers by GC/MS-SIM. Two methods for the analysis of petroleum biomarkers are contained herein, viz., quantitative and qualitative. The difference between these two analyses is that quantitative analysis produces absolute concentrations of target analytes whereas qualitative analysis produced pattern, or fingerprints, only. The proposed target analyte list for quantitative biomarkers is provided in **Table 1.1e.** This list may be expanded if warranted. This method is discussed in further detail in:

  Murphy, Brian L. and Robert D. Morrison (Editors). 2007. *Introduction to Environmental Forensics*, 2nd Edition. Chapter 9, p. 389 – 402;

  Wang, Z., Stout, S.A., and Fingas, M. (2006) Forensic fingerprinting of biomarkers for oil spill characterization and source identification (Review). *Environ. Forensics* **7(2)**: 105-146.

- Qualitative biomarker patterns may also be acquired using GC/MS-SIM with monitoring of selected ions (m/z) as provided in **Table 1.1f**. Since no concentration data are generated by qualitative analysis the results are reported as hardcopy PDF files of each ion over the appropriate retention time(s) and scale and included in the hardcopy data package produced by the laboratory.

- Corexit indicator compounds can be identified and (semi-) quantified by conventional GC/MS-SIM. The indicator compounds presently identified include: 2-butoxyethanol, three closely-eluting glycol ether isomers (reported together as a single analyte), and bis(2-ethylhexyl)fumarate (the latter of which is a thermal degradation product of DOSS

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[4] Note that the term TEH is being used for the total hydrocarbon analysis. The term "Total Petroleum Hydrocarbon" (TPH) may be used to refer to TEH, in some instances. For this QAP, the term TEH is used to avoid confusion with state-regulated gasoline or diesel determinations, rather TEH is used to refer to the sum of hydrocarbons from C_9 to C_{44}.

formed in the GC injection port). These indicator compounds can be identified in samples prepared for alkylated PAH analysis using conventional solvent extraction and preparation. These indicator compounds can be analyzed for concurrently with the alkylated PAHs during the same GC/MS acquisition by adding appropriate ions to the file. Suggested ions for monitoring are listed in **Table 1.1.g**. Indicator compound identifications are confirmed by analyzing a Corexit standard (i.e., a mixture of Corexit 9500 and 9527) under the same conditions as used for samples by comparing ion patterns and GC retention times. Semi-quantitative results for these indicator compounds can be based on a normalized response factor of 1 (without surrogate correction), and then the concentrations reported flagged by the laboratory as semi-quantitative.

- Corexit 9500/9527 dispersant (DISP) by liquid chromatography (LC)/MS for quantitative assessment, particularly dioctyl sulfosuccinate sodium salt (DOSS). Proposed measurement performance criteria are presented in **Table 6.1g**. Because the method is under development the laboratory may develop appropriate performance criteria based on past method performance.

- GC/MS may be used for qualitative assessments of solvent package components (e.g. glycol ethers) or primary degradation products of DOSS (alkyl diesters), pending further method development. Standard methods are not available for either technique but provisional analytical criteria and detection limits are under development.

- Total metals in sediments and tissues by inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) and total mercury in sediments and tissues by cold vapor atomic absorption (CVAA) or cold vapor atomic fluorescence spectrometry (CVAFS). The analytical procedures are based on EPA SW-846 Methods 6010C, 6020A, 7470A, 7471A, 7471B, 7474, and 7742. The target analyte list and target reporting limits (RLs) for each matrix are included in **Table 1.1h**. In order to meet the target RLs, if may be necessary to use an increased sample size to account for the high moisture content in marine sediments.

- PCB congeners by GC/MS. The GC/MS analysis can either be low resolution or high resolution mass spectrometry (LRMS or HRMS), provided that the target reporting limits can be met for the selected congeners of interest. Analytes and target detection limits are listed in **Table 1.1i.**

Analyses will include a number of different sample matrices. Matrices that will be analyzed will be determined in sampling plans and may not include all analyses for each matrix. The following table provides a summary of which analyses may be applicable to each matrix (analyses may be added or deleted as warranted over time).

Matrix	PAH	SHC/TEH	BIOMARK	DISP	VOC	Metals	PCB
Water	X	X	X	X	X		
Filters	X	X	X				
Sediment/Soil	X	X	X	X	X	X	X
Tissue	X		X	X		X	X
Vegetation	X	X	X	X			
Inert Sorbent Materials	X	X	X	X	X		
Oil/Oily Debris	X	X	X	X	X		

TABLE 1.1a
Extended PAH (Parent and Alkyl Homologs) and Related Compounds

	Compound	RF Source[5]		Compound	RF Source		Compound	RF Source
D0	cis/trans-Decalin		PA4	C4-Phenanthrenes/Anthracenes	P0	BEP	Benzo[e]pyrene	
D1	C1-Decalins	D0 or tD0[6]	RET	Retene	RET or P0	BAP	Benzo[a]pyrene	
D2	C2-Decalins	D0 or tD0	DBT0	Dibenzothiophene		PER	Perylene	
D3	C3-Decalins	D0 or tD0	DBT1	C1-Dibenzothiophenes	DBT0	IND	Indeno[1,2,3-cd]pyrene	
D4	C4-Decalins	D0 or tD0	DBT2	C2-Dibenzothiophenes	DBT0	DA	Dibenz[a,h]anthracene[7]	
BT0	Benzothiophene		DBT3	C3-Dibenzothiophenes	DBT0	GHI	Benzo[g,h,i]perylene	
BT1	C1-Benzo(b)thiophenes	BT0	DBT4	C4-Dibenzothiophenes	DBT0			
BT2	C2-Benzo(b)thiophenes	BT0	BF	Benzo(b)fluorene	BF or FL0	4MDT	4-Methyldibenzothiophene	DBT0
BT3	C3-Benzo(b)thiophenes	BT0	FL0	Fluoranthene		2MDT	2/3-Methyldibenzothiophene	DBT0
BT4	C4-Benzo(b)thiophenes	BT0	PY0	Pyrene		1MDT	1-Methyldibenzothiophene	DBT0
N0	Naphthalene		FP1	C1-Fluoranthenes/Pyrenes	FL0 or PY0	3MP	3-Methylphenanthrene	P0
N1	C1-Naphthalenes	N0	FP2	C2-Fluoranthenes/Pyrenes	FL0 or PY0	2MP	2-Methylphenanthrene	P0
N2	C2-Naphthalenes	N0	FP3	C3-Fluoranthenes/Pyrenes	FL0 or PY0	2MA	2-Methylanthracene	P0
N3	C3-Naphthalenes	N0	FP4	C4-Fluoranthenes/Pyrenes	FL0 or PY0	9MP	9/4-Methylphenanthrene	P0
N4	C4-Naphthalenes	N0	NBT0	Naphthobenzothiophenes		1MP	1-Methylphenanthrene	P0
B	Biphenyl		NBT1	C1-Naphthobenzothiophenes	NBT0		2-Methylnaphthalene	
DF	Dibenzofuran		NBT2	C2-Naphthobenzothiophenes	NBT0		1-Methylnaphthalene	
AY	Acenaphthylene		NBT3	C3-Naphthobenzothiophenes	NBT0		2,6-Dimethylnaphthalene	
AE	Acenaphthene		NBT4	C4-Naphthobenzothiophenes	NBT0		1,6,7-Trimethylnaphthalene	
F0	Fluorene		BA0	Benz[a]anthracene				
F1	C1-Fluorenes	F0	C0	Chrysene/Triphenylene				
F2	C2-Fluorenes	F0	BC1	C1-Chrysenes	C0		**Other**	
F3	C3-Fluorenes	F0	BC2	C2-Chrysenes	C0		Carbazole	
A0	Anthracene		BC3	C3-Chrysenes	C0		C30-Hopane[8]	
P0	Phenanthrene		BC4	C4-Chrysenes	C0			
PA1	C1-Phenanthrenes/Anthracenes	P0	BBF	Benzo[b]fluoranthene	BBF[9]			
PA2	C2-Phenanthrenes/Anthracenes	P0	BKF	Benzo[,k]fluoranthene	BKF			
PA3	C3-Phenanthrenes/Anthracenes	P0	BAF	Benzo[a]fluoranthene	BKF or BAF			

	Target Method Detection Limit Range
Sediment/Soil =	0.1 – 0.5 ng/g dry weight
Tissue =	0.2 – 1.0 ng/g wet weight
Water =	1 – 5 ng/L
	Target Reporting Limit
Oil =	2.0 mg/kg

[5] Response factor (RF) to be used for quantitation. If blank, compound is included in the calibration mix.

[6] tD0 = transD0 (used if cis/trans in separate standards).

[7] Dibenz(a,c)anthracene commonly coelutes with dibenz(a,h)anthracene. Laboratories should verify if the coelution is occurs and report accordingly.

[8] Quantitative concentrations of C29-hopane and 18α-oleanane may be provided if laboratories are calibrated to do so; the C30-hopane is a minimum requirement.

[9] Benzo(j)fluoranthene may coelute with benzo(b)fluoranthene or benzo(k)fluoranthene, depending on specific laboratory instrument conditions. Laboratories should verify the specific co-elutions for their analytical conditions.

TABLE 1.1b
Saturated Hydrocarbons (Alkanes/Isoprenoids Compounds) and Total Extractable Hydrocarbons

Abbr.	Analyte
nC9	n-Nonane
nC10	n-Decane
nC11	n-Undecane
nC12	n-Dodecane
nC13	n-Tridecane
1380	2,6,10 Trimethyldodecane
nC14	n-Tetradecane
1470	2,6,10 Trimethyltridecane
nC15	n-Pentadecane
nC16	n-Hexadecane
nPr	Norpristane
nC17	n-Heptadecane
Pr	Pristane
nC18	n-Octadecane
Ph	Phytane
nC19	n-Nonadecane
nC20	n-Eicosane
nC21	n-Heneicosane
nC22	n-Docosane

Abbr.	Analyte
nC23	n-Tricosane
nC24	n-Tetracosane
nC25	n-Pentacosane
nC26	n-Hexacosane
nC27	n-Heptacosane
nC28	n-Octacosane
nC29	n-Nonacosane
nC30	n-Triacontane
nC31	n-Hentriacontane
nC32	n-Dotriacontane
nC33	n-Tritriacontane
nC34	n-Tetratriacontane
nC35	n-Pentatriacontane
nC36	n-Hexatriacontane
nC37	n-Heptatriacontane
nC38	n-Octatriacontane
nC39	n-Nonatriacontane
nC40	n-Tetracontane

TEH	$\Sigma(C_9\text{-}C_{44})$ Integration of the FID signal over the entire hydrocarbon range from n-C9 to n-C44 after silica gel cleanup.
TEM	$\Sigma(C_9\text{-}C_{44})$ Integration of the FID signal over the entire hydrocarbon range from n-C9 to n-C44 no silica gel cleanup.

	Target Method Detection Limit
Sediment (Alkanes) =	0.01 µg/g dry weight
Sediment (TEH) =	1 µg/g dry weight
Water (Alkanes) =	0.8 µg/L
	Target Reporting Limit
Oil (Alkanes) =	200 mg/kg
Oil (TEH) =	200 mg/kg
Water (TEH/TEM) =	200 µg/L

TEH = Total Extractable Hydrocarbons with silica gel "clean-up"
TEM = Total Extractable Matter with no extract "clean-up"

TABLE 1.1c
Standard Volatile Organic Compounds

Analyte
1,2,4-Trimethylbenzene
1,3,5-Trimethylbenzene
4-Isopropyltoluene
Benzene
Ethylbenzene
Isopropylbenzene
m,p-Xylenes
Naphthalene[10]
n-Butylbenzene
n-Propylbenzene
o-Xylene
sec-Butylbenzene
Styrene
tert-Butylbenzene
Toluene

	Target Method Detection Limit Range
Sediment/Soil =	0.1 – 1 ng/g
Water =	0.05 – 0.5 µg/L
	Target Reporting Limit
Oil =	2 mg/kg

[10] Naphthalene is also included on the **Table 1.1a** target analyte list of PAH compounds. The PAH analysis is the preferred method, rather than this volatile method. Thus, if a sample location is analyzed for both PAH and VOC the result from the PAH analysis will be noted in the database as the preferred result.

