

**QUALITY ASSURANCE PROJECT PLAN
eDNA MONITORING OF BIGHEAD AND SILVER CARP**

Prepared for:

**U.S. Fish and Wildlife Service
USFWS Midwest Region
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ACRONYMS/ABBREVIATIONS

Table 1. Common acronyms/abbreviations commonly referenced in this document.

Acronym/Abbreviation	Definition
COC	Chain-of-custody
CSO	combined sewer overflow
DI	deionized (water)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates, also known as nucleotides
eDNA	environmental Deoxyribonucleic acid
FWCO	Fish and Wildlife Conservation Office
GL	Great Lakes
GPS	Global Positioning System
LDB	left descending bank
MRWG	Monitoring and Response Work Group
SDS	Safety Data Sheets
PCR	polymerase chain reaction
QAPP	Quality Assurance Project Plan
RDB	right descending bank
Taq	<i>Thermus aquaticus</i>
USACE	United States Army Corps of Engineers
USFWS	United States Fish and Wildlife Service
UV	Ultraviolet light
WGL	Whitney Genetics Lab

SECTION 1 PROJECT DESCRIPTION AND PERSONNEL REQUIREMENTS

1.1 Background

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Within Illinois, the man-made Chicago Sanitary & Ship Canal (CSSC), constructed in the early 1900s, provided an unnatural portal for invasive species dispersal between the geologically separated Mississippi River and Great Lakes drainage basins. In 2002, in an effort to curtail the spread of invasive species between the two basins, the U.S. Army Corps of Engineers (USACE), constructed a dispersal barrier system within the CSSC. The primary objective of the barrier system was to stop the dispersal of the invasive Round Goby into the Mississippi River basin; however, the Round Goby had surpassed the barrier before its completion. Since then, a new threat to the Great Lakes from the Mississippi River basin has become the primary objective of the dispersal barrier system. Invasive Asian carps, including Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp (*H. molitrix*) have been steadily dispersing upstream through the Mississippi, Illinois, and Des Plaines rivers. Their potential dispersal through the dispersal barrier system within the CSSC poses a potential threat to the Great Lakes ecosystem.

In the past, traditional fishery techniques were used to detect the leading edge of the Asian carp population; however, this method was somewhat ineffective at targeting these species at low densities. The University of Notre Dame, with funding from the USACE, developed a method that detected “environmental” DNA (eDNA) left behind in the aquatic system by the targeted species. Environmental DNA enters the system through a variety of mechanisms, some of which include sloughing of external epidermal cells into the water, sloughing of internal epidermal cells into feces and into the water, and as tissue residues following injury or predation. The detection of eDNA in water samples is based on whole DNA extraction from particulate organic and inorganic matter found in the water and polymerase chain reaction (PCR) assays for species-specific mitochondrial DNA markers.

Use of this method is to provide detection of Asian carp at low densities and to serve as an early detection system of the spread of Asian carp into previously uninhabited areas. The original Quality Assurance Project Plan (QAPP) detailing the eDNA monitoring process, including methodologies and quality controls, was requested from the U.S. Army Engineer Research and Development Center (ERDC) as the USACE assumed eDNA monitoring responsibility from the University of Notre Dame. This modified QAPP will be followed by the U.S. Fish and Wildlife Service (USFWS) offices responsible for field sampling and lab processing of eDNA samples. Fish and Wildlife Conservation offices (FWCO) will handle field sampling and the Whitney Genetics Lab (WGL) at the Midwest Fisheries Center will process eDNA samples since the USFWS assumed responsibility for monitoring Asian carp in the Chicago Area Waterway System (CAWS) and other water bodies in 2013.

This version of the QAPP has been modified to be a general guide to field and laboratory methods that should be employed during eDNA monitoring programs. Specific sampling plans and schedules for particular water bodies will be developed by regional sampling agencies and their partners, as well as overseeing governing bodies (such as the Asian Carp Regional Coordinating Committee and Great Lakes Fishery Commission). These plans are available online on various agency websites and will not be included in this document.

1.2 General Requirement

USFWS Midwest Region requires a Quality Assurance Project Plan (QAPP) for eDNA monitoring. Full-scale eDNA monitoring commenced in September 2010 by the USACE, and based on consultation with experts in processing of litigable DNA evidence (which applies to eDNA monitoring), a final, comprehensive QAPP was not in place within that time frame. Instead a provisional QAPP was used

during the first year of eDNA monitoring, to be followed later by a finalized version under a different scope of work. The USFWS will continue to maintain and update a QAPP for use in eDNA monitoring programs that allows for inclusion of any beneficial technical or strategic modifications that become apparent from past monitoring events, research conducted by the eDNA calibration research team, or research published in the literature and vetted by the eDNA calibration team or other USFWS genetics laboratories.

1.3 Project History

The University of Notre Dame, Department of Biological Sciences, Center for Aquatic Conservation, prepared a Standard Operating Procedure (SOP) in 2010. The SOP provided details regarding eDNA monitoring protocol and was given to USACE in May 2010 in compliance with Cooperative Ecosystem Study Unit agreement #W912HZ-08-2-0014, modification P00007. On 15 and 16 December 2009, a technical and quality systems audit of the Center for Aquatic Conservation Lodge Laboratory at the University of Notre Dame was conducted by the US Environmental Protection Agency. The laboratory audit report dated 5 February 2010 was provided to USACE in addition to the eDNA monitoring protocol. These documents served as the basis for the QAPP followed by ERDC. Additionally, USACE submitted the eDNA methodology for an Independent External Peer Review (IEPR), which is a requirement to examine decision documents and supporting work products where there are public safety concerns, significant controversy, a high level of complexity, or significant economic, environmental and social effects to the nation. Released fall of 2011, the eDNA IEPR report, conducted by objective panelists with technical expertise in genetics and population ecology, confirmed eDNA sampling and testing methodology is sound for detecting Silver and Bighead carp DNA but cannot indicate the source of Asian carp DNA (information on the size, gender, age and number of individuals present and cannot distinguish between pure Silver or Bighead carp and their hybrids). In 2013, the WGL received the QAPP and made changes so that it would be applicable to the new field sample processing and eDNA processing personnel and their specific locations. It was also modified according to results of the eDNA Calibration Studies (ECALS) and lab validations carried out by the interagency ECALS team. Annual modifications (see section 1.8) to the QAPP will be approved by regional field staff, the USFWS eDNA team, and leadership of USFWS Midwest Region. Changes to sample analysis will undergo peer-review by external experts and validation in specifically designed studies carried out in at least three different laboratories.

1.4 Objective

The objective of this QAPP is to provide detailed procedures for Asian carp eDNA sample collection, sample processing (including filtering, centrifuging, DNA extraction, PCR, biomarker analysis, DNA sequencing), data reporting, and quality control/quality assurance procedures to ensure that data are as technically defensible, consistent, and usable as possible. The specific goals and objectives of sampling plans for particular bodies of water are currently directed by the appropriate governing body. For example, the Chicago Area Waterway sampling plan has been issued by the Asian Carp Regional Coordinating Committee's Monitoring and Response Workgroup in the Monitoring and Response Plan. The Great Lakes, Ohio River, and Upper Mississippi River plans will be or have been issued by USFWS Midwest Region in conjunction with state and other partners.

Use of this QAPP for all aspects of eDNA monitoring for the early detection of Silver and Bighead carp is strongly encouraged if resource agencies would like to compare results to FWS results. Any samples processed that do not follow this protocol cannot be directly compared to FWS monitoring results.

1.5 Project Personnel

The eDNA monitoring project in USFWS Midwest Region has formed an eTeam, with members of the Regional Office, all field stations and the Whitney Genetics Lab. An official document defining roles and responsibilities is attached in Appendix A, and the names and contact numbers should be updated annually.

The eDNA monitoring program of USFWS Midwest Region must have personnel appointed to the following positions, and it is highly recommended for other agencies interested in eDNA monitoring: Field sampling point of contact for each field station responsible for sampling and field processing. For each sampling trip, specific personnel will differ for each role as listed in the QAPP, but each role will be filled on each sampling trip

- eDNA Project Coordinator
- Field Operations Manager
- Sampling Leader
- Sampling Quality Assurance Specialist
- Sample Processing Leader
- Sample Processing Quality Assurance Specialist
- DNA Processing Leader
- DNA Processing Quality Assurance Specialist
- Data Documentation & Reporting Specialist
- eDNA Database Coordinator
- Supporting Agency contacts

The minimal responsibilities of the above positions are detailed below. Specific personnel assigned to the project are listed in Appendix A. Sampling and Sample Processing roles may NOT be filled by the same person and must each be filled on every sampling trip.

Prior to each trip, the eDNA Program Coordinator and the eDNA Processing Leader need the name and mobile phone number for the field sampling and sample processing leaders. After each trip, a brief summary report (Exhibit 2) covering quality assurance issues and any changes in personnel roles should be provided to the eDNA Program Coordinator.

These roles assume all staff are USFWS Midwest Region Staff who have the minimum requirements and have been trained in the QAPP methods. If other agency personnel are assisting in the field or lab, they must have read and understood the QAPP and have Exhibit 15 and their CV on file with the lead FWS office. When personnel from other agencies or volunteers do not meet the minimum requirements or have equivalent requirements (e.g. state boat operator training), this must be documented on Exhibit 2 as a deviation from the QAPP and a brief explanation provided that explains the situation.

eDNA Project Coordinator: USFWS Midwest Regional Office staff specialist responsible for development, coordination and implementation of the USFWS eDNA monitoring program for Asian carps. Assumes principal responsibilities for initiating, leading, facilitating, integrating, coordinating, and communicating necessary monitoring work and activities using eDNA of the Midwest Region's Fisheries Program through the cooperative conservation community. Provides guidance for implementing and utilizing eDNA activities at the field level. Provides technical leadership to plan, conduct and lead other biologists as well as coordinate activities to identify and coordinate surveillance areas in water bodies for the early detection of Asian carp eDNA. Ensures the best scientific practices are used during the development and implementation of management plans to monitor, control, and eradicate Asian carp.

Implements and coordinates the eDNA collection program; eDNA processing and interpretation of data; collection and dissemination of research information from institutions and research agencies on findings and new developments in eDNA collection and interpretation of data and the development and implementation of Asian carp management plans. Responsible for the overall QAPP and its implementation at the field and lab levels. Responsible for communication between the lab and Regional office. Integration with interagency ECALS team and other relevant entities.

Field Operations Manager: Serve as lead biologist to implement the eDNA monitoring program for the watersheds assigned to their respective field office. Under the supervision of the Project Leader, develops and implements eDNA sampling plans for waters assigned and serves as a point of contact, with their Project Leader, for partners to coordinate all eDNA field activities. Responsible for reporting results to Sample Processing and DNA Processing Team Leaders, the eDNA Program Coordinator, as well as other designated USFWS personnel

Sampling Leader: Responsible for obtaining water samples for eDNA monitoring and providing those to sample processing team. Responsible for reporting results to Field Operations Manager.

Sampling Quality Assurance Specialist: Responsible for knowing all quality assurance/quality control (QA/QC) measures for eDNA sampling efforts. Advises Sampling Leader on any potential QA/QC problems. Reviews procedures, field logs, data collection methodology; ensures that all agencies participating in sampling are conforming to procedures, and documents this after each sampling trip (Exhibit 2). Recommends corrective actions for non-conformities.

Sample Processing Leader: Responsible for centrifuging water samples for eDNA monitoring and providing those processed samples to eDNA Processing team. Responsible for reporting Field Operations Manager.

Sample Processing Quality Assurance Specialist: Responsible for knowing all QA/QC measures for filtering/centrifuging efforts. Advises Sample Processing Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for processing; ensures all personnel are conforming to procedures, and documents this after each sampling trip (Exhibit 2). Recommends corrective actions for non-conformities.

DNA Processing Leader: Responsible for processing eDNA samples through DNA extraction, PCR, and sequencing. Responsible for reporting results to eDNA Program Coordinator, as well as other designated USFWS personnel.

DNA Processing Quality Assurance Specialist: Responsible for knowing all QA/QC measures for eDNA processing efforts. Advises eDNA Processing Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for DNA processing; ensures all personnel are conforming to procedures. Recommends corrective actions for non-conformities.

Data Documentation & Reporting Specialist: Assists DNA Processing Leader in maintaining laboratory database for eDNA sample processing. Performs data completeness and data verification checks, and ensures that all data are documented completely.

eDNA Database Coordinator: Assists the eDNA Project Coordinator and Field Operations Managers with eDNA database QA/QC, data collection via the Collector App, and result map generation. Updates and maintains the ArcGIS Collector App Instructional and ArcMap eDNA data editing/proofing manual.

Assigned Project Leaders and Specialists: Others serving on the project may include researchers, technicians, and budgetary personnel. Sampling may employ personnel from other agencies in

coordinated efforts. All personnel must meet a minimum standard for training and/or experience before independently conducting any portion of the eDNA monitoring protocol. Each station's lead eDNA personnel will attend an annual QAPP review training (train the trainer). These lead eDNA staff will then train any other staff assisting with eDNA work. The supporting agency contacts are given in Appendix A. Minimum personnel training requirements are given below.

1.5.1 Personnel Training Requirements

Minimum training and/or experience requirements for the different major components of the eDNA monitoring protocol are detailed below.

Boat Operator

- Must meet USFWS boat operator requirements as a minimum.

Sampling

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid and/or boating safety course.
- Minimum 1 year experience in collecting field samples for biological analyses.

Sample Processing

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid training.
- Facility-specific safety training.
- Minimum one semester college level laboratory experience, plus eDNA-specific training.

DNA Processing

- A minimum BA/BS degree or its equivalent in biology or related area and successful completion of college coursework (graduate or undergraduate level) covering the subject areas of biochemistry, genetics, and molecular biology (molecular genetics, recombinant DNA technology) or other subjects that provide a basic understanding of the foundation of DNA analysis, as well as coursework and/or training in PCR amplification as it applies to eDNA analysis.
- A minimum of 6 months of general DNA laboratory experience, including experience with DNA extraction and PCR. Additionally, 2 weeks of training on Asian carp eDNA protocols.
- Successful completion of a qualifying test demonstrating effective execution of eDNA-type assays before beginning independent work on the project.

1.6 Reporting

- All agencies, need to submit resumes (Curriculum Vitae) for proposed staff to the eDNA Program Coordinator, to be reviewed and approved by the eDNA Program Coordinator. As new personnel are acquired and trained, their resumes must be filed with the eDNA Program Coordinator. Additionally each proposed staff member will review the QAPP annually and sign Exhibit 15. Both forms of documentation may be provided hard copy or in electronic format to the eDNA Program

Coordinator. CVs of returning personnel should be updated as position titles or job descriptions change.

- For each sampling event, a pre-trip summary (Exhibit 1) of the sampling sites, dates, personnel roles, and contact information should be supplied to the eDNA Program Coordinator, eDNA Database Coordinator, other USFWS personnel as needed, and partner agencies as appropriate.
- Specific staff member names conducting the various activities must be documented on all official records.
- Pre-trip communications with eDNA Program Coordinator may be via email or text.
- To provide proof of Quality Assurance, upon completion of all sampling events, the post-sampling summary report (Exhibit 2) must be filled out and filed with the eDNA Program Coordinator.
- The lab will file weekly reports with the eDNA Program Coordinator that contain up-to-date information on the progress of samples within each case, and the complete case log with detailed notes about lab processing.
- The lab will update the eDNA Program Coordinator with information regarding presumptive positive samples so that timely handling of final results can be mapped and communicated internally and with other offices and partners as needed.
- The lab will file final reports for each case that contains all of the processing information and final positive and negative results with the eDNA Program Coordinator.
- The eDNA Program Coordinator will track eDNA sampling events and processing in a master case file that will be shared with USFWS personnel and partner agencies.

1.7 Case number assignment and management

All eDNA field and lab data will be collated and housed in a geo-referenced database maintained at the Regional Office. The database is organized around a regional unique reference ID (RUID) for each individual sample, thus a unique identifier is required for each eDNA sample.

- Each sampling event is centered around a specific water body, and will be assigned a case number consisting of a 5-digit integer (00000). Sample ID numbers within cases will be in consecutive numerical order, and will consist of a 3-digit integer (000). Each sample will be identified by the 8-digit number consisting of the case **and** 3-digit sample ID number, without spaces or punctuation (00000000).
- WGL will create a master case list each season and assign case numbers as needed to each FWCO. FWCOs will assign case numbers to sampling events so that the lab remains blind for sample processing.
- A table of case numbers will be provided by WGL to the eDNA Project Coordinator so that case numbers will never be repeated among sampling years.
- Sampling priority will be finalized by the eDNA Project Coordinator and appropriate FWS and partner personnel.
- Lab processing priority (which may differ from sampling priority) will be finalized by the eDNA Project Coordinator and appropriate USFWS and partner personnel.

1.8 QAPP maintenance and modifications

Early detection of aquatic species by eDNA is a new technique that is simultaneously undergoing extensive development on the research front as it is applied on the ground in monitoring efforts. This results in rapid advancement of the technique that should be applied to monitoring efforts to not only improve use of the tool in management applications, but also to improve efficiency.

- The USFWS works closely with collaborating agencies such as the USGS and the USACE to keep up

with current research so that the eDNA monitoring program can be updated regularly.

- The QAPP will be updated annually to reflect advancements in eDNA research as well as to improve efficiency in field and lab efforts based on lessons learned from the previous year of implementation.
- Annual updates to the QAPP may be implemented by two mechanisms.
 - Changes to field methods and anti-contamination measures by the eTeam. In 2014 USFWS FWCOs, WGL, and the Midwest Regional Office formed an eDNA team (eTeam, see Appendix A) to work on maintaining the QAPP.
 - These changes are suggested by field staff, and reviewed by the eTeam, and upon approval, implemented into the QAPP. In general, these changes are to accommodate a more-widely implemented monitoring program spatially to include a much greater area than the CAWS. It also accommodates implementation by several different FWCO offices, instead of a fairly limited crew used in the CAWS.
 - None of these changes reduce the level or rigorousness of quality control measures so that the QAPP maintains the high level of quality control that was approved by several external reviews.
 - Changes to lab methods as recommended by collaborating research agencies such as the USGS or USACE or lab staff. New techniques and products must be validated through rigorous studies conducted in at least two labs, preferably three labs.
 - Again, these changes incorporate the same level of rigorous quality control as the original QAPP.
- Other agencies that would like to contract with other labs for eDNA monitoring may want to use the QAPP are encouraged to do so. However it is likely that partnering with other labs will lead to procedures that are not covered in the QAPP. In this case, it is up to each agency to decide on their own requirements needed for eDNA sampling and lab processing.
 - The USFWS recommends a minimum of following closely sections 1-4, 6-10 to ensure rigorous quality control and sample integrity.
 - Use of other labs should be carefully reviewed by the contracting agency staff and each agency should ensure that labs are validated and can process samples with at least the same level of quality control and in lab situations that minimize contamination in highly sensitive PCR applications.
 - It is likely that other labs will use assays with different PCR markers and even different PCR techniques such as digital PCR. It is up to the management agency contracting the work to ensure that these assays have been rigorously tested and validated for use in sensitive eDNA monitoring programs. At a minimum, the assay methods should be published or validated in a rigorous, round-robin lab study.
- List of updates to QAPP
 - 2013
 - Section 2.1.5 added guidance for expanding sampling into Great Lakes tributaries.
 - Section 2.1.6 added for eDNA processing trailer maintenance and cleaning.
 - Section 2.4 added guidance for wading and non-motorized sampling procedures.
 - Section 3.3 added detailed procedures for centrifuging samples as an alternative to filtering. Filtering was the only method allowed when objectives were early detection and surveillance for Asian carp. Centrifugation was allowed in special cases when approved by Region 3 RO staff.
 - Section 5.5.5 added extraction procedures for using the Qiagen DNeasy Blood and Tissue kit. All official 2013 monitoring samples were processed with the Qiagen kit. The original Power water kit methods were moved to appendix H in 2015.
 - A blind, round-robin validation study was conducted in three laboratories to ensure

- the Qiagen kits performed as well as or better than the Power Water kits (Amberg, J.J., S.G. McCalla, E. Monroe, R. Lance, K. Baerwaldt, and M.P. Gaikowski. 2015. Improving Efficiency and Reliability of Environmental DNA Analysis for Silver Carp. Accepted. Journal of Great Lakes Research. doi:10.1016/j.jglr.2015.02.009.).
- All exhibits were updated with customization for Whitney Genetics Lab
 - Appendices A-G were added or updated to function within the US Fish and Wildlife Service.
- 2014
 - Section 5.6, 5.7 and 5.8 were updated with new qPCR markers, new methods for determining presumptive positive and confirmed positive results, and protocols for data reporting and management. Markers published in: Farrington, H.L., C.E. Edwards, X. Guan, M.R. Carr, and R.F. Lance. 2015. Mitochondrial Genome Sequencing and Development of Genetic Markers for the Detection of DNA of Invasive Bighead and Silver Carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in Environmental Water Samples from the United States. PLoS ONE 10(2): e0117803. doi: 10.1371/journal.pone.0117803. A blind, round-robin validation study was conducted in three laboratories to document marker performance. Final report and publication to follow. All 2014 samples were screened with these new markers.
 - Original sections regarding amplification with old cPCR markers, and methods for determining results were moved to appendix G.
 - 2015
 - Section 1.5 had an additional role added for field stations, Field Operations Manager.
 - Section 3.3 Centrifugation was approved for use in all eDNA applications based on results of WGL study, Validation of centrifugation vs. filtering for eDNA sample collection (Appendix K). Report filed with eDNA Coordinator in Regional Office. Filtering methods were moved to appendix H. References to bottles in other sections were either moved to Appendix H or changed to reflect centrifuging materials and methods.
 - Section 5.6-8 were enhanced with the addition of methods using an internal positive control to assess and measure inhibition of PCRs.
 - Section 5.6.5 added details regarding quantitative PCR standard control materials and methods.
 - 2016
 - Roles and Responsibilities updated with current staff names and contact information.
 - The internal positive control standard curves were changed to a 5-point 5X curve instead of a 10X curve, and the number of copies spiked to field samples to measure inhibition was raised to 250 from 100 copies, since there was no longer a 100-copy standard to use for spiking.
 - 2017
 - Roles and Responsibilities updated with current staff and new (eDNA database coordinator) roles defined.
 - Clarification on sample preservation made to ensure final concentration of preservative is 95% if ethanol and 70% if isopropyl.
 - Clarification in text describing the internal positive control to improve understanding of methods.
 - Implementation of Collector App to record field data electronically in the field. Removed Database Instruction Manual from Appendix D, since the SDE database cannot be directly linked to the ArcGIS Collector App due to PIV card issues. Instead,

individuals will be uploading data directly to the FWS ArcGIS Online Cloud under the FWS Fisheries eDNA Online GIS group. The SDE database will be used as a separate backup location for eDNA data.

- o 2018
 - Roles and Responsibilities updated with current staff.
 - Removed bleach baths for sample tubes in the trailer, and changed procedure to simplify handling and reduce bleach fumes for staff, but maintain sample integrity.
 - Collector App Manual updated and provided as a separate document.
- o 2019
 - Roles and Responsibilities updated with current staff.
 - Updated field sampling with minor changes to increase clarity of guidance.
 - Changed criteria for determination of a presumptive positive to increase detection probability.
 - Remove Internal Positive Control based to economize lab capacity.
 - Exhibit 1 and Exhibit 16 updated and replaced by Exhibits 1 and 2.
 - Removed Appendix on Mobile Trailer Maintenance to be a stand-alone document.

1.9 eDNA Security Plan(s)

- A detailed eDNA security plan for the Midwest Fisheries Center has been developed due to the co-location of the La Crosse FWCO and the WGL. Staff in the FWCO often work in Asian carp contaminated waters and conduct field work where Asian Carp are directly handled. Thus, this plan includes detailed procedures for decontaminating field equipment as well as boats, trailers, and trucks used for all field work including eDNA sample collection. It is a stand-alone document included as Appendix G.
- A DNA security plan for each field or lab station involved in eDNA sampling or processing should be developed using the Midwest Fisheries Center eDNA security plan as a model, but adapted for each station's unique situation. This plan should be kept on file at the regional office. The documents should be signed by all personnel at the field or lab station and approved by the eDNA Project Coordinator(s).

SECTION 2 SAMPLE COLLECTION

Prior to any field sampling work, all field employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Field employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Field employees will also review the sampling safety plan (separate station-specific document) and participate in a safety briefing.

2.1 Pre-Trip Planning and Site Selection

Refer to the internet for annual monitoring plans issued by the agency responsible for each particular body of water. For example, the CAWS monitoring plan can be found at www.asiancarp.us.

2.1.1 Purpose

Accurate planning of a general collection site is necessary to effectively manage the time of crews collecting samples, as well as to ensure complete and correct sampling procedures are used.

2.1.2 Pre-trip Planning Procedure

Refer to the annual monitoring plans to properly plan for sampling. The sampling agency should use interactive aerial imagery software (i.e., Google Earth) to scope out reaches to be sampled, placement of samples, and unique features that should be targeted during sampling.

(1) File pre-trip plan (Exhibit 1) per section 1.6.

(2) Spatial planning

- a. Use of aerial maps detailed enough to show unique features (e.g., barge slips, factory, bridge pilings etc.) that can be identified in the field and used as markers for location when sampling. The recommended minimum scale is 1':500', if that is not available, use the best scale possible.
- b. Aerial maps should be marked with sample locations and should ensure spatial coverage and overall representativeness of the sample area.
- c. Target specific areas (backwaters, island side channels, pooled areas, below and around structures, and confluence of tributaries).
- d. Print map(s) with detailed sample plan.
- e. Locate access points for boat launch and acquire permission to use if necessary. If sampling around locks, or if sampling will require lockage, notify the Lockmaster at least 1 day before sampling.
- f. Coordinate sample plan with sampling crew, eDNA Program Coordinator, eDNA Database Coordinator, or any partner agency.

(3) Temporal planning - Biology and ecology of Asian carp should be considered when timing sampling events throughout the year.

- a. Time sampling events to low water, cooler temperatures and to coincide with fish aggregation areas to increase detection probability. While spawning may release gametes, high water events that trigger spawning also dilute eDNA and transport it rapidly downstream. Water temperatures above 25 °C cause faster degradation of DNA.
- b. If timing of sampling cannot be optimized around fish biology, then consideration should be given to determine the effectiveness of sampling. If sampling is to proceed, then larger sample sizes will be necessary to overcome lower detection probability in high flow or warm water

conditions.

(4) General planning

- a. Sampling crew should comprise three people at a minimum: one boat operator, one lead sampler, and one sampling assistant.
- b. Processing crews should consist of two people.
- c. River stage and weather forecast should be checked and taken into account prior to and throughout sampling.

(5) In the event of a significant rainfall event (more than 1.5 inches in a 24-hr period), the Field Operations Manager must assess the situation and determine if sampling should be pursued. The first consideration is crew safety. Once it is determined that sampling can occur safely, sampling may be pursued. However, because effects of high rainfall events will differ among systems, it is important for experienced field staff to weigh in with discussions with their Project Leaders and the eDNA Program Coordinator to avoid unnecessary travel if the high water would prevent sampling. If it is deemed safe for sampling to occur, the sampling crew, Project Leader and eDNA Program Coordinator need to discuss the effects of high water on the ability to detect eDNA, because increased runoff will dilute the signal or transport it too rapidly downstream, and thus decrease the chances of detecting target DNA. This call must be made on a case-by-case basis. If sampling must occur, sample size should be increased. In general, and especially in the CAWS, sampling should be avoided within 3 days of a combined sewer overflow (CSO) event, due to adverse health effects from raw sewage. Weather data and river stage for the area to be sampled can be checked at: <http://waterdata.usgs.gov/nwis>.

(6) In general sampling should be avoided in water that has obvious disturbance (e.g. prop-wash) from the sampling vessel that increases the turbidity and suspended sediments in the water. If possible, those areas should be avoided for sampling and care should be taken to prevent sediment disturbances as much as possible. This is because eDNA can be stored in sediments for long time periods, which confounds early detection monitoring goals, and too much sediment prevents DNA extraction by methods outlined in the QAPP.

(7) Ensure that the designated data recorders for the field crew and processing trailer have read and understood the ArcGIS Collector App Instructional Manual for eDNA Monitoring of Bighead and Silver Carp. All participating offices have been provided with a digital and hard copy of the manual. A copy of the manual can be requested from Jeena Credico by e-mail (jeena_credico@fws.gov).

2.1.3 Field equipment list

- (1) Waterbody-appropriate vessel. If possible, vessel and associated equipment should be dedicated to eDNA collections to minimize risk of DNA contamination of samples from other sources. If boat dedication is not possible, or eDNA collection occurred in known carp positive waters, procedures for decontaminating boats and equipment are included in Section 2.2.4. These procedures will also work for converting a previous carp sampling boat to a dedicated eDNA sample collection vessel.
 - a. eDNA-only dedicated personal flotation devices (PFD) for each crew member, using the type of devices listed in the safety plan. Minimum PFD requirements are Type I within the Safety Zone near the existing electric barrier (on the Chicago Sanitary and Ship Canal) or Type II for other areas. NOTE: In some cases, eDNA sampling may occur in Asian carp-infested waters, avoid using clean eDNA dedicated field gear for these trips to prevent contamination for later use in non-infested waters.

- b. It may be necessary to sample eDNA in carp-infested waters. In this case, crews should carefully consider which personal protective gear should be used. It may be prudent to avoid exposing clean eDNA gear to carp-infested waters and instead other “relatively” clean gear should be used instead. If there is uncertainty or questions, call the eDNA Program Coordinator.
- (2) If centrifuging: enough 100-qt coolers capable of holding 5 50-ml centrifuge tubes per planned field sample. See Appendix D for tube handling plan.
- (3) If centrifuging: Sterile, chemical-free polypropylene 50-ml centrifuge tubes, that are rated to withstand 6000 x g, labeled by sampling agency. Tubes will not be reused, but WGL will return shipping containers to be reused by sampling agencies if requested.
- (4) 3-gallon sprayer (low pressure hand sprayer for spraying down boats in the field), hot water pressure sprayer (for decontaminating boats where water and electrical hookups are available) or cold water high pressure sprayer with low pressure detergent injection (may be portable for use in the field). Car washing brushes or mops may be used in lieu of low pressure hand sprayer for chemical decontaminant applications.
- (5) Habitat measurement equipment (Global Positioning System [GPS], Digital Depth Sounder).
- (6) Pre-Sampling Summary (Exhibit 1)
- (7) Charged I-Pad inside a LifeProof cover or similar cover equipped with ArcGIS Collector App.
- (8) Charged GNSS Receiver inside a clear waterproof bag or container.
- (9) Chain-of-custody forms (Exhibit 3).
- (10) Sharpie® permanent marker in black.
- (11) Powderless nitrile or latex gloves.
- (12) Ice for coolers with samples.
- (13) Safety plan – the USFWS plan represents minimum requirements; agency-specific alternative plans are allowable as long as all hazards are addressed and minimum requirements are met.
- (14) Bleach (any commercially available brand or strength), detergent for use with cold water pressure sprayer, or Virkon Aquatic: decontamination chemicals will only decontaminate clean surfaces, thus clean any solid surface well to completely remove any film or biological build up before application of decontaminating agents. If using cold water pressure sprayer to apply detergent in lieu of hot water pressure sprayer or bleach/Virkon, surfaces must be pre-cleaned before application of the detergent (3-5 min contact time) with a 10-second high pressure rinse.
 - a. Bleach may be mixed in two strengths: 10% solutions require 10-minute contact time but 20% solutions require 5-10 second contact time (and require extra caution when spraying).
 - b. The oxidative feature of bleach deteriorates with exposure to organic material and over time, so bleach solutions must be made fresh daily in order to decontaminate DNA.
 - c. Virkon must be applied in a 2% solution for DNA decontamination or reduction methods. Equipment must be fully submersed in a 2% Virkon solution for 30 minutes for complete decontamination. A 2% Virkon spray or swab application for a 10-minute contact will reduce DNA to negligible levels. Virkon mixed in solution is good for one week.
- (15) Water sources: water used to mix bleach or Virkon solutions can be any source of potable water, it does not have to be DI water. Tap water may be used to wipe down surfaces or to rinse equipment such as filter funnels after bleach soak. DI water is only required when the water used will come into contact with a filter that will be processed in the lab.