TABLE 1.1d
C5-C13 Volatile Compounds for PIANO Forensic Assessment

Abbrev.	Analyte
IP	Isopentane
1P	1-Pentene
2M1B	2-Methyl-1-butene
C5	Pentane
T2P	2-Pentene (trans)
C2P	2-Pentene (cis)
TBA	Tertiary butanol
CYP	Cyclopentane
23DMB	2,3-Dimethylbutane
2MP	2-Methylpentane
MTBE	MTBE
3MP	3-Methylpentane
1HEX	1-Hexene
C6	Hexane
DIPE	Diisopropyl Ether (DIPE)
ETBE	Ethyl Tertiary Butyl Ether (ETBE)
22DMP	2,2-Dimethylpentane
MCYP	Methylcyclopentane
24DMP	2,4-Dimethylpentane
12DCA	1,2-Dichloroethane
CH	Cyclohexane
2MH	2-Methylhexane
B	Benzene
23DMP	2,3-Dimethylpentane
THIO	Thiophene
3MH	3-Methylhexane
TAME	TAME
1H	1-Heptene/1,2-DMCP (trans)
ISO	Isooctane
C7	Heptane

Abbrev.	Analyte
MCYH	Methylcyclohexane
25DMH	2,5-Dimethylhexane
24DMH	2,4-Dimethylhexane
223TMP	2,2,3-Trimethylpentane
234TMP	2,3,4-Trimethylpentane
233TMP	2,3,3-Trimethylpentane
23DMH	2,3-Dimethylhexane
3EH	3-Ethylhexane
2MHEP	2-Methylheptane
3MHEP	3-Methylheptane
T	Toluene
2MTHIO	2-Methylthiophene
3MTHIO	3-Methylthiophene
1O	1-Octene
C8	Octane
12DBE	1,2-Dibromoethane
EB	Ethylbenzene
2ETHIO	2-Ethylthiophene
MPX	p/m-Xylene
1N	1-Nonene
C9	Nonane[11]
STY	Styrene
OX	o-Xylene
IPB	Isopropylbenzene
PROPB	n-Propylbenzene
1M3EB	1-Methyl-3-ethylbenzene
1M4EB	1-Methyl-4-ethylbenzene
135TMB	1,3,5-Trimethylbenzene
1D	1-Decene
1M2EB	1-Methyl-2-ethylylbenzene

Abbrev.	Analyte
C10	Decane[11]
124TMB	1,2,4-Trimethylbenzene
SECBUT	sec-Butylbenzene
1M3IPB	1-Methyl-3-isopropylbenzene
1M4IPB	1-Methyl-4-isopropylbenzene
1M2IPB	1-Methyl-2-isopropylbenzene
IN	Indan
1M3PB	1-Methyl-3-propylbenzene
1M4PB	1-Methyl-4-propylbenzene
BUTB	n-Butylbenzene
12DM4EB	1,2-Dimethyl-4-ethylbenzene
12DEB	1,2-Diethylbenzene
1M2PB	1-Methyl-2-propylbenzene
14DM2EB	1,4-Dimethyl-2-ethylbenzene
C11	Undecane[11]
13DM4EB	1,3-Dimethyl-4-ethylbenzene
13DM5EB	1,3-Dimethyl-5-ethylbenzene
13DM2EB	1,3-Dimethyl-2-ethylbenzene
12DM3EB	1,2-Dimethyl-3-ethylbenzene
1245TMP	1,2,4,5-Tetramethylbenzene
PENTB	Pentylbenzene
C12	Dodecane[11]
N0	Naphthalene[12]
BT0	Benzothiophene[12]
MMT	MMT
C13	Tridecane[11]
2MN	2-Methylnaphthalene[12]
1MN	1-Methylnaphthalene[12]

	Target Method Detection Limit Range
Sediment/Soil =	0.1 – 10 ng/g
Water =	0.2 - 2.0 µg/L
	Target Reporting Limit
Oil =	2 mg/kg

[11] These compounds are also included on the **Table 1.1b** target analyte list of saturate hydrocarbons. Because of the extraction technique, the GC/FID method for hydrocarbons is the preferred method, rather than this volatile method. Thus, if a sample location is analyzed for both saturate hydrocarbons by GC/FID and VOC the result from the GC/FID analysis will be noted in the database as the preferred result.

[12] These compounds are also included on the **Table 1.1a** target analyte list of PAH compounds. Because of the extraction technique, the PAH analysis is the preferred method, rather than this volatile method. Thus, if a sample location is analyzed for both PAH and VOC the result from the PAH analysis will be noted in the database as the preferred result.

TABLE 1.1e
Petroleum Biomarkers for Quantitative Analysis

Compound *	Quant Ion m/z
C23 Tricyclic Terpane (T4)	191
C24 Tricyclic Terpane (T5)	191
C25 Tricyclic Terpane (T6)	191
C24 Tetracyclic Terpane (T6a)	191
C26 Tricyclic Terpane-22S (T6b)	191
C26 Tricyclic Terpane-22R (T6c)	191
C28 Tricyclic Terpane-22S (T7)	191
C28 Tricyclic Terpane-22R (T8)	191
C29 Tricyclic Terpane-22S (T9)	191
C29 Tricyclic Terpane-22R (T10)	191
18a-22,29,30-Trisnorneohopane-Ts (T11)	191
C30 Tricyclic Terpane-22S (T11a)	191
C30 Tricyclic Terpane-22R (T11b)	191
17a(H)-22,29,30-Trisnorhopane-Tm (T12)	191
17a/b,21b/a 28,30-Bisnorhopane (T14a)	191
17a(H),21b(H)-25-Norhopane (T14b)	191
30-Norhopane (T15)	191
18a(H)-30-Norneohopane-C29Ts (T16)	191
17a(H)-Diahopane (X)	191
30-Normoretane (T17)	191
18a(H)&18b(H)-Oleananes (T18)	191
Hopane (T19)	191
Moretane (T20)	191
30-Homohopane-22S (T21)	191
30-Homohopane-22R (T22)	191
T22a-Gammacerane/C32-diahopane	191
30,31-Bishomohopane-22S (T26)	191
30,31-Bishomohopane-22R (T27)	191
30,31-Trishomohopane-22S (T30)	191

Compound	Quant ion m/z
30,31-Trishomohopane-22R (T31)	191
Tetrakishomohopane-22S (T32)	191
Tetrakishomohopane-22R (T33)e	191
Pentakishomohopane-22S (T34)	191
Pentakishomohopane-22R (T35)	191
13b(H),17a(H)-20S-Diacholestane (S4)	217
13b(H),17a(H)-20R-Diacholestane (S5)	217
13b,17a-20S-Methyldiacholestane (S8)	217
14a(H),17a(H)-20S-Cholestane/ 13b(H).17a(H)-20S-Ethyldiacholestane (S12)	217
14a(H),17a(H)-20R-Cholestane 13b(H),17a(H)-20R-Ethyldiacholestane (S17)	217
Unknown sterane(S18)	217
13a,17b-20S-Ethyldiacholestane (S19)	217
14a,17a-20S-Methylcholestane (S20)	217
14a,17a-20R-Methylcholestane (S24)	217
14a(H),17a(H)-20S-Ethylcholestane (S25)	217
14a(H),17a(H)-20R-Ethylcholestane (S28)	217
14b(H),17b(H)-20R-Cholestane (S14)	218
14b(H),17b(H)-20S-Cholestane (S15)	218
14b,17b-20R-Methylcholestane (S22)	218
14b,17b-20S-Methylcholestane (S23)	218
14b(H),17b(H)-20R-Ethylcholestane (S26)	218
14b(H),17b(H)-20S-Ethylcholestane (S27)	218
C26,20R- +C27,20S- triaromatic steroid	231
C28,20S-triaromatic steroid	231
C27,20R-triaromatic steroid	231
C28,20R-triaromatic steroid	231

* Peak identification provided in parentheses.

	Target Reporting Limit
Sediments/Soil =	2 ug/Kg dry weight
Waters =	10 ng/L

	Target Reporting Limit
Oil =	2 mg/Kg

TABLE 1.1f
Suggested Hydrocarbon Groups and Petroleum Biomarkers for Qualitative Analysis

n-Alkycyclohexanes (m/z 83)
n-Alkanes (m/z 85)
Diamondoids (m/z 135, 187)
Sesquiterpanes (m/z 109, 123)
Isoprenoids (m/z 183)
Triterpanes (m/z 191)
Regular Steranes (m/z 217)
Rearranged β,β-steranes (m/z 218)
Methyl steranes (m/z 232, 245)
Methyl and triaromatic steroids (m/z 231)
Monoaromatic steroids (m/z 253)
Diasteranes (m/z 259)

TABLE 1.1g
Corexit Indicator Compounds for Qualitative Analysis in Water Only
(monitoring mass/charge ion)

2-Butoxyethanol (m/z 87, 75)
Glycol ether Isomers (m/z 59, 103)
Bis-(2-ethylhexyl) fumarate (m/z 112, 211)

TABLE 1.1h
Metals Target Analyte List

Analyte	Method	Target Reporting Limits (RL)	
		Sediment (mg/Kg) dry weight	Tissues (mg/Kg) wet weight
Aluminum	ICP/ICP-MS	10	5
Antimony	ICP/ICP-MS	0.05	NA
Arsenic	ICP-MS	0.5	0.5
Barium	ICP/ICP-MS	0.1	0.05
Beryllium	ICP/ICP-MS	0.05	NA
Cadmium	ICP/ICP-MS	0.05	0.02
Calcium	ICP/ICP-MS	100	NA
Chromium	ICP/ICP-MS	0.2	0.2
Cobalt	ICP/ICP-MS	0.05	0.05
Copper	ICP/ICP-MS	0.1	0.1
Iron	ICP/ICP-MS	10	2
Lead	ICP/ICP-MS	0.05	0.02
Magnesium	ICP/ICP-MS	50	NA
Manganese	ICP/ICP-MS	0.2	0.2
Mercury	CVAA/CVAFS	0.01	0.01
Nickel	ICP/ICP-MS	0.5	0.5
Potassium	ICP/ICP-MS	100	NA
Selenium	ICP/ICP-MS	0.1	0.1
Silver	ICP/ICP-MS	0.05	0.05
Sodium	ICP/ICP-MS	100	NA
Strontium	ICP/ICP-MS	2.00	NA
Thallium	ICP/ICP-MS	0.1	NA
Vanadium	ICP/ICP-MS	0.5	0.5
Zinc	ICP/ICP-MS	1	0.5

Method detection limits (MDL) should be at least 3 times lower than the target reporting limits.

TABLE 1.1 i
PCB Congener Target Analyte List

PCB Congeners (Using Ballschmiter-Zell Numbers[13])								
BZ# 1	BZ# 25	BZ# 49	BZ# 73	BZ# 97	BZ# 121	BZ# 145	BZ# 169 *	BZ# 193
BZ# 2	BZ# 26	BZ# 50	BZ# 74	BZ# 98	BZ# 122	BZ# 146	BZ# 170	BZ# 194
BZ# 3	BZ# 27	BZ# 51	BZ# 75	BZ# 99	BZ# 123 *	BZ# 147	BZ# 171	BZ# 195
BZ# 4	BZ# 28	BZ# 52	BZ# 76	BZ# 100	BZ# 124	BZ# 148	BZ# 172	BZ# 196
BZ# 5	BZ# 29	BZ# 53	BZ# 77 *	BZ# 101	BZ# 125	BZ# 149	BZ# 173	BZ# 197
BZ# 6	BZ# 30	BZ# 54	BZ# 78	BZ# 102	BZ# 126 *	BZ# 150	BZ# 174	BZ# 198
BZ# 7	BZ# 31	BZ# 55	BZ# 79	BZ# 103	BZ# 127	BZ# 151	BZ# 175	BZ# 199
BZ# 8	BZ# 32	BZ# 56	BZ# 80	BZ# 104	BZ# 128	BZ# 152	BZ# 176	BZ# 200
BZ# 9	BZ# 33	BZ# 57	BZ# 81 *	BZ# 105 *	BZ# 129	BZ# 153	BZ# 177	BZ# 201
BZ# 10	BZ# 34	BZ# 58	BZ# 82	BZ# 106	BZ# 130	BZ# 154	BZ# 178	BZ# 202
BZ# 11	BZ# 35	BZ# 59	BZ# 83	BZ# 107	BZ# 131	BZ# 155	BZ# 179	BZ# 203
BZ# 12	BZ# 36	BZ# 60	BZ# 84	BZ# 108	BZ# 132	BZ# 156 *	BZ# 180	BZ# 204
BZ# 13	BZ# 37	BZ# 61	BZ# 85	BZ# 109	BZ# 133	BZ# 157 *	BZ# 181	BZ# 205
BZ# 14	BZ# 38	BZ# 62	BZ# 86	BZ# 110	BZ# 134	BZ# 158	BZ# 182	BZ# 206
BZ# 15	BZ# 39	BZ# 63	BZ# 87	BZ# 111	BZ# 135	BZ# 159	BZ# 183	BZ# 207
BZ# 16	BZ# 40	BZ# 64	BZ# 88	BZ# 112	BZ# 136	BZ# 160	BZ# 184	BZ# 208
BZ# 17	BZ# 41	BZ# 65	BZ# 89	BZ# 113	BZ# 137	BZ# 161	BZ# 185	BZ# 209
BZ# 18	BZ# 42	BZ# 66	BZ# 90	BZ# 114 *	BZ# 138	BZ# 162	BZ# 186	
BZ# 19	BZ# 43	BZ# 67	BZ# 91	BZ# 115	BZ# 139	BZ# 163	BZ# 187	
BZ# 20	BZ# 44	BZ# 68	BZ# 92	BZ# 116	BZ# 140	BZ# 164	BZ# 188	
BZ# 21	BZ# 45	BZ# 69	BZ# 93	BZ# 117	BZ# 141	BZ# 165	BZ# 189 *	
BZ# 22	BZ# 46	BZ# 70	BZ# 94	BZ# 118 *	BZ# 142	BZ# 166	BZ# 190	
BZ# 23	BZ# 47	BZ# 71	BZ# 95	BZ# 119	BZ# 143	BZ# 167 *	BZ# 191	
BZ# 24	BZ# 48	BZ# 72	BZ# 96	BZ# 120	BZ# 144	BZ# 168	BZ# 192	
Total Monochlorobiphenyls (BZ# 1 – BZ# 3)					Total Hexachlorobiphenyls (BZ# 128 – BZ# 169)			
Total Dichlorobiphenyls (BZ# 4 – BZ# 15)					Total Heptachlorobiphenyls (BZ# 170 – BZ# 193)			
Total Trichlorobiphenyls (BZ# 16 – BZ# 39)					Total Octachlorobiphenyls (BZ# 194 – BZ# 205)			
Total Tetrachlorobiphenyls (BZ# 40 – BZ# 81)					Total Nonachlorobiphenyls (BZ# 206 – BZ# 208)			
Total Pentachlorobiphenyls (BZ# 82 – BZ# 127)					Decachlorobiphenyl (BZ# 209)			
Total PCBs								

*** Congener is one of the 12 toxic co-planar PCB congeners**

PCB Congener Target Method Detection Limit Range

Sediments/Soil = 0.1 – 0.5 ng/g dry weight
Tissue = 0.1 – 0.5 ng/g wet weight

PCB Homolog and Total PCB Target Reporting Limit

Sediments/Soil = 1 ng/g dry weight
Tissue = 1 ng/g wet weight

[13] PCB congeners are biphenyl structures that have one or more chlorine substitutions. PCB congeners can range from one (monochlorinated biphenyls) to ten (decachlorinated biphenyl) chlorine substitutions. PCB congeners are often referred to using the "BZ" number, after a numbering scheme originally published by Ballschmiter et. al. in 1980 and revised in 1992, or by using the 'BZ' number with 'PCB'. For example, BZ# 1 (or PCB 1) is 2-chlorobiphenyl, and BZ# 209 (or PCB 209) is 2,2',3,3',4,4',5,5',6,6'-decachlorobiphenyl. The use of the BZ and/or PCB congener number naming scheme greatly reduces transcription errors.

2.0 PROJECT ORGANIZATION AND RESPONSIBILITIES

2.1 Assessment Manager

Greg Baker
Office of Response and Restoration
NOAA
345 Middlefield Road, MS-999
Menlo Park, CA 94025
(650)329-5048 FAX (650)329-5198
greg.baker@noaa.gov

The Assessment Manager is the designated natural resource trustee representative who is responsible for the review and acceptance of specific work plans and associated QA plans.

2.2 Project Coordinators

The Project Coordinators are responsible for administration of the contracts with the laboratory(ies). The Project Coordinators will oversee the proper scheduling and transmittal of the data from the time of sampling to data reporting.

Project Coordinator for Battelle:

Dennis Beckmann
Data and QA Manager
Gulf Coast Restoration
BP America, Inc.
501 Westlake Park Boulevard
Houston, TX 77079
dennis.beckmann@noaa.gov

Project Coordinator for Alpha Analytical, Columbia Analytical Services, and NOAA NW Fisheries Science Center:

Tony Penn
Deputy Division Chief
NOAA Assessment and Restoration Division
1305 East West Highway
Building SSMC4
Silver Spring, MD 20910
tony.penn@noaa.gov

2.3 Quality Assurance

Ann Bailey is the QA Coordinator reporting directly to the Assessment Manager. Ms. Bailey is responsible for the implementation of this Analytical QA Plan. She will receive assistance in the coordination and performance of laboratory technical audits and independent data validation from the QA Contractor (EcoChem). The QA Coordinator has the authority and responsibility to cease or temporarily halt activities not in keeping with this QA Plan. The QA Coordinator will work closely

with laboratory representatives and the project team to assure that project and data quality objectives are met. The QA Coordinator may be reached at:

Ann Bailey
EcoChem, Inc.
1011 Western Avenue, Suite 1011
Seattle, WA 98104
(206)233-9332 x106 FAX (206)233-0114
abailey@ecochem.net

Cheryl Randle is a QA Reviewer conducting data validation on behalf of BP America. Ms. Randle is responsible for working closely with the Assessment Manager's QA Coordinator to assure the validity of the final data in accordance with this Analytical QA Plan. The QA Reviewer will conduct spot validation of up to 25 percent of the reported data, unless substantial problems are discovered in which case up to 100 percent validation may be performed. The QA Reviewer may be reached at:

Cheryl Randle
Cardno ENTRIX, Inc.
1000 Hart Road, Suite 130
Barrington, IL 60010
(847)277-2865 FAX (847)381-6679
cheryl.randle@cardno.com

2.4 Analytical Laboratories

The laboratories planned to be contracted at this time for analytical work in support of the NRDA are TDI-Brooks B&B Laboratories (B&B), Newfields/Alpha Analytical (Alpha), and ALS Environmental [formerly Columbia Analytical Services (CAS)]. The laboratory project managers are responsible for assuring that all analyses performed meet project and measurement quality objectives. The Laboratory Project Managers are:

Juan Ramirez
TDI-Brooks B&B Laboratories
1902 Pinon
College Station, TX 77845-5816
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As additional analytical laboratories are brought under contract, this QAP will be updated to include their names and project managers.

3.0 SAMPLE HANDLING AND CHAIN OF CUSTODY PROCEDURES

Chain of custody procedures will be used for all samples throughout the analytical process and for all data and data documentation, whether in hard copy or electronic format. Sampling procedures, including sample collection and documentation, are part of the work plans of the individual projects and as such, are not considered here.

3.1 Sample Preservation and Holding Times

Sample preservation and field treatment of samples for analyses should be described in relevant field work plans. Based on EPA guidance, "advisory" sample holding times prior to analysis and holding times for the extracts are presented in **Table 3-1**. These holding times may be extended or preservation guidance amended, as options are assessed.

3.2 Chain of Custody

Chain of custody records will be completed in ink.

A sample is considered in "custody" if:

- it is in the custodian's actual possession or view, or
- it is retained in a secured place (under lock) with restricted access, or
- it is placed in a container and secured with an official seal(s) such that the sample cannot be reached without breaking the seal(s).

Samples are kept in the custody of designated sampling and/or field personnel until shipment.

3.3 Sample Shipping

Any transfer or movement of samples will use chain of custody procedures. The original signed and dated chain of custody record accompanies the sample(s); a copy is retained by the sample shipper. All shipments will comply with DOT regulations (*49CFR, Parts 172 and 173*).

TABLE 3-1
Sample Holding Times

Matrix/Analysis	Storage for Samples	Holding Time to Extraction	Holding Time to Analysis
VOC Analyses			
Water	Refrigeration 4°C ±2° with no headspace; Optional: Preserved with HCl in the field in VOA vial.	Not applicable	7 days if not acid preserved; 14 days if acid preserved
Sediment	Refrigeration 4°C ±2° For preservation requirements, see SW-846 Method 5035A.	Not applicable	14 days
Oil	Above freezing to 30°C	Not applicable	No holding time
Oily Debris	Refrigeration <6°C	Not applicable	No holding time
PAH, SHC/TEH, Biomarker Analyses			
Water	Refrigeration 4°C ±2°; Optional: Preserved with 1:1 HCl to pH<2	7 days if not acid preserved; 14 days if acid preserved	40 days from extraction[14]; except biomarkers no holding time
Filters	Frozen (-20°C ±10°C)	4 Years	40 days from extraction[14]; except biomarkers no holding time
Sediment/Soil (also total solids, grain size and TOC)	Frozen (-20°C ±10°C), except Grain Size should not be frozen – store at 4°C ±2°	4 Years, except not applicable for Grain Size, Total Solids, and TOC	40 days from extraction[14]; except biomarkers grain size, total solids and TOC no holding time.
Tissue (Total Extractable Organics aka Lipids)	Frozen (-20°C ±10°C)	4 Years	40 days from extraction[14]; except biomarkers and TEO no holding time.
Vegetation	Frozen (-20°C ±10°C)	4 Years	40 days from extraction[14]; except biomarkers no holding time
Inert Sorbent Material	Frozen (-20°C ±10°C)	4 Years	
Oil	Above freezing to 30°C	No holding time	
Oily Debris	Refrigeration <6°C	No holding time	
Dispersants (DOSS) Analyses			
Water	Frozen (-20° ±10°C), 15mL plastic centrifuge tubes	Not established	Not established
Sediment and Tissue	Frozen (-20° ±10°C), glass jars	Not established	Not established
PCB Congener Analyses			
Sediment and Tissue	Frozen (-20° ±10°C)	4 Years[15]	40 days from extraction[14]
Metals Analyses			
Water	Preserve with HNO_3 to pH <2	Not applicable	6 months except Mercury: 28 days
Sediment and Tissue	Frozen (-20°C ±10°C)	Not applicable	2 years except Mercury: 1 year[16]

[14] 40 days is an advisory extraction holding time. Extracts should be held at -20C in the dark, and may be analyzed past 40 days and results not qualified if surrogates are within criteria.

[15] There is no holding time limitation specified in most methods. The cited holding time is an AQAP defined limit; however, if the samples are properly maintained they may be stored indefinitely.

[16] Holding time for metals, except mercury, is based on *Puget Sound Dredged Disposal Analysis Data Quality Guidance Manual* (PTI July 1988). Holding time for mercury is based on *Appendix to Method 1631 Total Mercury in Tissue, Sludge, Sediment, and Soil by Acid Digestion and BrCl Oxidation* (EPA-821-R-01-013, January 2001)

3.4 Sample Receipt

Immediately upon receipt of samples, the recipient will review the shipment for consistency with the accompanying chain of custody record and sample condition, before signing and dating the chain of custody record. Sample condition(s) will be noted on the laboratory's sample receipt form and maintained with the chain of custody records. If there are any discrepancies between the chain of custody record and the sample shipment, the recipient will contact the sample shipper immediately in an attempt to reconcile these differences. Reconciliation of sample receipt differences will be maintained with the chain of custody records and discussed in the laboratory narrative which accompanies the data report.

3.5 Intra-Laboratory Sample Transfer

The laboratory sample custodian or designee will maintain a laboratory sample-tracking record, similar to the chain of custody record that will follow each sample through all stages of laboratory processing. The sample-tracking record will show the name or initials of responsible individuals, date of sample extraction or preparation, and sample analysis.

3.6 Inter-Laboratory Sample Transfer

Transfer of samples from one analytical laboratory to another, e.g. for grain size or TOC analysis, will follow chain of custody, sample shipping and receipt procedures described above. Transfer of samples between laboratories will be noted in the laboratory case narrative which accompanies the data report.

3.7 Sample Archival

All unanalyzed samples and unutilized sample aliquots or extracts will be held by the laboratory in a manner to preserve sample integrity at a secure location with chain of custody procedures for one (1) year after the QA Contractor has validated the data package for that particular set of samples. All archived materials will be accessible for review upon request. At the end of the archival period, the laboratory shall contact the QA Coordinator to obtain directions for handling remaining samples. The samples will not be disposed of by the laboratory unless provided with written approval from the Assessment Manager.

3.8 Data and Data Documentation

The laboratories will provide the QA Contractor with hardcopy data tables, QC documentation and instrument printouts suitable for QA assessment/data validation. Required laboratory deliverables are listed in **Table 7.1**. Data packages will include all related instrument print-outs ("raw data") and bench sheets. A copy of the data and data documentation developed by the laboratory for a given data package will be kept by the laboratory in a secure location using chain of custody procedures for five (5) years after the QA Contractor has validated that data package. All archived data and documentation will be accessible for review upon request. These materials will become the responsibility of the Assessment Manager upon termination of the archival period.

The original data will be transferred from the laboratory to the QA Contractor by means such that a signature is required at the time of document delivery. The QA Contractor will document receipt of packages and maintain a record of the method and date of data submittal with the complete data package. The QA Contractor will maintain the copy of the data packages and related validation documentation in a secure location for a period of one (1) year from the date of validation. These materials will become the responsibility of the Assessment Manager upon termination of the archival period.

4.0 LABORATORY OPERATIONS

All laboratories providing analytical support for the MC252 Damage Assessment must have the appropriate facilities to store and prepare samples, and appropriate instrumentation and staff to provide data of the required quality within the time period dictated. Laboratories are expected to conduct operations using good laboratory practices, including:

- Training and appropriate certification of personnel.
- A program of scheduled maintenance of analytical balances, laboratory equipment and instrumentation.
- Routine checking of analytical balances using a set of standard reference weights (ASTM class, NIST Class S-1, or equivalents).
- Recording all analytical data in secure electronic system with date and associated analyst identification, and/or logbooks with each entry signed and dated by the analyst.
- Monitoring and documenting the temperatures of cold storage areas and freezer units.

Laboratory operations may be evaluated by the QA Coordinator through technical systems audits, performance evaluation studies, and performance in a NIST-managed intercomparison program. Personnel in any laboratory performing analyses for this damage assessment should be well versed in good laboratory practices, including standard safety procedures. It is the responsibility of the laboratory manager and /or supervisor to ensure that safety training is mandatory for all laboratory personnel. The laboratory is responsible for maintaining a current safety manual in compliance with the Occupational Safety and Health Administration (OSHA) or equivalent state or local regulations. Proper procedures for safe storage, handling and disposal of chemicals should be followed at all times; each chemical should be treated as a potential health hazard and good laboratory practices should be implemented accordingly.

4.1 Quality Assurance Documentation

All laboratories must have the latest revision of the MC 252 NRDA Analytical QA Plan. In addition, the following documents and information must be current and available to all laboratory personnel participating in the processing of MC 252 samples:

- Laboratory Quality Assurance Management Plan
- Laboratory Standard Operating Procedures (SOPs) – Detailed instructions for performing routine laboratory procedures.

- Control charts or data tables – These must be developed and maintained throughout the project for appropriate analyses and measurements, including:
  - Alkyl PAH pattern book for MC252 reference oil.

4.2 Laboratory Systems Audits

Prior to or during sample analysis, QA systems audits will be performed. The laboratory audits will be conducted by the QA Coordinator or designee. The checklists used for the laboratory audits are based on requirements outlined in "Good Laboratory Practice Standards" (*40 CFR Part 792*) and audit procedures of the EPA National Enforcement Investigations Center, "NEIC Procedures Manual for the Contract Evidence Audit and Litigation Support for EPA Enforcement Case Development" (*EPA 330/9-89-002*). The Laboratory Project Managers will be informed of the findings and recommendations of the audit before the auditors leave the facility. A written report discussing the audits will be submitted to the Assessment Manager.

Additional laboratory audits may be performed at any time throughout the duration of the NRDA.

4.3 Participation in Intercomparison Exercises

Each analytical laboratory performing analysis will be required to participate in potential intercomparison exercises that may be organized by NS&T and/ or NIST during the duration of the laboratory's participation in this NRDA analytical program. A variety of samples including sample extracts and representative matrices (e.g., sediment or tissue samples) may be utilized in these exercises. Laboratories are required to analyze only those matrices or analytes that they are providing in like manner for the NRDA analytical program. When participating in the intercomparison exercise, the laboratory should analyze the sample(s) in the same manner as routinely performed for this NRDA and as specified in this Analytical QA Plan. Laboratories which fail to achieve acceptable performance will be required to provide an explanation to the QA Coordinator and/or undertake appropriate corrective actions.

5.0 ASSESSMENT OF DATA QUALITY

The purpose of this Analytical QA Plan is to develop and document analytical data of known, acceptable, and defensible quality. The quality of the data is presented as a set of statements that describe in precise quantitative terms the level of uncertainty that can be associated with the data without compromising their intended use. These statements are referred to as Data Quality Objectives (DQOs) and are usually expressed in terms of precision, bias, sensitivity, completeness, and comparability.