(16) Garbage bags

2.1.4 Lotic System Site Selection Procedure

Specific eDNA monitoring plans that explicitly explain where and how many eDNA samples will be collected for particular water bodies will be created each year by the sampling office or agency in coordination with state partners and other agencies. These plans will contain specific sampling locations, numbers of samples, temporal and spatial sampling details. These plans are outside the scope of this document; however, there are some critical considerations that should be followed.

- (1) In lotic systems, sampling should occur in a downstream to upstream direction to minimize the potential for surface water disturbance caused by the vessel's wake within the sample reach. The only exception where sampling may occur in an upstream to downstream direction would be if the nearest boat launch is upstream of the reach to be sampled or if it is not feasible to sample a system in an upstream direction (for example by kayak). If sampling direction does not follow the QAPP, this should be noted on the post-sampling summary (Exhibit 2), and include a brief justification.
 - (2) Samples should be collected by using a grid system within targeted habitats or areas of sampling. Based on research of eDNA over the last several years, transects across any high flow, main- or side-channel area should no longer be collected, due to rapid transport of eDNA downstream. Instead, select sample sites with lower flow. If low flow areas are not part of the river system to be sampled, sampling should be focused along near-shore areas in depositional bends or in eddies where eDNA may accumulate. When collecting samples near the bank, be observant of wake-disturbed surfaces. To compensate for the wake created by a passing boat, samples may need to be collected 2–3 ft off the bank to obtain displaced surface film. If sampling along near-shore areas is the best available site in the system, total sample number should be increased to compensate for lower detection probabilities and care should be taken to avoid sampling during high flow conditions.
- (b) Targeted sampling is collecting samples in the most probable places of eDNA accumulation, such as (but not limited to):
- Backwater areas
 - Island side channels
 - Effluent areas
 - Eddies or pooled areas
 - Near structures that create slack-water (e.g., sunken barges)
 - Bays
 - Marinas
 - Wind driven scumlines
 - Other areas where organic material has accumulated on the water surface

Avoid sampling under or downstream of bird rookeries or storm sewer outflows since these have been shown to be vectors of eDNA from sources other than a live fish.

2.1.5 Lentic System Site Selection Procedure

- (1) Lake and bay samples can be divided into blocks, and random blocks selected for sampling. Each block should be sampled in a grid pattern.
- (2) Each block to be sampled should have boundaries defined, total surface area calculated. Blocks with less than 1.0 km² should have a minimum of 40 samples. Larger blocks should be sampled at 1

sample per 0.01 km². Blocks should be divided by an evenly spaced grid, and random grids selected for sampling.

- (3) Water collection should be done in each grid, targeting areas where eDNA will accumulate as allowed in each grid site. If no obvious accumulation areas are observable, samples may be taken anywhere within the site.
 - a. Targeted areas include areas below and around structures such as piers, docks, as well as any areas where floating debris accumulates. Juvenile and age-0 Asian carp prefer shallow, productive areas like those adjacent to and within wetlands. Areas to be considered for sample collection include those with locally high productivity, such as sewage system effluents.

2.1.6 eDNA Trailer Preparation and Field Considerations

Routine trailer maintenance is covered in the Trailer Maintenance manual saved on the Google Drive eDNA folder. Prior to departure the trailer should be cleaned, decontaminated and then stocked with all supplies needed for field sampling and lab processing.

- (1) Upon arrival at the sample processing site, select an area to park the trailer that has limited risk for contamination from other activities on-going in the area. For example, avoid parking next to boat washing stations where commercial carp fishing boats will be sprayed clean. Decontaminate surfaces and floors with bleach. For 20% bleach solutions, allow it to sit a minute or two before rinsing clean. For a 10% bleach solution, allow it to soak for 10 minutes, then rinse to prevent build-up of bleach salts.
- (2) UV lights should be used for 30 minutes at the beginning of each day.

2.2 Equipment Preparation

2.2.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, vessels and equipment must be decontaminated in accordance with the following protocols to eliminate introduction of outside DNA sources in the sampling regime. Bleach will only decontaminate clean surfaces, so be sure to clean any solid surface well to completely remove any film or biological build up so the bleach can destroy any DNA.

Cautions: Precautions should be made to avoid direct skin contact with bleach; bleach solution may also stain clothing or other materials. Be aware of pollutants in the aquatic environment and related health hazards.

Water used for bleach solutions may be any source of clean, potable water. Water used to rinse equipment, such as the filtering funnels or sample container interior that comes into contact with the actual eDNA sample should be DI water to avoid introducing any inhibitors to the samples.

2.2.2 Equipment Procedure for Coolers

- (1) Bleach solution (10 or 20%) with water will be prepared in a 3-gallon low-pressure sprayer that is dedicated to the project. The bleach solution must be prepared immediately prior to use, and each time decontamination activities will be occurring. Use caution if spraying 20% bleach to avoid creating an inhalation hazard.
- (2) Remove mud and other biological residues from surfaces by rinsing and scrubbing. Equipment surfaces must be free of debris and other material before decontaminating.

- (3) Sample transport coolers will be decontaminated with freshly made bleach solution in the low pressure sprayer. Use the low-pressure sprayer to thoroughly cover the inside and outside surfaces. For 10% bleach, allow 10 min of contact time before rinsing with water. A 20% bleach solution only needs to sit a few minutes before rinsing. Coolers may be left to air dry, or dried using clean paper towels while wearing clean gloves.
- (4) Purchase sterile, chemical-free disposable 50-ml polypropylene tubes with maximum RCF of at least 6,000 x g. Even sterile tubes can still have traces of the chemical used to free the plastic tubes from the metal forms during production. Some manufacturers sell sterile tubes that do not have this residual chemical in them, and these should be used to avoid introducing PCR-inhibitors from the sample tube.
- (5) Sample labels can be printed by the sampling agency. Sample containers will be labeled with a unique barcode ID or agency generated code (Section 1.7 for case and sample numbering) that does not indicate location (to allow blind processing). Labels will be printed on Rite-N-Rain® or some type of waterproof labels and affixed to the outside of the sample tubes.
- (6) Once tubes have been labeled, they will be placed in the sterilized sample coolers in numerical order. Sample containers will be stored in the sterilized coolers until use, and will be transported only in the coolers. Although the sample ID number of the samples is not relevant except for identification purposes, collecting in consecutive order will aid in determining where samples were taken in case of a recording error.
- (7) A minimum of 10% of the number of samples collected should be field blanks. For offices working with the WGL, all field blanks must be filled by WGL. For other agencies, blanks may be filled with any city-provided tap water (If water source is a well or you are unsure of the source, use distilled or deionized water). Note, if the sampling design for your particular body of water requires a specific sample size in order to meet a precise detection probability, then containers for control samples to meet the 10% will be added to the total number of sample containers dictated by your sampling plan.

2.2.3 Boat and Field Equipment

This section applies to all motorized or hand-powered boats, paddles, and any associated field equipment used to collect eDNA.

- (1) A set of field equipment used by staff collecting eDNA such as personal flotation devices, rain gear, hats, sunglasses, etc., should be dedicated for eDNA field work only, to avoid contamination risks. This is especially important if the same staff are involved in field work in rivers with well-established populations of Asian Carp, or if staff conduct field work where they come into direct contact with Asian Carp. This dedicated gear should be stored and transported in designated containers (such as totes) so that contamination from trucks or boats contaminated with Carp DNA is avoided. After the trip, gear and transport containers should be decontaminated according to section 2.2.4.
- (2) It is preferable to have designated vessels, trailers, and trucks set aside for eDNA work if possible. Even if there is designated equipment, because eDNA can be moved among sites on vessels, boats and equipment must be decontaminated prior to sampling, and between sampling sites. If complete decontamination cannot be performed between cases while in the field, choose the most preferred method of DNA reduction available in between cases (Appendix E). Upon return to the field station office, a complete DNA decontamination for all associated equipment must be

performed before returning to the field.

2.2.4 Boat and Equipment Decontamination Procedure

Follow steps 1-12 for decontamination or reduction of DNA on equipment surfaces before and between collections of eDNA samples from any site. Refer to Appendix E for a list of recommended decontaminants. Use personal protective equipment (PPE) and read SDS before use of any product. Follow equipment safety instructions and read equipment manual before using an industrial hot water pressure washer:

- (1) Put on appropriate PPE. Decontamination PPE should be designated, stored separately and decontaminated after each use to prevent reintroduction of DNA to equipment, and transfer of splashed DNA around your facility.
- (2) Remove equipment from boats, trucks, etc. and lay them out separately so that all surfaces of equipment will be exposed to treatment. Treat one side and then flip if necessary.
- (3) Remove any environmental debris such as plant material, mud, or fish slime with brushes or gloves. If possible, perform this step at the sampling site to leave as much DNA material behind as possible. Buckets and brushes or a water pump can help rinse boat surfaces of blood and slime before leaving the water.
- (4) Rinse surfaces with the highest pressure water available for the location. Surfaces must be clean for decontaminant/DNA reducers to work.

Choose a decontamination method that is appropriate for the equipment, location and services available (Proceed to steps 5, 6, 7 or 8). If equipment has been exposed to Asian carp DNA use one high pressure sprayer method ***in conjunction*** with one chemical method (steps 5 or 6 ***and*** 7 or 8) for decontamination:

- (5) Use an industrial hot water pressure washer set at 212 F to decontaminate appropriate surfaces. Minimum exposure time for decontamination is 10 seconds.
- (6) Apply detergent at low pressure to saturate surfaces with an industrial cold water pressure sprayer with detergent injector. Wait 3 to 5 minutes, then rinse at high pressure for 10 seconds.
- (7) Mix a 10% solution of household bleach (5-8% sodium hypochlorite before mixing) in tap water in a hand pressure sprayer (low pressure saturation) or large tub (immersion bath). Spray or swab to saturate at low pressure or immerse all appropriate surfaces. Exposure time for complete decontamination is 10 minutes. Rinse with fresh water and allow surfaces to dry. Mixed solution good for one day.
 - a. As an alternative to step 6 for smaller items, prepare a 20% bleach solution in a small tub and completely immerse items for 10 seconds. Rinse and allow surfaces to dry.
- (8) Immerse smaller equipment in a 2% Virkon bath for 30 minutes. Metals should be immersed no longer than 10 minutes. For larger equipment, prepare a 2% Virkon solution in a low-pressure sprayer or swab and saturate surfaces. Minimum exposure time is 10 minutes. Rinse with fresh water and allow surfaces to dry. Caution: Do not aerosolize this product. Use at the largest droplet setting to avoid respiratory exposure. Mixed solution good for one week.

In absence of availability of methods for decontamination previously mentioned in this document, rinse equipment with copious amounts of water at the highest pressure available and allow to dry. Exposure to the sun or UV radiation and heat will help reduce residual DNA. Follow complete decontamination procedures beginning at step 1 upon return to field station office or at first availability.

Upon completion of methods above, follow steps 10-12 to complete eDNA clean-up:

- (9) Use DNA Away and paper towels to decontaminate pens, hats, notebook surfaces, electronic equipment surfaces, truck interior and other non-saturable equipment.
- (10) Contain any equipment that was not treated in bags or totes for later decontamination.
- (11) Wash hands, launder or change soiled clothing.
- (12) Remove PPE and decontaminate before storing separately for next use.

2.3 Motorized Sample Collection Procedure

2.3.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, samples must first be collected from the appropriate aquatic environment in accordance with the following protocols. Each morning prior to sampling, collection staff and processing staff will have a morning briefing that will discuss where and how the sampling is occurring. At this time a float plan and GAR model will be filed with the boat operator and collection crew and processing lead. Once the meeting is complete the processing lead will email those documents to the lead agency's main office project leader and any other necessary personnel.

Cautions: Lifejackets must be worn at all times in any watercraft used for eDNA sample collection. Additionally, disposable powder-free latex or nitrile gloves must be worn when collecting water samples. Be aware of pollutants in the aquatic environment and related health hazards. Field crews should have separate and designated field gear and outerwear that is for eDNA sampling only. This gear should be stored separately from other field gear that may come in contact with Asian carp biological material. Gear should be transported in a clean eDNA gear box so that it is not contaminated in vehicles, boats, or trailers. Follow Boat and Equipment Decontamination Procedures (above section 2.2.3) between sampling sites.

Gear (such as PFDs and hats) should be decontaminated between sampling events according to section 2.2.3. However, if any gear is compromised during sampling (accidentally falling in, or a spill, etc.) and staff feel that it may contribute to contamination of the samples, the gear should be decontaminated as soon as possible at the end of the work day, or replaced with new gear as soon as possible.

2.3.1 Water Collection Procedure

- (1) Prior to launch, crew members will have reviewed this QAPP, will have signed the QAPP certification form (Exhibit 15), and will understand their assigned roles in the sample collection procedure. All sampler identification information and other field data will be recorded on the pre-sampling summary (Exhibit 1).
- (2) The transport vessel will be launched from an appropriate area that allows access to the reaches to be sampled.
- (3) Sampling will commence at the first site located at the downstream end of the reach to be sampled and will proceed in an upstream direction. The only exception to this protocol is when the boat launch is located upstream of the sampling reach. Then sampling will commence at the first site located at the upstream end of the reach to be sampled and will proceed in a downstream direction. The direction traveled for sampling should be recorded on the pre-sampling summary (Exhibit 1).
- (4) It is imperative to avoid disturbing sediments, and avoid collecting samples where the sediments have been stirred up. If sediments are accidentally disturbed by the boat motor (prop-wash), it is required that the driver, re-position the boat in a new area without disturbing sediments. The data

recorder must make a note on the data sheet and record actual GPS coordinates where the sample was taken. (Sediments add to the inhibition load of the sample and could increase the rate of false negative results for either filtered or centrifuged samples).

- (5) When arriving at a sample location the lead sampler and sampling assistant will put on new gloves (powderless latex or nitrile). **REMINDER – Gloves must be changed before each new sample is taken to prevent cross contamination. The same gloves may be worn when collecting blank samples in tandem with a regular sample in a site.**
- (6) Going in consecutive numerical order based on the tube labels, remove a set of labeled sample tubes from the sample cooler.
- (7) Just prior to collecting the sample, unscrew and remove the lid(s) from the sample containers. Do not under any circumstances touch the interior of a sample container, even with a clean glove. Lids should be held in hand and kept clean, or placed in a container designated for holding lids while water is collected. To remove lids from holding container, dump them into a clean gloved hand, do not reach into the container to remove the lids.
- (8) Collect water by reaching over the upstream side or the bow of the transport vessel with the sample container and fill the tubes by skimming the surface of the field water. The sample container must not be submerged or dipped beyond the upper 2 inches of the surface water for sample collection, since the intent of the sampling is to collect floating organic matter that is on the water surface. Avoid collecting large organic debris such as twigs, leaves, seeds, etc., because they cause problems in extraction. A small amount of duckweed is fine. To avoid contamination, the individual collecting the sample should avoid touching any other surfaces with the clean gloves (i.e. the gunwale) and should only handle the sample container and cap.

Note: Centrifuge samples for early detection and monitoring are comprised of 5 replicate 50-mL tubes, which may be collected in two ways: 1) to minimize variation among offices, a sampling device may be used to collect all 5 tubes at once, as long as there is a clean place to store five caps while sampling is occurring. 2) To collect samples across a larger accumulation zone, tubes may be collected one at a time across a larger sample area within the accumulation area or spaced around a block, to increase detection probability. This option should be covered in the specific site plan, where specific guidance on the methods should be explicit for field crews.

In some cases, eDNA research projects may be conducted outside of the early detection effort. For these studies, centrifuge samples may also be collected in already carp-infested waters to detect or confirm spawning events, or to find areas where carp congregate to be used in control efforts. In these applications, samples are a single 50-ml tube. Cases of these types are to be arranged by the field station in advance with the eDNA Program Coordinator.

- (9) Once the sample container is filled, the lead sampler will screw the lid back on until it is tight. The closed container will then be placed in a cooler. Tubes may be filled all the way, there is no reason to measure water, and as long as all tubes are filled the same, they will be safe to use in the centrifuge.
- (10) While the water sample is collected, the sampling assistant will record habitat measurements in the Collector App (see data fields in Appendix X Data Management for proper format).
- (11) Field blanks (water filled prior to trip) will be opened to expose the container contents to the atmosphere for 5 sec, and resealed. Fully submerge the container in the field water, and return the

place the sample with the other samples. Blanks are taken in tandem with the next actual sample and do not replace a sample in that location, in other words, , the boat will remain at the same location for the blank and an actual sample.

- (12) Steps 1 through 10 will be repeated at each sampling location until sampling has been completed for the targeted reach.
- (13) Once a cooler is full, add ice to completely cover all containers. Replace ice as it melts, removing excess water only as needed, since ice water will provide better cooling than ice alone.
- (14) Chain-of-custody (COC) forms (Exhibit 3) will be completed for every sample and every cooler. All samples, including blanks, will be logged onto COC forms. The forms will be collected and signed whenever the coolers are transferred between parties. If you must split samples into different configurations than listed on the original COC, make an entry for those samples on the original COC and create a new COC for any new transfer or shipping containers. Be sure that each container has its own COC that has records for exactly and only the samples contained within.

2.4 Wading and Non-motorized Sample Collection Procedure

2.4.1 Purpose

Collection of water samples for eDNA analysis may be conducted in areas not accessible by motorized watercraft and alternative means must be employed. In order to provide accurate and creditable eDNA results, watercraft and equipment must be decontaminated in accordance following section 2.2.3 protocols to eliminate introduction of outside DNA.

Cautions: Precautions should be made to avoid direct skin contact with bleach solution, which may also stain clothing or other materials. Be aware of pollutants in the aquatic environment and related health hazards. When canoeing or kayaking two people must be together either in separate kayaks or in one canoe with PFDs worn at all times and a float plan and GAR model on file with sample lead, processing lead, sample quality lead and processing quality lead and sampling agency's main office. If wading PFDs must be worn.

2.4.2 Wading Water Collection Procedure

- (1) Collection of water samples while wading should commence at the most downstream accessible sampling site and proceed upstream. Carefully approach each sampling site slowly to avoid disrupting sediments and accumulation areas. When taking the sample the collector's position should always be down current of the targeted area.
- (2) When there is no access to the downstream most site except from an upstream location field crew may enter at that point and proceed downstream carefully. Sampling will commence at the downstream most site of the reach to be sampled and proceed upstream. The entire sampling route, including direction, should be recorded on the Field Collection Summary (Exhibit 1). When traveling downstream stay out of the water as much as possible and avoid disturbing any areas of accumulation or sampling sites.
- (3) The sample collector will put on clean gloves, remove the first container from the cooler and wade to an area of accumulation to collect the first sample. After the sample is taken, the collector will return to shore and replace the sealed container to the cooler. Do not under any circumstances touch the interior of a sample container, even with a clean glove. If you cannot grasp the container with one hand, use both hands to hold the container. Since the lid should not be set down, either

hand the lid to the sampling assistant or hold the lid with the inside toward the container alongside the container as you collect the sample, keeping the lid out of the water.

- (4) When approaching an accumulation area moving downstream make a “J” like approach to disturb the water and sediment as little as possible. If approaching an accumulation area moving up stream, make an “L” like approach to not disturb sediment along the bank.
- (5) Change gloves and remove the next sample number going in consecutive order and proceed to the next accumulation area repeating steps 1 and 2.
- (6) If sampling in a remote location a backpack container may be used. The backpack should be either a hard plastic pack/trapper’s basket or a frame pack with a hard plastic storage container attached. Any backpack containers used must be able to withstand the bleach decontamination protocols and not absorb/retain water. Clean sampling container should be placed in a large plastic bag (i.e., heavy duty garbage bag) and closed prior to loading into the backpack. Another plastic bag placed in the backpack will be for full water samples. The person wearing the backpack will serve as the data recorder if a third person is not available. The collector will put on new gloves prior to opening and removing a sample container from the clean bag. Ensure the clean bag is sealed after removing a container. After collection, place the container in the bag designated for water samples, seal the bag, and remove contaminated gloves. The box of clean gloves should be sealed in a plastic bag/container between uses. Place used gloves in a separate waste bag. Place water samples on ice as soon as possible and within two hours or less of collection.

Note: Centrifuge samples are comprised of 5 replicate 50-mL tubes, which may be collected in two ways: 1) to minimize variation among offices, a sampling device may be use to collect all 5 tubes at once, as long as there is a clean place to store five caps while sampling is occurring. 2) To collect samples across a larger accumulation zone, tubes may be collected one at a time across a larger sample area within the accumulation area or spaced around a block, to increase detection probability. This option should be covered in the specific site plan, where specific guidance on the methods should be explicit for field crews.

2.4.3 Non-motorized Water Collection Procedure

- (1) Kayaking/Canoeing will commence at the most upstream site (collecting samples in an upstream to downstream fashion). As you are moving downriver, try to maintain a reasonable distance from the shoreline to reduce wave action to the shoreline and accumulation areas.
- (2) Clean sampling containers (up to seven) can be placed either loosely within the boat or in a plastic trash bag depending on type and design of craft. Sample numbers of containers should be in consecutive order and collected in order.
- (3) When approaching an accumulation area moving downstream kayak #1 will make a “J” like approach not to disturb the water and sediment as little as possible. After the sample is collected and kayak #1 has moved from the collection spot kayak #2 will move into the spot to record depth, water temperature and GPS coordinates. Do not under any circumstances touch the interior of a sample container, even with a clean glove. If you cannot grasp the container with one hand, use both hands to hold the container. Since the lid should not be set down, either hand the lid to the sampling assistant or hold the lid with the inside toward the container alongside the container as you collect the sample, keeping the lid out of the water.
- (4) After 5-7 containers have been collected kayak #1 will switch roles with Kayak #2 (i.e., kayak #2 is the collector and kayak #1 is the data recorder). The eDNA trailer or a filter staff member will

meet the kayakers at a location downstream that is accessible to place collected water samples into a cooler with ice.

- (5) At this time the kayakers will take the last of the containers in the cooler and continue collecting within the collection reach repeating steps four and five as needed until all locations are sampled.
- (6) If sampling location is in slow or no current areas (e.g., impoundments, shallow channels, etc.) sampling collection protocols should follow the lentic procedures in section 2.4.2. Craft may proceed upstream and also should collect from downwind to upwind if possible and approach all accumulation sites with minimal to no disturbance.

SECTION 3 SAMPLE PROCESSING

3.1 Purpose

In order to isolate eDNA from water samples collected in the field, particulate matter must be concentrated. As of March 2015, centrifuging will be used by the USFWS. Filtering methods used in the past have been moved to appendix H.

Centrifugation of water samples collects matter at the bottom of the tubes, which can be collected on sterile cotton swabs and eDNA can be extracted from the swab. Validation of this method has shown that a maximum of five tubes can be swabbed with a single swab.

Cautions: Wear powder-free latex or nitrile gloves when handling samples (a glove change is required for each sample when opening and decanting the samples. One pair of gloves can be used to load and unload centrifuge batches since none of the tubes are opened). Be careful not to touch commonly used items in the laboratory when wearing sample gloves (i.e., writing utensils, stationary lab equipment). If in doubt, change your gloves!

3.2 Centrifuging Procedure

Water samples collected in the field need to be centrifuged and preserved with ethanol within 12–16 hours after the last field sample is collected.

Equipment needed:

- Sterile, chemical-free centrifuge tubes (Midwest Scientific #TP91050) made of polypropylene that can withstand 6000 x *g* with caps and labels (recommended Avery laser white weatherproof address labels #5520).
- Paper towels
- 95% ethanol (non-denatured) or 70-99% isopropyl alcohol (mix prior to trip with purchased molecular grade water). It is IMPERATIVE only pure ethyl or isopropyl alcohols are used and diluted with molecular grade water. There can be NO methanol in the mix, as methanol damages DNA so that it cannot be amplified in PCR or sequenced.
- A reusable bottle-top re-pipettor for dispensing alcohol
- Black permanent markers (e.g., Sharpie®)
- Powderless latex or nitrile gloves
- Refrigerated centrifuge(s) with rotors and adaptors for 50-ml tubes (Fisher item # listed for: Sorvall Legend XTR centrifuge; rotor GS25F7087G item #75-033-607; 750 ml round bucket GS25F7087G item #75-003-608; 50-ml tube adaptors item #75-003-638)
- Bleach
- Dedicated lab equipment cleaning sink

- Waste water disposal location such as non-specified-use sink
- Clean bench paper or clean paper towels
- Dedicated water bottles: one for DI water; one for bleach solution

3.2.1 Laboratory Preparation

- (1) Hands must be washed thoroughly prior to starting. A dedicated plastic wash bottle with 10% (or 20%) bleach solution, or commercially available product such as DNAaway, should be prepared for wiping down lab tables and other surfaces prior to processing samples. All equipment must be cleaned with bleach, DNAway, or Clorox wipes between sampling events.
- (2) Each workstation must be rinsed with bleach solution and the surface covered with one or more clean paper towels or bench paper prior to beginning the centrifuging process. . One set of gloves may be used to move tubes from the cooler to the centrifuge adaptors and to load adaptors in to centrifuges. A new set of gloves should be used for each set of tubes comprising one sample any time the tube is opened. Prior to centrifuging samples, each workstation should have pre-printed labels or one black, waterproof permanent marker for labeling sample tubes, a wastewater container (with lid), and a dedicated wash bottle with 10% bleach solution, and a dedicated alcohol bottle with 95% ethanol or 70% isopropanol.
- (3) Refrigerated centrifuge(s) set at 4⁰ C. If in mobile trailer, check horizontal alignment, trailer should be as level as possible.
- (4) Clean paper towels, or if preferred commercial bleach wipes.
- (5) Centrifuge tube racks should be available for organizing and working with sample tubes.

3.2.2 Sample Preparation

New powder-free latex or nitrile gloves must be worn prior to handling each sample set, any time tubes will be opened. Batch transfer of tubes that are not opened may be done with one set of gloves.

Process tubes in sets of replicates, until a full centrifuge batch is prepared.

A centrifuge equipment control comprised of 50 ml of tap water should be included with each batch of tubes in each centrifuge. It should be labeled with the range of sample numbers included in the batch. For example, centrifuge equipment control sample number 1000001-1000005 was included in the centrifuge with field samples 1 through 5 from case number 10000. This equipment control is in addition to the field control, and should be made by the sampling office or agency while processing samples in the trailer.

3.2.3 Centrifuging the Samples

- (1) 50 ml tubes should be removed from the transport cooler, caps checked and tightened, wiped dry with either a clean paper towel or a clean bleach wipe, and placed into the centrifuge adaptors. The same towel or wipe may be used for all 5 tubes in a sample, unless they become saturated and do not completely dry the tubes. Tubes with heavy soiling on the outside can be rinsed with plain water in the non-dedicated sink and wiped dry.
- (2) Place filled adapters into a refrigerated centrifuge set at 4⁰C. Tubes must be evenly distributed within the centrifuge to maintain the rotor balance. NOTE: Always follow the manufacturer's guidelines for centrifuge operation. Remember to include one equipment control tube with each full centrifuge. It is also important to keep replicate tubes for each sample together in batches. Do not

split a sample between batches, otherwise it is too difficult to trace equipment control results through the lab.

- (3) Once 50-ml tubes are in position, close and secure centrifuge lid. Set centrifuge to spin the samples for 30 min at max speed ($\sim 4500\text{-}5000 \times g$) and begin centrifuging the samples. During this period the other tubes may be dried and placed in a clean rack. Change gloves after loading racks.
- (4) Once samples have been centrifuged the eDNA will be on the bottom of the tube. Use a new pair of gloves for each set of tubes comprising one sample, carefully remove cap and GENTLY pour off water into a wastewater container. It is imperative to decant as much water as possible while ensuring pellet retention. A carboy is useful to prevent splashing, or a plain bucket with bleach in the bottom will also work. Change gloves after each sample set.
- (5) Add ~ 20 ml of 95% ethanol or 70% isopropanol to the tube. The alcohol is required to stabilize the eDNA, and if too little is used, the DNA will degrade before it is detected. Replace cap and swirl alcohol around tube covering the entire internal wall. Centrifuge the samples with alcohol for 10 minutes (or more as needed if pellet is too loose) at max speed ($\sim 4500\text{-}5000 \times g$). Blanks should be handled exactly the same as samples.
- (6) Decant excess alcohol until the level of the alcohol is within the conical end of the tube. If it was difficult to decant water in step (3) and more than 3 ml water remained, then the alcohol used in step (4) will not have provided a 95% alcohol preservation level, so a second addition of alcohol will be required at this time. Add another 5-10 ml of alcohol, swirl gently, and leave excess alcohol in the tube. Place tubes back into a sterilized rack, keeping samples organized by sample number and ensure control samples are labeled so that they can be paired with their processing batches. Change gloves between sample sets.
- (7) When a rack is full, secure lids to tubes with tape (for shipping requirements), place racks in plastic bags so that the tubes are secured in the rack. Tape bags closed and sign across the tape where it overlaps. Place in box to be shipped. NOTE: If a stabilizing solution (i.e., ethanol) is not used eDNA samples must be frozen (traditional commercial freezer or -20 °C freezer) or placed in cold storage. To conserve dry ice, samples may be placed in a cooler filled with ice during the working day. At the end of the day, quickly transfer all samples into a dry ice cooler for long-term storage. Be sure to monitor temperature twice a day and replenish dry ice as needed.
- (8) Prior to centrifuging the next batch of 50 ml samples, remove the 50 ml tube adaptor inserts and examine for any water or debris. If anything is found, place in 10% bleach bath for 10 minutes, rinse well to remove all residual bleach, dry with new paper towels, and replace. If adaptor inserts are clean and dry only perform this step during daily lab cleanup. (It may be helpful to have two full sets of inserts so that processing can proceed while any dirty inserts are being cleaned). Be sure to record the time centrifuging was completed in the Collector App and the initials of the person that finished processing the sample. This should be the initials of the individual who handled the sample during and the second decanting step. If more than one person processed a sample, the Collector App and database only allow for one set of initials to be recorded. Therefore, the processor who closed the sample for the last time should record their initials, and the other processor(s) are already documented on the field plan and will be documented on Exhibit 2 for a trip report.
- (9) Steps 1–9 should be repeated until all samples have been processed.

When the wastewater collection carboy is full, it should be disposed of in a sink separate from the one used to bleach equipment. If a second sink is not available, dispose of the wastewater in any drain that is connected to a sewage treatment facility or system.

If a sample is accidentally spoiled during the centrifuging process (e.g., the pellet was lost during decanting, bleach was squirted into a tube, or cross-contamination is suspected), it should immediately be thrown away. Record the appropriate sample ID as well as the reason for the ruined sample in the

Collector App and make a note on the COCs.

- (10) File post trip and QA/QC summary (Exhibit 2). IF the trip was executed as planned, use the check box provided on Exhibit 2 and continue page 3. If any deviations from this QAPP occurred during water sample collection in the field or during processing of samples, clearly describe the deviations on Exhibit 2. If deviations will affect lab processing, the eDNA Program Coordinator will contact the eDNA Processing Leader. **Even if there were no deviations from the QAPP, Exhibit 2 forms should be sent to the eDNA Project Coordinator** following a sampling event to satisfy reporting requirements in section 1.6. Some of this information may seem duplicated from the Collector App, but it is necessary to provide quality assurance information to the Coordinator and GIS specialist at the time lab data is joined with field data to ensure accuracy. These documents standardize information for a long-term monitoring program implemented by several different offices with frequent staff turnover across a large geographic area.

SECTION 4 SAMPLE SHIPMENT

4.1 Purpose

Samples must be maintained accordingly depending on sample type. Non-preserved centrifuge samples must be on dry ice or in a -80 freezer until they can be shipped to the DNA processing lab (WGL lab in Onalaska, WI) as soon as possible. Temperature should be recorded twice a day during sample storage and immediately prior to sealing the shipping container (Exhibit 7). If samples were centrifuged, samples do not need to be cooled. All samples should be shipped as soon as logistics in the field allow. There is no longer a 24-hour maximum time limit on shipping, but that does not mean that several cases may be saved up to be delivered or shipped at one time. Since the lab has to open and account for each individual sample and equipment control, samples should be shipped in no more than 400 total samples (unless a single case is larger than this, then ship by case). The Processing Leader is responsible for ensuring that samples are properly packed and shipped according to the procedure below.

There will be no air shipments accepted on Fridays at WGL. Experience in the past with the rare failure of FedEx to deliver on Friday has resulted in loss of samples. If samples cannot be shipped by Wednesday, then they must be held, with temperatures recorded if applicable, by the sampling agency (according to step 3.3.3 (7)) until the following week. If shipping flammable samples by ground, WGL will accept Friday deliveries.

Please note: the COC forms are as important as the samples themselves. If COC forms are not filled out properly, then sample integrity is lost and the samples should not be processed because their custody cannot be accounted for. Therefore, please be sure to accurately and completely fill out the COC forms. If you have questions, do not hesitate to call the eDNA Program Coordinator or the eDNA laboratory. COC forms are to be filled out according to good laboratory practices, which includes proper correction notation. Mistakes are to be left legible for any future audit by crossing out the error with a single line. The error must be initialed and dated, and a brief explanation provided near the error if there is room. If more room is needed, notate the error with a small superscript numeral and provide a clear explanation in the margins, using the same superscript numeral. Erasing or using white out to correct errors is prohibited, since the mistake cannot be seen and understood during an audit.

Cautions: Wear gloves and use caution when working with dry ice.

4.2 Shipping Procedure for samples on dry ice

- (1) Centrifuge tubes must be shipped organized in racks, secured in plastic bags that will keep tubes in the racks, and shipped in containers that will hold the tubes and prevent damage to the tubes.

Be sure tubes are securely closed to prevent leaking. If necessary, twist caps counter-clockwise to properly seat the threads indicated with a “click”. Tighten. If tubes have been chilled, lids may become loose, so double-and triple-check before packing. Place tubes back into the rack in numerical order. Place the rack of tubes back into a plastic bag, seal or wrap it tight to keep tubes from falling out, tape it closed. Pack racks of tubes into boxes, using packing material to keep them from shifting. With use of liner bags for the entire shipping box, it is acceptable to only sign the closed, taped, large gusseted liner bag.

- (2) Fill out a COC form for each shipping container where the most recent listing lists exactly and only the samples shipped in that particular container (equipment controls are separate samples from the field samples, so they must be accounted for on COC forms in the containers in which they are

shipped). The individual employee packing and sealing the containers should list their name in the “from” line, be sure to include agency and print clearly. The container should be packed and released on the same date. Sign and place COC forms (Exhibit 3) in a clean 1-gal resealable bag, place evidence tape across the seal, and place the bag on top of the cooler before closing the corrugated shipping box and sealing with packing tape. **Each box MUST** have a separate signed COC form included to document the specific samples included therein. If you must split samples into different configurations than listed on the original COC, make a new entry for those samples going into one container on the original COC and create a new COC for any new shipping containers.

- (3) Fill out a Federal Express (FedEx) air bill shipping label with appropriate information. Be sure to affix a dry ice warning label (can be purchased in rolls from FedEx, or printed from the internet) with the amount of dry ice in each cooler on the cardboard shipping container. On the label be sure to designate FedEx Overnight Express (delivery is usually the following day between 8 and 10 AM) as well as to identify the weight of the dry ice in the package. When ready, drop off at FedEx or call FedEx (1-800-463-3339) for pickup. Be sure to tell the operator that the package contains dry ice and ask for an approximate pickup time. Be sure to record tracking numbers for all boxes being shipped.
- (4) Items will be shipped to the following address:

Midwest Fisheries Center
Whitney Genetics Lab
555 Lester Avenue
Onalaska, WI 54650
608-783-8444

4.3 Shipping Procedure for samples preserved with 70% isopropyl or 95% ethanol

- (1) Centrifuge tubes must be shipped organized in racks, secured in plastic bags that will keep tubes in the racks, and shipped in containers that will hold the tubes and prevent damage to the tubes, as well as meeting regulations for shipping “small quantities” of flammable liquids according to 49 CFR 173.4. Note, these samples may only be shipped ground, and if using FedEx, you must call for pick-up, they will NOT accept drop-offs at any office.
- (2) Be sure tubes are securely closed to prevent leaking. If necessary, twist caps counter-clockwise to properly seat the threads indicated with a “click”. Tighten. Use a single piece of tape to secure the lid to the tube. Place tubes back into the rack in numerical order. Place the rack of tubes back into a plastic bag, seal or wrap it tight to keep tubes from falling out, tape it closed, signing across the tape.
- (3) Construct shipping box, double-taping all seams. Box must meet federal guidelines; two options can be purchased from Uline: model #S-4798 (holds 120 samples or 600 tubes) or model #S16458 (holds 90 samples or 450 tubes).
- (4) Use two sections of absorbent material (BLU-102 from newpig.com) and make a cross over the box. Push the center of the cross into the bottom of the box, making sure to press the matting into the bottom corners.
- (5) On top of the matting, place a 24x24x48 gusseted bag.
- (6) Place racks in a 3x2 configuration (3 layers high) for a 22x18x16 box. They only fit like this one way. 18 racks in total. A 22x22x22 box will be able to add another layer, for 4 layers of 3x2 racks. Add

more matting or use spare zip bags to fill in the side space left by the larger box.

- (7) Twist tie shut the 24x24x48 gusseted bag to fully enclose all the tube racks. Use tamper tape to lay the twist knot as flat as possible. You can tuck most of it into the space created where two racks meet.
- (8) Fold tag ends of matting onto each other to fully enclose the top of the box with matting.
- (9) Place a 20x20 divider on top of everything to make a flat surface.
- (10) Fill out a COC form for each shipping container where the most recent listing lists exactly and only the samples shipped in that particular container (equipment controls are separate samples from the field samples, so they must be accounted for on COC forms in the containers in which they are shipped). The individual employee packing and sealing the containers should list their name in the "from" line, be sure to include agency and print clearly. The container should be packed and released on the same date. Sign and place COC forms (Exhibit 3) in a clean 1-gal resealable bag, place evidence tape across the seal, and place the bag on top of the divider before closing the corrugated shipping box and sealing with packing tape. **Each box MUST** have a separate signed COC form included to document the specific samples included therein. If you must split samples into different configurations than listed on the original COC, make a new entry for those samples going into one container on the original COC and create a new COC for any new shipping containers.
- (11) Finally, double tape the top seams to close the box. Clearly write "This package conforms to 49 CFR 173.4 for domestic highway or rail transport only" on one side of the box. Be sure to draw arrows on all sides of the box, indicating which side is the top of the box.
- (12) Fill out a Federal Express (FedEx) GROUND shipping label with appropriate information and request a pick-up. FedEx will only ship flammables if you request a pick up, you will not be allowed to drop boxes off at FedEx.

(13) Items will be shipped to

Midwest Fisheries Center, Whitney Genetics Lab
555 Lester Avenue
Onalaska, WI 54650
608-783-8444

4.4 WGL contacts

- (1) Once items have been shipped, at least one person at WGL must be contacted and notified of approximate delivery date and time. At least one of the following personnel should be contacted via telephone and email regarding shipment, continue calling down the list until you speak with a human on the phone. Following shipment, email approximate delivery time, date and tracking numbers to the person you talked with and the person who will be receiving the delivery at WGL:

Aaron Johnson
Phone: 608-783-8419
Email: [Aaron E Johnson@fws.gov](mailto:Aaron.E.Johnson@fws.gov)
Nick Grueneis
Phone: 608-783-8404
Email: [Nikolas Grueneis@fws.gov](mailto:Nikolas.Grueneis@fws.gov)
Kyle Von Ruden

Phone: 608-783-8419

Email: Kyle.VonRuden@fws.gov

Zeb Woiak

Phone: 608-783-8424

Email: zebadiah_woiak@fws.gov

Maren Tuttle-Lau

Phone: 608-783-8403

Email: maren_tuttle-lau@fws.gov

Erica Mize

Phone: 608-783-8449

Email: erica_mize@fws.gov

Katie Bockrath

Phone: 608-783-8442

Email: katherine_bockrath@fws.gov

Emy Monroe

Phone: 608-783-8402

Email: emy_monroe@fws.gov

- (2) Upon receipt of the samples at WGL Personnel receiving the shipment must immediately sign the COC form, scan it and email it back, within one business day, to the sender of the COC and that station's Field Operations Manager (see Appendix A for eDNA contacts by office). Make sure to cc the Data Reporting Specialist, the eDNA Processing Coordinator and the eDNA Project Coordinator so that proper documentation can be recorded. Receiving personnel should also call the sender and Field Operations Manager if they do not have email access in the field. (from Appendix A: eDNA Point Of Contact's: Alpena, Anjanette Bowen; Ashland, Jason Ross; Cartersville, Neil Gillespie; Columbia, Kristen Towne; Green Bay, Cari-Ann Hayer; La Crosse, Jenna Merry; Lower Great Lakes (Region 5), Ted Lewis; Wilmington, Nathan Evans.
- (3) eDNA Field Operations Managers must QA/QC data in database within 10 business days of returning from the field unless other arrangements have been made with the eDNA Project Coordinator and eDNA Database Coordinator. If the eDNA Field Operations Manager cannot complete QA/QC, other personnel that were in the boat may also proof the data.

SECTION 5 DNA ASSAYS

5.1 General Quality Assurance and Chain-of-Custody Considerations

- Prior to any sample processing or analysis work, all laboratory employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Laboratory employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Laboratory employees will also review the laboratory safety plan (separate station-specific document) and participate in a safety briefing.
- Any change to described DNA handling, storage, or processing procedures must not result in reduction of eDNA sensitivity relative to current values and must be cleared with the eDNA Processing Leader. The original protocol produced the following results at different concentrations of purified DNA amplicons ¹ in sterile water:

Table 2. Different concentrations of purified DNA amplicons ¹ in sterile water for Bighead and Silver Carp.

Species	Purified DNA amplicon (copies/ μ l water)	Purified DNA amplicon (ng/ μ l water)
Bighead Carp	207	3.30×10^{-8}
Silver Carp	7	7.25×10^{-10}

New markers developed and then tested and verified via the Marker Validation Study were found to be more sensitive than the original markers:

Table 3. Marker copies per/ μ l water and marker length (bp) for both Silver and Bighead Carp individually, as well as combined.

Marker (species)	Copies/ μ l water	Marker length (bp)
ACTM 1 (both, cannot differentiate)	3	145
ACTM 3 (both, cannot differentiate)	3	133
SCTM 4 (Silver)	3	168
SCTM 5 (Silver)	3	98
BHTM 1 (Bighead)	3	144
BHTM 2 (Bighead)	3	96

The new markers are located throughout the mitochondrial (mt) DNA loop (figure 1), a suite of markers will detect and confirm the presence of carp DNA in the water samples, rather than a single locus per species. Each pair of markers can be analyzed in duplex, a single reaction, and when positive for markers, indicate 2 loci within the mt genome are present. The initial screening reaction will detect the presence of two loci within the cytochrome oxidase 1 (COI) gene in the mtDNA of both Silver and Bighead carp. This locus is particularly powerful, as it is used as the universal barcode of life, a region of mtDNA used to identify specimens to species for the entire animal kingdom. In order to be deemed presumptive positive, either of the COI loci can be detected in at least one replicate reaction. The confirmation of each species

¹ As reported in Jerde et al., 2012. Response to Casey et al.'s sensitivity of detecting environmental DNA comment. Conservation Letters 0:1-2.

will then be demonstrated by detecting at least one additional locus on the mtDNA genome. For Silver carp, two markers are in the ND2 and ND6 genes, and for Bighead carp in the ND2 and ND4 genes. The presence of one or the other locus in at least one replicate will confirm the presence of either Silver or Bighead carp DNA in the sample.

Detecting at least two different loci within the mt genome provides confidence in the detection of carp DNA in the water sample, and sequencing is not required since the entire amplicon (marker length in table above) is required to match the primers and probe indicating high specificity.

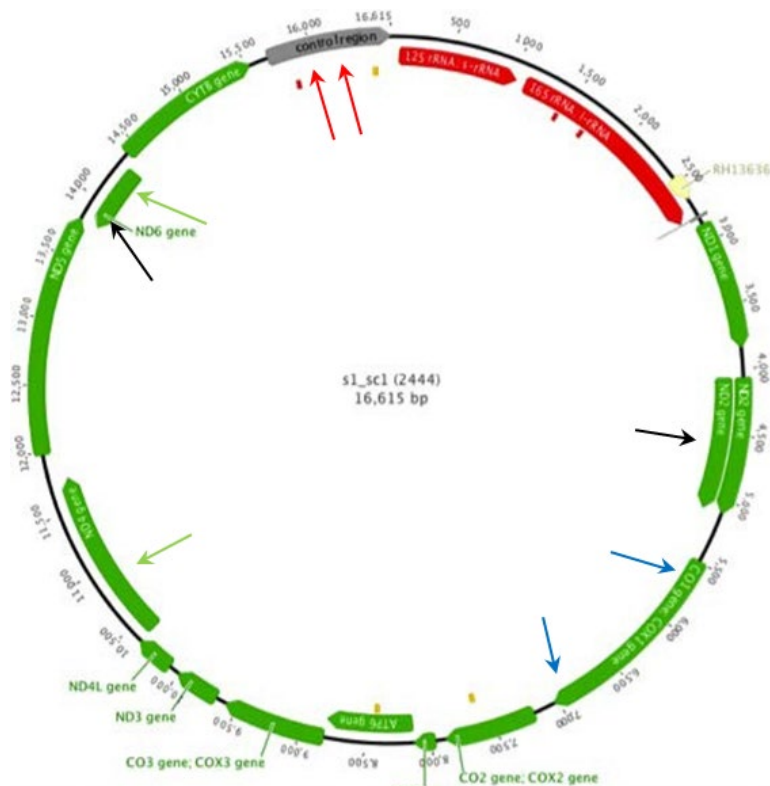


Figure 1. Map of entire Asian carp mitochondrial genome which was determined at ERDC with 33 bighead carp and 29 silver carp collected from across the United States (Farrington et al. 2015). Marker locations are indicated with arrows. The original markers (red) are located within the control region (D loop). The ACTM 1 and 3 markers (blue) are on either end of the COI gene. Bighead markers BHTM 1 and 2 (green) are in the ND4 and ND6 regions, and Silver markers SCTM 4 and 5 (black) are in the ND6 and ND2 regions, respectively.

²Farrington, H.L., C.E. Edwards, X. Guan, M.R. Carr, and R.F. Lance. 2015. Mitochondrial Genome Sequencing and Development of Genetic Markers for the Detection of DNA of Invasive Bighead and Silver Carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in Environmental Water Samples from the United States. PLoS ONE 10(2): e0117803. doi: 10.1371/journal.pone.0117803

- Each stage of eDNA genetic processing procedures (eDNA sample extraction, PCR setup, and post-PCR processes) should be performed in a separate room in order to minimize the risk of sample cross-contamination. If separate rooms are not available, extractions and PCR setup can be combined in one room, ONLY if PCRs are set up in a PCR hood with a hepa filter and UV light. All post-PCR processing *must* be in a separate room.
- Every effort should be made to ensure that equipment, work areas, and solutions are free from DNA contamination. All surfaces should be wiped clean with 10% bleach solution (or commercial DNA eliminating solution such as DNA Away) before and after use. If equipped with UV lamps, clean lab rooms or PCR hoods should be irradiated with UV light 30 minutes at the beginning and end of the

work day.

- Non-sterile, non-PCR ready microcentrifuge tubes (MCT) and glassware used in the lab must be autoclaved at 121°C for 20 min before being used. Any re-used items must be soaked in a freshly made 10% bleach solution for 10 minutes followed by a thorough rinse. (Sterile, certified DNA-free MCT do not need to be autoclaved prior to use).
- Good housekeeping policy should be practiced at all times. Reagents that have passed expiration dates should not be used, unless they have been tested in the lab. Reagents that have been kept at incorrect storage temperatures for a significant length of time should not be used. All reagents, reaction tubes, etc., must be clearly labeled. Records of batch numbers of all reagents used in individual assays should be made whenever reagents are signed out from the designated freezer. The temperatures of cold storage units must be monitored once a day (week days), and recorded in the Survey123 application. Temperature files will be downloaded at the end of each season and appended to the case log.
- Positive and/or negative reactions should be used to test all new batches of critical components prior to or concurrent with their application to eDNA samples.
- Standard sterile techniques should be used in the DNA laboratory to prevent the unintended transfer of DNA between surfaces, and to prevent cross-contamination between samples. Contamination can adversely affect the outcome of a case; therefore, it is essential that the laboratory have procedures in place to limit, recognize, and address contamination.
- Gloves (e.g., powder-free nitrile or latex) must be worn throughout sample processing. At a minimum, gloves should be changed at the completion of each step of the process. If gloves become contaminated or if contamination is suspected, discard them and replace them with new ones. Clean gloves should be used when using or handling keyboards, notebooks, pens, telephones, etc. and must be replaced immediately before recommencing bench work.
- Centrifuge tubes before opening the reagents. Uncap and close tubes carefully to prevent aerosol contamination.
- Ensure that centrifuges are always balanced when centrifuging samples.
- Ensure that all equipment, including paper, pens, and lab coats, are dedicated for use only in that particular laboratory (e.g., laboratory coat for each stage of procedure rooms. Workbooks that have been in contaminated areas shall not be taken into clean PCR areas. A Project Lab Book should be kept in a room separate from the DNA Extraction Room and DNA PCR Room. Each room (Extraction, PCR, and Post-PCR) should have note-taking materials (e.g., loose-leaf paper, networked tablet PCs) that can be transported or viewed for consolidation in the Project Lab Book. Other solutions for preventing contamination of sensitive areas via lab notes may be used following approval by the eDNA Processing Leader. Any changes should be incorporated into a revised QAPP. Laboratory notes/notebooks should:
 - Be written or printed with tamper-proof procedures
 - Have lab book identification, with consecutive numbering, dates, and signatures (of the note-taker) on each page.
 - Be made using permanent blue ink. Special pens may be required for certain paper types.
 - Have any changes to notebooks be dated and initialed by the person who made the change. Any incorrect information should have a single line drawn through it and not be completely obscured.

- Contain all data images (e.g., gel photographs, denaturing curves, and DNA sequence electropherograms). Images should be permanently affixed to the notebook and signed across both the edge of the insert and the page.
 - Be kept in a locked drawer or cabinet with restricted access when not in use.
- A log of all batches of critical components should be kept. This log should include material safety data sheets (SDS) and product information sheets. Dates of receipt, opening, testing, and disposal for each component should be recorded in the log.

5.2 Quality Control for Sample Custodian Procedure and Storage

- An internal log book or electronic file should be kept for all samples. The log book or file should be kept in a locked room when not in use.
- Separate freezers should be designated for storage of (a) concentrated water samples, (b) DNA extracts, (c) PCR and sequencing product, and (d) PCR, cloning, and sequencing kit components. A dedicated refrigerator should also be maintained for any PCR, gel electrophoresis, cloning, and sequencing kit components that require 4°C storage.
- Maps or other designations of the location of samples within freezers should be maintained.
- All items in freezers should have indelible ink identifications.
- All freezers should have non-universal locks or marine brackets attached that can be used with keyed locks, or be housed in a secure facility.
- All samples placed into or removed from freezers should be signed for on freezer log or ambient storage log (see Exhibit 7).

5.3 Physical Separation of Pre-PCR and Post-PCR Assay Stages

5.3.1 The eDNA Extraction Room

- Extraction of DNA must be performed where PCR products and stocks of cloned material are not handled.
- A PCR hood with a built-in ultraviolet (UV) light and HEPA filter may be used to further isolate DNA extraction kit solutions and elutes from ambient DNA.
- A complete separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for use in DNA extraction.

5.3.2 Pre-PCR Room

- To prevent carry-over of amplified DNA sequences, PCR reactions should be set up in a separate room from that used for post-PCR manipulations.
- A completely separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for the specific use of pre-PCR manipulations.
- Reagents and supplies should be taken directly from clean storage into the PCR area and should never be taken or shared with areas in which post-PCR analyses are being performed. Equipment such as pipettes should never be taken to the post-PCR area after use with amplified material.
- A sign-in log system should be implemented for use of the thermal cyclers (PCR machines), including the run name, and the number of thermal cycler heads.

5.3.3 PCR Product Analysis Room

This is the post-PCR Room where post-PCR manipulations are performed, including agarose gel electrophoresis of products and sequencing of presumptive positives.

- This room is a contaminated area; therefore, **no** reagents, equipment, laboratory coats, etc. from this room should be used in any of the other lab areas.
- A biological or PCR-type hood may be used for setting up cloning or sequencing reactions.

5.4 Receipt of Tubes

5.4.1 Source

Water samples have been taken in the field according to Section 2 and centrifuged according to Section 3. Samples have been shipped to WGL and personnel assigned to the WGL eDNA Team have received packages from the overnight service. NOTE: alcohol-preserved centrifuged samples are shipped at ambient temperature.

- (1) Upon receipt of samples from the eDNA sample filtering team, the shipped box(es) should be opened and if shipment was on dry ice, the temperature inside each box recorded. The general condition of the box(es) should also be recorded.
 - (a) If samples were shipped on dry ice only: measure temperature upon opening the box, either (1) place a glass thermometer inside the Styrofoam container, replace lid, and leave the thermometer in place for at least 2 min before removing it and immediately recording temperature or (2) immediately aim an infrared laser thermometer at the samples and press the MEASURE button to record the temperature inside the cooler.
- (3) Place all samples in sample storage freezer (-80°C) and log samples on freezer sheet (Exhibit 7). Centrifuged samples can be stored with preservative at ambient temperatures for up to three weeks, post receipt at the lab. After three weeks, samples must be decanted, dried, and frozen. Emergency storage of samples in -20°C freezers is allowable until room in the -80°C can be made.
- (4) Sign and date the COC forms that accompanied the samples. Place them in a designated file; a copy should also be provided to sampling agency. If the forms were inside sealed bags, slit the bag to remove the COC forms. Note any condition issues (broken tape or seals, damaged containers or containers, etc.) with the samples on the COC forms. Note any samples that must be discarded due to condition issues and the reason for discard.
- (5) Enter sample data into internal sample log book (or LIMS), including noting any samples that are being discarded and that should not be analyzed, and create new WGL COC form for samples. Note any observations about samples such as condition issues. Store COC forms in a secure area.
- (6) Alert Sampling and Processing Leaders that samples have been received. cc their Project Leaders as well. Send e-mail or call; team contacts are listed in Appendix A. This reporting must be done within 1 business day of completing sample check-in. Be sure to scan and send COCs according to 4.2.

5.5 Preservative Decanting and DNA Extraction from Tubes

5.5.1 Source

Tubes from eDNA sampling have been received by WGL eDNA Team and logged. Centrifuged sample tubes may be in ambient storage or if they are not preserved with alcohol in designated -80°C freezer.

5.5.2 DNA Extraction Quality Assurance and Chain-of-custody

At this stage, a critical component of quality control should be to correctly label all sample extraction processing tubes so that there is no question about the origin of samples.

- (1) Bench areas in DNA extraction laboratory and PCR-type hood (if used) should be wiped before and after use with 10% bleach. Validated, commercially available sterilization reagents, such as LookOut® DNA Erase®, may be preferred. Extraction rooms should be irradiated with UV lights for 30 minutes prior to use.
- (2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach must be rinsed to avoid corrosion.
- (3) It is common practice for moisture barrier paper towels to be placed on the bench top while processing samples to act as a barrier. The paper barriers must be changed and the bench top cleaned between sample batches.
- (4) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for the extraction process should be cleaned before and after each use.
- (5) Instruments such as forceps and scissors should be cleaned just prior to use. Sterile disposable equipment should be opened just prior to sample processing and discarded after one use.
- (6) When using pipettors, use filtered tips and never allow the liquid in a pipette tip to rise up to the barrier.
 - Do not rest the pipette on a dirty surface.
 - Avoid cross-contamination by changing pipette tips after each use.
 - Watch that the tip, and only the tip, is allowed to go into a bottle of reagent, never the pipette itself.
- (7) Record all solution batch/lot numbers used for reactions in lab notes.
- (8) Always mix tubes by vortex or finger flicking, and then briefly spin down tubes before opening.
- (9) No deviations to the DNA extraction protocol are allowed without written approval by the eDNA Processing Leader. Any errors in processing should be noted in the laboratory log. Samples affected by errors in the extraction protocol should be clearly identified.

5.5.3 Alcohol evaporation from centrifuged samples procedure

- (1) Centrifuged samples may need to be re-spun to re-concentrate sample at the bottom of the tube after shipping. Swirl liquid to re-capture any observable debris from the side of the tube wall.
- (2) Centrifuge at max speed for 5 minutes. This entire procedure can be done in advance, and samples stored at -80 until ready for processing.
- (3) Centrifuged samples preserved with alcohol must have the alcohol evaporated away before extracting the samples. Prepare laminar flow hood by wiping down the work surface with 10% bleach or DNA away and/or use the UV lamp for 15 minutes.
- (4) Remove samples from freezer or ambient storage; note on freezer/storage and sample log (see Exhibit 7).

- (5) Move samples in tube racks to the laminar flow hood. Carefully remove tube lids and place in the same order as the samples next to each rack. Turn on the air flow and leave the samples to dry until all traces of ethanol or isopropanol smell are gone, because these are both PCR inhibitors. Do not let samples sit at room temperature longer than it takes to evaporate the preservative, and never over a weekend. If some samples in a case are taking longer than the rest to dry, put dry samples in the freezer, and allow the remaining samples to evaporate until they are dried. If working in advance, evaporated samples should be stored at -80 until extraction.
- (6) Include a hood negative control sample for each hood containing samples set out to dry. There should be at least one hood control for each case, and there can be multiple hood controls for a single case if samples from the same case were placed in multiple hoods. Make a negative hood control by placing an empty, sterile centrifuge tube in the racks with the lid removed alongside the field samples.
- (7) Positive and negative extraction controls should be added to each eDNA extraction procedure batch.
 - Before proceeding with extraction, a positive control swab is prepared by pipetting 200 µl of diluted Silver and Bighead carp cell lines or tissue slurry directly onto a sterile swabs in 1.5-ml MCT. Alternative species (e.g. sturgeon or bluegill) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA. A batch of extraction positives can be prepared ahead of time and frozen at -20°C.
 - Additionally, an extraction negative control sample should be prepared by pipetting 200 ul of sterile lab DI water onto a sterile swab in a 1.5-ml MCT. A batch of extraction blanks can be prepared in advance and kept frozen at -20°C
 - For every extraction batch of filter samples or centrifuged samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control and one prepared positive control.
- (8) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below.

Cautions: As with all components of eDNA processing, quality control and sterilization procedures must be carefully followed in order to avoid contamination of downstream procedures.

5.5.4 Centrifuged samples shipped on dry ice procedure

Centrifuged samples may need to be re-spun to re-concentrate sample at the bottom of the tube after shipping. Thaw 5 samples of tubes, and work quickly to avoid DNA decomposition. When that set of 5 samples is finished, place them back into the freezer and take out the next five samples. Since samples are being placed back into the freezer, do not worry about entering this action into the freezer log, but be sure to note the dry date and initials in the case log. Only fill out the freezer log when samples are removed to extraction.

- (1) Swirl water to re-capture any observable debris from the side of the tube wall. Consolidate pellets into a single tube, rinse sides with additional water if needed, using a squirt bottle, and taking care to avoid contaminating the tip. Be sure to change gloves between samples.
- (2) Centrifuge at max speed for 5 minutes. Decant as much of the water as possible so that the entire sample can be extracted, including the remnant water.
- (3) This entire procedure can be done in advance, and samples stored at -80 until ready for processing.

- (4) Remove samples from freezer, note on freezer log (see Exhibit 8).
- (5) Samples frozen with water can be directly extracted.
- (6) Positive and negative extraction controls should be added to each eDNA extraction procedure batch.
 - Before proceeding with extraction, a positive control swab is prepared by pipetting 200 μ l of diluted Silver and Bighead carp cell lines or tissue slurry directly onto a sterile swabs in 1.5-ml MCT. Alternative species (e.g. sturgeon or bluegill) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA. A batch of extraction positives can be prepared ahead of time and frozen at -20°C .
 - Additionally, an extraction negative control sample should be prepared by pipetting 200 μ l of sterile lab DI water onto a sterile swab in a 1.5-ml MCT. A batch of extraction blanks can be prepared in advance and kept frozen at -20°C
 - For every extraction batch of filter samples or centrifuged samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control and one prepared positive control.
- (7) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below.

Cautions: As with all components of eDNA processing, quality control and sterilization procedures must be carefully followed in order to avoid contamination of downstream procedures.

5.5.5 IBI Scientific gMAX Kit Procedure for centrifuged samples

- (1) Obtain one Qiagen **lyse and spin** per sample to be extracted, open and organize tubes in rack. Label both caps of each sample. You will have time to label the rest (1 lab-supplied 1.5-mL MCT, 1 GD spin column, and 3 IBI collection tubes) during the 30 minute incubation. Be sure to add positive and negative extraction controls to each eDNA extraction procedure batch.
- (2) Add 350 μ L **GSB** to each lyse and spin basket tube. If precipitate has formed in GSB buffer, dissolve by incubating at 60°C for 10 minutes, before dispensing into tubes.
- (3) Add 35 μ L **proteinase K** to the GSB.
- (4) Move dried samples in 50 ml centrifuge tubes to the extraction room. Make sure each tube is dry.
- (5) Remove a sterile swab from pack, and dip it into a numbered tube of GSB/proteinase K mix. Use the moistened swab to swab the bottom of each tube included in the field sample (e.g. there is 5 tubes per sample, swab the bottom of all 5 tubes with the moistened swab). If the swab becomes covered with debris, rinse swab in tube of GSB/proteinase K solution for that sample, and proceed swabbing the rest of the replicates. Be careful to avoid cross-contamination at this step.
- (6) Place the swab back into the GSB mixture, break the wooden stick as close to the swab as possible, and close the tube. Change gloves between samples. For particularly dirty samples, add mixed GSB and ProK to any samples that appear dry or low so that the level appears to be about 350 μ l. Ensure maximum extraction of the sample.
- (7) Incubate at 60°C for 30 minutes. Label the rest of the tubes, place GD spin columns in your tube rack (same amount as your sample size), and print final archive labels for extracts. Also during this time place the Elution Buffer into the 60°C bead bath.
- (8) Remove lyse and spin tube from incubator and centrifuge at max speed for 1 minute. IF samples are particularly dirty, spin at least 3 minutes. . Archive tube with swab at -80°C , until the case is closed, then dispose. If the basket is clogged, use a pipette to remove the supernatant from the sample, recovering as much as you can. Make notes in the case log.
- (9) Add 500 μ L **ethanol** (molecular grade) to the extract. Mix thoroughly by vortexing. If liquid collects

around the cap, spin briefly before opening to reduce contamination risk.

- (10) Transfer about half of the mixture by pipette into a GD spin column placed in a 2 mL collection tube. Centrifuge at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (11) Transfer the remaining mixture by pipette onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (12) Place GD spin column in a new 2ml collection tube. Add 400 μ L Buffer **W1**. Centrifuge at $6000 \times g$ for 30 seconds. Discard flow-through and collection tube.
- (13) Place spin column in a new 2 mL collection tube. Add 600 μ L **Wash Buffer**. Centrifuge at $18,000 \times g$ for 3 minutes. Discard flow-through and collection tube.
- (14) Transfer the spin column to a new 1.5 mL MCT.
- (15) Add 200 μ L of the pre-heated **Elution buffer** to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at $\geq 18000 \times g$ for 30 seconds.
- (16) Discard the spin column and store the eluted DNA samples at -20°C . If DNA is to be immediately used for PCR, keep in 4° refridgerator.

5.6 PCR Amplification of eDNA Samples

5.6.1 Purpose

In order to determine if the DNA of a specific species is present in the concentrated water samples taken in the field, the total DNA extracted from the filtered samples must be amplified using species-specific primers. New in 2014, real-time PCR markers will be used for detecting presumptive positive samples as well as confirming Silver or Bighead carp DNA. All samples will be first assayed with two general Asian carp markers that detect two loci within the cytochrome oxidase I gene of both Silver and Bighead carp mitochondria (ACTM 1 and ACTM 3). Any sample that has at least one replicate PCR positive for either loci will be considered presumptive positive.