5.1 Precision

Precision is the degree of mutual agreement among individual measurements of the same property under prescribed similar conditions, such as replicate measurements of the same sample. Precision is concerned with the "closeness" of the results. Where suitable reference materials (RMs) are available,

precision will be expressed as the relative standard deviation (RSD) for the repeated measurements. This use of RMs allows for the long-term measurement of precision but does not include homogenization as a source of analytical variability.

In addition to the tracking precision of replicate RM analyses, precision will be expressed as the relative percent difference (RPD) between a pair of replicate data from environmental samples prepared and analyzed in duplicate.

5.2 Bias

Bias is the degree of agreement of a measurement with an accepted reference value and may be expressed as the difference between the two measured values or as a percentage of the reference value.

The primary evaluation of bias will be through the use of RMs. RMs with certified values (from NIST or a similar source) will be used if they are available. The laboratory will maintain control charts to track the RM performance. Spiked matrix samples will also be analyzed to assess bias for those analytes that are not available in suitable reference materials.

5.3 Comparability

Comparability expresses the confidence with which one data set can be evaluated in relationship to another data set. Comparability of the chemical analytical data is established through the use of:

- Program-defined general analytical methodology (e.g., low resolution MS), detection limits, bias and precision requirements and reporting formats;
- NIST-traceable calibration materials;
- Reference material with each sample batch;
- Analysis of a common "reference oil".

5.4 Completeness

Completeness is a measure of the proportion of data specified in the sampling plan which is determined to be valid. Completeness will be assessed by comparing the number of valid sample results to the total number of potential results planned to be generated. The DQO for completeness is 95%, i.e. no more than 5% of the analytical data missing or qualified as unreliable (rejected).

6.0 QUALITY CONTROL PROCEDURES

No particular analytical methods are specified for this project, but the QA/QC requirements will provide a common foundation for each laboratory's protocols. This "common foundation" includes: (1) the specification of the analytes to be identified and quantified and the minimum sensitivity of the analytical methods and (2) the use of NIST reference materials, and (3) the use of a common MC252 Reference Oil.

Prior to the analysis of samples, each laboratory must provide written protocols for the analytical methods to be used; calculate detection limits for each analyte in each matrix of interest and establish an initial calibration curve in the appropriate concentration range for each analyte. The laboratory must demonstrate its continued proficiency by participation in refereed intercomparison exercises (as available) and repeated analyses of reference materials, calibration checks, and laboratory method blanks. Laboratories will be expected to take corrective actions promptly if measurement quality objectives described in this plan are not met.

A laboratory may be audited at any time to determine and document that they have the capability to analyze the samples and can perform the analyses in compliance with the QA plan. Independent data validation will be undertaken promptly after analyses of each sample batch to verify that measurement quality objectives are met. The data validator will discuss any unacceptable findings with the laboratory as soon as possible, and assist the laboratory in developing a satisfactory solution to the problem.

6.1 Standard Operating Procedures for Analytical Methods

Prior to the analysis of field samples, each laboratory is required to submit to the QA Coordinator for review and approval, written Standard Operating Procedures (SOPs) detailing the procedures used in sample receipt and handling, sample preparation and analysis, data reduction and reporting. Once approved, the SOPs for each analytical method and from each analytical laboratory will be archived with this plan as part of the QA documentation.

6.2 Determination of Method Detection Limit, Quantitation Range, and Reporting Limits

The analytical laboratory will establish and report a method detection limit (MDL) for each analyte of interest in each matrix, with the exception of oil for which MDLs cannot be accurately determined. The target detection ranges or limits are specified in **Tables 1.1a – 1.1e and 1.1h – 1.1i**. The actual MDLs will be established by following the method in *40CFR part 136*. The quantitation limit (QL) will be defined as the concentration that is equivalent to five times the MDL result, or equivalent to the lowest concentration standard analyzed as part of the initial calibration. Results that are less than 5X the MDL or less than the lowest calibration standard will not be required to meet the measurement quality objectives (MQOs) for precision and bias, because these results may be outside the "quantitation range". Thus, these results may be flagged by the laboratory with a J, to indicate the results are possibly an estimate and have not been required to meet the MQOs. If the analyte is not detected in a sample, the result will be reported as non-detected at the MDL and flagged with a "U".

Note that for the Query Manager electronic data deliverable (EDD), there are two fields: detection limit and reporting limit. The detection limit field is equivalent to the MDL, except for those cases where no MDL value exists (for example, oils). If no MDL value exists, the detection limit field is populated with the quantitation limit (QL). The reporting limit field is always populated with the QL value.

Reporting limits for the supporting analyses (percent moisture, percent total extractable organics [TEO], and total organic carbon) will be 0.01%. Reporting limit for grain size will be 0.1% or lower. The reporting limit will be demonstrated by the laboratory to be greater than 5X the detection limit.

Target detection limits, as shown at the bottom of **Tables 1.1a through 1.1e** and in **Tables 1.1h – 1.1i,** may not be met due to required dilutions, interferences, and/or limited sample size. If a laboratory MDL does not meet the target detection limit, the reason for the elevated detection limits should be discussed in the laboratory case narrative.

At the discretion of the analytical laboratory, detected analytes at concentrations less than the MDL may be reported, provided that the compound meets the established identification criteria and the peak height is greater than or equal to three times the background noise level. These results will be "J" flagged by the laboratory. During validation, these results will be qualified as "F" (found) to indicate that the value is less than the MDL (see Table 7.2).

6.3 Quality Control Criteria

MQOs and required minimum frequency of analysis for each QC element or sample type are summarized in **Tables 6.1a – 6.1i**. The analytical laboratory will determine when MQOs have not been met, and perform appropriate corrective actions before continuing the analyses or reporting of the data. If the "Corrective Action" in the Method Performance Criteria table states "Resolve before proceeding", the laboratory must perform an adjustment to the analytical process and subsequently demonstrate the criteria will be met before proceeding with analysis for project samples. In addition, if results associated with a non-compliant QC element have been obtained, the laboratory must repeat those analyses until acceptable QC results are obtained. If the laboratory determines the non-compliance does not affect the quality of the data, the laboratory will discuss the non-compliance and the rationale, used to conclude the data are not affected, in the case narrative which accompanies the data report. If the laboratory determines the non-compliance is due to interferences or circumstances outside the laboratory's control, the laboratory will discuss the reason for the non-compliance in the case narrative and the results reported.

NIST released a Certificate of Analysis for the MC252 Reference Oil on January 30, 2012, and labeled the reference oil SRM 2779. At the initiation of the project work, vials of this material (SRM 2799) were provided to the participating laboratories and analyzed as indicated in Tables 6.1a through 6.1e and Table 6.1g, and labeled "MC 252 Reference Oil". As of Version 3.1 of this AQAP, QC criteria for the SRM 2779 analysis has been inserted for the extended PAH analysis. The criteria apply to work subsequent to the date of Version 3.1 of the AQAP, and to those analytes that are calibrated against authentic standards and within the calibration range of the analytical run. Because SRM 2779 is now certified, the analysis of the Oil SRM 1582, which was originally required for analysis with each batch of samples, is no longer required. No certified values are currently provided for SHCs or biomarkers in SRM 2779; however, analysis of this SRM for each batch of samples for these tests is still required as listed in Tables 6.1b, 6.1d, and 6.1e, so the results can be used for comparability purposes. In addition, experience with the reference oil has indicated that SRM 2779 need not be run with the VOC analysis (Table 6.1c), nor with the DOSS analysis (Table 6.1g) as the oil may cause undue carryover. No certified values are currently available for VOCs or DOSS in SRM 2779; therefore, analysis of this SRM for these analytes was deleted from these tables

TABLE 6.1a
Method Performance Criteria for Extended PAH (Parent and Alkyl Homologs) and Related Compounds

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Tuning	Prior to every sequence	Tune as specified in laboratory SOP	Resolve before proceeding.
Initial Calibration (All parent PAH and selected alkyl homologue PAH)	Prior to every sequence, or as needed based on continuing calibration/verification check.	5-point calibration curve over two orders of magnitude %RSD ≤ 20	Resolve before proceeding.
Continuing Calibration (CCAL)	Every 12 hours or every 12 field samples	%D ≤ 25 for 90% of analytes %D ≤ 35 for 10% of analytes	Perform instrument maintenance. Re-analyze affected samples.
Initial Calibration Verification (Second Source or can be met if CCAL is second source)	Per initial calibration	%R target analytes 80-120%	Resolve before proceeding.
Matrix SRM 1941b for sediment; SRM 1974c for tissue	One per batch/every 20 field samples	Within ±30% of NIST 95% uncertainty range for analytes within the quantitation range. The results for 2 analytes may be greater than the 30% limit; however, the average %D must be <35%[17]	Resolve before proceeding.
Oil SRM 1582 (Oil and Water only)	Optional	Within ±20% of NIST 95% uncertainty range for analytes within the quantitation range. The results for 2 analytes may be greater than the 30% limit; however, the average %D must be <35%	Resolve before proceeding.
SRM 2779 Gulf of Mexico Crude Oil (originally referred to as MC 252 Reference Oil)	One per batch/every 20 field samples	Peak resolution >80% of 9-methylphenanthrene from 1-methylphenanthrene (m/z 192). Within ±20% of NIST 95% uncertainty range for analytes within the quantitation range. The results for 2 analytes may be greater than the 20% limit; however, the average %D must be <35%	Resolve before proceeding.
Matrix Spike/Matrix Spike Duplicate (Sediments, Soils, Tissues only)	One per batch/every 20 field samples	%R 50% - 125% for target analytes detected <5X the spiked amount; RPD ≤30%, except biphenyl (40%-140%) and decalin (25%-125%)	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Blank Spike/Blank Spike Duplicate (Aqueous Samples)	One per batch/every 20 field samples	%R 50% - 125% for target analytes, RPD ≤30%, except biphenyl (40%-140%) and decalin (25%-125%)	Resolve before proceeding.
Procedural Blank	One per batch/every 20 field samples	No more than 2 analytes to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedance'.
Sample Duplicate (not required for water matrix)	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Mass Discrimination	Initial calibration and CCVs (mid-level)	Ratio for the concentration of Benzo[g,h,i]perylene to phenanthrene ≥0.70	Resolve before proceeding.
Internal Standard (IS)	Every sample	50% - 200% of the area of the IS in the associated calibration standard	Resolve before proceeding.
Surrogates	Every sample	%R 40-120% except d12-perylene which is 10-120%	Re-extract affected samples. Evaluate impact to data, discuss with manager, if corrective action is needed.

[17] Except for fluorene in SRM 1941b, extend the low end to 40%.

TABLE 6.1b
Method Performance Criteria for Alkanes/Isoprenoids Compounds and Total Extractable Hydrocarbons

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Initial Calibration (Standard solution - all target analytes, except phytane, and C_{31}, C_{33}, C_{35}, and C_{39} n-alkanes)	Prior to every sequence, or as needed based on continuing calibration/verification check.	5-point calibration curve %RSD ≤ 20	Resolve before proceeding.
Continuing Calibration (CCAL)	Every 12 hours or every 12 field samples	%D ≤ 15 for 90% of analytes %D ≤ 20 for 10% of analytes	Perform Instrument Maintenance. Re-analyze affected samples.
Initial Calibration Verification (Second Source or can be met if CCAL is second source)	Per initial calibration	%R target analytes 80-120%	Resolve before proceeding.
SRMs - no SRMs for SHC or TPH are available at this time			
SRM 2779 Gulf of Mexico Crude Oil (originally referred to as MC 252 Reference Oil)	One per batch/every 20 field samples	Peak resolution >80% of n-C17 from pristane; Additional criteria to be developed.	Resolve before proceeding.
Matrix Spike/Matrix Spike Duplicate (Sediments, Soils, Tissues only)	One per batch/every 20 field samples	%R 50% - 125% for target analytes detected at <5X the spiked amount; RPD ≤ 30%.	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Blank Spike/Blank Spike Duplicate (Aqueous Samples)	One per batch/every 20 field samples	%R 50% - 125% for target analytes, RPD ≤ 30%.	Resolve before proceeding.
Procedural Blank	One per batch/every 20 field samples	No more than 2 analytes to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedances'.
Duplicate Sample Analysis (not required for water matrix)	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Mass Discrimination	Initial calibration and CCVs (mid-level)	Ratio for the raw areas of n-C36 / n-C20 ≥0.70	Resolve before proceeding.
Surrogates	Every sample	%R 40-125%	Re-extract affected samples. Evaluate impact to data, discuss with manager, and determine if corrective action is needed.

TABLE 6.1c
Method Performance Criteria for VOCs

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Tuning	Prior to every sequence	Per SW846 8260B	Resolve before proceeding
Initial Calibration (ICAL)	Prior to every sequence, or as needed based on continuing calibration/verification check.	Minimum of 5 concentration levels %RSD ≤ 25% for 90% of analytes %RSD ≤ 35% for all analytes >C6	Resolve before proceeding.
Continuing Calibration (CCAL)	Every 12 hours or every 12 field samples	%D ≤ 25% for 90% of analytes %D ≤ 35% for all analytes >C6 Except t-butanol <50%	Perform Instrument Maintenance. Re-analyze affected samples.
Initial Calibration Verification (Second Source or can be met if CCAL is second source)	Per initial calibration	%R target analytes 80-120%. Except 2 analytes can be at 60 - 140%	Resolve before proceeding.
SRMs – No SRMs are available at this time			
Matrix Spike/Matrix Spike Duplicate (Sediments, Soils)	One per batch/every 20 field samples	%R 50% - 130% for target analytes detected at <5X the spiked amount; RPD ≤30%.	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Blank Spike/Blank Spike Duplicate (Aqueous Samples)	One per batch/every 20 field samples	%R 50% - 130% for target analytes, RPD ≤30%.	Resolve before proceeding.
Procedural Blank	One per batch/every 20 field samples	No more than 2 analytes to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedances'.
Sample Duplicate	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Internal Standard (IS)	Every sample	50% - 200% of the area of the IS in the associated calibration standard	Resolve before proceeding.
Surrogates	Every sample	%R 70-130%	Re-extract or re-analyze affected samples. Evaluate impact to data, discuss with manager, and determine if corrective action is needed.