5.6.2 Source

eDNA samples have been received by WGL eDNA Team and DNA has been extracted. DNA elutes from samples should either be located in designated -20°C freezer or carried from the DNA extraction room to PCR room. Be sure the room is irradiated with UV light for 30 minutes prior to use. Ensure thermalcyclers are available before mixing the master mix. Master mix with or without template DNA cannot sit longer than it takes to prepare the samples for cycling.

5.6.3 PCR Quality Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to contamination and, subsequently, inaccurate results. Carefully follow quality control and COC steps listed below:

- (1) PCR-type hood bench should be wiped before and after use with 10% bleach. Validated commercially available sterilization reagent such as LookOut[®] DNA Erase[®] may be used. PCR room should be sterilized using built-in UV lights if available.
- (2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach should be rinsed to avoid corrosion.

- (3) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for PCR amplification should be cleaned before and after each use.
- (4) Use autoclaved, filtered, or commercially sterile molecular grade water prior to use for setting up PCR reactions.
- (5) Aerosol-resistant pipette tips should be used. Place the sterile tip on the pipette immediately prior to use. If the pipette is set down with the tip on, discard the tip. A new pipette tip must be used for the addition of each reagent to a sample tube.
- (6) Use PCR clean or autoclaved sample tubes for PCR master mix.
- (7) Close each tube immediately after labeling and after the addition of sample or reagents to prevent cross-contamination.
- (8) Be sure to only touch the tip of the MCT cap or use a tube opener, clean Kimwipe®, or other suitable barrier to open MCT.
- (9) Record all solution batch numbers used for reactions in lab notes.
- (10) PCR reagents should be aliquoted (a portion of the original stock) to avoid excessive repetitive freeze-thaw cycles and to protect stock reagents if contamination occurs.
- (11) Lightly vortex (quick touch, because vigorous vortexing can damage *Taq*) to mix sample and quick-spin/centrifuge tubes before opening the reagents to avoid splashes or drips from cap when opening. Uncap and close tubes carefully to prevent aerosol contamination.
- (12) Any revisions to the DNA amplification protocol must be approved by the eDNA Project Leader and documented in writing.

Cautions: Wear powder-free latex or nitrile gloves throughout the DNA amplification and gel electrophoresis procedures. Ethidium bromide, used in DNA gel electrophoresis to visualize DNA, is a known mutagen that affects biological processes.

Note for Exhibit 10-11: Plate maps have been included for scenarios most processed at WGL. 384-well plate sheets with inhibitor PCR components are included for initial screening of each case, but 96-well plate sheets are also included for processing any equipment controls for confirmed positive samples. Likewise, 96-well sheets are included for confirmation assays using silver and bighead PCR components, but if needed, 384-well sheets may be used instead.

5.6.4 Procedure

- (1) If DNA samples (extraction elutes) are removed from freezer, note this on sample log (Exhibit 7).
- (2) Use pre-printed plate map (Exhibits 10-11) or build plate map to determine which samples will be pipetted into which wells. Clearly mark plate identification on bottom edge skirt of plate. Write plate identification information (ddMONyy_initials_ASSAY_case(sample#-sample#)_Plate#) in lab notes.
- (3) Make sure sample map for each plate is entered into the pipetting robot or attached to lab notes and a signature is written across the map and lab book page.
- (4) Follow DNA amplification protocol detailed below.
- (5) Primers for two loci (ACTM 1 and ACTM 3) within the cytochrome oxidase I gene of the mitochondrial genome will detect either *Hypophthalmichthys molitrix* (Silver Carp) or *H. nobilis* (Bighead Carp), or both to screen eDNA samples and detect DNA present in the eDNA samples by

PCR. The PCR programs used to amplify the extracted DNA are the same for all three pairs of duplex real-time markers. The PCR protocol has been optimized to utilize TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA) in the eDNA screening. If other brands of real-time PCR master mixes are used, optimization of the recipe and thermal profiles must be executed. Eight reactions are set up for each sample, in addition to negative (DNA blank) and positive (DNA extracted from cell lines or tissue) controls for each master mix. The PCR reactions are prepared as follows:

- (6) Wipe lab bench area with 10% bleach, 75% Ethanol, or commercial DNA sterilization wipes. Also wipe down work area in PCR hood. Use built-in UV lamps to radiate clean room for 30 min prior to PCR set-up.
- (7) Electronic pipettors should be wiped down with one of the solutions or wipes listed in Step 1. Liquid
- (8) In the clean reagent room, obtain all PCR master mix reagents (using only those that have not expired and that have been tested and found viable).
 - a. TaqMan Environmental Master Mix 2.0
 - b. One tube with two forward and two reverse primers at 10 μ M working dilution.
 - c. One tube with a FAM-labeled probe for ACTM 1 and one HEX-labeled (or another appropriate fluorophore for your instrument) probe for ACTM 3 diluted to 2.5 μ M. (we prefer double-quenched probes that include a ZEN quencher located about 9 basepairs from the fluorophore as well as an Iowa Blackhole Quencher on the 3' end of the probe, but regular TAMRA-quenched probes may also be used).
 - d. Sterile molecular grade water (commercially sterile or Millipore filtered, autoclaved)
- (9) Allow reagents to thaw. Do not vortex primers or Taq too violently. Briefly spin down tubes to minimize aerosolization.
- (10) Record in lab notebook the lot number and expiration date of all reagents used.
- (11) Prepare PCR master mixes in clean reagent room. The master mix volume can be adjusted according to the number of samples to be processed. In order to make sure that master mix does not run out prior to supplying all the desired reactions (this may occur as a result of minor errors or variations in pipetting volumes), it is generally helpful to make more than enough master mix than is needed for the desired number of reactions. For example, make enough master mix for 100 reactions when actually preparing for 96 reactions. NOTE: If positive extraction controls consist of a different species of DNA, be sure to make a small separate master mix for those samples and use primers specific to the content of the control sample. Negative extraction controls should be amplified with the Asian carp master mix and a second time with the extraction positive control assay.

Each Initial PCR 1X reaction should contain:

- 10.0 μ L TaqMan Environmental Master Mix 2.0
- 1.0 μ L primer mix (10 μ M each, working dilution)
- 1.0 μ L probe mix (2.5 μ M each, working dilution)
- 5.0 μ L sterile water (*7.0 μ L in standard curve mix)
- 3.0 μ L sample extract as template (*1.0 μ L gblock template in standards)

NOTE: The TaqMan Environmental Master Mix is very viscous, and requires careful pipetting to ensure accuracy, and a new tip for each aliquot removed. The tip must be left in the mix for a few seconds after aspiration to ensure complete aspiration of the desired volume, and upon dispensing, a pause is required to allow the mix to pool in the tip before the final plunge to dispense the last drop of mix. If this mix is pipetted too quickly, there will be too little volume to fill the required wells. The electronic pipetting epMotion 5075s were specifically calibrated for this viscous mix. If other mixes are validated for use, new programs for the epMotion 5075s must also be developed.

- (12) Mix master mix well by slowly inverting the tube 17 times. Avoid shaking or inverting too quickly

to prevent creating bubbles. Move prepared mix from reagent room into PCR room.

- (13) Remove DNA extracts from freezer or fridge (fill out sample logs as needed), vortex (quick touch) and quick-spin down the extract tubes. Take them into the PCR room. Place the 96-well PCR plate onto a clean surface, positioned from left to right.
 - a. NOTE: WGL now has epMotion 5075s (automated liquid handling robots) that will fill plates for cycling. If these are unavailable, detailed directions for loading plates follows. Fill all plate wells with 17.0 μL PCR mix. FOR STANDARD WELLS only, add 2.0 μL water. To avoid creating bubbles, which interferes with data collection, place the pipette tip gently in the very bottom of the wells. Fill wells from the bottom up. Do not dispense air into the mix.
- (14) Carefully pipette 3.0 μL of each extract (or 1.0 μL gblock + 2.0 μL water in standards) to be screened onto the side of each well of a column, changing the pipette tip between each sample. Again, placement of the tip is important to avoid creating bubbles, so set the tip against the side of the well, above the level of master mix, and then deposit the aliquot of template.
- (15) Each column of eight wells should be filled with the same sample (i.e., eight replicates per sample to be tested). 46 columns of the PCR plate can test 46 different samples, the last remaining column is for PCR negative and positive controls and standard curve dilutions. Pipette 3.0 μL of water into wells G, H, O, and P to serve as PCR negative samples. Five-point, five-fold standard curves with 10, 50, 250, 1250, and 6250 copies/ μL will be used. The highest standard should be placed in well B followed by the next lowest standards through wells 7, and again in wells J-N. Place a mixed sample of both Silver and Bighead carp cell extract into wells A and I to serve as PCR positive samples. Note that standard wells need extra water (2.0 μL) added to the mix to account for only 1.0 μL of standard template added to each well compared to 3.0 μL DNA template added to each well for samples.
- (16) Extraction negatives will be run as a sample, in order as extracted in the Asian Carp assay because they were extracted with the sample batch, but may be plated in consecutive order in the extraction positive assay plate.
- (17) Extraction positives and negatives will be removed and run on separate plates with a bluegill marker assay (Takahara et al. 2013).
 - Each Bluegill PCR 1X reaction should contain:
 - 13.4 μL TaqMan Environmental Master Mix 2.0
 - 1.8 μL forward primer (10 μM each, working dilution)
 - 1.8 μL reverse primer (10 μM each, working dilution)
 - 1.0 μL probe (2.5 μM , working dilution)
 - 2.0 μL of extraction positive extract as template.
- (18) A new tip should be used for each template or standard delivered in each well. Carefully observe the volume of liquid in the pipette tip is accurate. All templates or standard material is delivered on the side of the well, as described in (15).
- (19) If filling plates by hand in 96-well formats, before the PCR plate is sealed, check every well for bubbles. Bubbles disrupt the accuracy of the camera detecting fluorophores and need to be removed before the plate is sealed and put into the machine. To remove bubbles, remove the straw from an isopropanol bottle and use the fumes from the alcohol to break the surface tension of the bubble. If a bubble is trapped in the bottom of the well, seal the plate and spin for an extended period of time. Use care to avoid touching tip to any surface of the plate or wells.
- (20) If filling plates on the epMotion robots, place PCR film over the PCR plate and use an automatic plate sealer to ensure the edges of all wells are sealed. Lightly vortex the sealed plate to mix contents, spin down the plate in the plate spinner to ensure mixture is in the bottom of wells and there are no bubbles.
- (21) WGL has a BioRad brand plate sealer. Use the seal guard to keep the seal from shifting. Turn on

the sealer and ensure it is up to temp. Seal 96 well plates at 180°C for 3 seconds and 384 well plates at 167°C for 3 seconds. Inspect the seal to ensure it is not loose, and all wells are sealed. If necessary to obtain reliable seals, temperature and time may be adjusted.

- (22) If you do not have a plate sealer, use adhesive seals and a plate roller or paddle to ensure a good seal. It is imperative to have a good seal to prevent evaporation which will prevent data collection, and potentially contaminate the lab with amplified DNA.
- (23) Place the PCR plate in the thermal cycler, close and secure lid, and select the appropriate PCR thermal program. The thermal programs for the ACTM 1 & 3 duplex reaction is:

Initial denaturation at 95°C for 10 min
Followed by 40 cycles of:
95°C for 15 sec
60°C 1 min

- (24) Record the plate ID, thermal cycler unit or head, and run times for the PCR plate in the PCR log (Exhibit 8).
- (25) Place cycled PCR plates and product in trash without opening the seal. Remove plates promptly.
- (26) Under no circumstances should you open or uncover PCR plates that have been cycled in the PCR room.

5.6.5 Standard curve material, preparation and storage

The use of standard curves in quantitative PCR applications allows for calculation of the initial starting copy number of target DNA in the aliquot of template used in each reaction. Standard curve materials will be synthetic strands of DNA purchased from a reliable production company. WGL will purchase G-block product from Integrated DNA Technologies, but other suitable vendors are allowed. To get reproducible and high quality standard curve data, it is important to store materials properly, and make serial dilutions fresh each week. Storing longer produces inaccurate results.

- (1) gblock standard curves
 - a. gblock is purchased so that it contains all 6 qPCR marker targets.
- (2) gblock is hydrated with TE buffer to make an initial dilution that is 1.00 E+10 copies per microliter. (an excel stock dilution calculator spreadsheet is on the shared drive at WGL).
 - a. Volume TE to add = $fmoles * 0.0000000000000001 * 6.022E+23 / 1.00E+10$
 - i. Mix well. .
 - b. Immediately use 1.0 µl of the hydrated gblock into 999.0 µl of TE buffer to make storage stock with 1.0 E+7 copies/µl.
 - i. Make several aliquots of this stock and store in screw-cap tubes with o-rings to prevent evaporation in the freezer. Freeze remainder of stock from step a.
- (3) Add 1.0 µl of 1.0 E+7 copies/µl stock to 31.0 µl 100 ng/µl yeast tRNA in water. This working stock has 312,500 copies/µl.
- (4) Serial dilutions for use in assays should be made fresh weekly with the stock and 100 ng/µl tRNA as a diluent. They may be stored frozen and thawed as needed throughout the week.
 - a. A **new** pipette tip **is required for each** dilution for accurate results.
 - b. After each addition, use the pipette to mix a **minimum** of 17 times.
 - c. Currently, a 5X curve is used at WGL.
 - i. 30 µl working stock into 270 µl tRNA. Mix 17 times = 31,250 copies
 - ii. 60 µl of 31,250 mix into 240 µl tRNA, mix 17 times = 6250 copies.
 - iii. 60 µl of 6250 mix into 240 µl tRNA, mix 17 times = 1250 copies
 - iv. 60 µl of 1250 mix into 240 µl tRNA, mix 17 times = 250 copies

- v. 60µl of 250 mix into 240 µl tRNA, mix 17 times = 50 copies
- vi. 60 µl of 50 mix into 240 µl tRNA, mix 17 times = 10 copies

5.7 Positive Sample Determination and data management

5.7.1 Purpose

Once real-time data has been collected, the results must be documented, interpreted (i.e., scored), and archived.

5.7.2 Source

Following cycling, data files can be exported from the cyclers, analyzed with R-statistical package code, and results confirmed by visual examination of the trace data. Following data processing, samples determined as presumptive positive must be assayed with two additional reactions, one each for Silver and Bighead confirmation.

5.7.3 Presumptive Call Documentation and Storage Assurance

- (1) Immediately upon opening a BioRad result file, use the default baseline correction function (Apply Fluorescence Drift Collection found under SETTINGS menu in CFX software). Assay quality must be assessed before data is accepted. Data must be exported as a *.txt file and saved into a folder named with the case number. File names must be carefully entered without any mistakes in spacing or coding. File names will follow this format: ddMONyy_initials_ASSAY_case#(sample#-sample#)_Plate#_cyclerSN. There should not be any spaces. Sample range should be integers separated with a dash. Plate numbers are designated as P1, P2, P3.....and no plate numbers should be repeated within an analysis day. Therefore, if there are 3 rounds of PCR, plate numbers should go from 1 to 24. Dates must be as depicted with a two-digit day, three-letter CAP month, and two digit year. Initials are not case sensitive, but assay MUST BE CAPS.
- (2) Requirements for a successful analysis are as follows:
 - a. Minimum requirements for accepting the data are $E=80-120\%$ and $R^2 \geq 0.95$. If necessary, errant standard curve points may be dropped to recover curve statistics, but there must be at least one 3-point curve remaining. This is acceptable, since the standard curves are for assessing assay performance only, and not for quantification of starting copy number. If the standard curves fail, the plate must be re-amplified.
 - b. Clean negative controls, an extraction negative sample with a single ACTM marker in a single reaction should be considered presumptive and included in the confirmation assays. If it is confirmed, then that batch of samples is rendered unreportable. A positive PCR negative requires a plate re-run unless the entire plate is negative.
 - c. Extraction positive samples requires a minimum of 1 replicate positive.
 - d. PCR Positive controls must have at least one of two reactions positives.
- (3) Once text files are in the folder, the program R should be used analyze the data. Output will include the following:
 - a. Summary statistics (StandardCurves.csv) on assay quality including the efficiency (E) and R^2 of the duplicate standard curves on each plate.
 - b. Summary data for the total number of negative controls that were clean and the total number of positive controls that were positive, sorted into extraction vs PCR controls (AnalysisLog.txt). If there are any issues, the samples and controls must be re-processed at

the step of failure. For example, if PCR controls fail, the plate must be re-amplified. If the problem is resolved with the re-do, the re-run data should be used. If the problem still persists, then the data should be rejected and not reported. Document failed analyses on the data sheets and in the case log file.

- i. Rerun data should be exported as a txt file with the same naming convention, but saved within a folder nested within the folder with case results. The folder with re-run data must be named "reruns", all lowercase one word.
 - c. A list of samples that had cycle thresholds less than or equal to 15 cycles that were changed to negative.
 - d. A list of presumptive positive samples (Presumptives.csv).
 - e. A table of data including the number of octets positive per sample and the average and standard deviation copy number for positive samples (DataSummary1.csv).
- (4) Enter results and quality control summary data into the database in an Excel file.
- a. Notify the eDNA Processing Leader immediately of any cases with presumptive positive results.
- (5) All reports will reviewed by the eDNA Processing Leader before being reported.
- (6) A paper copy of the report should be held in the files for 5 years.
- (7) Electronic copies of all reports should be held for 5 years or longer, as space permits.
- (8) Any substantive revisions to the DNA amplification protocol must be approved by the eDNA Processing Leader and approved by the eDNA Coordinator. Any such changes must be incorporated into a revised QAPP.

5.7.4 Confirmation of Presumptive Positive Samples

- (1) Repeat section 5.6.4 for only the presumptive positive samples with the SCTM markers and with the BHTM markers. Recipes for the master mix are the same, except substitute the appropriate primers and probes. The PCR program is the same as well. Be sure to use silver carp positives for the SCTM marker and bighead positives for the BHTM marker assay.
- (2) Repeat section 5.7.3. to assess assay quality before accepting data.
- (3) All equipment controls for confirmed positive samples must be dried and extracted with the same method as the samples, and assayed with ACTM 1 and 3, and as in section 5.6.4. If any equipment controls test positive, the result for that sample will not be included. A new hood control is not necessary at this stage.
- (4) Final confirmed results are in the output file: DataSummary2.csv. This file is formatted so that if sorted by sample ID, the results may be copied and pasted into the case log file. Be sure to proof alignment of the paste by checking sample ID numbers randomly the full length of the case.
- (5) Notify the eDNA Processing Leader immediately of any cases with confirmed positive results.
- (6) Ensure that all raw data, output files from the thermocyclers and data analysis are in the case folder on the LIMS computer.

5.8 Communication of eDNA Assay Results

5.8.1 Purpose

To convey to Service designated personnel the progress and results of eDNA assays.

5.8.2 Source

WGL keeps a record of each samples progress through the eDNA assay procedure. These records are summarized for each batch for reporting to the eDNA Coordinator in USFWS Midwest Region.

Each field sampling agency keeps a record of field data. These records are summarized and reported to the eDNA Coordinator in USFWS Midwest Region via the eDNA database.

The eDNA Database Coordinator in USFWS Midwest Region collates field and lab data for reporting results to partners and the public.

5.8.3 Quality Control

The WGL eDNA Processing Leader will provide updates and reports to the USFWS Midwest Region eDNA Coordinator. At this time, Emy Monroe is the WGL eDNA Processing Leader and Kelly Baerwaldt is the USFWS Midwest Region eDNA Coordinator. Any permanent or temporary changes to either position should be communicated immediately to the assigned USFWS senior executive. Any permanent changes should be incorporated into a revised QAPP.

Any revisions to the reporting procedures must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.8.4 General Procedure (for details see Appendix C for SOP)

The USFWS eDNA Processing Leader should, on every Friday during the period over which the WGL eDNA Team is processing samples, provide updates on all sample batches to the USFWS eDNA Coordinator. Reports should be organized by case and should consist of spreadsheets showing the stage of processing for each sample. Additionally, following approval by the eDNA Processing Leader, the WGL PRR should convey final results for each case (all samples confirmed as positive or negative for AC eDNA) within 24 hours of completion of processing for the last sample within a case.

The USFWS eDNA Coordinator may request updates from the eDNA processing leader at any point. The WGL eDNA Processing Leader is expected to respond as soon as possible during normal working hours.

SECTION 6 INTERNAL QUALITY CONTROL CHECKS

Details on quality control are found within each of the various protocol sections. In summary, however, quality control relative to sample contamination is covered by the transport (or cooler), equipment, DNA extraction, PCR, and sequencing blanks or negative controls. Quality control for efficacy of methodology, solutions, etc. is covered by positive controls for each sample handling step (extraction, PCR, and sequencing) of the eDNA protocol. Furthermore, each new solution or kit to be used in eDNA processing will be tested with positive and negative controls before or during the first use. Quality Assurance staff will review and recommend changes or updates to improve processes as needed or when new technology is adopted.

The WGL eDNA processing facility and protocols was reviewed by an ERDC audit shortly after completion of the transition plan and full deployment of Asian carp eDNA dedicated equipment.

6.1 Laboratory Quality Control Evaluation Criteria

Quality control is measured in two ways:

- If transport, filtering, centrifuge, hood, extraction, PCR, and DNA sequencing negative controls show product (e.g., DNA copies greater than zero), the associated positive data are negated and, when possible, samples are reprocessed. Contamination of DNA extract will require that any positive samples be removed from consideration. If all other samples are negative, contamination was only an issue in the controls, and negative results may be reported.
- Positive controls are currently employed for extraction, PCR, and sequencing. If the positive controls fail to behave as expected, any sample showing an apparent lack of results will be rerun at the same time or following rerunning of the positive controls. This will be done until all positive controls produce the expected results.

SECTION 7 PROCEDURES TO ASSESS DATA PRECISION, ACCURACY, AND COMPLETENESS

7.1 Field Methods and Laboratory Data

Use of quantitative PCR techniques provides regular and on-going assessment of accuracy and precision of laboratory analyses. The integrity of each plate analyzed is confirmed when the standard curves pass minimum requirements in 5.7.3.2a. Each standard curve represents a 5-fold dilution of carp DNA down to 10 copies.

Thus, the following 3 month assay no longer needs to be executed to document quality of data produced in the lab.

SECTION 8 CORRECTIVE ACTIONS

Corrective actions may be required for two classes of problems: analytical/equipment problems and noncompliance problems. Analytical and equipment-related problems may develop during sampling and sample handling, sample preparation, laboratory instrumental analysis, and data review. Noncompliance issues arise when eDNA sampling, processing or lab procedure execution deviates from procedures described in the QAPP.

In the case of analytical/equipment problems or deviations from set procedures (as outlined in QAPP), the responsible lead will determine if the problem or deviation will impact the accuracy of the resulting data. If it is determined that the problem or deviation does impact data accuracy, two courses of action may be followed:

- (1) If possible, the procedure is repeated until it is performed without any problem or deviation, or
- (2) The sample or samples are removed and not processed any further.

In either case, a corrective action report must be completed (Exhibit 2). Careful notes of any corrective actions and what incident led to them, as well as the resolution or preventative measure(s) identified will be carefully noted in the corrective action report, which must be provided electronically to all Leaders (Project Leader, Sampling Leader, etc) as an after action report. The paper copy of the corrective action report will be maintained in the project file as a long-term record.

In the case that the responsible lead determines that data accuracy is not affected by the analytical/equipment problem or deviation from procedure, the sample or samples may continue to be processed. The responsible lead will make careful note of the incident in project records and include the rationale for continuing processing.

Laboratory problems and issues are documented first in the paper laboratory notebooks, and transferred to the electronic case log at the end of each processing batch for all phases of lab work. The Quality Assurance specialist will verify lab notes and electronic notes, and the electronic record will serve as the report and corrective action note for any future audit. If any problem renders a result non-reportable, this will be highlighted in the weekly report made by the Data Documentation Specialist, and the eDNA Processing Lead will provide notation in the final case report so that samples with no results are easily seen by the eDNA Coordinator and the eDNA Database Coordinator.

SECTION 9 PREVENTATIVE MAINTENANCE PROCEDURES

9.1 Field Equipment/Instruments

Hand-held or console installed sonar: Batteries will be changed at least once a month in hand-held units (if not required sooner) to ensure accurate readings of the instrument. In addition, reading accuracy should be checked prior to sampling season for all units. Depth readings may be checked by filling a container of a known depth with water and submerging the instrumentation. Temperature readings of the sonar may be checked against a thermometer. These records should be kept in ink in a bound notebook, where the originals are kept on site in a secure location and copies are sent to the eDNA Coordinator at the end of each sampling season.

iPads and GNSS Receiver: Charged daily and ensure data points are uploaded to the cloud as soon as a signal is available.

GPS equipment: Batteries will be changed at least once a month (if not required sooner) to ensure accurate readings of the instrument. In addition, coordinate accuracy will be checked against known benchmarks.

DI equipment in the Trailer: Keep a bound hard-copy notebook in the trailer to record the total dissolved solids present each day the trailer is used. It should read zero.

Centrifuges: Annual service is a good idea if they are heavily used.

All other laboratory equipment will be inspected monthly and undergo proper maintenance to maintain their ideal working condition. Any equipment not performing accurately or to established standards will be disposed of and replaced.

9.2 Laboratory Instruments

Pipettes: Annually all pipettes will be inspected, calibrated, and certified. Any pipette failing inspection and certification will be disposed of and replaced.

Any thermal-cycler head that fails the manufacturers self-test upon instrument startup will be removed and replaced with the manufacturer's certified replacement part.

Equipment maintenance contracts, with annual maintenance check-ups, will be used for any appropriate equipment (i.e., DNA sequencer).

SECTION 10 PERFORMANCE AND SYSTEM AUDITS

10.1 Field Audits

Internal audits of field crew performance and quality controls for sampling and filtering will be made semi-annually by the FWCO field staff to make sure that all procedures in the sample collection portions of the QAPP are being followed. On sampling trips where more than two FWCO offices are on the team, the visiting FWCO eDNA leader will serve as the sampling auditor. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures). If there are no trips with coordinating offices, then the representative on the eTeam for USFWS Midwest Region may serve as the auditor for their office.

10.2 Laboratory Audits

Internal audits of WGL laboratory performance and quality controls will be made semi-annually by the DNA Processing Lead to make sure that all procedures in the DNA processing portions of the QAPP are being followed. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures. Participation in validation studies by the lab may suffice as internal audits, and reports of such studies may be substituted and filed with the eDNA Program Coordinator. Every 2 years an external review of WGL eDNA processing will be undertaken. The review panel or consultant(s) will be selected by the Project Lead. The DNA Processing Lead may assist the Program Coordinator in identifying one or more potential reviewers.

SECTION 11 EXHIBITS

Exhibit 1. Pre-Trip Field Plan Summary

NOTE: fillable forms for use are stored in the google drive folder. Do not use this sample form.

Pre-Trip Plan for eDNA Field Collection and Processing

Exhibit 1a must be completed and sent to the eDNA Program Coordinator and the Database Manager at least one business day prior to the start of the event. Please attach maps of the target sampling areas.

Case: Basin: Dates:

PRE-TRIP PLAN

Target Waterbody	Samples	Blanks	Total	Sample #s
			0	0
			0	
			0	
			0	
			0	
			0	
			0	
			0	
TOTAL	0	0	0	

PERSONNEL:

Field Operations Manager: Phone #:

Sampling Crew:

Boat Operator:
Sampling Lead:
Sampling Assistant:
Data Recorder:
QA/QC Specialist:

Processing Crew:

Processing Lead:
QAQC Specialist:

SAMPLING SCHEDULE:

Day 1

Date: [redacted]

Boat ramp(s) Coordinates (dec. deg): [redacted]

Number of Samples: [redacted]

Number of blanks: [redacted]

Notes: [redacted]

Day 2

Date: [redacted]

Boat ramp(s) Coordinates (dec. deg): [redacted]

Number of Samples: [redacted]

Number of blanks: [redacted]

Notes: [redacted]

Day 3

Date: [redacted]

Boat ramp(s) Coordinates (dec. deg): [redacted]

Number of Samples: [redacted]

Number of blanks: [redacted]

Notes: [redacted]

Day 4

Date: [redacted]

Boat ramp(s) Coordinates (dec. deg): [redacted]

Number of Samples: [redacted]

Number of blanks: [redacted]

Notes: [redacted]

Day 5

Date: [redacted]

Boat ramp(s) Coordinates (dec. deg): [redacted]

Number of Samples: [redacted]

Number of blanks: [redacted]

Notes: [redacted]

Exhibit 2. Post-Trip Field Plan Summary

NOTE: fillable forms for use are stored in the google drive folder. Do not use this sample form.

Post-Trip & QA/QC Summary for eDNA Field Collection and Processing

Exhibit 1b must be completed and sent to the eDNA Program Coordinator and the Database Manager within 10 business days of event completion.

Case: Basin: Dates:

1. POST TRIP SUMMARY

- Sampling plan was completed as described in Exhibit 1a (Check box if applicable)
- Sampling plan was NOT completed as described in Exhibit 1a (Check box if applicable, describe below)

Description of deviations from Exhibit 1a (List and describe deviations, include reasoning)

Field Operations Manager: Print Sign _____ Date

2. QUALITY CONTROL AUDIT

Sampling Quality

I observed the collection of eDNA samples for case # [redacted]. I compared the actions of the sampling crew to the QAPP and witnessed adherence to the QAPP in the following (check the appropriate response for each item; if No, explain below):

Yes No Boat decontamination in the field

Yes No Sample collection procedure

Yes No Navigation to sample sites

Explanation of deviations (if applicable):

Sampling QAQC Specialist: Print [redacted] Sign _____ Date [redacted]

Processing Quality

I observed the processing of eDNA samples for case # [redacted]. I compared the actions of the processing crew to the QAPP and witnessed adherence to the QAPP in the following (circle the appropriate response for each item; if No, explain below):

Yes No Decontamination of processing equipment

Yes No Sample processing procedure

Explanation of deviations (if applicable):

Processing QAQC Specialist: Print [redacted] Sign _____ Date [redacted]

Exhibit 3. Chain of Custody

NOTE: Do not use this form, use the PDF stored on the google drive.

US Fish & Wildlife Service		CHAIN-OF-CUSTODY RECORD		
Date and time of collection:		Collected by (first and last names):		
Notes:				
Case Number:				
All control samples must also be listed and accounted for. The chain of signatures should be continuous.				
Sample Numbers	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Sample Numbers	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Sample Numbers	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Sample Numbers.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Sample Numbers	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Sample Numbers	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: • FedEx • In Person • Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	

Exhibit 4. USFWS WGL Sample Receipt Checklist

USFWS WGL Sample Receipt Checklist

Case Number:	Receipt Date:
Other:	Rec'd by:

Were samples shipped? Yes, FEDEX/UPS/Other No, Courier pickup/ <u>hand</u> delivered	Comments:
Cooler temp upon arrival C/NA	
Chain-of-custody (COC) present? Yes/ <u>no</u> Complete? Yes/no	
Custody seals present on <u>cooler</u> ? Yes/no Samples? Yes/no	
Were sample containers intact? Yes/no	
Samples and COC match? Yes/no	
If any problems, was project manager notified? Yes/no By whom? _____	
Appropriate sample containers? Yes/no	
Date/time of collection on COC Yes/no	
Location and ID of sample storage: Freezer _____ Refrigerator _____	
Temperature log updated for <u>storage</u> ? Yes/no	

Exhibit 5. Laboratory Case Log

Screen capture of Excel log file. First worksheet in workbook lists all sampling events or cases which correspond to samples from a particular system. Subsequent worksheets are created for each case and allow for sample tracking through the eDNA lab. This is our Laboratory Information Management Tracking system. "REF" in cells is due to formulae already entered into the form, which will work once data are entered.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
										Presumptive Positive			(number of samples)		Quality Control Success (Y/N)		
	Priority	Case Number	Case Type	Number of Samples	Check-in Date	Shelf Storage Expiration Date	Days You Have to Move Samples to Freezer	Sample Progress	Status (Open/Close)	ACTM 1	ACTM 3	BOTH	Silver Carp	Bighead Carp	Extraction Control	PCR Control	Equip Control
3	1					1/21/1900	FREEZE										
4	2					1/21/1900	FREEZE										
5	3					1/21/1900	FREEZE										
6	4					1/21/1900	FREEZE										
7	5					1/21/1900	FREEZE										
8	6					1/21/1900	FREEZE										
9	7					1/21/1900	FREEZE										
10	8					1/21/1900	FREEZE										
11	9					1/21/1900	FREEZE										
12	10					1/21/1900	FREEZE										
13	11					1/21/1900	FREEZE										
14	12					1/21/1900	FREEZE										
15	13					1/21/1900	FREEZE										
16	14					1/21/1900	FREEZE										
17	15					1/21/1900	FREEZE										
18	16					1/21/1900	FREEZE										
19	17					1/21/1900	FREEZE										
20	18					1/21/1900	FREEZE										
21	19					1/21/1900	FREEZE										
22	20					1/21/1900	FREEZE										
23	21					1/21/1900	FREEZE										
24	22					1/21/1900	FREEZE										
25	23					1/21/1900	FREEZE										
26	24					1/21/1900	FREEZE										
27	25					1/21/1900	FREEZE										
28	26					1/21/1900	FREEZE										
29	27					1/21/1900	FREEZE										
30	28					1/21/1900	FREEZE										
31	29					1/21/1900	FREEZE										
32	30					1/21/1900	FREEZE										
33	31					1/21/1900	FREEZE										
34	32					1/21/1900	FREEZE										
35	33					1/21/1900	FREEZE										
36	34					1/21/1900	FREEZE										
37	35					1/21/1900	FREEZE										
38	36					1/21/1900	FREEZE										
39	37					1/21/1900	FREEZE										
40	38					1/21/1900	FREEZE										
41	39					1/21/1900	FREEZE										
42	40					1/21/1900	FREEZE										
43	41					1/21/1900	FREEZE										
44	42					1/21/1900	FREEZE										
45	43					1/21/1900	FREEZE										
46	44					1/21/1900	FREEZE										
47	45					1/21/1900	FREEZE										
48	46					1/21/1900	FREEZE										
49	47					1/21/1900	FREEZE										

Exhibit 7. Sample Storage Logs

Sample Chain-of-Custody logs used in the lab. Samples are CHECKED IN to storage area once, and then each successive removal or return are logged in the CHECKED OUT spaces across the line.

-80 °C Sample Log

Freezer ID and Location: _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.			

Example of ambient temperature (alcohol-preserved centrifuge samples only) sample storage log. Once samples are checked out of ambient storage into cold storage, the entry is closed in the ambient log.

Bench-top Shelving Log

Room _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.			

Exhibit 9. Extraction Data Sheet

"COPY" watermark is grey on original.

Extraction data sheet: to be filled out and taped into Extraction room data book

Analyst _____ Room# _____ Date _____ Sample batch _____

Reagents used: note lot ID and expiration date for kit components

Reagent/tubes	Lot #	Expiration date
GSB Buffer		
Proteinase K		
Ethanol		
W1 Buffer		
Wash Buffer		
Elution Buffer		

Tube ID	Sample ID	Tube ID	Sample ID
1		25	
2		26	
3		27	
4		28	
5		29	
6		30	
7		31	
8		32	
9		33	
10		34	
11		35	
12		36	
13		37	
14		38	
15		39	
16		40	
17		41	
18		42	
19		43	
20		44	
21		45	
22		46	
23		47	
24		48	

Write notes for any deviations from QAPP or lab blunders on facing page of notebook. Be sure to add the same notes to the case log.

|

Exhibit 10. qPCR sheet, 384-well plate general assay

ERDC AC TM1/3 NO IPC qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ date _____ Kit or sample batch _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

SAMPLE PLATE MM

Reagent name	Volume per rxn	Volume for rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10.0 µl	µl		
AC TM1/3 Primer Mix(10 µM)	1.0 µl	µl		
AC TM1/3 Probe (2.5 µM)	1.0 µl	µl		
ddH ₂ O	5 µl	µl		

Samples: 3.0ul template + 17.0ul master mix

Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A																								POS	
B																									6250
C																									1250
D																									250
E																									50
F																									10
G																									NTC
H																									NEG
I																									POS
J																									6250
K																									1250
L																									250
M																									50
N																									10
O																									NTC
P																									NEG

Cycler ID _____

Did cycle complete Y N

Plate Name _____

ddMONVv Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								POS
B																								6250
C																								1250
D																								250
E																								50
F																								10
G																								NTC
H																								NEG
I																								POS
J																								6250
K																								1250
L																								250
M																								50
N																								10
O																								NTC
P																								NEG

Cycler ID _____

Did cycle complete Y N

Plate Name _____

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								POS
B																								6250
C																								1250
D																								250
E																								50
F																								10
G																								NTC
H																								NEG
I																								POS
J																								6250
K																								1250
L																								250
M																								50
N																								10
O																								NTC
P																								NEG

Cycler ID _____

Did cycle complete Y N

Exhibit 11. qPCR sheet; 96 well plate general assay

ERDC AC TM 1/3 NO IPC qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ date _____ Kit or sample batch _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

SAMPLE PLATE MM

Reagent name	Volume per rxn	Volume for ___ rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10.0 µl	µl		
AC TM1/3 Primer Mix(10 µM)	1.0 µl	µl		
AC TM1/3 Probe (2.5 µM)	1.0 µl	µl		
ddH ₂ O	5 µl	µl		

Samples: 3.0ul template + 17.0ul master mix

Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12
A											POS	POS
B											6250	6250
C											1250	1250
D											250	250
E											50	50
F											10	10
G											NTC	NTC
H											NEG	NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____

Did cycle complete Y N

Exhibit 12. qPCR sheet silver carp assay

ERDC SC TM4/5 qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ date _____ Kit or sample batch _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

SAMPLE PLATE MM

Reagent name	Volume per rxn	Volume for ___ rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10 µl	µl		
SC TM 4 Primers (10 µM)	1.0 µl	µl		
SC TM 5 Primers (10 µM)				
SC TM 4/5 Probes (2.5µM)	1.0 µl	µl		
ddH ₂ O	5 µl	µl		

Samples: 3.0ul template + 17.0ul master mix

Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12
A											POS	POS
B											6250	6250
C											1250	1250
D											250	250
E											50	50
F											10	10
G											NTC	NTC
H											NEG	NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____

Did cycle complete Y N

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

Exhibit 13. qPCR sheet bighead carp assay

ERDC BH TM1/2 qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ date _____ Kit or sample batch _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

SAMPLE PLATE MM

Reagent name	Volume per rxn	Volume for ___ rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10 µl	µl		
BH TM 1 Primers (10 µM)	1.0 µl	µl		
BH TM 2 Primers (10 µM)				
BH TM 1/2 Probes (2.5µM)	1.0 µl	µl		
ddH ₂ O	5 µl	µl		

Samples: 3.0ul template + 17.0ul master mix
Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12
A											POS	POS
B											6250	6250
C											1250	1250
D											250	250
E											50	50
F											10	10
G											NTC	NTC
H											NEG	NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____

Did cycle complete Y N

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

Exhibit 14. qPCR sheet bluegill assay, 384-well plate

Bluegill qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ Date _____ Samples _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

Reagent name	Volume per rxn	Volume for ____ rxns	Lot or ID #	Expiration date
TaqMan Env. Master Mix	13.4 µl	µl		
Forward Primer 10µM	1.8 µl	µl		
Reverse Primer 10µM	1.8 µl	µl		
Probe (2.5µM)	1.0 µl	µl		

2 µl template

Plate Name _____
ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								POS
B																								
C																								
D																								
E																								
F																								
G																								NTC
H																								NEG
I																								POS
J																								
K																								
L																								
M																								
N																								
O																								NTC
P																								NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____ Did cycle complete Y N

Exhibit 15. qPCR sheet bluegill assay, 96-well plate

Bluegill qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ Date _____ Samples _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

Reagent name	Volume per rxn	Volume for ___ rxns	Lot or ID #	Expiration date
TaqMan Env. Master Mix	13.4 µl	µl		
Forward Primer 10µM	1.8 µl	µl		
Reverse Primer 10µM	1.8 µl	µl		
Probe (2.5µM)	1.0 µl	µl		

2 µl template + 18.0ul master mix

Plate Name _____

ddMONyy Initials ASSAY case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12
A											POS	POS
B												
C												
D												
E												
F												
G											NTC	NTC
H											NEG	NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____

Did cycle complete Y N

Exhibit 16. QA Certification Statement

Quality Assurance Project Plan Certification Statement

I, the undersigned, certify that I have read and that I understand the Quality Assurance Project Plan (QAPP) for the eDNA monitoring of Invasive Asian Carp. I further certify that I will follow the procedures listed in this QAPP.

Signed:

Name	Agency	Date
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SECTION 12 APPENDICES

Appendix A: Roles and Responsibilities & Annual Staff Assignments

As of March 2019, the following staff are assigned to the eDNA monitoring project.

eDNA Program Coordinator: Kelly Baerwaldt, USFWS, 608-498-8435

eDNA Processing Leader: Emy Monroe, WGL, 608-783-8402

DNA Processing Quality Assurance Specialist: Maren Tuttle-Lau, WGL, 608-783-8403

Data Documentation & Reporting Specialist: Zeb Woiak, WGL, 608-783-8424

eDNA Database Coordinator: Jeena Credico, MFC, 608-783-8412

Fish and Wildlife Conservation Office Points of Contact:

Alpena: Scott Koproski, 989-356-5023

Ashland: Mark Brouder, 715-682-6185 x11

Cartersville: Neil Gillespie, 608-997-6869 x 106

Columbia: Kristen Towne, 573-234-2132

Green Bay: Cari-Ann Hayer, 920-866-1763

La Crosse: Jenna Merry, 608-783-8428

Lower Great Lakes: Sandra Keppner, 585-948-7039 and Ted Lewis, 585-948-7035

Wilmington: Nathan Evans, 815-423-5327

USFWS Midwest and Northeast Region eDNA Roles and Responsibilities, March 2018

ROLES

Regional Office

Region 3 Deputy RD/National Asian Carp Lead: Charles Wooley

Fisheries ARD: Todd Turner

Deputy ARD/FWS National Asian Carp Plan Coordinator: Aaron Woldt

R3 AIS Coordinator: Amy McGovern

R5 AIS Coordinator: Sandra Keppner

R3 Asian Carp/eDNA Coordinator: Kelly Baerwaldt

R3 FWCO Program Supervisor: Brian Elkington

Midwest Fisheries Center

Center Director: Teresa D. Lewis

Whitney Genetics Lab

Project Leader: Emy Monroe

Fish and Wildlife Conservation Offices

Project Leaders: Alpena, Scott Koproski; Ashland, Mark Brouder; Cartersville, Sam Finney; Columbia, Jason Goeckler; Green Bay, Susan Wells; La Crosse, Rebecca Neeley

eDNA Point Of Contact's: Alpena, Anjanette Bowen; Ashland, Jason Ross; Cartersville, Neil Gillespie; Columbia,

Kristen Towne; Green Bay, Cari-Ann Hayer; LaCrosse, Jenna Merry; Lower Great Lakes (Region 5), Theodore Lewis; Wilmington, Nathan Evans.

RESPONSIBILITIES

Deputy ARD/National Asian Carp Plan Coordinator

Regional oversight of all Asian carp and eDNA activities. Senior level authority on all decisions on eDNA sampling program.

Region 3/5 Aquatic Invasive Species Coordinators

National Aquatic Invasive Species Subject Matter Expert on development and implementation of processes and strategies for aquatic invasive species (AIS) management, providing regional project leadership on DOI initiatives addressing AIS management. Provides technical assistance for development and application of new scientifically based concepts and improved technology in AIS prevention and control. Leads interagency and inter regional efforts to develop and implement programs for early detection and rapid response processes. Region 3 AIS Program Supervisor is direct supervisor of the Midwest Fisheries Center Director.

Region 3 Asian Carp/eDNA Coordinator

Regional Office staff specialist responsible for development, coordination and implementation of the Midwest Region's eDNA monitoring program for Asian carps. Reports to the Deputy Assistant Regional Director. Assumes principal responsibility for initiating, leading, facilitating, integrating, coordinating, and communicating necessary monitoring work and activities using eDNA of the Midwest Region's Fisheries eDNA Program through the cooperative conservation community. Provides guidance for implementing and utilizing eDNA activities at the field level. Provides technical leadership to plan, conduct and lead other biologists as well as coordinate activities to identify and coordinate selection of surveillance areas in water bodies for the early detection of Asian carp eDNA. Ensures the best scientific practices are used during the development and implementation of management plans to monitor, control, and eradicate Asian carp. Implements and coordinates the Regional eDNA collection program; eDNA processing and interpretation of data; collection and dissemination of research information from institutions and research agencies on findings and new developments in eDNA collection and interpretation of data and the development of Asian carp management plans. Responsible for the QAPP and coordinating its implementation with the FWCOs and lab, as well as ensures that field offices and laboratory staff receive proper training to adhere to the procedures identified within. Responsible for communication between the lab, field offices, and Regional office. Responsible for external communication with partners regarding eDNA programmatic changes (eg. marker development, extraction techniques), collections, and results. Regularly communicates with the FWCO Program Supervisor, Supervisory Molecular Geneticist, and Center Director to ensure a high level of quality and scientific rigor with respect to field operations.

Region 3 FWCO Program Supervisor

Responsible for the implementation and delivery of the FWS fisheries program through the Fish and Wildlife Conservation field offices of the Midwest region. Direct supervisor of the Project Leaders of FWCOs. Responsible for developing overall program direction and budget needs for FWCOs. In addition to managing the assigned field stations, serves the role of coordinating and integrating their operations with other Fishery Program operations, and ensuring cooperative and productive partnerships with the Upper Midwest and Great Lakes, Eastern Tallgrass Prairie and Big Rivers, Plains and Prairie Potholes, and Ozark Plateau Landscape Conservation Cooperatives and between the Midwest and Northeast Regions.

Midwest Fisheries Center Director

Responsible for the implementation and delivery of the FWS fisheries program through the Midwest Fisheries Center. Direct supervisor of the Supervisory Molecular Geneticist and the La Crosse FWCO Project Leader. In addition to managing the Fish Technology Center, serves the role of coordinating and integrating their operations with other Fishery Program operations, and ensuring cooperative and productive partnerships with the Upper

Midwest and Great Lakes, Eastern Tallgrass Prairie and Big Rivers, Plains and Prairie Potholes, and Ozark Plateau Landscape Conservation Cooperatives and between the Midwest and Northeast Regions.

Supervisory Molecular Geneticist, Whitney Genetics Lab

Responsible for planning and implementing operations of the Whitney Genetics Lab (WGL) and provides lab services to federal and state entities nationwide. Reports to the Midwest Fisheries Center Director. Supervises a WGL staff of geneticists, fish biologists and technicians performing a variety of procedures in support of early detection and monitoring of aquatic species and in meeting fisheries management objectives. Responsible for all processing of eDNA samples and reporting of results to eDNA Coordinator and Supervisor. Primary author and editor of all processing related portions of the QAPP. Regularly communicates with the Region 3 Asian Carp/eDNA Coordinator to ensure a high level of quality and scientific rigor.

FWCO Project Leaders

Responsible for overseeing the delivery implementation of the eDNA program at FWS aquatic conservation, management, and aquatic invasive species programs within the field level which includes: collection, processing (filtering or centrifuging), and shipping samples assigned to their station. Responsible for and supervises all aspects of administration of the Aquatic Nuisance Species Act in the states or geographic area of responsibility assigned to the respective office. Will report any eDNA related issues/questions to Asian carp/eDNA coordinator and/or National Asian Carp coordinator.

eDNA POC's

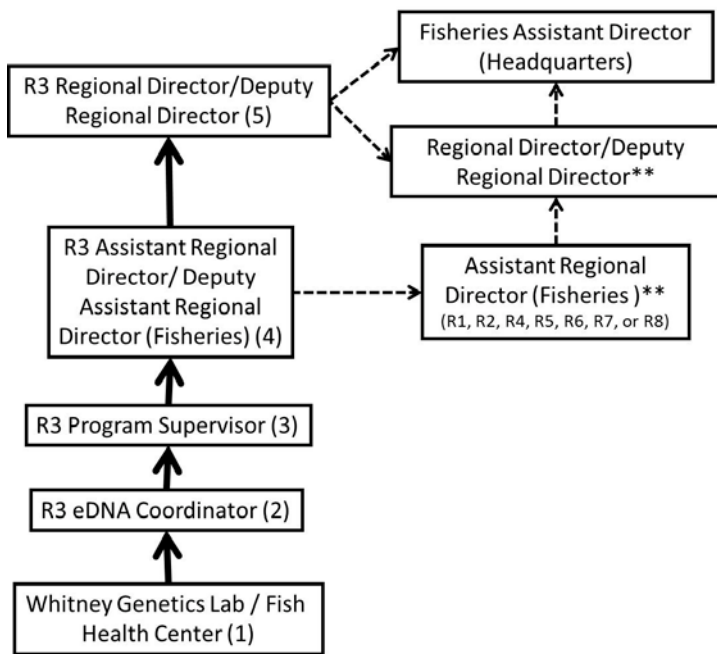
Serve as lead biologist to implement the eDNA program for the watersheds assigned to their respective field office. Under the supervision of the project leader, develops and implements eDNA sampling plans for waters assigned and serves as a point of contact, with their PL, for partners to coordinate eDNA activities.

Appendix B: Internal Communication of Results SOP

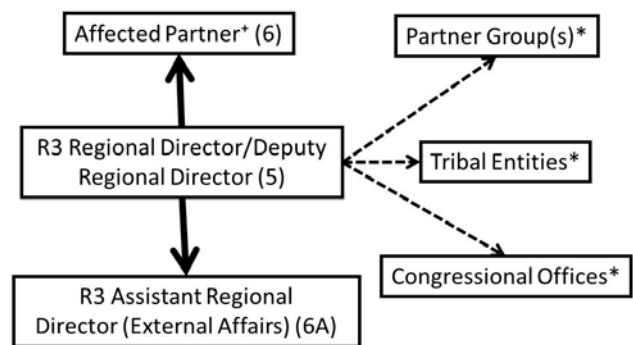
Standard Operating Procedure Notification of U.S. Fish and Wildlife Service eDNA Results

This Standard Operating Procedure (SOP) is intended to guide U.S. Fish and Wildlife Service (Service) employees involved in the sampling and analysis of environmental DNA (eDNA) as they disseminate the results of early detection and monitoring for Bighead and Silver Carp genetic surveillance. Included is a flowchart (Figure 1) depicting how information will be transmitted through the Midwest Region, as well as specific guidance relating to the format and types of information that will be crucial to include as eDNA results are communicated. Further guidance regarding Service eDNA sampling can be found in the Asian Carp Regional Coordinating Committees Monitoring and Response Plan and other regionally specific plans. All eDNA sampling should be completed in accordance with the *Quality Assurance Project Plan; eDNA Monitoring of Bighead and Silver Carp* (QAPP; updated annually).

Internal Flow of Results



External Flow of Results



*Affected Partner refers to an entity, such as a state, province, or tribe, who has management authority of the body of water being sampled.

*Partners Group(s), Tribal Entities, and Congressional Offices communication will be carried out as determined appropriate by the Region 3 (R3) Regional Director/Deputy Regional Director. See "Post Analysis Coordination" section for further detail.

**Information sharing cross regionally within the Service, communication will be carried out as determined appropriate by the R3 Assistant Regional Director and R3 Regional Director/Deputy Regional Director.

- - - Dashed lines indicate situation dependent lines of communication based on case specific information.

Data sharing from FWCO's and Service Whitney Genetics Laboratory (WGL)

- Once sample processing is complete and positives identified, results will be communicated through the flowchart (Figure 1) within Region 3 (R3) Fisheries to the Assistant Regional Director (ARD) level. Reporting internally to the eDNA Coordinator level as the results are determined by the Whitney Genetics Lab (WGL) will help streamline the process and allow time for the eDNA Coordinator to

prepare for disseminating the results up and out to the affected partner

- eDNA results information is to be kept confidential, being transmitted as shown in the flow chart (Figure 1). Any potential communication with partner groups or other entities outside the Service will not occur prior to informing the affected partner. For further information on the dissemination of results to others, see the “Post Analysis Coordination” Section below.
- eDNA results information will be formatted as described in the Results Procedure Section (below) by the eDNA Coordinator and prepared as a briefing for the Fisheries Deputy Assistant Regional Director (DARD), and ARD.
- Once briefed, the R3 Fisheries ARD will brief the R3 Regional Director (RD), or Deputy Regional Director (DRD), which will allow the RD or DRD to provide input and disseminate the results as appropriate to affected partner and partner group(s) as outlined in the Post Analysis Coordination Section (below).
- The communication and briefing process from eDNA Coordinator to RD and/or DRD should occur quickly. This will allow R3 to disseminate results promptly and maintain our responsiveness to affected partner and partner group(s).
- When communications are made which transmit results or data from one step to the next in the flowchart (Figure 1), the person sending it must confirm receipt of the information via email response to ensure the message was received and maintain a record of the exchange.
- Communication of results beyond the eDNA Coordinator will only be made once all samples from the sampling event are fully analyzed.

Results Procedure

- The sampling event data will be packaged together by the eDNA Coordinator in a uniform template to be used to inform the affected partner. Sampling events are defined by the geographic boundaries sampled.
- Field data related to the sampling event are automatically uploaded to the eDNA database and are proofed by the collecting FWCO.
- The analysis results associated with each sampling event will be provided to the eDNA Coordinator by the WGL once processing at the lab is completed.
- The eDNA Database Coordinator, as part of gathering this information, will compare the datasets provided by the WGL and the FWCO to ensure the data matches each other, i.e. labeling and unique identifiers match, and combine them to create the final data package to be sent the eDNA Coordinator.
- The data package to be sent to the affected partner will include:
 - Geo-referenced map indicating all sites eDNA samples were taken, highlighting those sites that were determined to be positive for eDNA (Example included as Appendix A).
 - Digital Excel file with each sample uniquely identified. Data columns will include: Sample ID, Latitude and Longitude in decimal degrees, Date of Collection, Water Body Name, Silver Carp Results, and Bighead Carp Results.
 - A transmission memo from R3 Fisheries ARD to the affected partner relating a summary of the information that has been collected and our proposed next steps (Memo template attached as Appendix B). This memo will be sent via email.
 - A press release template with eDNA specific language will be provided upon request

Post Analysis Coordination

- The data package of results will be emailed from the Deputy Regional Director (delegated to Assistant Regional Director or Deputy Assistant Regional Director as appropriate) directly to the affected partner point of contact (as determined by the affected partner).
- Following transmission of the data package to the affected partner we will be prepared to work with them, to assist in interpreting the results or supporting further sampling if possible,

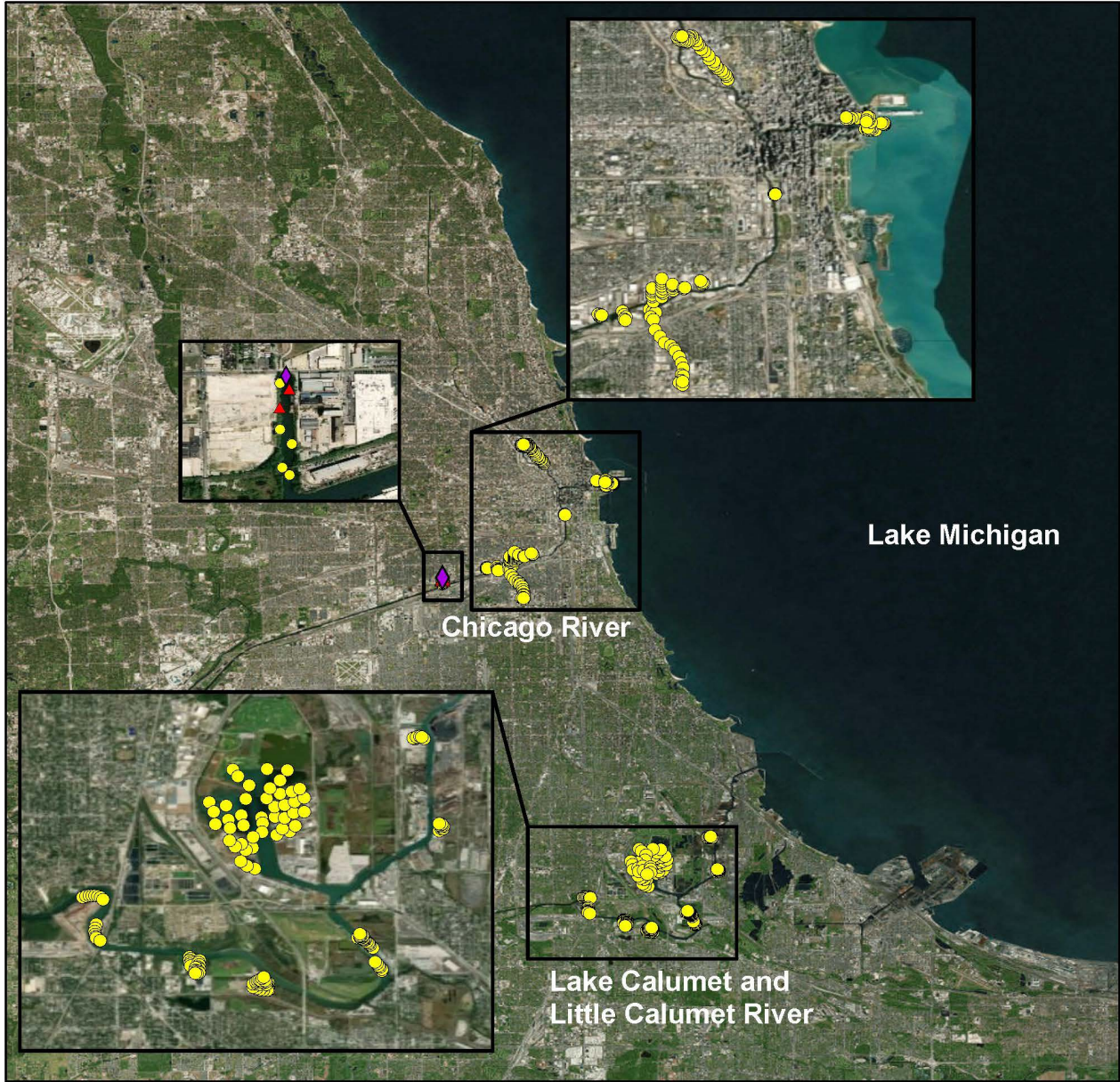
understanding that in some cases the affected partner may not decide to carry out follow up investigation. We will also assist as requested in a partner led press release sharing the results with the general public.

- When notifying the affected partner, the data package will also be transmitted via email to our website manager and External Affairs Office. The Service External Affairs team will be prepared to assist the affected partner with outreach or press release materials upon request. This transmission will start a 5 business day waiting period at the end of which the results will be posted to a Service website. However, upon written request from an affected partner, the Service will extend the timeframe for up to an additional 5 business days. The request will specify the reason(s) for the extension (e.g. new geographic area of eDNA detection, high number of positive results, etc.), the name and position of the requesting official, and the number of additional days requested. See appendix D for further information regarding Service External Affairs role in eDNA results notification process.
- The website manager will review the materials and format them as needed to prepare them for posting on the FWS public website: www.fws.gov/midwest/fisheries/eDNA.html
- Results will be posted concurrent with any press releases issued by affected partner, **OR** by 3 PM CST on the fifth business day after delivery of the data package to the affected partner, whichever comes first, unless more time is requested by the partner. This is to ensure transparency in the system and to not restrict information sharing.
- Immediately upon posting the results online, the website manager will inform the ARD/DARD of the posting.
- In addition, Partner Groups, Tribal Entities and Congressional Member Offices will be notified on a case dependent basis.
 - Partner Groups – Groups, such as the Asian Carp Regional Coordinating Committee, the Council of Great Lakes Fisheries Agencies, the Affected Great Lakes Fishery Commission Lake Committee, the Mississippi River Interstate Cooperative Resource Association, etc. will be notified via email and/or phone call from the DRD (delegated to ARD as appropriate) to their chairperson upon issuance of a press release by the affected partner.
 - Tribal Entities – Notifications will be made to each Tribe’s leadership respectively as an affected partner when sampling has taken place within or in direct proximity to tribal lands. In this case, they will receive the same data package via email as any other affected partner (See Results Procedure section). If the samples were taken in treaty waters, but not in proximity to tribal lands, their leadership will be notified via email and/or phone call from the DRD (delegated to ARD as appropriate) either upon issuance of a press release by the affected partner or when the results are posted on the FWS website.
 - Congressional Member Offices – Congressional Office contacts will be notified upon issuance of a press release by the affected partner on their behalf. Notification will be made via email and/or phone call from DRD (delegated to ARD as appropriate).

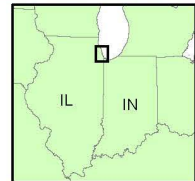
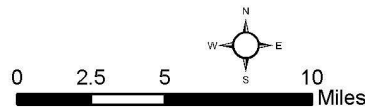
Communication SOP

Example geo-referenced map of results.

Bighead and Silver Carp eDNA Early Detection Results:
Chicago Area Waterway System
Sampling Period: Week of October 29, 2018
Number of Samples Collected: 311



eDNA Sample Points
● Negative
▲ Positive Silver Carp
◆ Positive Bighead AND Silver Carp



Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

ESRI base maps used must be acknowledged: Map image is the intellectual property of Esri and is used herein under license. Copyright © 2017 Esri and its licensors. All rights reserved.

Communication SOP

Dear (Insert name of Partner),

Enclosed with this letter you will find the U.S. Fish and Wildlife Service (Service) Whitney Genetics Lab results relating to the recent Environmental DNA (eDNA) sampling which occurred in (Enter Name of Sampling Location or Water Body). These results are presented geographically to provide an overview of the locations where each individual sample was collected, as well as indicating those that were found to be positive for Asian Carp eDNA (include if positives are found). Additionally, there is a table provided which identifies each individual sample collected by unique identifier and its corresponding data.

If one or more samples were positive for Asian carp eDNA, use this paragraph:

Of the X samples taken, positive results were found in X samples. Of those, X were positive for silver carp and/or X were positive for bighead carp. Working with you as our partner, we would recommend that...

If no samples were positive for Asian carp eDNA, use this paragraph:

After reviewing the data, you will see that none of the water samples collected in this effort were found to be positive for Asian carp eDNA. We will keep you apprised as further information is collected through future sampling efforts.

Please be aware that these results will be posted on our public website no later than (Enter approximate time (noon) and date of posting if no press release if issued), 3 business days after the transmission of this message. Upon your request, we will work with you to interpret these results and issue a press release.