TABLE 6.1d
Method Performance Criteria for Quantitative Biomarkers

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Tuning	Prior to every sequence	Tune as specified in laboratory SOP	Resolve before proceeding.
Initial Calibration	Prior to every sequence, or as needed based on continuing calibration/verification check.	5-point calibration curve over two orders of magnitude %RSD ≤ 20	Resolve before proceeding.
Continuing Calibration (CCAL)	Every 12 hours or every 12 field samples	%D ≤ 25 for 90% of analytes %D ≤ 35 for 10% of analytes	Perform instrument maintenance. Re-analyze affected samples.
Oil SRM 1582 (Oil and Water only)	Optional	Biomarker concentrations are not certified; Peak resolution (*m/z* 191) of: (a) oleanane (T18) from hopane (T19); (b) C26 Tricyclic Terpane stereoisomers 22R (T6b) from 22S (T6c) and from C24 Tetracyclic Terpane (T6a)	Resolve before proceeding.
SRM 2779 Gulf of Mexico Crude Oil (originally referred to as MC 252 Reference Oil)	One per batch/every 20 field samples	Peak resolution (*m/z* 191): 30-Norhopane (T15) from 30-Norneohopane (T16) from Diahopane (X). Add'l. criteria To Be Determined.	Resolve before proceeding.
Method Blank	One per batch/every 20 field samples	No more than 2 analytes to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedance'.
Sample Duplicate	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Internal Standard (IS)	Every sample	50% - 200% of the area of the IS in the associated calibration standard	Resolve before proceeding.
Surrogate	Every sample	%R 50-130%	Evaluate impact to data, discuss with manager, if corrective action is needed.

TABLE 6.1e
Method Performance Criteria for Qualitative Biomarkers

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Oil SRM 1582 (Oil and Water only)	Optional	Peak resolution (m/z 191) of: (a) oleanane (T18) from hopane (T19); (b) C26 Tricyclic Terpane stereoisomers 22R (T6b) from 22S (T6c) and from C24 Tetracyclic Terpane (T6a)	Resolve before proceeding.
SRM 2779 Gulf of Mexico Crude Oil (originally referred to as MC 252 Reference Oil)	One per batch/every 20 field samples	Peak resolution (*m/z* 191): 30-Norhopane (T15) from 30-Norneohopane (T16) from Diahopane (X). Add'l. criteria To Be Determined.	Resolve before proceeding.
Method Blank	One per batch/every 20 field samples	No interference with biomarker patterns	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedance'.
Sample Duplicate	One per batch/every 20 field samples	Qualitative comparison meets laboratory SOP	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.

TABLE 6.1f
Method Performance Criteria for General/Conventional Chemistry

Conventional Sediment Parameters: Total Organic Carbon (TOC), Grain Size, Total Solids
Tissues: Total Extractable Organics (TEO)

QC Element or Sample Type	Minimum Frequency	Acceptance Criteria	Relevant Parameter(s) Reference Methods*
Initial Calibration	Prior to analysis (method and instrument specific procedures & number of standards)	For multipoint calibration, Correlation coefficient (r) >0.995	TOC
Continuing Calibration	Must start and end analytical sequence and every 10 samples	%R 90%-110%	TOC
Method Blanks	One per batch/every 20 field samples	Not to exceed QL	TOC, TEO
Blank Spike Samples	One per batch/every 20 field samples	%R 75% - 125%	TOC
Matrix Spike Samples	One per batch/every 20 field samples	%R 75% - 125% If MS/MSD analyzed, RPD ≤ 25%	TOC
Replicate Analyses[18]	Each sample must be analyzed at least in duplicate. The average of the replicates shall be reported.	RPD or %RSD < 20% for concentrations > QL	TOC
Sample Duplicates[19]	One per batch/every 20 field samples	RPD ≤ 25% for analyte concentrations greater than QL	TOC, Grain Size, TS, TEO
Reference Materials TOC NIST 1941B TEO NIST 1974B	One per batch/every 20 field samples	Values must be within ±20% of NIST uncertainty range	TOC, TEO

*** Reference Methods**

TOC Plumb 1981 or SW 846 Method 9060A or Standard Methods 5310C or ASTM D4129-82M, or equivalent

Grain Size ASTM D422 or PSEP 1986 Particle Size. If using sieve analysis only, report as percent gravel, coarse sand, medium sand, fine sand, very fine sand, and silt/clay. If using sieve with hydrometer or sieve with pipette, report as percent gravel, coarse sand, medium sand, fine sand, very fine sand, silt, and clay. Additionally, grain size must be reported as "True" for sediment treated with hydrogen peroxide prior to analysis or "Apparent" for sediment not treated with hydrogen peroxide.

TS (percent) EPA 160.3

Method 9000 series - analytical methods from SW-846 (U.S. EPA 1986) and updates
The SW-846 and updates are available from the web site at: http://www.epa.gov/epaoswer/hazwaste/test/sw846.htm
Plumb (1981) - U.S. EPA/U.S. Army Corps of Engineers Technical Report EPA/CE-81-1:
http://yosemite.epa.gov/r10/CLEANUP.NSF/ph/T4%20Technical%20Documents/$FILE/Plumb.pdf
PSEP. 1986. "Recommended Protocols for Measuring Conventional Sediment Variables in Puget Sound." Prepared for the Puget Sound Estuary Program.

[18] Method SW9060 requires quadruplicate analyses, however duplicate or triplicate analyses are acceptable. Standard Method 5310C requires that injections be repeated until consecutive measurements within 10% are obtained for a water matrix, however, duplicate analyses < 20% RPD are acceptable based on a sediment matrix.

[19] Method SW9060 requires a duplicate spike. A matrix spike and sample duplicate are acceptable in lieu of matrix spike/matrix spike duplicates. For grain size, RPD criteria only applied if fraction is greater than 5%.

TABLE 6.1g
Method Performance Criteria for Analysis of Dioctylsulfosuccinate sodium salt (DOSS)

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Initial Calibration	Prior to every sequence, or as needed based on continuing calibration/verification check.	5-point calibration curve over two orders of magnitude %RSD ≤ 20	Resolve before proceeding.
Continuing Calibration (CCAL)	Every 12 hours	%D ≤ 30	Perform instrument maintenance. Re-analyze affected samples.
Initial Calibration Verification (Second Source or can be met if CCAL is second source)	Per initial calibration	%R target analytes 70-130%	Resolve before proceeding.
Matrix Spike/Matrix Spike Duplicate (Sediments, Soils, Tissues only)	One per batch/every 20 field samples	%R 50% - 125% if sample concentration detected at <5X the spiked amount; RPD ≤30%	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Blank Spike/Blank Spike Duplicate (Aqueous Samples)	One per batch/every 20 field samples	%R 50% - 125; RPD ≤30%	Resolve before proceeding.
Method Blank	One per batch/every 20 field samples	Not to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding.
Sample Duplicate (not required for water matrix)	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Internal Standard (IS)	Every sample	50% - 200% of the area of the IS in the associated calibration standard	Resolve before proceeding.
Surrogates	Every sample	%R 40-120%	Re-extract affected samples. Evaluate impact to data, discuss with manager, if corrective action is needed.

TABLE 6.1h
Measurement Quality Objectives for Metals by ICP-AES & ICP-MS and Mercury by CVAA/CVAFS

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
ICP-MS Tune	Daily at the beginning of each 24 hour shift. Must start each analytical sequence.	Tuning solution must contain elements spanning all the mass regions of interest (see EPA methods 200.8 & 6020). Analyze 5 times with RSD ≤ 5% Resolution <0.9amu at 10% peak height Mass calibration < 0.1 amu difference from target mass	Resolve before proceeding
Initial Calibration	Daily prior to sample analysis.	Minimum of a 2 point curve for ICP-AES/ICP-MS (1 blank + 1 standard containing all target analytes) Min 5 point curve for CVAA/CVAFS r > 0.995 for multi-point curves	Resolve before proceeding
Independent (Initial) Calibration Verification (ICV)	Analyzed immediately after calibration and prior to samples	Different source than calibration standards Concentration near mid-point of calibration curve Must contain all target analytes to be reported %R = 90% - 110%	Resolve before proceeding
Initial Calibration Blank (ICB)	Must be analyzed after each ICV	ICB < RL for all target analytes	Resolve before proceeding
Reporting Limit Standard (CRI)	Daily prior to sample analysis if initial calibration did not contain a low-level standard at the RL for each target analyte. If initial calibration includes the RL as the low-level standard in the initial calibration curve, then RL Std is not required.	Prepare using same source as calibration standards all target analytes at a concentration = RL %R = 70% - 130%	Resolve before proceeding unless all target analytes in associated samples are > 10x RL
Interelement Interference Check Standards (ICSA & ICSAB)	Daily prior to sample analysis	See EPA methods for ICSA & ICSAB concentrations of interferents and other analytes; for ICP-AES checks on background points and instrument interelement interference corrections; for ICP-MS checks on isobaric interference corrections. ICSA & ICSAB: %R = 80% - 120%	Resolve before proceeding
Continuing Calibration Verification (CCV)	Must be analyzed before samples, after every 10 samples, and at end of each analytical sequence	CCV concentration should be near mid-point of calibration curve and contain all target analytes %R = 90% - 110%	Perform instrument maintenance. Re-analyze affected samples.
Continuing Calibration Blank (CCB)	Must be analyzed after each continuing calibration verification (CCV)	CCB < RL for all target analytes Unless: analyte not detected in associated sample(s) or sample analyte concentrations are >10x the blank value	Resolve before proceeding
Method Blank	Every batch (max. 20 field samples).	No analytes to exceed the reporting limit unless analyte not detected in associated sample(s) or detected in samples at >10x the blank value	Resolve before proceeding

TABLE 6.1h
Measurement Quality Objectives for Metals by ICP-AES & ICP-MS and Mercury by CVAA/CVAFS

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Laboratory Control Sample or Reference Material Possible sediment RMs: NRCC MESS-3 or PACS-2 NIST 1646A*, 1944*, 2702 ERA 540 Possible tissue RMs: NRCC DOLT-4, DORM-3, or TORT-2; NIST 2976 or 1947	Every batch (max. 20 field samples).	Reference Material or laboratory control sample must be matrix-matched to the field samples and prepared/analyzed with the sample batch. Aqueous: %R = 80% - 120% Sediment & Tissue: Values must be within ±30% of the vendor 95% confidence limits for true values >RL	Resolve before proceeding
Matrix Spike (MS)	Every batch (max. 20 field samples).	Must be performed on a NOAA sample from same preparation/analysis batch. Must contain all target analytes to be reported. Sediment/Tissue: %R = 70% - 130% (For native conc. < 4X spike added)	If a MS %R is <30%, a post digestion spike should be analyzed and fall within 75%-125%. See EPA Method 6010C and 6020A for details on spike levels and evaluation. Report QC exceedance in data package narrative.
Sample Duplicate (or matrix spike duplicate)	Every batch (max. 20 field samples).	Sediment/Tissue: RPD ≤ 30% if value > RL	Report this QC exceedance in data package narrative.
Internal Standards (ICP-MS only)	Every sample (QC & field samples)	See EPA methods 200.8 and 6020 for recommended IS elements.Relative intensity of IS %R = 70% - 130% compared to IS of standard in calibration curve (or mid-point standard of calibration for multi-level curve).	Check for instrument drift. If IS in assoc. CCB is acceptable, then dilute sample 5X and re-analyze until IS in control for affected analyte(s). If instrument drift is indicated, recalibrate and re-analyze.
General Reporting	Every sample	• Non-detected values should be reported to the sample-specific MDL or RL to achieve the target sensitivity levels listed in Table 1.1h for each target analyte (using all preparation/dilution factors). • Reporting of detected results less than the RL must be qualified "J" as estimated values. • Results > the linear range must be diluted to within the LR; the diluted result will be reported for the affected analyte.	Include explanation of all non-compliances observed in sample receipt, holding times, preparation, or analysis in the laboratory narrative of the data report.

* These SRMs do not have a certified value for Mercury

TABLE 6.1i
Method Performance Criteria for PCB Congeners

Element or Sample Type	Minimum Frequency	Measurement Quality Objective/ Acceptance Criteria	Corrective Action
Instrument Tuning	Prior to every sequence	Tune as specified in laboratory SOP	Resolve before proceeding.
Resolution Checks (if appropriate)	Prior to every sequence	As specified in laboratory SOP	Resolve before proceeding.
Initial Calibration (ICAL)	Prior to every sequence, or as needed based on continuing calibration/verification check.	5-point calibration curve %RSD ≤ 20% for target analytes 10% of targets may have %RSD >20 but ≤30 %RSD ≤ 35% for labeled compounds (if analyzed)	Resolve before proceeding. Recalibrate as required by method.
Continuing Calibration (CCAL)	Every 12 hours or every 12 field samples	%D ≤ 25% for target analytes 10% of targets may have %D >25 but ≤35 %D ≤ 35% for labeleds (if analyzed)	Perform instrument maintenance. Re-analyze affected samples.
Initial Calibration Verification (Second Source or can be met if CCAL is second source)	Per initial calibration	%R target analytes 80% - 120% 10% of targets may have %R >20 but ≤30	Resolve before proceeding.
Matrix SRM 1941b for sediment; SRM 1974c for tissue	One per batch/every 20 field samples	Within ±30% of NIST 95% uncertainty range for analytes within the quantitation range. The results for 2 analytes may be greater than the 30% limit; however, the average %D must be < 35%	Resolve before proceeding.
Matrix Spike/Matrix Spike Duplicate (if analyzed)	One per batch/every 20 field samples	%R 50% - 125% for target analytes where spiked amount >5X the detected concentration RPD ≤ 30%	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Blank Spike (OPR)	One per batch/every 20 field samples	LRMS: %R 50% - 125% for targets HRMS: %R 60% - 135% RPD ≤ 30% (if duplicate analyzed)	Resolve before proceeding.
Procedural Blank	One per batch/every 20 field samples	No more than 2 analytes to exceed 5x target MDL unless analyte not detected in associated samples(s) or analyte concentration >10x blank value	Resolve before proceeding. QA coordinator may be contacted to resolve issues surrounding 'minor exceedance'.
Sample Duplicate	One per batch/every 20 field samples	RPD ≤ 30% if analyte concentration is greater than QL	Evaluate impact to data, discuss with manager, and determine if corrective action is needed.
Surrogates	Every sample	LRMS: %R 40% - 120% HRMS: %R 25% - 150% except monochlorobiphenyls at 15% - 150% or laboratory limits defined in SOP	Re-extract affected samples. Evaluate impact to data, discuss with manager, if corrective action is needed.
Internal Standards	Every sample	LRMS: 50% - 200% of the area of the IS in the associated calibration standard HRMS: same as extraction standards	Resolve before proceeding.
Window Defining/Column Performance Mix (HRMS)	Before every initial and continuing calibration	Valley < 40% for PCB 34 & 23 and 187 & 182. PCB 156 & 157 must elute within 2 seconds of each other	Resolve before proceeding.