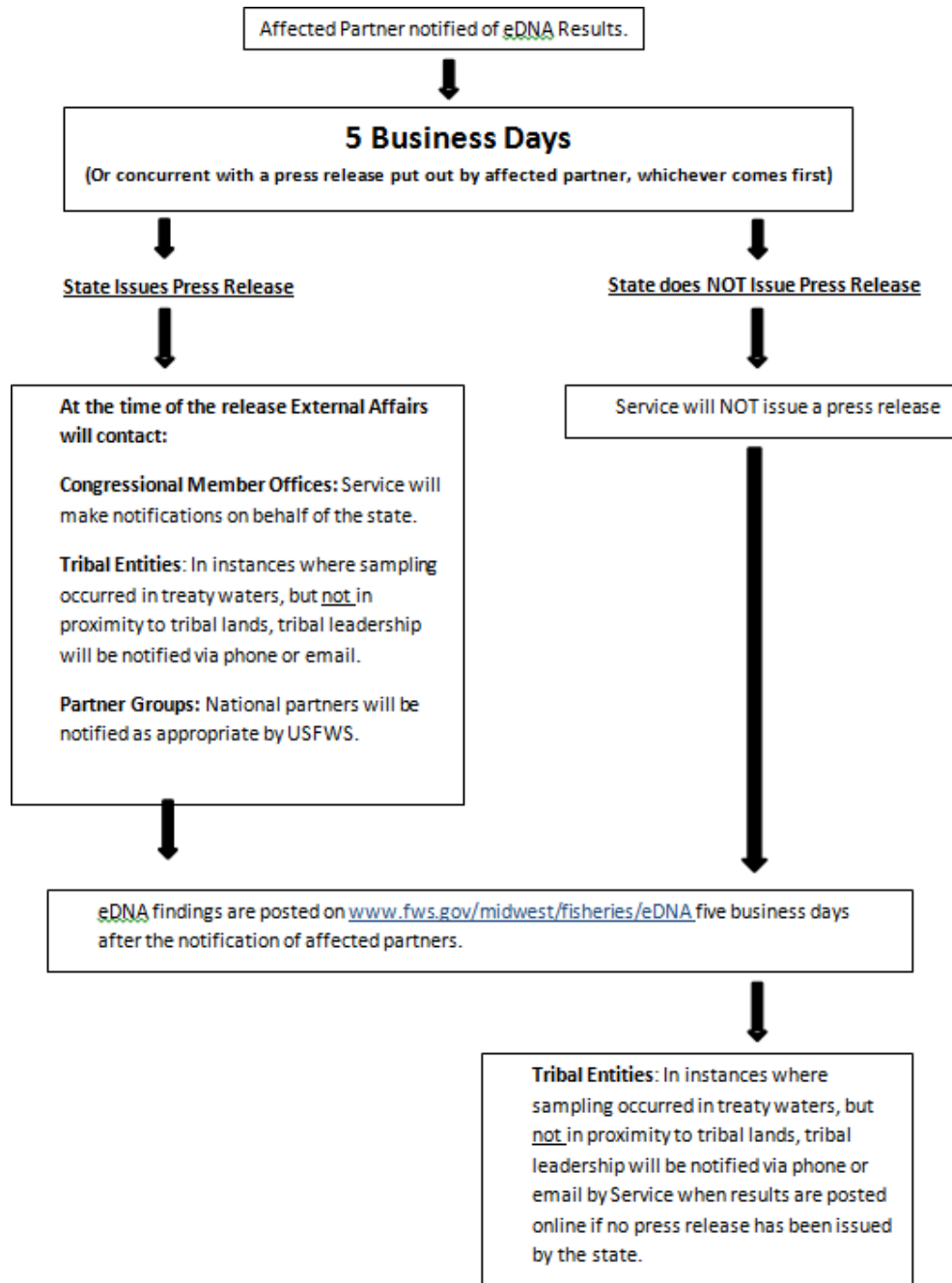
Include if more than one affected partner:

This information has also been sent to (Enter names of other affected partner) as affected partners. Please work together with the Service and the other affected partners to coordinate next steps in responding to these results and providing information to the public to ensure a clear and consistent message.

Please contact eDNA Coordinator XXXX at (Phone and email) if you have any questions.

Communication SOP

U.S. Fish and Wildlife Service External Affairs Flow Chart



Appendix C: Data Management

1. Purpose

In order to keep accurate records of eDNA sample collections, personnel associated with sampling and processing of collections, and data associated with a specific collection sample, data sheets associated with sample collections must be kept in accordance with the following protocols for quick reference and to prevent loss. This appendix describes procedures for data reporting and data management specific to USFWS.

2. Data management

- (1) Field data will be collected via the ArcGIS Collector App on I-Pad devices, and are the responsibility of the FWCO. Data fields available in the Collector App are provided in the table below.
 - a. FWCO staff are responsible for entering field data, proofing data as soon as possible after the trip is completed, and maintaining data at their location. Hard copy and electronic data should be backed up regularly.
 - b. Original COC forms are sent to WGL with the samples. FWCOs will receive a scanned COC that should be printed and filed in a project binder and the electronic copy saved with electronic field data and scanned data sheets.
 - i. The original COC forms are kept in a project binder at WGL, and will be given to the eDNA Project Coordinator at the end of each sampling year.
- (2) Lab data and data sheets are the responsibility of WGL.
 - a. WGL will maintain hard copies of laboratory notebooks and the electronic project data file cataloging each case in an excel file. Raw data files from the real-time instruments will be processed with standardized R-code, and all result files will be kept in a folder labeled with the case number. These files will be backed up on the lab LIMS server and on the center's server.
 - b. WGL will scan data notebooks into PDFs by cases and these will be saved on the lab networked computer with a back-up as well as on the station's shared drive which is also backed up by IT staff.
 - c. The electronic files and case log serve as the Laboratory Information Management System to track cases and samples through the lab workflow. The LIMS is be backed up a minimum of 4 times on separate hard drives.
 - i. Final reports will be generated as each case is completed, sent to the eDNA Program Coordinator as well as saved electronically in the lab network and on the station's shared drive.
 - ii. The PDFs of the scanned field COC as well as all data files will be maintained in electronic copy on site with back-up.
- (3) Collated field and lab data in geo-referenced database managed at the Regional Office
 - a. FWCOs are responsible for entering (automatic with ArcGIS Collector App) and proofing field data. The ArcMap eDNA data editing manual and ArcGIS Collector App Instructional manual can be used to enter data and facilitate QA/QC of the data. If possible, QA/QC data should be uploaded into the database within 10 business days after collection or before the next sampling event of a previously sampled location to reduce the confusion between previously sampled points and new sample points when entering processing information. Data cannot be cleared from the ArcGIS Collector App map until data has been QA/QC and synced back to the database. If other field work prevents QA/QC of data uploaded to the database, FWCOs must notify the eDNA Database Coordinator and eDNA Program

Coordinator.

- b. The eDNA Database Coordinator will backup and maintain the eDNA sample data in at least three backup locations. The eDNA Database Coordinator will also routinely back up eDNA sample data from the ArcGIS Online eDNA Fisheries Group once a week. Field offices should still download and maintain the data collected by their office (per 1.a. above).
- c. WGL is responsible for filing final case reports of results with the eDNA Program Coordinator within 24 hours of result confirmation.
- d. USFWS Midwest Region will maintain a database complete with field and lab data as well as electronic copies of reports provided to partner agencies and the public.

Table 1. Field descriptions for the eDNA_Sample point feature class.

Field Name	Alias	Type	Length	Description
RUID	Regional Unique ID	Long Integer	8	Composite number that has a 5-digit "case" number, a series of unique numbers assigned by the Whitney Genetics Lab (WGL) to each Fish and Wildlife Conservation Office (FWCO) at the start of the sampling year and provided for each individual planned sampling event, followed by a 3 digit sample ID number assigned by the FWCO numerically as they take samples in the field (no punctuation, #####)
FWCO_ID	Sampling Station ID Code	Text	3	Alpena FWCO – ALP Ashland FWCO – ASH Carterville FWCO – CAR Columbia FWCO – COL Green Bay FWCO – GRB La Crosse FWCO - LAX Lower Great Lakes FWCO - LGL
STATE	State	Text	2	Two letter state code in which samples were taken.
BASIN	Basin	Text	4	LH = Lake Huron LM = Lake Michigan LE = Lake Erie LS = Lake Superior LO = Lake Ontario CAWS = Chicago Area Waterway System UMR=Upper Mississippi River OHR=Ohio River MOR=Missouri River OTHR=Other (applicable to older eDNA field data) -Other designations to be added as needed by Fisheries Data Steward
WATERBODY	Waterbody	Text	100	Waterbody name of where sample was taken
DATE_COLL	Date Collected	Date	n/a	Date of collection (MM/DD/YYYY)
WIND_DIR	Wind Direction	Text	20	Abbreviated wind direction, "Calm", "Variable", or shorthand (NE, NW, SE, SW, N, S, E, W)
LATITUDE	LATITUDE	Double	n/a	WGS 84 Decimal Degrees, 5 decimals
LONGITUDE	LONGITUDE	Double	n/a	WGS 84 Decimal Degrees, 5 decimals
TEMP_F	TEMP_F	Double	n/a	Temperature of the water in degrees Fahrenheit (1 decimal)
DEPTH	DEPTH	Double	n/a	Depth where sample was taken in Feet (1 decimal)
BLANK	BLANK	Text	5	Yes/No (Control: Tube filled with distilled water)
HABITAT	HABITAT	Text	30	Habitat type where sample was taken, i.e. LDB (Left Descending Bank), RDB (Right Descending Bank), MC (Main Channel), SC (Side Channel), RAB (Right Ascending Bank), LAB (Left Ascending Bank), LAB-SC (Left Ascending Bank-Side Channel), RAB-SC (Right Ascending Bank-Side Channel), MC-SC (Main Channel-Side Channel), OW-B/BW/M (Open Water-Bay/Backwater/Marina), SH-B/BW/M (Shoreline-Bay/Backwater/Marina), DAM-TW (Dam Tailwaters), OTHER (Other), IMP (Impoundment)
COLLECT_TIME	Collection Time	Long Integer	4	Time stamp in military time, no punctuation

Field Name	Alias	Type	Length	Description
FILTER_TIME	Processing Time	Long Integer	4	Time stamp in military time, no punctuation
PROCESSOR	PROCESSOR	Text	3	Initials of person who filtered/processed the sample

Table 2. Field descriptions for eDNA_Results table.

Field Name	Alias	Type	Length	Description
RUID	Regional Unique ID	Long Integer	8	Composite number that has a 5- digit “case” number, a series of unique numbers assigned by the Whitney Genetics Lab (WGL) to each Fish and Wildlife Conservation Office (FWCO) at the start of the sampling year and provided for each individual planned sampling event followed by a 3 digit sample ID number assigned by the FWCO numerically as they take samples in the field (no punctuation, #####)
BIGHEAD	Bighead_Result	Text	3	Yes/No (Yes for positive Bighead Carp eDNA sample, No for negative Bighead Carp eDNA sample)
SILVER	Silver_Result	Text	3	Yes/No (Yes for positive Silver Carp eDNA sample, No for negative Silver Carp eDNA sample)

Appendix D: Centrifuge Tube Management Plan

As of 2015, samples will now be collected in 50-ml centrifuge tubes and concentrated in the field via centrifugation. Even though tubes come sterile and ready to use, the Regional Office has decided that WGL will serve as the single preparation facility for creating field blanks used in the Asian carp eDNA monitoring program.

WGL will fill new, sterile centrifuge tubes, package them in racks to be secured in a clean plastic sleeve, and then either deliver or ship them to field offices as needed.

Cooler (field) blanks:

- (1) Plastic bags fit to racks of filled tubes will be purchased by WGL
- (2) Filled tubes will be securely closed, and placed in the bag.
- (3) Bags will be twisted closed with wire closures or tape.
- (4) Filled tubes will be distributed at the annual field training meeting so that each field station will start out the season with the total estimated number of field blanks needed for the sampling season.
- (5) Field stations can store these prepared field blanks as long as needed, ideally in the field trailers. Bags should be re-sealed after removal of blanks to keep contamination risk low.
- (6) Field stations will prepare coolers according to the QAPP, in as clean of an area as possible at the station.
 - a. Anytime boxes are opened, staff should be in clean clothes and use a new pair of clean gloves for actually handling the tubes. For example, you may open the box with un-gloved hands. Then, put on gloves to open the bag and touch the tubes.
- (7) Once clean coolers are ready, boxes may be opened, tubes labeled, and placed into clean coolers.
- (8) After use, field staff should return the racks to WGL, but dispose of all used tubes

A note on labels: Avery laser white weatherproof address labels #5520 hold up good in all but an ice bath.

Appendix E: Elimination of eDNA on Boats and Equipment

Recommended methods for reduction of residual or environmental DNA on boats and other equipment associated with environmental conservation work in the field. For boats and equipment that have been previously exposed to carp DNA, choose one high pressure sprayer method *in conjunction* with one chemical method to reduce DNA levels to negligible or below LOD. Read MSDS and Use personal protective equipment (PPE). Read job hazard assessment (JHA) for applicable methods.

Method	Active Ingredient	Contact Time	Advantages	Disadvantages	Cautions
Steam + Pressure Washer @ 212 F	Pressure, Heat and Water	10 sec	Environmental safety	Need electrical and water hookups Can melt or tear materials	Use PPE – need proper safety training, can cause burns, cuts, air embolisms
Cold Water High Pressure Sprayer with Low Pressure Detergent Application	Detergent, Pressure and Water	3-5 min Detergent contact/ 10 sec high pressure rinse	Environmental safety	Need water hookups Can tear materials	Use PPE – need proper safety training, can cause cuts, air embolisms
10 % Household Bleach Low Pressure Saturation	Sodium hypochlorite (5-8% before mixing)	10 min	No hookups necessary – can use off station	Not preferred for environmental safety Single day use in solution	Use PPE and avoid breathing fumes
20 % Household Bleach Immersion Bath	Sodium hypochlorite (5-8% before mixing)	10 sec	No hookups necessary – can use off station	Not preferred for environmental safety Single day use in solution	Use PPE and avoid breathing fumes
2% Virkon Immersion Bath	Potassium Peroxymonosulfate and Sodium Chloride	30 min	Lasts 1 week in solution Environmental safety	Corrosive to metals when exposed longer than 10 min	Use PPE – wear a dust mask when mixing powder
2% Virkon Low Pressure Saturation	Potassium Peroxymonosulfate and Sodium Chloride	10 min	Lasts 1 week in solution Environmental safety	Not quite as effective as bleach in laboratory study; equal or better than bleach in field (boat) equipment study	Use PPE – wear a dust mask when mixing powder. Do Not Aerosolize! Use a low pressure dispenser (hose attachment sprayer at largest droplet setting)



Preventing the Spread of Pathogens, Bacteria and Invasives on/in Boats, Motors, Trailers and Equipment

Developed by the U.S. Fish and Wildlife Service Midwest Region

Disinfection Techniques and Options:

Boat, motor, trailer, and gear must have all aquatic vegetation, visible organisms/animals, soil, and water drained and removed BEFORE TRANSPORT. Upon leaving a water-body possibly infected with pathogens or invasive species, a proper disinfection must be completed before re-use of boat, motor, trailer, and any exposed gear in another waterway. **Contact time** is crucial for complete disinfection. **Contact time** reflects exposure of air, water, or disinfectant to a specific area, and not the total amount of time spent disinfecting. For example, if you are using 50C water to disinfect your boat, you must apply 50C water to each area for ten minutes or longer (see options and procedures below). **Read MSDS, wear personal protective gear (ppg), and comply with federal and state regulations.**

Disinfection is MANDATORY for all exposed equipment and gear!

Methods	Procedures	Positives	Negatives
Heat + Air (Drying in hot sun/air)	30C (86 F) 24 hours minimum (time at temp contact period crucial) (exposure to hot sun/ air while dry)	Chemical free Effective, but only if properly done under ideal conditions	Time consuming Weather/Temperature criteria critical to reliable results.
Heat + Water Spray &/or immerse	50C (122 F) contact time 10 minutes (time and temp contact crucial) (source of very hot water needed)	Chemical free Same as above	Must maintain high water temp/contact; hotter than normal tap or carwash. Use personal protective gear (ppg).
Steam Spray <small>*(approved for eDNA decontamination)</small>	100C (212 F) contact time 10 seconds (time and temp contact crucial) (steamer washer/ sprayer needed)	Chemical free Same as above	Must maintain very high water temp/contact; (i.e. steamer washer/sprayer). Risk of burns use ppg.
Virkon Aquatic <small>*(approved for eDNA decontamination in a 2% solution for 10 - 30 min)</small>	Follow product directions for proper mixture and minimum contact time (apply directly, maintain saturation and rinse thoroughly)	Environmentally friendly Designed for aquatic use Quick inactivation time Sewer compatible	Follow MSDS directions for health risks and use ppg when mixing. Chemical based. Corrosive in concentrate form.
Quaternary Ammonium + Water (family of products)	Follow product directions for proper mixture and minimum contact time (apply directly, maintain saturation and rinse thoroughly)	Effective, user friendly Low health risks Sewer compatible	Follow MSDS directions for health risks and use ppg. Chemical based.
Sodium Hypochlorite + Water <small>*(approved for eDNA decontamination)</small>	200 ppm for pathogens; 5000 ppm for eDNA - contact time: 10 minutes (apply directly, maintain saturation and rinse thoroughly)	Widely available Effective	Follow MSDS directions for health risks and use ppg. Highly Corrosive.

USFWS Contacts: Corey Puzach, Fish Health Center: corey_puzach@fws.gov, Dave Wedan, Watercraft Safety Coordinator: dave_wedan@fws.gov

*for eDNA decontamination details contact Jen Bailey, Whitney Genetics Lab: jennifer_bailey@fws.gov

updated: April 2014

Appendix F: eDNA Security Plan for Midwest Fisheries Center

Standard Operating Procedure for Minimizing Risk of Invasive Carp DNA Contamination within the Midwest Fisheries Center

eDNA Security Team: Jen Bailey , Jenna Merry, Zeb Woiak

Project Leaders: Rebecca Neeley, Ken Phillips, Emy Monroe

Safety Officer: Nikolas Grueneis

Quality Assurance and Control Officers: Maren Tuttle-Lau, Ken Phillips

Center Director: Teresa Lewis

Effective Date: April 20, 2018

Review Date (annually): April 2019

Purpose: The shared location of three U.S. Fish and Wildlife Service (USFWS) Offices, La Crosse Fish and Wildlife Conservation Office (LFWCO), La Crosse Fish Health Center (LFHC), and Whitney Genetics Laboratory (WGL) presents a unique challenge to the USFWS Environmental DNA (eDNA) Monitoring Program. eDNA sample processing is a sensitive process, inherently susceptible to contamination from outside sources. Processing of eDNA samples requires strict adherence to laboratory hygiene and specific protocols for reduction of risk factors that could cause contamination of samples. Sample analysis for the USFWS eDNA monitoring program is performed at the Whitney Genetics Laboratory with procedures and protocols outlined in the Quality Assurance Project Plan (QAPP). Laboratory staff minimizes risk by following QAPP procedures designed to reduce introduction of DNA to the laboratory that may be on surfaces of sample packages, sample bottles, clothing and footwear. To compound inherent risk factors, a mission of the co-located LFWCO is to monitor invasive carp populations directly, which includes collection of invasive carp and invasive carp tissue samples as well as collecting the water samples for eDNA analysis. Cross-contamination between eDNA samples and gear and carp sampling gear would be very likely if protocols were not in place to help staff address contamination challenges. The co-located LFHC also receives whole carps and/or carp tissue samples for diagnostic fish health cases, tissue samples for ploidy verification and reproductive risk assessment and maintains several invasive carp tissue cell lines for research purposes as part of its mission. Each of these activities introduces risk for contamination and transfer of DNA to Whitney Genetics Laboratory.

Midwest Fisheries Center (MFC) staff, collaborators, volunteers, and associated sample containers, laboratory and field equipment have a large potential for acting as vectors of contamination by carrying invasive carp DNA to and within the co-located field station and indirectly to WGL. Due to the unique challenges that co-location of these offices presents, stringent protocols must be followed to prevent contamination of water samples during collection, sample receipt, movement through the building and shared spaces (e.g. parking lot), and sample processing. Following these isolation and decontamination protocols will reduce unintentional transfer of invasive carp DNA to facilities, sampling gear, eDNA water samples and sample containers, field and lab personnel, and other surfaces.

Scope: This document (1) identifies high risk zones for movement of personnel, collaborators, volunteers, visitors and fish or tissue samples throughout the facility, (2) sets up communication practices for personnel to help contain known contamination risks so that they may be isolated and eliminated, and (3) provides directions for the isolation and decontamination of field and laboratory equipment, samples, and packages.

Responsibilities: MFC employees and volunteers are responsible for following procedures outlined in this document and reviewing updates to this document annually. The eDNA Security Team will prepare

an official copy for Midwest Fisheries Center staff and volunteers to review, sign and date annually. Staff and volunteers are also responsible for reading and understanding associated MSDS and Job Hazard Analysis documents and for following safety procedures related to their duties. Visitors and collaborators will be notified of procedures and safety precautions applying to the specific activities of their visit. Project Leaders are responsible for ensuring employees and volunteers have reviewed appropriate documents, adhere to the procedures and will review annual updates to the document. Safety Officers are responsible for reviewing the document for adequate safety procedures and providing necessary safety training and equipment if required. Quality Assurance and Control Officers are responsible for making sure procedures are followed correctly in laboratories.

Definitions Relating to This Document:

Invasive carp = silver, black, grass, and bighead carp. (**NOTE:** All four species are a contamination risk)

Established invasive carp populations = waters where all life stages of Asian carp have been observed and reproduction is occurring. In established areas, Asian carp can be observed and captured relatively easily. There is a high risk of coming into contact with individual, live specimens and their DNA in established waters.

Risk Zones: The facility is divided into zones of contamination risk for personnel or equipment dependent on the activities that may take place within (Figure 1). Each Risk Zone has specific personal protective gear (PPG) required for entry into and work performance in these Risk Zones (Table 1). All contaminated PPG must be disposed of or decontaminated before re-entry to other zones. Each Risk Zone is described below.

- **Zone A** is the area of highest contamination risk. This area includes the gated outdoor area directly in front of the three south garage bays bordered by fences to the south and east, and a path from the entry gate to this area. Direct contact boats, vehicles, sampling gear, and nets may be stored in this area. This zone will also serve as the decontamination area for all traditional sampling gear and equipment.
- **Zone B** is the area with a moderate risk of contamination. Areas with continuous moderate risk include the three LFWCO garage bays and the fisheries laboratory. Predominantly decontaminated equipment and gear will be housed in the garage area, fisheries laboratory, although in special circumstances direct contact gear, direct contact equipment, and invasive carp or their parts may be present. Specific protocols are in place to reduce contamination threats from these sources. Floors and other surfaces should be decontaminated (mop with 2% Virkon) after exposure to known DNA contamination risks.
- **Zone C** is the area with a periodic risk of contamination. These areas include the Fish Health Center garage bay and areas in the parking lot not in Zone A, the flow cytometry laboratory, necropsy laboratory, virology laboratory, wetlab, and the lobby. During wild fish surveys equipment may be contaminated with invasive carp DNA, or sometimes equipment with low levels of contamination from other programs will be decontaminated in this area. Equipment, floors, and surfaces will be decontaminated with 2% Virkon or DNA Away after these instances to minimize any DNA residues left behind. Invasive carp tissue cells are cultured in the virology laboratory and present a periodic risk during weekly tissue cell culture maintenance. Aseptic techniques are applied to methods in all tissue cell culture procedures, which will minimize risk for escape of DNA from this laboratory. In addition, 10% bleach (10 min exposure) or DNA away will be applied as needed to equipment and laboratory surfaces when working with invasive carp tissue cells. Any small equipment that is not disposed of after cell splitting should be immersed in a 10% bleach

bath for 10 min before being added to the detergent soaking bath. The front door and reception area where fish and/or tissue samples may arrive for fish health diagnostic cases or research purposes presents a periodic risk. In this case, diagnosticians will remove sample packages to the LFHC histology laboratory for processing. Specific protocols are in place to contain contamination threats and to communicate risks to building staff. In the rare case that a partner or member of the public in possession of an invasive carp brings carcass(es) into the building through the front entrance for reporting, MFC staff will contain the risk and notify building personnel of the specific contamination potential associated with each individual case. Floors and other surfaces should be decontaminated (mop with 2% Virkon) after exposure to known DNA contamination risks. **La Crosse FHC staff will inform visiting staff and volunteers of current risk factors in laboratories and quarantine a contaminated area while working with invasive carp tissues or cells. MFC staff and volunteers will inform others while working with contaminated equipment or samples and quarantine a working area until the risk is eliminated.**

- **Zone D** is the area with a low contamination risk. This area includes the interior of the LFWCO, LFHC, the office and hallway space of WGL, and the remainder of the gated parking lot aside from Zone A and periodic Zone C just outside the door from the FHC garage bay. Predominantly only decontaminated personnel, equipment, and gear will be housed in this zone. Under special circumstances, contaminated data sheets, personnel, and invasive carp or their parts may be present. Specific protocols are in place to reduce contamination threats from these sources.
- **Zone E** is the area where there is a very small risk of outside contamination. This area includes the laboratories of WGL. Personnel and equipment entering Zone E must adhere to strict protocols prior to and upon entry or exit to reduce the potential of outside contamination into the lab. If WGL staff has been working directly with invasive carp or invasive carp tissues, or may have been exposed to a known contamination risk outside of WGL labs, they should not enter the labs unless they were wearing protective gear during exposure, and the risk has been minimized by disposal or decontamination of that gear. If they were not wearing PPG during exposure to invasive carp cells or tissues, they should then put on PPG before entering the lab, and should not enter DNA extraction, amplification, imaging or sequencing rooms until the next day.

Table 1. This table shows the personal protective gear that is required within each Risk Zone of the Midwest Fisheries Science Center building.

Risk Zone	Required Personal Protective Gear
Zone A & Zone B	Raingear or Tyvek suits, high quality boot protectors (remove upon reentry to other zones) or dedicated boots that do not enter other Zones until decontaminated, gloves. WGL Staff mandatory boot covers at all times. Volunteers or Visitors- boot covers when contamination risk is present
Zone C	Raingear, Tyvek suit, or dedicated laboratory coat, high quality boot protectors (remove upon reentry to other zones or decontamination of work area), gloves. Volunteers or Visitors- boot covers when contamination risk is present
Zone D	None
Zone E	WGL Staff has own requirements, Non-WGL Staff: boot covers and Tyvek suits or lab coats if they may have been exposed to contamination from other areas.

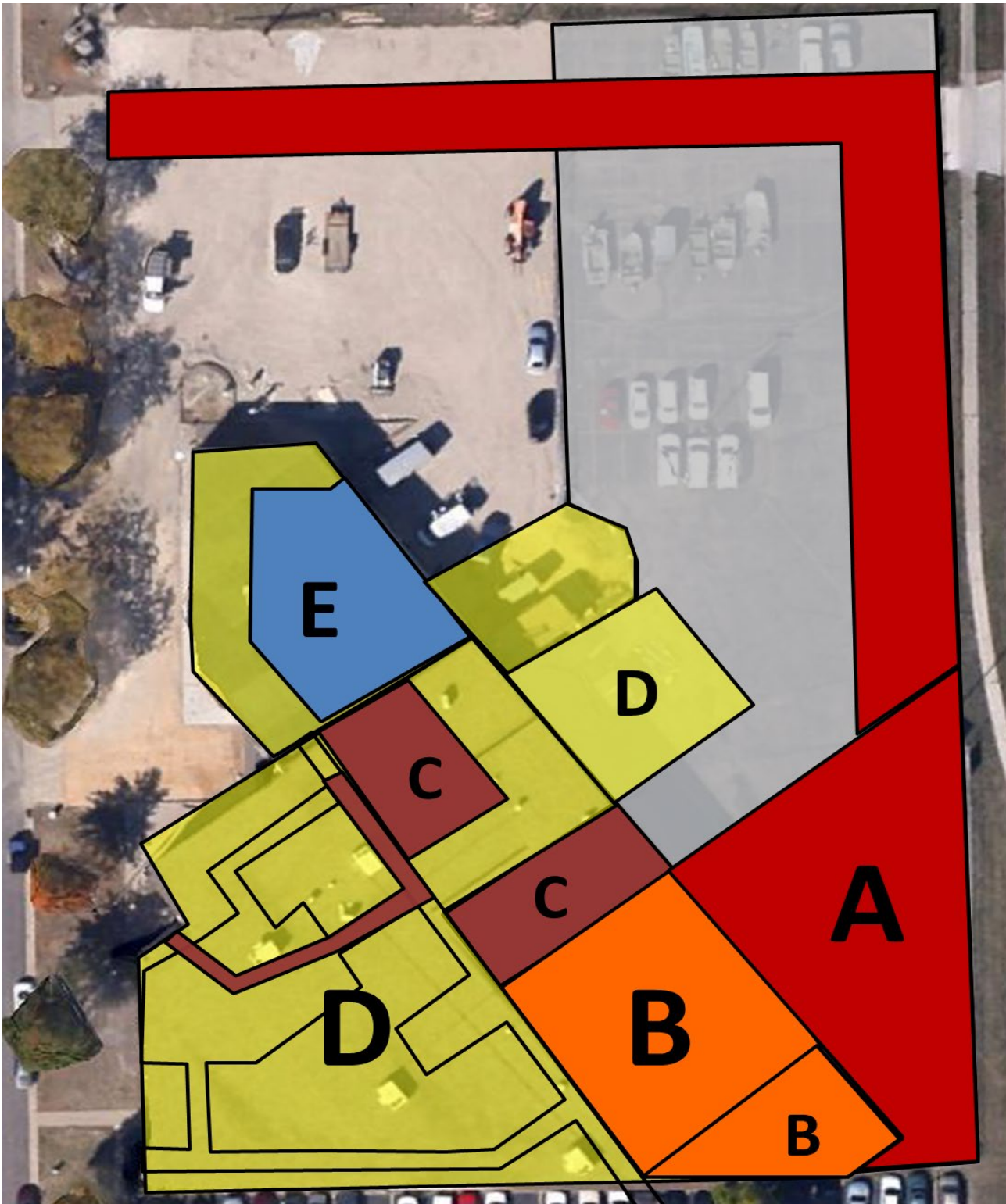


Figure 1. Zone map of the Midwest Fisheries Center Facility. Zones indicate the likelihood of invasive carp DNA being present, from A (red) the highest to E (blue) the lowest.



Figure 2. Sign may be printed to warn others that a known contamination risk exists in Zone.

Movement of samples, equipment and people: Each office has outlined specific procedures to minimize contamination threats for tasks that occur within respective programs and may not carry over to the other co-located offices (see procedure details below). However, many times tasks and group projects are shared or overlap between offices, so staff, volunteers, and collaborators must have procedures in place that encompass a wide variety of tasks and projects that fall into gray or shared areas. All participants must be aware of decontamination or risk minimization procedures that apply to daily duties as well as shared or overlapping duties. All staff, collaborators and volunteers are responsible for knowing Risk Zones and procedures that apply to entering each risk zone. Staff is also responsible for making collaborators, volunteers and visitors aware of procedures that apply to them and their corresponding activities. If staff or volunteers from one office will be working in the workspace of another office, or collaborating with another office, they must be familiar with SOP's pertaining to the work they are performing. Samples and equipment must be handled according to protocols, no matter who receives samples or packages, or which staff member is using the equipment. Much of the equipment in use for the Center is shared. Be aware of procedures to follow when borrowing or using shared equipment.

Decontamination Gear and PPE: There will be two separate decontamination zones, with dedicated equipment to remain in each zone. One zone will be for medium to low risk gear, and one for high risk gear. Clean sets of personal protective equipment (PPE) will be stored in cabinets in each zone (or nearby) that include boot covers, Tyvek suits, lab coats, and decontamination chemicals (Virkon, etc). This PPE should be worn while decontaminating gear, and then removed before re-entry into low risk areas. In Zone C of the garage, a "clean" cold water pressure washer with attachments for degreasing

detergent will be installed for use by Center staff to decontaminate medium to low risk field equipment and gear such as eDNA boats and equipment and boats and equipment used in Wild Fish Health Survey or other sample collections where invasive carp populations are not established. A duplicate set of decontamination equipment and PPE for use with highly contaminated boats, equipment, etc will be kept near the hot Zone A. Do not use this equipment for low-contamination equipment. This equipment and associated PPE will stay in Zone A. Keep “clean” and “dirty” equipment separate and do not move outside of their appropriate Risk Zones. Boot covers and Tyvek suits will be kept at entrances and transition areas for use as needed to prevent transfer of DNA on surfaces, boots, and employees.

Communications: Specific communications protocols for certain tasks are outlined in SOP’s (below), but many communications are essential for maintaining the integrity of Risk Zones, samples, packages, and shared spaces. Please inform Center staff of risks they may become involved in to make sure they are aware and follow appropriate procedures to minimize risk. Inform Project Leaders and the eDNA and Biosecurity Team members (Jenna Merry, Jen Bailey, Zeb Woiak) of known or unusual risks that are not covered in Procedures listed below. Team Members will develop a plan to address the specific situation and inform staff members of steps to take. Inform staff members who will be working with equipment, samples, or in areas that may become affected of any procedures they may follow to reduce DNA contamination and prevent spread throughout the building. If this may also include a safety risk, inform Safety Officers as well.

La Crosse Fish Health Center Standard Operating Procedures: The La Crosse Fish Health Center (FHC) follows guidelines established in the American Fisheries Society Fish Health Section Bluebook, Section 3: Quality Assurance/Quality Control, which establishes procedures for handling samples. The section provides basic guidance to prevent contamination in the laboratory and between samples.