LRMS – low resolution mass spectroscopy
HRMS – high resolution mass spectroscopy

6.3.1 Initial Calibration

Acceptable calibration (initial and continuing) must be established and documented before sample analyses may begin. NIST-traceable calibration materials must be used where available in establishing calibration. Initial calibrations will be established according to the criteria in **Tables 6.1a – 6.1d and 6.1f - 6.1i**. A specific requirement for this project is to use methodology (and tune instrumentation) for low detection limits, therefore, samples with analytes above the calibration range will be diluted and reanalyzed. If samples require a dilution, results from the initial analytical run that were within the calibration range should be reported. Results from the diluted analyses should be reported for only those analytes which exceeded the calibration.

6.3.2 Continuing Calibration Verification

Continuing calibration verification (CCV) standards will be run at the beginning (opening) and end (closing) of each analytical sequence, and at the frequencies indicated in **Tables 6.1a – 6.1d and 6.1f - 6.1i**. If CCV results do not meet the specified criteria, then the instrument must be re-calibrated and all samples analyzed since the last acceptable CCV must be re-analyzed.

6.3.3 Reference Materials

Reference materials of a matrix appropriate to the samples being analyzed, will be analyzed every 20 samples throughout the analytical program, if available. The data resulting from the analysis of these samples will be reported in the same manner as that from the field samples. These data will be the prime materials used to determine and document the accuracy and precision of the associated field sample data. The reference materials to be used are listed in the criteria tables.

Accuracy is computed by comparing the laboratory's value for each analyte against either end of the range of values reported by the certifying agency. The laboratory's value must be within 30% of either the upper or lower end of NIST's 95% uncertainty range for SRM 1941b and SRM 1974b except the low end for fluorene for 1941b is extended to 40%. SRM 2779 (MC 252 Reference Oil) is not extracted, but only diluted and analyzed on the instrument, thus the laboratory's value must be within 20% of the NIST uncertainty range for the applicable extended PAH analytes (Table 6.1a). Chromatographic resolution criteria of selected peak pairs in SRM 2779 are indicated in **Tables 6.1a, 6.1b, 6.1d, and 6.1e**.

6.3.4 Method Blanks

Method (procedural) blanks are laboratory derived samples which have been subjected to the same preparation or extraction procedures and analytical protocols as project samples. A method blank will be analyzed with every 20 field samples analyzed. Acceptance criteria are provided in **Tables 6.1a – 6.1i**. Failure to meet acceptance criteria requires definitive corrective action to identify and eliminate the source(s) of contamination before the subsequent reanalysis and re-extraction of the blank and affected samples. Sample results will not be blank corrected.

6.3.5 Sample Duplicates

A duplicate sample aliquot from a representative matrix will be prepared and analyzed with every 20 field samples, except for water samples, filters, and inert sorbent materials for SHC/TEH, PAH, and PCB congeners. Water samples, filters and inert sorbent materials for SHC/TEH, PAH, and PCB

congeners will not be analyzed in duplicate because of the difficulty in subsampling representative aliquots. If duplicate VOA vials are collected, then volatile organic analyses may be performed in duplicate. Acceptance criteria are provided in **Tables 6.1a – 6.1i**.

6.3.6 Matrix Spike/Matrix Spike Duplicates or Blank Spike/Blank Spike Duplicate

Matrix spike/matrix spike duplicates (MS/MSDs) will be analyzed every 20 samples, except for water samples, filters and inert sorbent materials. MS/MSDs will not be analyzed with the water sample batches because of the difficulty in subsampling representative aliquots from a sample container. Instead, blank spike/blank spike duplicates (BS/BSDs) will be analyzed with each batch of water samples. Samples will be spiked prior to extraction. Spike solution concentrations for the MS must be appropriate to the matrix and anticipated range of contaminants in the sample; that is 2 to 10 times analyte concentration. However, because it is not possible to know the concentration of contaminants prior to analysis, professional judgment may be exercised in choosing concentrations that are reasonable under the circumstances. Acceptance criteria are provided in **Tables 6.a – 6.1c, 6.1g - 6.1i.** Acceptance criteria for conventionals matrix spike and blank spike samples are provided in **Table 6.1f**.

6.3.7 Internal Standards

All samples will be spiked with internal standards prior to analysis, when required by the analytical method. Control criteria for internal standard recovery are listed in **Tables 6.1a, 6.1c, 6.1d, 6.1g, and 6.1i**.

6.3.8 Surrogates

All field and QC samples will be spiked with surrogates prior to extraction, as required by the analytical methods. Control criteria for the surrogate recovery are listed in **Tables 6.1a – 6.1d, 6.1g, and 6.1i**. For the PAH and saturated hydrocarbon analyses, the target analyte concentrations will be corrected for surrogate recovery as specified in the laboratory SOPs. For the PCB congeners, if isotope dilution analysis is performed, the target analyte concentration will be corrected for the labeled compound recovery, as specified in the analytical method and laboratory SOPs.

7.0 DATA REDUCTION, VALIDATION AND REPORTING

7.1 Data Reduction

Data reduction is the process whereby raw data (analytical measurements) are converted or reduced into meaningful results (analyte concentrations). This process may be either manual or electronic. Primary data reduction requires accounting for specific sample preparations, sample volume (or weight) analyzed, and any concentrations or dilutions required.

Primary data reduction is the responsibility of the analyst conducting the analytical measurement and is subject to further review by laboratory staff, the Laboratory Project Manager and finally, independent reviewers. All data reduction procedures will be described in the laboratory SOPs. Any deviations from the laboratory SOPs will be discussed in the laboratory case narratives.

- Concentrations will be reported as if three figures were significant.

- Data generated from the analysis of blank samples will not be utilized for correction of analyte data.
- Surrogate compounds, matrix spikes, and spike blanks will be evaluated as %R.
- Reference materials will be reported in units indicated on the certificate of analysis.
- Continuing calibration factors will be presented as %D
- Duplicate sample results will be expressed as RPD.

7.2 Data Review and Validation

Data review is an internal review process where data are reviewed and evaluated by personnel within the laboratory. Data validation is an independent review process conducted by personnel not associated with data collection and generation activities.

Data review is initiated at the bench level by the analyst, who is responsible for ensuring that the analytical data are correct and complete, the appropriate SOPs have been followed, and the QC results are within the acceptable limits. The Laboratory Project Manager has final review authority. It is the Laboratory Project Manager's responsibility to ensure that all analyses performed by that laboratory are correct, complete, and meet project data quality objectives.

External and independent data validation will be performed for all samples by the QA Contractor using a full data package containing sufficient information to allow the independent validation of the sample identity and integrity, the laboratory measurement system, and resulting quantitative and qualitative data. The required information with associated instrument print-outs are listed in **Table 7.1**.

TABLE 7.1 Laboratory Data Deliverables Per Sample Batch

Chain-of-Custody/ Sample Receipt Checklist	
Sample Data:	Result summaries including spiked compound recoveries, percent total solids, dilutions, etc
Standards Data:	Target MDL data based on the method in *40 CFR, 136* Calibration summaries: Initial calibration data, standard curve equation, correlation coefficient or %RSD, continuing calibration %D.
Quality Control Data (Method Blanks, CRMs, Duplicates, Matrix Spikes, Spike Blanks):	Results summaries including spiked compound recoveries and RPD, as applicable.
Case Narrative:	Special handling or analysis conditions. Any circumstance that requires special explanation such as an exception to QA/QC conditions or control criteria, dilutions, reanalysis, etc. Corrective actions/procedure alterations
Chromatograms and Extracted Ion Profiles	Appropriately scaled (1) GC/FID chromatograms for samples and associated QC analyzed for extractable hydrocarbons; (2) GC/MS EIPs for samples and associated QC analyzed for qualitative biomarkers
Electronic Data Deliverable:	As specified in laboratory contract.

Three levels of data validation will be performed (see USEPA, *Guidance for Labeling Externally Validated Laboratory Analytical Data for Superfund Use.* EPA-540-R-08-005. January 2009 for definitions): full (stage 4), summary (stage 2B), or cursory (stage 2A) validation. Full validation will consist of a review of the entire data package for compliance with documentation and quality control criteria for all the following items, plus recalculations of instrument calibration curves, sample and QC results. Summary validation will consist of a review of all the following items, but without recalculations. Cursory validation will consist of a review of only the starred (*) items:

- Package completeness*
- Holding times from extraction to analysis*
- Instrument calibration, initial and continuing
- Blank results*
- Instrument performance
- Spike recoveries*
- Standard reference material results*
- Laboratory duplicate results*
- Reported detection limits*
- Compound quantitation
- Compound identification
- Verification of electronic data deliverable (EDD) against hardcopy (10% verification)*

As the project proceeds and the quality of the data is verified and documented, the level of validation will decrease at the discretion of the QA Coordinator. At a minimum, cursory validation will be performed on all data packages, i.e., only the starred items will be reviewed.

Qualifiers (**Table 7.2**) may be assigned to individual data points by the QA Contractor. These validation qualifiers will not replace qualifiers or footnotes provided by the laboratory, but will be added to the data summary tables to inform the data user whether or not the data met all project quality objectives. Both sets of qualifiers will be maintained in the database.

TABLE 7.2 Data Validation Qualifier Codes

U	Analyte concentration is not significantly greater than the associated blank result. The result is judged to be the detection limit.
R	Unreliable result. Data should not be used.
N	The analysis indicates the present of an analyte for which there is presumptive evidence to make a "tentative identification".
NJ	The analysis indicates the presence of an analyte that has been "tentatively identified" and the associated numerical value represents its approximate concentration.
J	Reported concentration is an estimate with potentially more bias or less precision than an unqualified result, as determined by the associated quality control results.
UJ	Not detected. Detection limit is an estimate with potentially more bias or less precision than an unqualified detection limit as judged by the associated quality control results
DNR	Do not report; A more appropriate result is reported from another analysis or dilution.
F	Found. Analyte detected at less than the MDL, however, peak height is greater than 3 times the noise level and ID criteria are met.

All discrepancies and requests for additional corrected data will be discussed with the laboratory prior to issuing the formal data validation report. Review procedures and findings during data validation will be documented on worksheets. A validation report will be prepared for each data group/data package summarizing QC results, qualifiers, and possible data limitations. Only validated data with appropriate qualifiers will be released for general use. Data are not considered final until QA Coordinator has performed assessment and accepted the data.

In addition, the validated data will be reviewed by the QA Reviewer on behalf of BP America. The following process shall be used should the independent validation of the laboratory data results in a material difference in how qualifiers have been assigned or in the actual value itself:

- The QA Coordinator and QA Reviewer will meet to determine the source of the difference, and resolve. No changes to validated results will be made if the differences are considered immaterial to both the QA Coordinator and QA Reviewer.
- If the validated data have already been released by the QA Coordinator, then the data will be updated in accordance with the resolution and reposted.
- Should there be no agreement on how to resolve the difference, the QA Coordinator and QA Reviewer shall request further assistance from the Assessment Managers and BP America, respectively.
- The basis for all material changes to validated results will be documented along with the resubmitted validated data.

8.0 CORRECTIVE ACTION AND PROCEDURE ALTERATION

The analytical laboratories are required to adhere to the SOPs submitted by them to the QA Coordinator for this project. When the data from the analyses of any quality control sample exceeds the project specified control limits or indicates that the analytical method is drifting out of control, it is the

immediate responsibility of the analyst to identify and correct the situation before continuing with sample analysis.

A narrative describing the problem noted, the steps taken to identify and correct the problem and the treatment of the relevant sample batches must be prepared and submitted with the relevant data package. If the action indicates a revision to the accepted SOP is warranted, the laboratory will revise the SOP and resubmit the SOP to the QA Coordinator within 30 working days after the problem was noted. Until the revised SOP is approved, any data sets reported with the revised method will have the any changes to the method noted in the laboratory's case narrative.

9.0 QUALITY ASSURANCE REPORTS TO MANAGEMENT

Quality Assurance/Quality Control (QA/QC) reports will be submitted periodically to the Assessment Manager(s) by the QA Coordinator. These reports may be either formal or informal in response to the Assessment Manager's request. Upon termination of the analytical work for this damage assessment, a formal QA report will be submitted. This report will include:

- General compliance with QA objectives
- Summary of technical and performance evaluation audits
- Summary of data validation reports
- Summary of laboratory control charts

10.0 REFERENCES

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E. Retention of Samples and Solutions Generated during Toxicity Testing Memorandum

This appendix was not intended for public release and has been redacted. Note that information contained in this confidential memorandum is provided throughout Section 4 of the project QAPP.