The La Crosse FHC occasionally receives whole invasive carps and/or tissue samples from invasive carps as diagnostic cases or for flow cytometry. The La Crosse FHC also maintains several invasive carp tissue cell lines for research purposes. To minimize the spread within the Midwest Fisheries Center building of DNA from invasive carps, the La Crosse FHC has adopted additional procedures for handling invasive carps, tissue samples from invasive carps, and invasive carp tissue cultures. Procedures to be followed in addition to those described in Section 3 of the AFS-FHS Bluebook are:

1. La Crosse FHC staff will provide notification to Whitney Genetics laboratory staff that invasive carps and/or tissues are being worked with by posting a sign on the door of the laboratory where tissues or samples are being worked on. WGL staff will take appropriate precautions.
2. Any whole invasive carps or tissue samples from invasive carps received at the La Crosse FHC will be in a sealed container. The external surface of the container will be coated with an appropriate decontaminant (10 % bleach or DNA away) for a minimum of 10 minutes prior to opening. Isopropanol and quaternary ammonium products (Lysol Professional, Extra) are not acceptable decontaminants for eliminating DNA).
3. Whole invasive carps will be dissected/processed in FWCO’s Mark Steingraeber Lab following eDNA security procedures for working with whole carcasses and wearing minimum PPE for Zone B. Processed tissue samples will then be distributed to the appropriate laboratory(s) for pathogen screening, cell line development, other standard laboratory practices or research.
4. La Crosse FHC staff will don appropriate PPE when working with invasive carps, invasive carp tissues, or invasive carp tissue cell culture. Appropriate PPE will be rain gear, Tyvek, or other

designated clothing, gloves and boot covers when working with whole carp carcasses as appropriate for Zone B. Carcasses are disposed of by double bagging to contain contamination and stored in freezers for later transport to landfill. PPE used during whole carp processing will be removed after use and decontaminated or disposed of according to appropriate SOP for PPE with known contamination.

5. Laboratory coats and gloves are appropriate for tissue cell culture work and other laboratory work involving tissue samples in the LFHC laboratories. Laboratory coats, gloves and other PPE that may be contaminated with invasive carp DNA should not leave the laboratory until they have been washed, decontaminated or disposed of.
6. Immediately following completion of work involving invasive carps or their tissues, La Crosse FHC staff shall treat all laboratory surfaces, equipment and tools within three (3) feet of the designated work space with an appropriate decontaminant (10 % bleach or DNA away) for a minimum of 10 minutes. The sign indicating that invasive carp tissues are present shall not be removed until this time.
7. PPE shall be removed and placed in appropriate locations for disposal or cleaning. Employees shall thoroughly wash their hands with soap and water.
8. Any La Crosse FHC employee that worked directly with invasive carps and/or tissues from invasive carps, or may have potentially been exposed to invasive carp DNA, shall not enter Zone D until the following day.

Whitney Genetics Laboratory Standard Operating Procedures: Most of the procedures performed at Whitney Genetics Laboratory are specifically for eDNA monitoring of invasive carps and are incorporated into the Quality Assurance Project Plan (QAPP). Occasionally, duties are performed either in addition to or in support of the QAPP, or for programs not related to the QAPP such as flow cytometric analysis of grass and black carp samples. Samples are also occasionally processed at Whitney Genetics Laboratory in support of research and development of the QAPP, or for other USFWS programs. The following procedures will be followed in addition to QAPP procedures while in support of the eDNA program, performing research, or during development of new programs.

1. Any staff, collaborators, volunteers, or visitors to the WGL who are wearing clothing or footwear that has been exposed to any water where invasive carp are known to exist must wear Tyvek suits, lab coats, gloves or boot covers as appropriate before entering the lab. These are provided at the two main entrances at WGL. Any WGL staff or associated member who is leaving the laboratory for an area of known contamination should bring PPE with them to minimize risk of becoming contaminated while outside the lab. Dispose of contaminated gear before re-entry to the lab. Change footwear if possible.
2. When receiving samples, equipment, or packages that may be contaminated with invasive carp DNA, wear appropriate PPE to help contain contamination and dispose of PPE, gloves, and boot covers after each use. Stay in receiving room and don't remove samples or associated equipment to another room until all surfaces have been decontaminated.
3. Spray surfaces of containers or equipment with 10% bleach (10 min exposure) or DNA Away and wipe surfaces with paper towels. Remove to storage or to work area within the receiving room for processing.
4. When working with tissue samples from grass carp or black carp (i.e., not silver carp or bighead carp) use bench paper to define working space and keep associated laboratory instruments, etc, confined to workspace. After task is complete, decontaminate instruments with 10% bleach (10

min) or DNA Away and wipe entire area with either decontaminant. Contain contaminated waste products in a sealed waste bag and remove to trash bin.

5. Decontaminate surfaces of any sample containers saved for storage in WGL before removing to storage or other sample processing rooms.
6. Direct work with invasive carp tissue cells is important for preparation of extraction control samples for the eDNA monitoring program and sometimes for other purposes. If at all possible, perform this work in borrowed laboratory space from LFHC or another appropriate space. Wear protective gear to contain DNA contamination during sample processing, use 10% bleach or DNA Away before, during, and after processing procedures, and dispose of contaminated PPE. Follow all procedures outlined for the laboratory space you are working in.

In addition to step-by-step procedures, it is pertinent for all WGL employees to follow immaculate laboratory hygiene practices:

- Dedicate footwear to laboratory space. This footwear should not be worn in Risk Zones A or B and should be worn only in Risk Zone D if possible. If you must enter Risk Zone C during times of known contamination, wear boot covers while working in that area and dispose of later.
- Clothing or attire should not have been exposed to any potential contamination risks without laundering before entering WGL. This applies to hats, jackets, boots, shoes and regular clothing. Any attire that has been worn in areas of potential risk must be laundered using detergent or decontaminated with DNA Away before returning to the lab. If you have been sampling for the eDNA program, volunteering with other programs, recreating in areas of known contamination, or performing research duties do not return to the lab wearing the same clothes.
- Wash hands with copious amounts of soap and water if you have been exposed to invasive carp DNA.
- Be aware of any potential risks that may not be covered in these protocols and inform teammates of any concerns.

La Crosse Fish and Wildlife Conservation Office Standard Operating Procedures: The following procedures are specific to La Crosse Fish and Wildlife Conservation Office programs and projects.

Separation of Field Equipment: To reduce the threat of contamination, field crews will maintain two separate sets of personal field sampling gear including at a minimum PFD's and rain gear: 1) eDNA sampling gear and 2) Any other traditional field applications. Equipment used for eDNA sampling versus traditional sampling will also be maintained separately: 1) One vehicle and one boat with the necessary associated equipment (oars, fuel tanks, tool box, etc.) will be dedicated to eDNA sampling and 2) Remaining boats, vehicles, and other gear will be designated for any other use. All eDNA field equipment (boat, vehicle, PFD's, rain gear, etc.) and supplies (gloves, filters, centrifuge tubes, etc.) will be housed at a separate storage location. Contaminated traditional sampling equipment will be predominantly staged or stored in Zone A. Equipment that has been decontaminated and decontamination supplies will be housed in Zone B.

Decontamination Equipment List: This decontamination equipment will be maintained separately between eDNA sampling and traditional sampling

- Brushes
- Water pump and/or buckets
- DNA Away or equivalent
- Paper Towels
- Garden Hose

- Garden Sprayer attachment for hose
- Low Pressure hand pump sprayer and/or car washing brushes and swabs
- Pressure washer with steam option or detergent injection option
- Virkon Aquatic
- Household bleach
- >30 gallon decontamination tubs
- Personal Protective Equipment (PPE)

Decontamination Procedures:

Procedure A- Decontamination of eDNA sampling gear and equipment: This procedure refers to actions taken prior to leaving and upon returning to the station for collection of eDNA samples in the field. Equipment designated for eDNA gear and equipment decontamination will be used to perform any functions related to eDNA sampling. This equipment will be housed in Zone C of the garage. This procedure does not preclude additional protocols outlined in the Quality Assurance Project Plan (QAPP) performed prior to eDNA sampling. Due to the sensitivity of eDNA sampling, this must be done before and after each eDNA sampling trip.

1. Prior to leaving on a collection trip, use DNA Away or equivalent to decontaminate any personal items that will be used on the trip (watches, sunglasses, etc.)
2. Park truck and boat in Zone C.
3. Use hot pressure washer at 212F to rinse truck (including floor mats), boat, and any associated equipment capable of withstanding a high pressure rinse (oars, fuel tanks, tool box, totes, etc.). This could include PFD's, rain gear, or boots if they were heavily soiled.
4. Fill a pump sprayer or bucket with 2% Virkon Aquatic.
5. Spray or sponge 2% Virkon Aquatic on all surfaces of truck, boat, and other equipment until all surfaces are completely soaked. These surfaces must be coated for at least 10 minutes. **Important Note:** Metal surfaces are not to be coated for more than 10 minutes because corrosion will start to occur. Rinse when complete.
6. Mix a batch of 2% Virkon Aquatic according to recommendations in the decontamination tub.
7. Dip PFD's, rain gear, boots, floor mats, oars, or any equipment capable of withstanding a prolonged submergence without damage. Allow to soak for 30 minutes. Use proper PPE. Rinse thoroughly when complete.
8. Allow equipment to air dry on the designated drying racks prior to returning all eDNA equipment to the off-site facility.

Procedure B- Minimize Invasive Carp Residue Transport to Station: This procedure refers to returning from sampling that has occurred in waters with any reported invasive carp observations.

1. Prior to returning to the station, use water pump, buckets, and brushes to remove as much blood, slime, scales, sediments, etc. from the boat, equipment, and gear as possible.

Procedure C- Storage or Decontamination of Traditional Sampling Gear and Equipment: This procedure refers to returning from sampling that has occurred in water with any observed invasive carp. Equipment designated for decontamination of traditional sampling gear and equipment will be used to perform any functions. This equipment will be housed in Zone B of the garage. Boats, vehicles, gear and non-enclosed equipment that will be stored inside the facility will be decontaminated. Note: Gear and equipment from non-invasive carp infested waters are still subject to disinfection standards prior to entering a different body of water.

1. Park vehicle and boat in Zone A. These and any gear or equipment may remain in Zone A without

decontamination.

2. Personnel wearing contaminated direct contact clothes or shoes/boots should not proceed beyond Zone B.
3. Gill/trammel nets will be laid out to dry within Zone A. After drying, these will be stored in Zone A or enclosed with lids if storing in Zone B. Prior to entry to Zone B, the outside of the container will be decontaminated in step 6 of this procedure. Fyke/mini-fyke nets will be decontaminated in step 6 of this procedure prior to storage in Zone B.
4. Use hot pressure washer at 212F to rinse truck (including floor mats), boat, and any associated equipment capable of withstanding a high pressure steam rinse (oars, fuel tanks, tool box, totes, etc.). This could include PFD's, rain gear, or boots if they were heavily soiled.
5. Mix a batch of 2% Virkon Aquatic according to recommendations in the decontamination tub.
6. Dip PFD's, rain gear, boots, floor mats, oars, or any equipment capable of withstanding a prolonged submergence without damage. Allow to soak for 30 minutes. Use proper PPE. Rinse when complete.
7. Allow equipment to air dry on the designated drying racks.
8. Decontaminate cleaning supplies in tub and replace in Zone B
9. **Data sheets and clipboards will be kept in an enclosed container within FWCO offices when not in use or in the FWCO lab. After handling sheets for data entry or verification, replace into enclosed container, clean work area with DNA Away or equivalent, and wash hands.**

Procedure D- Net Mending: This procedure refers to mending gill nets, trammel nets, and fyke/mini-fyke nets that have been used in waters with any known invasive carp observations.

1. Prior to handling nets, personnel will don appropriate PPE (rain gear, Tyvex, or designated clothing and boots).
2. When possible, mending will take place in Zone A. Nets will be decontaminated via Procedure C-8 in the southern garage section of Zone B.
3. Prior to entering Zone C, any PPE will be removed and stored within the labeled hot zone and hands will be washed.
4. Upon completion of net mending, PPE and net mending tools will be decontaminated via Procedure C-8.
5. Remove any solids (leaves, scales, algae, etc.) that have accumulated on the floor.
6. Prepare a solution of decontaminate and mop floor.

Procedure E- Reduce Transport of DNA Contamination between Zones: This procedure refers to movement of personnel, gear, and equipment throughout the facility. Specific protocols must be followed to reduce the likelihood of spreading invasive carp DNA into zones of lower risk and ultimately WGL.

1. Personnel working with direct contact contaminated gear in Zone A or B will wear appropriate PPE. PPE will be removed and hands washed prior to re-entry into Zone C.
2. Personnel entering Zone D from Zone C will at minimum wash hands prior to entry.
3. It is recommended that personnel working in Zone E do not enter Zone A or B. If it is necessary, they will wear PPE to cover footwear and clothing prior to entering Zone A or B and remove upon exit.
4. Any potentially contaminated gear will be decontaminated via procedures above or enclosed prior to entry to Zone C or D.

Midwest Fisheries Center eDNA Security Plan Certification Statement

I, the undersigned, certify that I have read and that I understand the Midwest Fisheries Center eDNA Security Plan and the associated MDSs and Job Hazard Analyses. I further certify that I will follow the procedures listed in this document.

Name (Print)

Signature

Date

Appendix G: Methods from previous versions of QAPP

NOTE: Attempts to Exhibit references have been changed to NA, since the old Exhibits are no longer available. Remaining Exhibit references are no longer valid.

****Old 2.2 Equipment Procedure for Coolers and Sample Bottles**

- (1) A 10% bleach solution with water will be prepared in a 3-gallon low-pressure sprayer that is dedicated to the project. The bleach solution must be prepared immediately prior to use, and each time decontamination activities will be occurring.
- (2) Remove mud and other biological residues from surfaces by rinsing and scrubbing. Equipment surfaces must be free of debris and other material before decontaminating.
- (3) Sample transport coolers will be decontaminated with freshly made 10% bleach solution in the low pressure sprayer. Use the low-pressure sprayer to thoroughly cover the inside and outside surfaces. Allow 10 min of contact time before rinsing with water. Coolers may be left to air dry, or dried using clean paper towels while wearing clean gloves.
- (4) If filtering: Sterilized disposable 2L bottles may be used for sample collection or 2L autoclavable reusable bottles sterilized in the following way may be used:
 - (a) NEW 2L bottles may be used straight from the manufacturer. For subsequent sampling events, send bottles to the WGL for cleaning, and WGL will return clean bottles (refer to Appendix D). For sampling agencies not working with the WGL, follow procedures b-c to properly decontaminate bottles between uses
 - (b) If there is no autoclave available, use a 10% bleach bath to decontaminate sample bottles.
 1. In an area free of carp-contamination prepare a 10% bleach solution and soak bottles and caps for a minimum of 10 minutes. It is important to ensure that there are no air bubbles inside bottles or caps, which may require two 10-minute soaks, one with the bottle upright on each end of the closed bottle.
 2. Rinse each bottle by partially filling with water and shaking vigorously so that all areas of the bottle are rinsed, repeat a minimum of three times per bottle, but be sure to rinse until there are no traces of bleach.
 3. Air dry, cap, and place in sanitized coolers. NOTE: It is essential to rinse well since residual bleach would destroy any DNA collected and is a PCR inhibitor.
 - (c) If you have access to an autoclave, bottles need to be rinsed well with clean tap water and autoclaved.
 1. Rinse bottles by partially filling with clean water, shaking vigorously with the cap loosely held over the opening. Empty and repeat a minimum of two times.
 2. Place the threaded caps on the bottles and lightly screw on cap (cap will not be able to come off, but will still be able to move).
 3. Place into autoclave and set cycle for 1 hour at 121°C (15 psig or 1 bar).
 4. Once the autoclave cycle is complete, carefully remove bottles and allow them to cool to room temperature before fully tightening caps. If caps are tightened before allowing the bottles to cool, the bottles will warp and collapse.

If centrifuging: purchase sterile, chemical-free disposable 50-ml polypropylene tubes with maximum RCF of at least 6,000 x g. Even sterile tubes can still have traces of the chemical used to

free the plastic tubes from the metal forms during production. Some manufacturers sell sterile tubes that do not have this residual chemical in them, and these should be used to avoid introducing PCR-inhibitors from the sample tube.

- (5) Once all 2L bottles have been sanitized, sample labels can be printed by the sampling agency. Sample containers will be labeled with a unique barcode ID or agency generated code (Section 1.7 for case and sample numbering) that does not indicate location (to allow blind processing). Labels will be printed on Rite-N-Rain® or some type of waterproof labels and affixed to the outside of the sample bottles/tubes.
- (6) Once bottles/tubes have been labeled, they will be placed in the sterilized sample coolers in numerical order. Sample containers will be stored in the sterilized coolers until use, and will be transported only in the coolers. Although the sample ID number of the samples is not relevant except for identification purposes, collecting in consecutive order will aid in determining where samples were taken in case of a recording error.
- (7) A minimum of 10% of the total number of samples collected should be field blanks. For offices working with the WGL, all field blanks must be filled by WGL. For other agencies, blanks may be filled with any city-provided tap water (If water source is a well or you are unsure of the source, use distilled or deionized water). If filtering, a minimum of one blank per cooler is required, but more may be included. If centrifuging, collect blanks to meet 10% of total number of samples collected. Note, if the sampling design for your particular body of water requires a specific sample size in order to meet a precise detection probability, then containers for control samples to meet the 10% minimum will have to be added to the total number of sample containers dictated by your sampling plan.

**** Old 3.2 Filtering Procedure**

Water samples collected in the field need to be filtered within 12–16 hours after the last field sample is collected.

Equipment needed:

- Manifolds (Millipore 3-place stainless steel Hyrrosol Manifold, item #XX2504735)
- Magnetic 500-ml filter funnels (Pall item #4238; VWR item #28150-496)
- Glass fiber filters, 1.5 micron, 5.5 cm diameter glass fiber filter paper (Type 934-AH; Fisher item #1827-055)
- Forceps at least two pair, labeled with different colors or other identifier
- Carboy (3.5 gal or larger), for wastewater generated during filtering
- Rubber vacuum tubing (14-176-24, 1.25 in. inner diameter), double hole stopper (Fisher item #14-140P) that fits carboy opening, and glass connectors or plastic connectors (Fisher item #509557177) for connecting manifold to carboy and manifold to vacuum line
- Vacuum trap flask to protect vacuum pumps (plastic is fine), single hole stopper to fit flask and tubing to fit flask vent
- Sterile conical tubes (50mL) with caps and labels
- Sterile conical tubes (15mL) with caps and labels
- Paper towels
- Black permanent markers (e.g., Sharpie®)
- Powderless latex or nitrile gloves
- Vacuum system capable of -75 kPa vacuum
- Bleach

- 10% Bleach bath to sanitize filter funnels (use any clean, potable water source to mix bath)
- Dedicated lab equipment cleaning sink
- Waste water disposal location such as nonspecified-use sink
- Sterile bench paper
- Dedicated water bottles, one for bleach solution, one for DI water
- DI water for equipment blanks only
- Washbin for manifolds, such as a dedicated 10 qt plastic tub
- Plastic sealable bags (e.g. Ziplock®)

3.2.1 Laboratory Preparation

- (1) Wash hands thoroughly prior to starting. Prepare a dedicated plastic wash bottle with 10% bleach solution for wiping down lab tables and manifold surfaces prior to processing samples. Sanitize all equipment, in freshly made 10% bleach solution, prior to starting. Collect all supplies.
- (2) Rinse down each workstation with bleach solution prior to beginning the filtration process. Cover each workstation surface with sterile bench paper or clean paper towels. Bench paper must be changed between samples.
- (3) Put on new powder-free latex or nitrile gloves. Prior to filtering a sample, each work station should have one black waterproof permanent marker for labeling sample tubes (or pre-printed labels), one sterile 50-mL and one sterile 15-mL plastic conical tube, sterile filter paper, one set of sterile forceps for placing filter paper on filter apparatus, one set of forceps for handling used filter paper, dedicated wash bottle with freshly made 10% bleach solution.
- (4) At each workstation, connect a 3-place stainless steel filter funnel manifold to a large carboy bottle using rubber vacuum tubing, two-hole stopper, and plastic tubing connector. Connect carboy bottle to a trap flask, and the trap flask to the vacuum line with second piece of rubber tubing. Glass connectors may be used instead if available. Note, a trap flask should be between the carboy and vacuum to protect the pump from damage should the carboy get too full.
- (5) Sample containers should be dried to remove all external water so that nothing will drip when the sample is being filtered or they can also thoroughly rinsed and dried completely before being brought into the trailer. Ensure there is no water trapped in the grooves between the caps and bottles by tipping bottles and drying away any water dripping out.

3.2.2 Sample Preparation

- (1) Put on new powder-free latex or nitrile gloves prior to handling each sample.
- (2) Remove first sample from cooler of cleaned sample bottles and make sure it is dry.
- (3) Label one sterile 15-mL conical tube and one sterile 50-mL conical tube with sample number; indicate that the filter to be stored in the 15-mL tube is the equipment control by labeling this tube with a "C". Ensure gloves that come into contact with labeling marker are not used again for handling other samples. Alternatively, labels may be preprinted and used for each tube.

3.2.3 Filtering the Equipment Control

- (1) Put on new powder-free latex or nitrile exam gloves prior to processing each new sample.
- (2) Place bottom portion of sterile (section 3.2.6) magnetic filter funnel equipped with rubber stopper on manifold and open vacuum line.

- (3) Take designated forceps for handling clean filter paper, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion.
- (4) A sterile and clean magnetic filter funnel must be used for each sample. The cleaning process is described under the Equipment and Work Area Cleaning section 3.2.5.
- (5) Once the magnetic filter funnel top is secured to the bottom portion, pour 500 mL of DI water the magnetic filter funnel top as a control. In order to capture any potential contaminant DNA in the funnel, be sure to pour the DI water quickly so as to immerse all the internal surfaces of the magnetic filter funnel top with DI water. Once the DI water has been poured into the filter funnel top, turn the vacuum on to draw the water down quickly and filter the material as quickly as possible. Repeat with 500 mL.
- (6) Once the DI water has been filtered through the funnel, remove the filter funnel top, place it on clean bench paper dedicated for that sample. Take the forceps designated for that sample's used filter paper and grasp the edge of the filter paper. Roll or fold the filter paper until it is of a size to fit into the 15mL conical tube labeled as a control for the appropriate sample ID.
- (7) Place the control filter paper into the 15mL tube, screw on top, and place tube with control sample filter paper into cold storage. If work is at a laboratory, use a -20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. If using a cooler, be sure to use a clean sealable bag designated for control samples only to keep equipment control samples separated from field samples to avoid any contamination while in cold storage. Seal the plastic bag after each new sample is added. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.

3.2.4 Filtering the Sample

- (1) Take designated forceps for handling clean filters, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion. Avoid touching the funnel base with clean forceps. If forceps accidentally touch the base, get a new pair of forceps for handling clean filter paper. These designated clean forceps and the box of filters must be kept in a location far enough away from the filtering area to prevent them from becoming contaminated during filtering. If at any time water is spilled or splashed near the filters or forceps, get a new box of filters and a new pair of forceps.
- (2) Take the sample bottle and gently shake to distribute the contents within the sample evenly.
- (3) Once the magnetic filter funnel top is secured to the bottom portion, turn on the vacuum and then pour approximately one-third of the sample into the magnetic filter funnel top.
- (4) Once the one-third portion of the sample has been filtered through the funnel, *continue filtering until the filter is nearly dry*. Remove the filter funnel top. Take the forceps designated for used filter paper and grasp the edge of the filter paper. Neatly fold the filter paper once to fit into the 50mL conical tube labeled for the appropriate sample ID.
- (5) Repeat steps 1–4 at least two more times or until the entire sample has been filtered. After all the sample has been filtered, DI water from a wash bottle may be used to rinse any particulates attached to the sides of the magnetic filter funnel onto the filter paper. NOTE: since this water is

touching the DNA sample DI water is better than tap water.

- (6) Place the tube with field sample filter papers into cold storage. If work is at a laboratory, use a -20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. Use a sealable plastic bag designated for field samples only, to keep the sample tubes organized and separated from equipment control samples. Seal the plastic bag after each new sample is added. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.
- (7) The number of filters used to process the sample is up to the discretion of personnel processing the sample, however in the DNA lab, filters must be extracted one at a time. Therefore, if the water sample exhibits an excessively slow filtration rate, multiple filters should be used. Also up to the discretion of the personnel processing the sample is the amount of sample water to run through a single filter. A general rule is to run one-third of a 2L sample through a single filter; however, if the sample water is extremely turbid, for example, less water should be put through a filter. At the other end of the spectrum, if the sample water is extremely clear, more than one-third of a 2L sample may be run through a single filter. Do not place more than 10 filters into a single 50-mL tube. If more than 10 filters are generated, use additional 50-mL tubes, making sure they are clearly labeled with the sample ID and make a note on the data sheet.
- (8) On the field datasheet (Exhibit 2) next to the appropriate sample ID, mark the time (use AM PM or military time for clarity) of filter completion and the initials of the person that processed the sample.
- (9) Change gloves and sterilize the workstation between samples. Repeat steps 1–8 until all samples have been processed.
- (10) When filtering, if the water collection carboy becomes full, disconnect the carboy from the vacuum and manifold and dispense water in a sink separate from the one used to clean equipment. Once emptied, reconnect the carboy to the vacuum and manifold and proceed with the filtering process. **CAUTION: Be sure to open manifold valve and turn off the vacuum air supply when disconnecting and connecting the carboy so as to prevent explosion of the glass.**
- (11) If sample is accidentally spoiled during the filtering process (e.g., bleach was used to rinse filter funnel instead of DI water, forceps from previous sample used, etc.), immediately throw away ruined samples. If portions of the sample are still viable, place in 50mL conical tube. On the outside of the sample tube, label with the amount of the viable sample (e.g., 2/3 sample). On datasheet, label with the same information (e.g., 2/3 sample) next to appropriate sample ID. Note the reason for the ruined or diminished sample.

3.2.5 Equipment and Work Area Cleaning After Filtering Each Sample

- (1) Fill a 500mL glass beaker with 10% bleach solution. Forceps designated for used filter paper must be switched out for each sample. Used forceps will be placed in beaker with 10% bleach solution for a minimum of 10 min for sterilization. Once sterilized, remove forceps from bleach solution and rinse thoroughly before use.
- (2) Fill at least a 10-qt plastic tub (e.g., Rubbermaid® plastic storage bin) with 10% bleach solution. Once a sample has been processed, the filtering apparatus must be dismantled (i.e., the magnetic filter funnel should be separated into the upper and lower parts), rinsed to remove any particles

and/or film, and placed in the plastic tub with the 10% bleach solution for a minimum of 10 min for sterilization. Ensure there are no air bubbles preventing the bleach solution from fully contacting the entire filter cup interior. Once sterilized, remove the two parts of the magnetic filter funnel and thoroughly rinse before use. Rinsing should continue until all residues and scent of bleach can no longer be detected.

- (3) In between each sample, dispose of bench paper. Wipe down surface with 10% bleach solution using wash bottle and paper towels. CHANGE GLOVES! Cover work station with new bench paper.

If any deviations from this QAPP occurred during water sample collection in the field or during processing of samples, clearly describe the deviations on Exhibit 2. Even if there were no deviations from the QAPP, Exhibit 2 forms should be saved to the google drive OR sent to the eDNA Project Coordinator following a sampling event to satisfy reporting requirements in section 1.6. Be sure to note any changes in personnel from the pre-trip plan (Exhibit 1) on Exhibit 2 when the report is filed.

****Old 4.2 Shipping Filters**

- (1) If filters: Corrugated boxes (minimum outer dimensions 12" X 12" X 12") with styrofoam cooler inserts will be prepared for shipment. The number of boxes to prepare depends upon the number of samples collected (e.g., a 120-sample collection will require more boxes for shipping than a 50-sample collection). Filtering equipment controls should be placed into separate plastic bags during shipping, but centrifuge equipment controls can be mixed in with samples.

If centrifuge tubes: Any shipping containers that will hold the tubes and prevent damage to the tubes will work.

- (2) For filters or non-preserved centrifuge samples: The bottom of the coolers will be lined with solid blocks of dry ice (approximately 1–2 inch thickness). Oven-mitt type gloves must be worn by personnel that are handling dry ice to protect hands. If block dry ice is not available, pellets may be used, but avoid powder or flake.
- (3) For filtered samples: Remove the 15mL conical tubes with control filter paper from secure (i.e., locked) –20°C freezer and place in clean 1-gal resealable bag (e.g., Ziploc®). Multiple bags may be used if the entire sample does not fit in one bag. Seal over the opening of all bags used with tamper-evident tape (e.g., Evidence Tape – NC9709516). The individual packing the samples should sign the tape. Be sure to remove as much air as possible from all resealable bags used before sealing.

Be sure bags are secured so that they are tamper-evident. There are several ways to do this, here are two: 1) Close and seal bags, removing as much air as possible. Wrap tamper-evident tape or clear tape around the bag in two perpendicular directions so that the bag cannot slide out of the tape. If you don't use tamper evident tape, be sure to sign across where the tape meets itself with a permanent marker. 2) Close and seal bags, removing as much air as possible. Place tamper evident tape parallel to the bag seal, securing with three (3) staples. This option does not allow for clear tape with a signature.

When ambient temperature is above 60°F, more dry ice is needed for shipping than when it is cooler. If possible use both block and pellet dry ice to ensure sample integrity when shipping during warm weather. Place a ~10-lb block of dry ice on the bottom of cooler. Place a bag of samples on the block, layer approximately 1 in. of dry ice pellets on top of bag before placing another bag in the cooler. Repeat until only 2 inches of space is left at the top of cooler and fill the space with dry ice pellets. If pellets are not available, use a minimum of 20 pounds of block dry ice. Leave the bottom

block whole, but break up the other block and distribute among the bags of samples. Before closing the styrofoam cooler, record the inside temperature on the COC (Exhibit 3). Place the styrofoam lid on top of the sample contents and seal with tamper-evident tape, ensuring the tape crosses the lid and body of the cooler. If tamper-evident tape is not available, use packing tape and sign across where the tape overlaps as well as across where the lid meets the body of the cooler.

****Old 5.5 Qiagen DNeasy Kit (or equivalent kit) Procedure for *filters***

- (1) Remove samples that have been designated to be extracted from freezer.
- (2) Obtain one lyse and spin kit per filter to be extracted, open packages and organize tubes in rack. Label both caps of each tube per filter (one tube per filter). You will have time to label the rest (3 more 1.5mL lab supplied MCT, one spin filter tube, 3 Qiagen collection tubes) during the 1-hour incubation. Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.
- (3) Add 350 μ L ATL to the top of each basket tube.
- (4) Add 25 μ L protease K to the ATL in each tube.
- (5) Remove the appropriate filter sample, carefully tear off the edge of the filter so that it fits into the top of the basket tube. Only one filter can be extracted per MCT. If more than two extractions are required for one sample, combine products into a single tube once extractions have been completed (during step 17). Be sure to push the filter down into the ATL/proteinase K mixture (use a clean pipette tip), do not tap the lyse baskets on the counter or else the buffer will leak through.
- (6) Place the basket tube into the collection tube and incubate at 55°C for 1 hour. During incubation, label two more MCTs, and a spin-column assembly for each sample, and set up three additional collection tubes per sample. Print labels for archived extracts.
- (7) Remove from incubator and centrifuge at $\geq 16,000 \times g$ for 1 minute. Remove the assembly, archiving the filter only until the case is closed. Archive tube and filter at -80°C, until results are finalized, then dispose.
- (8) Add 375 μ L Buffer AL.
- (9) Add 375 μ L ethanol (96 – 100% molecular grade). Mix thoroughly by vortexing. If liquid gathers around the lid, briefly spin to reduce contamination.
- (10) Transfer about half of the mixture by pipette into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (11) Transfer the remaining mixture by pipette onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (12) Place spin column in a new 2 mL collection tube. Add 500 μ L Buffer AW1. Centrifuge at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (13) Place spin column in a new 2 mL collection tube. Add 500 μ L Buffer AW2. Centrifuge at 18,000 $\times g$ for 3 minutes. Discard flow-through and collection tube. Be careful to avoid sloshing the ethanol in the collection tube up onto the filter column. It is important to prevent ethanol from touching the filter because ethanol is a PCR inhibitor.
- (14) Transfer the spin column to a new 1.5 mL or 2 mL MCT.
- (15) If there are 8 or fewer filters per sample, elute the DNA by adding 200 μ L Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at \geq

6000 $\times g$ for 1 minute. If there are more than 8 filters, elute with only 100 μL Buffer AE so that all of the replicates can be pooled into one extraction tube.