F. Standard Operating Procedure – Fluorescence Spectroscopy to Verify Dilutions of Water Accommodated Fraction for Toxicity Testing

Preamble

When excited by UV light, substances containing conjugated double bonds will emit light (or heat) at longer wavelengths, causing fluorescence. The fluorescent properties of a substance are measurable and increase proportionally with concentration. PAHs are found in complex mixtures such as oil, and fluoresce when exposed to UV light. Although fluorescence cannot provide an exact measurement of total PAH (TPAH) or total petroleum hydrocarbons (TPHs) in water, fluorescence varies in direct proportion to the concentrations of aromatic compounds that fluoresce at specific excitation wavelengths. Therefore, assuming that these constituents are present in the same proportions in as the stock sample, fluorescence can be used to estimate relative concentrations across a dilution series of that stock.

Fluorescence has many benefits, including ease of use, low cost, minimal sample preparation, quick sample analysis, and the ability to analyze large numbers of samples on a daily basis. One primary benefit of this method is the ability to rapidly verify the relative PAH concentrations in the different dilution splits of a toxicity test. This allows near real-time data to ensure that the experiment has been set up properly. As described in detail in this SOP, this involves:

- Optimizing the excitation and emission wavelengths of the fluorescent spectrometer for the oil and WAF preparation type being tested
- Measuring the fluorescence of the test solutions prepared by diluting the stock solutions
- Verifying the accuracy of test dilutions by comparing to a standard curve (Figure F.1).

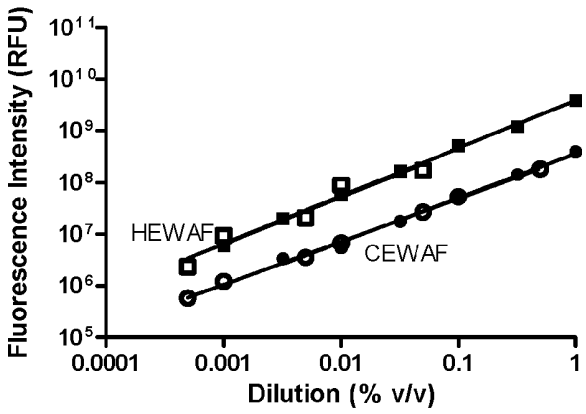

Figure F.1. Relationship between standard curves prepared from stock solutions of HEWAF and CEWAF of crude oil and dilution curves for toxicity test solutions prepared from the same stock solutions and sampled at Time 0, immediately after preparation. Open symbols represent standard curve data and closed symbols represent toxicity test dilutions. The data for toxicity test solutions fall on top of standard curves because test solutions were sampled immediately after preparation.

1. Wavelength optimization

a. Optimize excitation wavelengths *for each oil and WAF preparation type* or each time a composition change is suspected.
b. Use a high-concentration standard solution to optimize wavelength (e.g., 1 ppm).
c. Put the sample in the spectrometer.
d. Warm up the lamp according to manufacturer's instructions.
e. Open an emission scan. Enter an excitation wavelength of approximately 300 nm and an emission range of 310–460 nm. Ensure that the emission wavelength range always begin at least 5 nm higher than the excitation wavelength to avoid recapturing excitation light. The initial emission range should span 150 nm to ensure the entire peak is captured. This can be reduced to speed up analysis if the end of the range is determined to not be useful for measuring oil in water.
f. Set the step size to 2 nm to further increase analysis speed. Ensure that the step size does not exceed 2 nm; exceeding 2 nm will decrease the integrity of the analysis.
g. Start the emission scan. You will hear some clicking sounds as the monochromators are set to the wavelengths you selected.
h. After the scan is complete, record the wavelength at which the peak maximum occurs.

i. Open an excitation scan. Set the emission wavelength to the recorded peak wavelength from the previous step. The excitation range should span 150 nm. Set the highest wavelength in this range a minimum of 5 nm lower than the emission wavelength as mentioned above. Set the step size to 2 nm.

j. Start the excitation scan.

k. After the scan is complete, record the wavelength at which the peak maximum occurs. If this peak excitation is different from the excitation wavelength used in the first emission scan, use this new peak excitation wavelength for a new emission scan, and begin again at step e. The resulting curve should lie higher than the initial emission scan as the optimal wavelength range is approached.

l. Repeat this process of using peak wavelengths to run excitation and emission scans in tandem until the peak wavelengths do not differ from previous scans. The maximum peaks from both the optimal excitation and emission scans should be about the same height and the curves will look roughly symmetrical (Figure F.2).

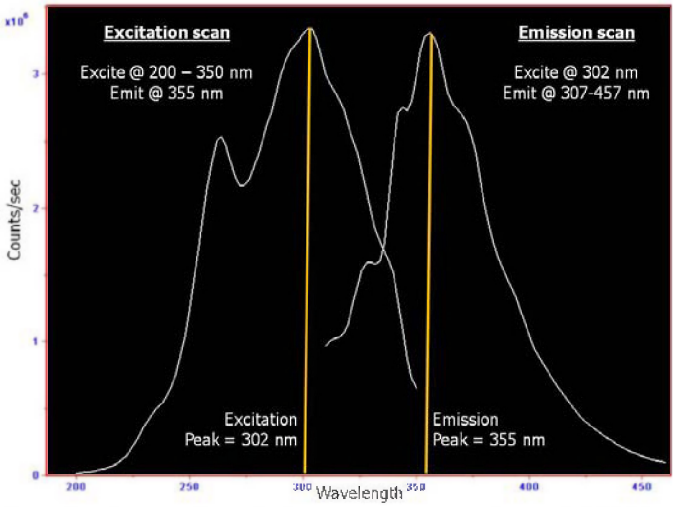

Figure F.2. Optimal excitation and emission scan curves for a 0.032% v/v CEWAF using Bunker C fuel oil.

2. Sampling and storage

Take a single 3.5-mL sample from each WAF stock and dilution series and add to 3.5 mL of ethanol in a 7-mL borosilicate scintillation vial with a foil- or Telfon-lined cap. Wrap Teflon tape or parafilm around the cap to minimize ethanol evaporation during storage. Analyze freshwater samples within 48 hours of sampling; analyze saline water samples within 1 week of sampling. Store samples in the dark at 4°C when not being handled for analysis. All samples, controls, exposure solutions, and standards should be prepared using the same water source.

3. WAF standard curve

A standard curve indicates the range of WAF concentrations over which the relationship between fluorescence and hydrocarbon concentrations are linear. It also demonstrates variance due to pipetting, and the reproducibility of test solutions from day-to-day and from experiment-to- experiment. Standard curves are characterized by their slopes, intercepts, and statistics related to "goodness of fit." With time, each laboratory will accumulate a database of standard curves that demonstrates the extent to which they vary, and the dilutions of each type of WAF needed to fall within a linear range. These data provide the confidence needed to reduce the frequency and number of dilutions on standard curves to streamline routine checks on preparation of test solutions.

a. Standard curves can be prepared for WAF, HEWAF, CEWAF, or any other solution that requires direct comparison. The WAF used for the standard curve should be the same WAF used for test dilutions.
b. Prepare the WAF and dilute in water to obtain a range of exposure concentrations. Add equal parts ethanol to preserve the sample.
c. Open an emission scan and enter the optimized excitation and emission wavelengths. Change the step size to 2 nm.
d. Before analyzing standards, measure the fluorescence of an ethanol control, water control, and a 50:50 ethanol:water solution control. Prepare the controls using the same water and ethanol source as the standards and samples. The ethanol and water controls are a check against contamination, The 50:50 ethanol:water control provides a baseline with a total area less than 1,000,000 relative fluorescence units (RFUs). Prepare new controls and re-run if contamination is suspected.
e. Prepare standards in 20-mL glass scintillation vials using test solution water. Add water to the vials first, then ethanol, and finally the sample.
f. Vortex each solution before sampling for dilution and after preparation. The dilutions (below) can be altered to reflect the test dilutions, time constraints, and the linear portion of the curve.

i. 100% v/v stock solution
ii. 50% v/v
    1. 5 mL of 100% v/v stock +5 mL EtOH
iii. 10% v/v
    1. 1 mL of 100% v/v stock + 4 mL water + 5 mL EtOH
iv. 3.2% v/v
    1. 320 µL of 100% v/v stock + 4.68 mL water + 5 mL EtOH
v. 1.0% v/v
    1. 100 µL of 100% v/v stock + 4.9 mL water + 5 mL EtOH
vi. 0.32% v/v
    1. 32 µL of 100% v/v stock + 4.968 mL water + 5 mL EtOH
vii. 0.1% v/v
    1. 10 µL of 100% v/v stock + 4.99 mL water + 5 mL EtOH
viii. 0.032% v/v
    1. 320 µL of 1% v/v stock + 4.84 mL water + 4.84 mL EtOH
ix. 0.01% v/v
    1. 100 µL of 1% v/v stock + 4.95 mL water + 4.95 mL EtOH

g. Run your standards on the fluorometer before the corresponding samples.
h. Re-run your standards each time the lamp is changed, major adjustments are made to the fluorescence spectrometer (slits adjusted, etc.), or daily if analysis will take several days.

4. WAF sample analysis

Sample preparation

a. Vortex samples for 5 seconds.
b. Sonicate samples for 3 minutes. Ensure that the sonicator heater option is not on. Note: When preparing samples of saline water for analysis, the addition of ethanol may cause the salt to precipitate, and the resulting turbidity can affect fluorescence readings. Thus, additional steps are needed to remove salt before analysis.
c. To remove precipitated salt from samples prior to fluorescence analysis, transfer the sample to microcentrifuge tubes. If using 1.5-mL microcentrifuge tubes, use two tubes for each sample in order to provide 3 mL for the fluorometer cuvette. Perform this step with a limited number of samples each time (e.g., four samples) to ensure the samples are not sitting in the microcentrifuge tubes for an extended period of time. Spin samples at 10,000 rpm for 10 minutes. A salt pellet will be visible at the bottom of the tube.
d. Remove the supernatant from the microcentrifuge tube while avoiding the pellet.

e. Transfer the sample to a cleaned quartz cuvette using a glass Pasteur pipette. The sample is ready to be analyzed.
f. Prepare and analyze all controls in the same manner as the samples.

Sample analysis

a. Before analyzing the samples, measure the fluorescence of an ethanol control, a water control, and a 50:50 ethanol:water control, as described above for standards. For controls with saltwater and ethanol, prepare the same way as saltwater samples.
b. Prepare new controls and re-run if contamination is suspected. Run a new 50:50 ethanol:water control after every 20 samples. This value is the baseline corresponding to the 20 samples following the control.
c. Open an emission scan. Enter the excitation and emission wavelengths determined from the wavelength optimization process above. Set the step size to 2 nm.
d. Wipe the cuvette on all sides with a Kimwipe and place the cuvette into the fluorescence spectrometer (ensure cuvette is always placed into instrument with the same orientation).
e. Label scan with the Sample ID.
f. Start emission scan.
g. Repeat the process with the next sample.
h. Save the file periodically while running samples.
i. Between each sample, rinse the cuvette twice with distilled or de-ionized water then rinse twice with ethanol.
j. At the end of the day, rinse the cuvette twice with distilled or de-ionized water and then once with ethanol. Use a cotton-tipped applicator to wipe the inner walls of the cuvette then rinse a second time with ethanol. Cuvettes are fragile and expensive – store in a box.

5. Determining the measured concentration

a. Subtract the fluorescence background (area of 50:50 ethanol:water control) from the fluorescence of each standard, and plot the log area of standards vs. the log of dilution (%v/v). The relationship can be described statistically by a log-log linear regression. If the R^2 value for the regression is less than 0.95, rerun your standards. The linear regression formula is the ***log area = intercept + (slope * log concentration)***. If the relationship is not linear, remove data points outside the linear range of the curve. If the linear portion of the curve is represented by fewer than 5 data points, rerun the standard curve with at least five concentrations within the linear range.

b. For each sample, subtract the background (area for 50:50 ethanol:water control) from the total area of the sample. Multiply this area by 2 to take into account the 50% dilution of samples with ethanol. Note: Standards should be prepared as exact concentrations/dilutions, therefore the fluorescence total area for standards should not be multiplied by 2.

c. Log-transform the resulting area and plot as a function of the corresponding log-transformed % dilution (%v/v). Compare the results to those obtained from the standard curve to determine if the test dilutions were prepared correctly. The relationship of area vs. concentration for samples should be identical to the standard curve (Figure F.1). Quick observations will determine which dilutions were not prepared correctly and the correct dilution can be estimated from the standard curve.

d. If standard curves are repeatable over time and among experiments with the same oil, this procedure can be shortened by measuring the fluorescence of one standard dilution and the fluorescence of freshly-prepared test solutions. The fluorescence readings of test samples should be predictable from the fluorescence of the one standard solution, using the average slope and intercept calculated from previous measurements of standard curves. ***This will only work within one oil and one method for preparing WAF***.

6. Supplemental information

a. Machine maintenance

   i. Each time the fluorescence spectrometer is used, fill in a usage log with the total time the lamp was turned on. This includes time for warming up, sample analysis, breaks, lunch, and other time increments.

   ii. The Xenon lamp life is about 500–700 hours. When changing out lamps, always wear gloves and eye protection because the bulb is under pressure. Never touch the quartz envelope with bare hands. This could cause the lamp to explode.

   iii. After the lamp is changed, optimize the signal by centering the lamp within the housing. Use a sample compound (e.g., retene) that has a clear and obvious peak, and run a time-based scan to detect the peak. Adjust the knobs on the lamp housing until the maximum intensity is reached.

b. Cuvettes

   i. Use high-quality quartz cuvettes for analyses. If more than one cuvette will be used for a set of samples, ensure that the cuvettes have been matched so that they can be used interchangeably. If the set of cuvettes changes at any point during analysis of a group of samples, a new standard curve to reflect the corresponding cuvettes.

ii. Between samples, rinse cuvettes twice with distilled water (essential for samples containing saltwater,) then twice with ethanol. Periodic control samples will ensure that the cleaning procedure was adequate. If necessary, use a cotton-tipped applicator to wipe the inner walls of the cuvette.

iii. Before placing a cuvette into the spectrometer, wipe the outside of the cuvette with a Kimwipe to remove any smudges or liquid that may interfere with the fluorescence reading.

c. Repetition of analyses

i. To conduct sample repeats, use a fresh sub-sample from the 7.0 mL original sample, or sample again.

ii. Because the fluorometer subjects each sample to a beam of high intensity light, many of the aromatic compounds that fluoresce will be partially or completely degraded. A repeated analysis of the same sample may give progressively lower fluorescence measurements.

G. Water Accommodated Fraction Filtration Standard Operating Procedure

Version 2 (February 4, 2014)

Purpose

This SOP describes the general techniques and procedures for filtration of laboratory-prepared WAFs [of both low energy (LEWAF) and high energy (HEWAF)] and chemically-enhanced WAFs (CEWAFs). This SOP can be used to filter samples for the purposes of determining the components in the "dissolved" phase of the WAF or CEWAF sampled, or it can be used to filter WAF or CEWAF exposure solutions for the purposes of testing these "dissolved" components of the WAF or CEWAF in a toxicity test.

Materials

1. Stainless steel (solvent-rinsed) Forceps.

2. 90-mm glass filter holder/funnel with sintered glass frit base (e.g., Sterlitech Item# 352100-KG90). We prefer sintered glass to stainless steel mesh or porcelain base; materials should be rinsed with solvent before each use.

3. Two glass aspirator flasks of appropriate volume(s), rinsed with solvent. One flask will be used for waste and the other for collecting filtrate. Using a filtrate aspirator flask appropriate for the volume required (i.e., to filter 1 L use a 2-L flask) reduces the chance of any potential contamination from the stopper coming into contact with the solutions or from the solution being pulled into the vacuum.

4. Filter holder/funnel to aspirator flask clamp.

5. Rubber stopper, cleaned with soap and warm water, and then rinsed with DI water. Do not clean the rubber stopper with solvents, as it can cause leaching of organic contaminants from the stopper.

6. Thick-walled rubber tubing to connect vacuum flask to pump or hydroaspirator. Tubing should be of appropriate diameter to securely fit all fittings without using adaptors.

7. No-oil vacuum pump with a very low setting (e.g., FisherSci Cat. No. 01-257-508) or access to a faucet with a hydroaspirator connection. We recommend vacuum pressure of no more than 5 cm Hg (2 in. Hg vac) for all preparations *except* for the 100% Slick A/B HEWAFs, for which we recommend 10 cm Hg vacuum pressure.

8. Two 0.3 µm pore size glass fiber filters[1] – 90 mm diameter (e.g., Sterlitech Item #GF7590100).[2]

9. Sample bottles, glass beakers, and/or designated containers rinsed with solvent.

10. WAF solutions and clean source water.

Procedure

1. Ensure all glassware and equipment is cleaned and decontaminated (as described in Appendix A) before starting. Ensure that solvents from all glassware have fully dried, so there is no solvent residue contamination.

2. Set up the filter apparatus with two stacked 0.3 µm glass fiber filters. To reduce the potential for contamination, use a pair of solvent rinsed forceps to transfer filters from the package to the frit.

3. Collect slightly more than the desired amount of WAF into an intermediate glass vessel (e.g., beaker). If filtering a WAF immediately following its preparation, WAF should not be collected until after its requisite settling time (as described in Appendix A.1). Also, when draining a settled WAF be sure not to use the top layer from the separatory funnel or aspirator bottle. Ensure that all collected solutions are homogeneous prior to filtering.

4. Place the first filter on the glass frit base. Add a small amount of clean source water (~ 3 mls) to moisten the filter so that it adheres to the glass frit. For source water, use the same seawater, or seawater with similar salinity and chemistry, that you used to prepare the WAF. Repeat this process after layering the second filter on top of the first one. Add the glass filter funnel on top of the filter papers and clamp it in place and assemble the

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1. In our current investigations, we have found that breakthrough of droplets can occur when using the typical 0.7 µm filters in the filtration of our CEWAFs. This may be caused by the very small droplet sizes produced when preparing a CEWAF. Using two stacked 0.3 µm filters reduces the breakthrough. For 100% stock oil HEWAFs (i.e., at 1 g oil / liter water), and depending on the volume needed, you may have to use multiple filters. For example, for source oil one set of two filters will filter at least 1 L, but for Slick A only 200 mls can be filtered at one time.

2. A table presenting comparable glass fiber filters of different brands can be found at the following website: http://www.sterlitech.com/glass-fiber-comparison-table.html.

filter apparatus with an aspirator flask designated for waste solutions, turn on the vacuum so that you can remove the excess source water from the filters.

5. Remove the waste aspirator flask and replace with a new, decontaminated filtrate aspirator flask of the appropriate capacity to collect required volume of filtrate and reassemble the filter apparatus.

6. Pour the WAF solution to be filtered into the funnel.

7. To start the vacuum, turn it onto a low setting (5–10 cm Hg vac) or slightly turn on the faucet connected to the hydroaspirator. Use gravity filtration if needed. If possible, record the vacuum pressure on the Analytical Sample Inventory Bench Sheet.

8. As the sample is filtered, add additional volumes of WAF to the funnel as needed to get the desired volume of filtrate. Do not let the filter run dry during filtration.

9. If the filtration starts to slow significantly, you may need to replace the filter paper. Do not increase the vacuum pressure or overload the filter paper; this may lead to droplet breakthrough. A 90 mm diameter filter is better than a smaller diameter filter, as the larger surface area helps reduce filter overload.

10. To replace filter, pour any residual WAF solution from the funnel, disassemble the filter apparatus to remove used filters, and then repeat steps 4 through 10, as needed.

11. Once you have filtered the desired volume of WAF, shut off the vacuum, make sure the sample is well mixed, and pour the filtrate into your designated container or sample bottle.

H. Shipping Instructions for Sample Retention

Each toxicity test may generate samples that need to be archived, including tissue samples and solvent extracts from in-house analyses, which must be retained as described in the sample retention order memorandum (Stratus Consulting, 2011). Archive samples requiring long-term cold storage can be sent to ALS Environmental. If frozen samples are being shipped using dry ice as a refrigerant, follow procedures outlined in Appendix A.6 (*Shipping Samples Using Dry Ice*).

When shipping archived samples to ALS Environmental for long-term storage, ship samples in separate coolers from samples being sent to ALS Environmental for chemical analysis. Each individual cooler should contain no more than 100 individual archive samples. Each sample in the cooler must be properly documented on the designated DWH toxicity testing COC form (Appendix C), including sample ID, date and time of sample collection and sample matrix. The samples should be organized in such a way as to allow for easy ID of samples once they arrive at ALS Environmental. For example, if 100 samples are going to be shipped, the samples will be divided by putting 10 samples in labeled zip-top plastic bags; indicate which samples are in which bag on respective COC form in the comments entry.

In some cases, only a small number of samples will fit in each cooler (e.g., when shipping large brood stock fish). For any cooler containing 10 or fewer samples, the sample ID numbers for each individual sample are sufficient, and the samples do not need to be further divided using the above approach. If shipping multiple coolers, then each cooler needs a separate COC form that lists only the samples included in the corresponding cooler.

In the special instructions section on the top of the COC form, include the phrase "all samples are for long-term storage only, no analysis required." Since these samples do not need any extractions or analyses done by ALS Environmental, do not check the "Analyses Requested" box on the COC form.

Indicate the storage temperature for the samples on the COC form (e.g., store at -20°C). Samples with different storage temperature requirements must be shipped separately from each other. ALS Environmental is only accepting archive samples that require storage at 4°C or -20°C. For archive samples that have different storage requirements, please contact Stratus Consulting for further instructions.

Ship coolers using FedEx Standard Overnight Express Package Service and retain the shipping receipt as described in Appendix A.2. Hand coolers over directly to a FedEx representative; do not leave them at a pick-up location where they may be unattended. Instructions for filling out the shipping label are below.

- 3. Recipient: ALS Environmental, 1317 South 13th Avenue, Kelso, WA 98626
  - Telephone: 800-695-7222
- 4a. Express Package Service: check "FedEx Standard Overnight" – do not fill out Section 4b
- 5. Packaging: check "Other"
- 6. Special Handling: leave blank, but check "No" for *Does this shipment contain dangerous goods?*
- 7. Payment: check "Sender"
- 8. Residential Delivery Signature Options: check "No Signature Required."

4. Toxicity Testing Quality Assurance and Quality Control Procedures

Prepared by: Jeffrey M. Morris, Claire R. Lay, Heather P. Forth, Michelle O. Krasnec, Fiona Garvin, Ryan Takeshita, Ian Lipton, Michael W. Carney, and Andrew K. McFadden, Abt Associates, Boulder, CO

4.1 Introduction

This report briefly describes quality assurance and quality control (QA/QC) procedures designed by Stratus Consulting (now Abt Associates) and implemented as part of the Trustee's toxicity testing program in support of the *Deepwater Horizon* (DWH) Natural Resource Damage Assessment (NRDA). These procedures apply to toxicity testing data entry, data processing, and analysis for tests described in this report.

4.2 Study Design and Principal Investigator Selection

The toxicity testing studies were designed to evaluate potential injuries to Gulf of Mexico reptiles, fish, and invertebrate species that were exposed to DWH oil, dispersed oil, and dispersants associated with the DWH incident. The study design outlined general objectives and specified anticipated activities for a variety of species, life stages, toxicants, and endpoints. During test design, we added, revised, and reviewed specific test design requirements periodically with the National Oceanic and Atmospheric Administration (NOAA) and the Trustees.

We identified qualified investigators and testing laboratories through familiarity from past collaborations, referrals from other scientists, and by searching literature to identify experts for certain species or test types. We solicited formal testing proposals from the investigators, then provided the investigators with feedback on initial draft proposals. The revised and finalized proposals provided an initial basis for our proposed toxicity testing program, and we used these proposal parameters to define work products for all subsequently identified testing laboratories.

Before starting definitive tests, we inspected each laboratory and communicated any findings that required corrective action to the principle investigator (PI) at the facility. We verified that testing laboratory staff had taken these corrective actions during periodic testing laboratory visits and communication with PIs. We required that each laboratory staff person working on the

project received project training on NRDA topics and QA project plans with a NOAA attorney involved in the training process.

4.3 Laboratory Data Collection and Entry

Each toxicity testing laboratory recorded data and observations on handwritten datasheets. In most cases, these datasheets were standardized, pre-printed datasheets that Stratus Consulting produced in Microsoft Excel. After recording initial data, testing laboratory personnel entered the data from the handwritten datasheets into matching templates in Excel. Following data entry, testing laboratory personnel (other than the person who originally entered the data) performed a 100% QC on the Excel datasheets to identify and correct any data transcription errors. Upon completion, the laboratory sent the electronic Excel files and either electronic scans or hardcopies of the handwritten datasheets to Stratus Consulting.

The testing laboratory sent chemistry samples under chain of custody to ALS Environmental (formerly Columbia Analytical Services, Kelso, WA) for analysis.

4.4 Data Intake, Tracking, and Initial Processing

Upon receipt of the handwritten and electronic datasheets, Stratus Consulting logged receipt in a tracking database and inspected the electronic datasheets to ensure that the testing laboratories filled out all required fields with information compatible with data processing (e.g., no text included in numeric data fields, no numeric data included in notes). Stratus Consulting conducted any necessary minor reformatting of these files and input these data into a database of preliminary results. Then, Stratus Consulting exported data from the database of preliminary results into a QA/QC Excel file template, which we termed the "QC file."

4.5 Quality Control Review

Stratus Consulting conducted a QC Review of the QC file described, above. In this QC review, in personnel either re-entered or visually inspected information from the original handwritten datasheets. The decision to re-enter or visually inspect data was made on a case-by-case basis, taking into account the type of each field on each datasheet. Any discrepancies between the original handwritten and processed electronic datasheets and the re-entered fields were flagged by a macro running on the Excel QC file. Stratus Consulting personnel who did not perform the double-entry QC inspected this QC file for flags and reconciled and corrected any discrepancies through reanalysis of the data sheets. Stratus Consulting considered the handwritten datasheets as the definitive record for any data-entry discrepancies. If the reviewer determined that he or she

could not reconcile a discrepancy using the handwritten datasheets, the PI was contacted and their response was used as the final determination. Following reconciliation, Stratus Consulting reformatted and read the QC file into a post-QC database and updated the tracking database to reflect completion of QC Review.

4.6 Analytical Data Intake, Tracking, and Validation

After receipt of samples from testing laboratories, ALS Environmental assigned a unique identification (ID) to the samples and sent a confirmation of receipt to Stratus Consulting; this information was entered in the tracking database. Analytical data packages produced by ALS were validated by EcoChem, Inc. (Seattle, WA), or Laboratory Data Consultants, Inc. (Sacramento, CA). Stratus Consulting imported data from validation packages into a validated chemistry database and updated the tracking database to reflect receipt of validation.

4.7 Final Data Verification

Stratus Consulting matched data describing samples and bioassay results in the post-QC database to corresponding analytical chemistry data from ALS Environmental and produced an Excel data review package consisting of bioassay and analytical results for QA/QC purposes. These review packages were returned to the toxicity testing PIs for final review. At that time, Stratus Consulting communicated any unresolvable issues identified during the entire QC process to the PIs for their review and resolution. Once the PIs approved a data review package for a test, the data became ready for distribution to the Trustees and the data packages were marked accordingly in the tracking database. Stratus Consulting incorporated any changes or corrections identified or approved by the PIs into a final Excel file, maintaining version control; this final file was subsequently included in the post-QC database.

4.8 Additional QA/QC Measures Associated with Toxicity Testing Activities

Before definitive testing, Stratus Consulting personnel visited each laboratory to confirm that the facilities had no issues of concern, as well as to ensure that the PIs had a clear understanding of all specified testing requirements. Additionally, an independent third-party consultant from Experimental Pathology Laboratories, Inc. (EPL), visited each laboratory during definitive testing to ensure that the laboratories were following specified protocols and procedures, as well as the test-specific protocols for particular tests (see Attachments 1 and 2).