(16) Discard the spin column, combine multiple extractions for a single sample if necessary. Store the eluted DNA samples at -20°C . If DNA is to be immediately used for PCR, keep on ice.

*** Old 5.5.5 Qiagen DNeasy Kit (or equivalent kit) Procedure for centrifuged samples**

- (1) Obtain one **lyse and spin** kit per sample to be extracted, open packages and organize tubes in rack. Label both caps of each sample. You will have time to label the rest (3 lab-supplied 1.5-mL MCT, 1 Qiagen spin tube, and 3 Qiagen collection tubes) during the 1-hour incubation. Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.
- (2) Add 370 μL **ATL** to the top of each basket tube.
- (3) Add 30 μL **proteinase K** to the **ATL**.
- (4) Remove sterile **swabs** from pack and place one swab into each tube of ATL/proteinase K mix.
- (5) Move dried samples in centrifuge tubes to the extraction room. Use the moistened swab to swab the bottom of each tube included in the field sample (e.g. if there are 5 tubes per sample, swab the bottom of all 5 tubes with the moistened swab). If the swab becomes covered with debris, rinse swab in ATL solution for that sample in the extraction tube and proceed swabbing the rest of the replicates. BE careful to avoid cross-contamination at this step.
- (6) Place the swab back into the **ATL** mixture, break the wooden stick and close the tube.
- (7) Incubate at 55°C for 1 hour. Label the rest of the tubes and print final archive labels for filters and extracts.
- (8) Remove from incubator and centrifuge at $\geq 18,000 \times g$ for 1 minute. Remove the assembly and keep the swab only until the case is closed. Archive tube with swab at -80°C , until the case is closed, then dispose.
- (9) Add 400 μL Buffer **AL**.
- (10) Add 400 μL **ethanol** (molecular grade). Mix thoroughly by vortexing. If liquid collects around the cap, spin briefly before opening to reduce contamination risk.
- (11) Transfer about half of the mixture by pipette into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (12) Transfer the remaining mixture by pipette onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at $\geq 6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (13) Place spin column in a new 2 mL collection tube. Add 500 μL Buffer **AW1**. Centrifuge at $6000 \times g$ for 1 minute. Discard flow-through and collection tube.
- (14) Place spin column in a new 2 mL collection tube.
Add 500 μL Buffer **AW2**. Centrifuge at $18,000 \times g$ for 3 minutes. Discard flow-through and collection tube.
- (15) Transfer the spin column to a new 1.5 mL or 2 mL MCT.
- (16) Add 200 μL Buffer **AE** to the center of the spin column membrane. Incubate for 1 minute at room temperature ($15 - 25^{\circ}\text{C}$). Centrifuge at $\geq 6000 \times g$ for 1 minute. (17) Discard the spin column and store the eluted DNA samples at -20°C . If DNA is to be immediately used for PCR, keep on ice.

****Old 5.6 Original PCR Procedure (used 2009-2013)**

- (1) If DNA samples (extraction elutes) are removed from freezer, note on freezer/ambient log (see Exhibit 7). Also note on sample log (Exhibit 6).
- (2) Use preprinted 96-well plate map (Exhibits 10-11) or build plate map in LIMS to determine which samples will be pipetted into which wells. Clearly mark plate identification on bottom edge skirt of plate. Write plate identification information (e.g. FY_case#_sample numbers_species_initials_date) in lab notes. Legible labels for use while working must be complete to allow for easy down-stream processing in the sequencing lab.
- (3) Make sure sample map for each plate is entered into the LIMS or attached to lab notes and a signature is written across the map and lab book page.
- (4) Follow DNA amplification protocol detailed below.

Primers specific to either *Hypophthalmichthys molitrix* (Silver Carp) or *H. nobilis* (Bighead Carp) are used to screen eDNA samples and amplify unique DNA sequences in each species potentially present in the eDNA samples by PCR. The PCR programs used to amplify the extracted DNA are specific to the primer set used. The PCR protocol has been optimized to utilize Platinum® *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA) in the eDNA screening. If other brands of *Taq* are used, optimization of the recipe and thermal profiles must be executed. Eight reactions are set up for each sample, in addition to negative (DNA blank) and positive (DNA extracted from tissue²) controls for each master mix. The PCR reactions are prepared as follows:

- (1) Wipe lab bench area with 10% bleach, 75% Ethanol, or commercial DNA sterilization wipes. Also wipe down work area with PCR hood. Use built-in UV lamps to radiate clean room for 30 min prior to PCR set-up.
- (2) Electronic pipettors should be wiped down with one of the solutions or wipes listed in Step 1.
 - In the clean reagent room, obtain all PCR master mix reagents (using only those that have not expired and that have been tested and found viable).
 - 10X PCR buffer (comes in *Taq* kit)
 - 10mM equally mixed dNTP solution (2.5 mM per nucleotide)
 - 50 mM Mg²⁺ solution (concentration in Platinum *Taq* kit; use what comes with your brand)
 - Species-appropriate forward primer
 - Species-appropriate reverse primer
 - *Taq* DNA polymerase (we use Platinum, but any hot-start *Taq* can be used)
 - Sterile molecular grade water (commercially sterile or Millipore filtered, autoclaved)

Allow reagents to thaw. Do not vortex primers or *Taq* too violently.

- (3) Record in lab notebook the lot number of all reagents used.
 - (a) Prepare PCR master mixes in clean reagent room. The master mix volume can be adjusted according to the number of samples to be processed. In order to make sure that master mix does not run out prior to supplying all the desired reactions (this may occur as a result of minor errors or variations in pipetting volumes), it is generally helpful to make more than enough master mix than is needed for the desired number of reactions. For example, make

² Extract from tissue using commercial DNA extraction kit (e.g., Qiagen DNeasy Blood & Tissue Kit), and manufacturer protocol. Run test PCR before relying on any DNA extract for eDNA assay positive controls.

enough master mix for 100 reactions when actually preparing for 96 reactions. NOTE: If positive extraction controls consist of a different species of DNA, be sure to make a small separate master mix for those samples and use primers specific to the content of the control sample. Negative extraction controls should be amplified with the Silver or Bighead Carp master mix.

(b) Each Initial PCR 1X reaction should contain:

- 2.5 μ L 10X PCR buffer
- 0.5 μ L dNTP (10 mM mixed dNTP)
- 0.75 μ L Mg²⁺ solution (50mM)
- 0.5 μ L forward primer (10 μ M working dilution)
- 0.5 μ L reverse primer (10 μ M working dilution)
- 0.25 μ L Platinum® *Taq* polymerase (= 1.25 U)
- 19.0 μ L sterile water.

Move prepared mix from reagent room into PCR room.

- (4) Remove DNA extracts from freezer or fridge (fill out logs as needed), vortex (quick touch) and quick-spin down the extract tubes. Take them into the PCR room. Place the 96-well PCR plate onto a clean surface, positioned from left to right.
- (5) Fill the plate wells with 24 μ l PCR mix. Carefully pipette 1 μ L of each sample to be screened into each well of a column, changing the pipette tip between each sample. Each column of eight wells should be filled with the same sample (i.e., eight replicates per sample to be tested). The first 11 columns of the PCR plate can test 11 different samples for one target species. Into the 12th column, pipette 1 μ L of sterile water into the bottom three wells (F, G, H) and pipette 1 μ L of each of the target species positive control DNA into each of the top four wells (A, B, C, D). Leave the intervening well (E) empty.
- (6) Place the positive control DNA back in to the appropriate -20°C freezer and change gloves immediately in order to reduce risk of contamination.
- (7) Place PCR film over the PCR plate and press firmly (or use an automatic plate sealer) to ensure the edges of all wells are sealed. Gently tap a few times on the lab bench to ensure thorough mixing of each reaction. Spin down the plate in the plate spinner to ensure all DNA is down in the master mix.
- (8) Place the 96-well PCR plate in the thermal cycler, close and secure lid, and select the appropriate PCR thermal program (thermal cycle programs for Silver Carp and Bighead Carp utilize different annealing temperatures). The thermal programs for the current eDNA markers (Jerde et al. 2011) both consist of:
 - Initial denaturation at 94°C for 10 minFollowed by 45 cycles of:
 - 94°C for 1 min,
 - 50°C for Silver Carp program or 52°C for Bighead Carp for 1 min
 - 72°C for 1.5 min.Followed by:
 - final extension at 72°C for 7 min
 - 4°C hold temperature until plate removed from thermal cycler.
- (9) Record the plate ID, thermal cycler unit or head, plate orientation, and run times for the PCR plate in the PCR log (Exhibit 8).

- (10) Place cycled PCR plates and product in designated -20°C freezer for long-term (more than overnight) storage, in the designated 4°C refrigerator for short- or mid-term storage (1–12 hours). If you leave for the night, the thermal cyclers are set to hold at 4°C forever. Remove plates promptly in the morning.
- (11) Under no circumstances should you open or uncover PCR plates that have been cycled in the PCR room. Only open cycled plates in the post-PCR rooms.

****Old 5.7 Gel Electrophoresis of eDNA PCR Assays (original method used 2009-2013)**

5.7.1 Purpose

Once amplified, the DNA samples should then be subjected to gel electrophoresis in order to visualize the amplified DNA. This method is useful in determining the presence of DNA from the target species in different aquatic environments.

5.7.2 Source

PCR product following amplification can be taken either from cold storage (see #11 above) or directly from the thermal cycler.

5.7.3 Gel Electrophoresis Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to pipetting error. It is also highly susceptible to mislabeling and, consequently, confounding of sample results.

Draw or otherwise produce a map of which sample will be electrophoresed on which gel and in which lane of the select gel.

Carefully pipette samples so as to avoid:

Injecting samples to incorrect wells.

Piercing the bottom of sample wells and losing PCR product.

Spill over from adjacent wells.

Record all solution batch numbers or name/date identification for stock solutions. Record precast gel batch identification number if appropriate.

Centrifuge plates, strip tubes, etc. before removing film or caps in order to prevent aerosol cross-contamination.

Any revisions to the DNA amplification protocol must be approved by the eDNA Project Leader (and documented) and incorporated into a revised QAPP.

****Old 5.6.6 Internal Positive Control (for indicating inhibition)**

The use of internal positive controls in qPCR is the most commonly used method to detect inhibition in laboratories spanning from clinical diagnostic settings, forensic settings to environmental research. WGL will use a novel mouse gene, HemT, which encodes for a hematopoietic cell-specific transcript. Known numbers of the HemT will be spiked into each well of the plate containing field samples, which will then be quantified using standard curves for that target. If fewer copies of the spike are measured in a sample, inhibition is assumed, and the extract will be cleaned up with a PCR clean-up kit (OneStep Inhibitor

Removal kit by Zymo Research, Irvine, CA), and re-amplified. Samples will then continue through the eDNA workflow. This method will not determine what particular inhibitor may be present, and will only test for inhibition due to blockage of taq activity.

- (1) At WGL, all primers, probes, and the target ultramer will be purchased from Integrated DNA Technologies, but other providers may be used.
 - a. HemT Forward Primer = 5' TCTGAGTGTCCCTCGAATCT 3'
 - b. HemT Reverse Primer = 5' GCAGTCCTTGAGAACATAGAGC 3'
 - c. HemT Probe = 5' Cy5/ZEN TGA CAG TCT CCT TTC GTG TGA ACA TTC G 3' IBQ
 - d. Hem T Ultramer = 5' CTA CAT AAG TAA CAC CTT CTC ATG TCC AAA GCT CTC TGA GTG TCC CTC GAA TCT CAG ACG CTG TAT GAC AGT CTC CTT TCG TGT GAA CAT TCG GCT CTA TGT TCT CAA GGA CTG CAC 3'
- (2) HemT primers should be hydrated, diluted to 5.0 μ M, and stored in small aliquots in the reagent room freezer.
- (3) The HemT probe should be diluted to 2.5 μ M and stored in small aliquots in the reagent room freezer.
- (4) HemT standard 5X curves will be run on each plate, with the ACTM1 and 3 markers. The table below indicates standard curve dilutions to be used. See Exhibit NA, page 1 for the PCR datasheet.

Standard dilution copy number of HemT IPC	Standard dilution copy number of ACTM 1 and ACTM 3 gBlock
6250	6250
1250	1250
250	250
50	50
10	10

- (5) The PCR cocktail recipe for the ACTM 1 and 3 duplex assay has been adjusted to account for the spike. All unknown field samples and the PCR negative and PCR no template control samples will be made from master mixes including 250 copies of HemT per reaction. The recipe per reaction follows:
 - a. TaqMan Environmental Master Mix 2.0 = 10.0 μ L
 - b. HemT Primer Mix (5.0 μ M) = 1.0 μ L
 - c. HemT Probe (2.5 μ M) = 0.75 μ L
 - d. AC TM1/3 Primer Mix (10 μ M) = 1.0 μ L
 - e. AC TM1/3 Probe (2.5 μ M) = 1.0 μ L
 - f. IPC spike template (250) = 1.0 μ L
 - g. ddH₂O = 2.25 μ L
- (6) 3.0 μ L of template is added to each reaction.
- (7) The PCR cocktail recipe for the standard curve samples differs because it does not include the IPC spiked sample. Only 1.0 μ L of each G-block standard dilution is used in these reactions.
 - a. TaqMan Environmental Master Mix 2.0 = 10.0 μ L
 - b. HemT Primer Mix (5.0 μ M) = 1.0 μ L
 - c. HemT Probe (2.5 μ M) = 0.75 μ L
 - d. AC TM1/3 Primer Mix (10 μ M) = 1.0 μ L
 - e. AC TM1/3 Probe (2.5 μ M) = 1.0 μ L
 - f. ddH₂O = 4.25 μ L
- (8) Analysis of results

- a. The standard curve of the IPC must meet efficiency of 80-100% and $R^2 > 0.95$.
- b. Determine inhibition.
 - i. Calculate the reference Cq for each plate:
 1. Determine the average the Cq for the 250-copy standard dilution replicates (N=2), the 2 no template controls, and the 2 negative controls (these controls have the spiked IPC in their master mix).
 2. Determine the difference between the minimum and maximum Cq values of the same 6 samples. Use this value (max-min) as the range allowed around the average Cq of these same samples. If it is less than 1.0, round up to 1.0.
 3. The value from #1 plus the value from #3 is the reference Cq.
 - ii. Inhibition will be defined as any field sample with a Cq for the IPC that is greater than the reference Cq.
- c. The entire remaining extract of samples determined to be inhibited will be cleaned up with the PCR inhibitor clean up kit according to the manufacturer's directions and re-assayed with the ACTM1 and 3 markers.
- d. Uninhibited results will enter the normal workflow, negative results reported, and presumptive positive results screened with the confirmation assays.

5.7.4 Option A Procedure

Prepare 2% agarose gels with SB (sodium hydroxide and boric acid) buffer and allow the gel to polymerize for a minimum of 25–30 min prior to loading samples. Gels can be prepared at any time prior to PCR or immediately after PCR. However, once you begin loading a gel finish loading and run the gel immediately.

To prepare PCR samples for gel electrophoresis, transfer 10µl from each well of the 96-well plate to new wells in an identically labeled 96-well plate. Add 2µl of loading dye (see recipe below) to each well with PCR product (loading dye = 500µl 6X loading dye, 500 µl DMSO, 1µl SYBR Green I)

Place the 2% agarose gel in the electrophoresis chamber that contains SB Buffer and remove the gel combs. In the first well of each row on the agarose gel, load 100bp DNA ladder/loading dye mix. Next, load 10µL of each sample mixture (i.e., each PCR reaction and loading dye), and negative controls into the remaining wells. Be sure 10 µl of a positive control PCR product is in the last lane of each row. Run electrophoresis at ~250V for ~45 min depending on migration times through the gel. Times and voltages required to run each gel are approximate (different buffers and higher content agarose gels will require different run times and voltages, use what works in your lab).

Be sure to annotate the gel loading map in the notebook as you observe the gel in the geldoc. Interpret positive samples and note successful positive and negative controls on each gel. After documentation (5.8), dispose of gel in the garbage.

5.7.5 Option B Procedure

Pre-cast 2% E-Gel® 48 gels (Invitrogen) are used with the E-Base™ system (Invitrogen).

Open E-gels and load into the bases.

1. Remove gel from the pouch. Remove comb from the gel.
2. Slide gel into the two electrode connections on the Mother E-Base™ or Daughter E-Base™. If gel is properly inserted, a fan in the base begins to run, a red light illuminates, and digital display shows 20 min.

3. Load each gel within 30 min of removing gel from the pouch and run within 15 min of loading.

Selecting Program on E-Base™

1. Connect the Daughter E-Base™ to a Mother E-Base™ or another Daughter E-Base™ if running multiple gels.
2. Select program EG by pressing and releasing the pwr/prg (power/program) button on the base.

Loading E-Gel® 48 Gels

1. Load 15µL PCR product into each well of an E-Gel® 48 gel. Keep all sample volumes uniform. Load with a multichannel pipettor.
2. Load appropriate DNA ladder (distinct bands between 0-500 bp) into far left well (labeled M) and positive control PCR product into the far right marker well. Ensure the marker salt concentration is similar to that of adjacent samples (2% gel uses 100 bp DNA Ladder).
3. Load 15µL of sample buffer containing the same salt concentration as the sample, or sterile water, into any empty wells.

Run Conditions

1. To begin electrophoresis, press and release the pwr/prg button on the Mother E-Base™ and Daughter E-Base™. The red light changes to green.
2. At the end of the run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button on the base to stop the beeping and flashing red light.
3. Remove gel cassette from the base and analyze results.

Note: if a gel is removed before a run is complete, a gel must be replaced and the unit allowed to run out until the timer counts down to zero. There is no option to reset the base.

**** Old 5.8 eDNA Gel Documentation and Storage (original method used 2009-2013)**

5.8.1 Purpose

Once eDNA gels have been visualized, the results must be documented, interpreted (i.e., scored), and recorded. In some cases, very light bands may be visible, making scoring difficult. Documentation, labeling and saving for sequence confirmation, and storage are critical for later quality control review.

5.8.2 Source

Following electrophoresis, agarose gels should be immediately documented. Following documentation, PCR products requiring sequencing must be labeled and organized for sequencing.

5.8.3 Gel Documentation and Storage Assurance and Chain-of-custody

Because of the difficult nature of scoring some results, careful records must be kept of all gels and results. These results must be maintained so as to minimize the risk of tampering or data loss.

Gel image quality must be assessed at the time images are obtained. Images should exhibit all bands on gels as clearly as possible, if this is not possible, it must be noted on the gel data sheet (Exhibit NA). All gel digital image files should be saved and should be archived at the end of each working day. All gel image data are referenced to the samples case number to make sure the consistency of the sample custody.

Gel score data should be entered and stored in the appropriate database in an Excel file and on the data sheet in the laboratory notebook, or in LIMS.

All reports should reviewed by the eDNA Processing Leader before being reported.

A paper copy of the report should be held in the files for 5 years.

Electronic copies of all reports should be held for 5 years or longer, as space permits.

Any substantive revisions to the DNA amplification protocol must be approved by the eDNA Processing Leader and approved by the eDNA Coordinator. Any such changes must be incorporated into a revised QAPP.

5.8.4 Procedure

After electrophoresis is complete, remove casting tray with gel from the electrophoresis chamber and place the gel onto the gel scanner (BioRad Molecular imager FX), select DNA ethidium bromide stain gel, set up scanning area, and then select 100 micrometer to start scanning the gel.

Alternatively, place the gel on a UV transilluminator equipped with a digital camera, such as the Alpha red Imager (Cell Biosciences, Inc.), and capture a digital photograph of the gel.

After the gel scanning is done, properly label file name and save the file on the hard drive immediately.

If not using LIMS, print out a picture of the gel image and insert into lab book. A copy should be kept with the Project Lab Book. Be sure to sign across the print out and the lab book page.

**** Old 5.9 Gel Interpretation (original method used 2009-2013)**

5.9.1 Purpose

Once a gel is visualized, the quality of the results and presence of potential positive bands must be assessed in order to determine which samples need to be further assayed.

5.9.2 Source

Immediately following the cessation of electrophoresis, the agarose gel containing the eDNA PCR products should have been visualized on either the UV-based imager or the laser-based imager. In both cases, the gel image should be captured (saved to hard disk) immediately and then printed.

5.9.3 Gel Interpretation Quality Assurance and Chain-of-custody

The positive controls should have bright bands at the appropriate migration distance (number of base pairs), indicating a positive reaction.

No bands at the targeted sizes (~200bp silver, ~300bp bighead) should be observed in the negative controls.

If any of the initial PCR reactions are positive (i.e., a visible band at the appropriate migration distance), the initial sample is designated a "presumptive positive".

Record the number of presumptive positive reactions for each sample both in the gel electrophoresis lab notebook and in the excel file on the lab computer.

Presumptive positive results will initiate a series of results confirmation mechanisms (see below). These mechanisms include screening the transport and equipment controls, and DNA sequencing.

Once presumptive positives are documented:

Fill in the quality control results in the eDNA sample log (Exhibit 6).

Notify the eDNA Processing leader.

Color in the plate seal above the wells containing presumptive positive samples on the PCR product plate. Move all plates requiring sequence confirmation into the freezer in the sequencing room. Plates without positives can be discarded.

**** Old 5.10 Confirmation of Positive Results (original method used 2009-2013)**

5.10.1 Purpose

These confirmation mechanisms are initiated if a sample returns as a positive for the PCR test (any number of the eight reactions, e.g., one of eight up to eight of the eight PCR reactions).

5.10.2 Source

Positive results for Asian carp eDNA require that those positive samples be further assayed. The original DNA elutes from samples should be located in designated -20°C freezer.

5.10.3 Gel Interpretation Assurance and Chain-of-custody

Presumptive positive assays (PCR reactions) are validated through DNA sequencing and testing of additional control samples.

Any revisions to the DNA QA/QC amplification protocol must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.10.4 Procedure

Conduct PCR assays of the paired equipment control for each presumptive positive. DNA extraction, amplification, documentation, and interpretation following protocols detailed above (Sections 5.6–5.9).

Ensure that the transport blanks (see 2.2.2 (6) and 2.3.2 (10)) have been tested for that sample group (i.e., from the same cooler in which the presumptive positive sample was transported).

For all presumptive positive samples, bidirectional sequencing confirmation is performed. This is done by using a commercially available gel extraction kit (e.g., Qiagen Qiaquick Gel Extraction kit) per the manufacturer's recommendations on the positive PCR reactions, or E-Gel® CloneWell Agarose Gels (Invitrogen) per manufacturer's recommendation.

If the equipment control and transport blanks test negative, the sample is designated a "confirmed positive."

The following sequencing reaction can be done either by cloning (TA TOPO cloning kit used for sequencing per manufacturer's recommendation) then sequencing, or by a direct Sanger sequencing method (ABI BigDye® Terminator v3.1 or v1.1 Cycle Sequencing Kit) modified by WGL. BigDye Terminator Reaction Master Mix for 1X reaction:

1 μL BigDye terminator mix,
4 μL 5X reaction buffer,
0.8 μL either SC/BH forward primer, and
10.2 μL of water.

Add 16 μL of master mix to the 4 μL of purified DNA. Total reaction size is 20 μL .
The positive control reaction of sequencing was done per manufacturer's recommendation.

For each pGEM control PCR master mix:

- 1 μL BigDye terminator mix,
 - 4 μL 5X reaction buffer,
 - 2 μL m13 primer,
 - 8 μL water, and
 - 2 μL pGEM.
- Add 20 μL to each control well.

Place the PCR plate on the thermal cycler and begin temperature cycling protocol. Program the thermal cycler as follows: 25 cycles of [96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min], then ramp to 4°C.

Option 1: To clean the BigDye reaction with EDTA precipitation:

1. Spin plate after removing from thermal cycler (just to make sure that everything is at the bottom of the well).
2. Add 5 μL of 125mM EDTA to each well.
3. Add 60 μL of 100% EtOH.
4. Seal the plate and mix by inverting four times.
5. Incubate at room temperature (RT) for 15 min.
6. Spin plate at 3000xg for 30 min (at 4°C) or at 2000 x g for 45 min. Proceed to next step immediately.
7. Invert the plate and spin at 185 x g for 1 min (time from when rotor starts).
8. Add 60 μL of 60% EtOH.
9. Spin at 1650 x g for 15 min at 4°C.
10. Invert plate and spin at 185 x g for 1 min.
11. Resuspend samples in 20 μL Hi-Dye.

Option 2: To clean the BigDye reaction with Sephadex in Centri-SEP spin columns or Millipore plates, use columns for 1-16 samples and plates for 17 or more.

Centri-SEP column procedure

Materials list:

- Spin columns (preloaded with dry sephadex, caps, and bottom caps), one per sample
- Pipets and tips
- Centrifuge with MCT rotor
- Reagent grade water
- Labeled 1.5-mL MCT and tube rack
- Vortexer

Column Hydration

1. Tap column on counter to settle sephadex gel into the bottom.
2. Remove cap and add 800 μL sterile ddH₂O. Replace cap, invert, and mix well on vortexer, ensuring all gel is hydrated. Invert again, vortex and place in rack.
3. Let sit 30 minutes at room temp in a tube rack.
4. While columns sit, label 1.5ml MCT with sample names for collecting cleaned product.
5. Remove excess water
6. Check each tube for bubbles, if there are bubbles in the gel, invert the tubes and vortex so all the slurry moves toward the cap.

7. Invert and gently place in tube rack, allowing gel to settle.
8. Remove caps, then bottom, and place column in collection tube to drain.
9. Allow to drain until about 200-250 μ l water has drained. If needed, gently apply pressure with a gloved finger to get the water draining.
10. Discard fluid, replace column in collection tube.
11. Place columns into centrifuge, making sure the indicator tabs are up.
12. Spin at 750xg(rcf) for 2 min
13. Discard the collection tube, blot any water off the bottom of columns and place column into labeled sample collection tube.
14. Sample processing
15. Hold the column up to eye level, and carefully place all of the cycle sequencing product onto the center of the packed column, being careful to not disturb the column. Don't touch the side of the column with the product or the pipette tip.
16. Place collection tube into centrifuge with caps open and facing inward and the indicator tab of the column facing up.
17. Spin at 750xg(rcf) for 2 min. Discard spin column.

If you have more than 32 samples, you must dry filtrates in the spin vac at $\sim 60^{\circ}$ for ~ 45 minutes. Once dry, you may freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.

Millipore plate sephadex procedure

Materials list:

Millipore filter plate with centrifuge alignment frame

- Plain 96-well plate to catch waste water (can reuse the same one)
- Sequencing plate (with bar code)
- Sephadex G50-fine
- Millipore column loader and tray
- Centrifuge with plate rotor
- Reagent grade water

Prepare sephadex plate:

1. Pour dry sephadex into the black metal loading plate which is in the plastic catchment tray.
2. Use the clear squeegee to level each well you need for cleanup (tape off unused wells).
3. Scrape excess powder into the catchment tray so you don't waste too much sephadex.
4. Invert a multiscreen plate over the metal tray and hold them together while you invert the tray to fill the wells with sephadex. Make sure you are filling the wells you wish to use! Tap gently to make sure all of the sephadex moves into the filter plate.
5. Pour excess sephadex from the tray back into the sephadex container and cap tightly.
6. Add 300 μ l of water to each well of the plate and let sit *3 hours at room temp*. These plates can be prepared ahead of time, wrapped tightly in plastic wrap, placed in a Tupperware with a moist towel and refrigerated for two weeks.
7. Remove excess water and pack columns prior to use:
8. Place the filter plate over a plain 96-well plastic plate, and load into the plate rotor. Don't use the interior lid because it does not fit over stacked plates.
9. Centrifuge at 910xg(rcf) for 5 min.
10. Dump the waste water into the sink and rinse catchment plate (set aside for another use).

11. Clean sequencing reactions with the columns.
12. Use the multichannel pipette to carefully add reactions to the *center* of each column.
13. Place the filter plate over a 96-well sequencer plate making sure wells A1 are aligned.
14. Centrifuge at 910xg(rcf) for 5 minutes.
15. Dry filtrates in the spin vac at ~60°C for ~20 minutes. You may seal plate and freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.
16. Once samples are dry, add 15 µl HiDi to each sample. Samples in Hi-Dye can be stored at 4°C overnight, but may not be left any longer than 12 hours.

To sequence:

1. Denature samples with HiDi for 5 min at 95°C in thermocycler (do not denature samples run wet)
2. Place immediately on ice
3. Load into sequencer plate and onto sequencer.
4. Resulting sequencing reactions that are successful are screened in GenBank (<http://www.ncbi.nlm.nih.gov/blast>) using the BLAST (Basic Local Alignment Search Tool) algorithm. If the resulting sequence is a positive match to the targeted species of Asian carp, the sample is designated a “confirmed positive – sequenced”.

References: User Manual, “BigDye® Terminator v3.1 Cycle Sequencing Kit”

http://www.ibt.lt/sc/files/BDTv3.1_Protocol_04337035.pdf

“TOPO TA Cloning® Kit for Sequencing” http://tools.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf

“QIAquick Gel Purification Kit”

http://molecool.wustl.edu/krolllab/Kroll_Lab_Protocols/Molecular%20Biology%20protocols/Cloning%20protocols%20folder/Gel%20extraction-Qiagen.pdf

**** Old 5.5.4 PowerWater Procedure for filters**

- (9) Remove samples from freezer if filters, note on freezer and sample logs (see Exhibits NA).
- (10) Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.
 - (b) Before proceeding with extraction, a positive control filter is prepared by pipetting 300 µl of a mixed slurry of homogenized Silver and Bighead carp tissue or cells directly onto a sterile filter paper. Alternative species (e.g. – sturgeon) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA and (2) can be run on the same thermocycler settings as carp samples.
 - (c) Additionally, an extraction negative control sample should be prepared by placing a new filter paper in a new sterile 1.5mL microcentrifuge tube (MCT) and pipetting 300 µl of sterile lab DI water directly onto the filter. A batch of extraction blanks can be prepared in advance and kept frozen at -20°C.
 - (d) For every extraction batch of filter samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control filter and one prepared positive control filter.
- (11) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below. For each filter, you will need one spin column and 4 1.5/1.7 ml lab supplied MCT and 5 Powerwater collection tubes.

This DNA extraction utilizes the PowerWater DNA Isolation Kit (MoBio Laboratories, MoBio Inc.) and the protocol is adapted from the manufacturer's protocol (<http://www.mobio.com/images/custom/file/14900.pdf>).

- (a) Place Solution PW1 in a 55°C water bath for 5–10 min to dissolve any precipitates that have formed at room temperature. Remove Solution PW1 from the water bath immediately prior to use.
- (b) Remove the appropriate filter sample from –20°C freezer and transfer the filter(s) to a labeled 5mL PowerWater Bead Tube. If DNA will be extracted from multiple filter samples, continue to remove each filter sample from –20°C freezer immediately prior to filter transfer. Each PowerWater Bead Tube will hold up to two filters per sample. If any filtered water samples required more than two pieces of filter paper, split the filters into two per PowerWater Bead Tube.

Note: Change gloves between the transfers of each sample to avoid cross-contamination of samples.
- (c) Add 1mL of Solution PW1 to the PowerWater Bead Tube and secure the cap tightly. Mount the tube on a vortex adaptor (MoBio Inc.) and vortex on high for 5–10 min, or until the contents of the bead tube appear liquefied. Times can vary depending on the number of filter papers being extracted. A bead beater can be used on a large batch of bead tubes if available.
- (d) Centrifuge the tubes at 4,000 x *g* for 8 min at room temperature. Ensure centrifuge is balanced before centrifuging. Transfer 650-800 µl supernatant using a 1mL pipette to a labeled 1.7mL lab supplied MCT.
- (e) Centrifuge tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled 1.7 mL lab supplied MCT. Be sure to avoid any beads or filter debris.
- (f) Add 200µL of Solution PW2, vortex briefly, and incubate at 4°C for 5 min. It should appear cloudy.
- (g) Centrifuge the tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled lab-supplied 1.7 mL MCT.
- (h) Add 650µL of Solution PW3 and vortex briefly. Load 650µL of supernatant onto a spin filter, place spin filter into a MoBio 2mL tube and centrifuge at 13,000 x *g* for 1 min. Discard the flow through and collection tube. Place spin filter into a new MoBio 2mL tube and load another 650 µl , centrifuge and repeat until all the supernatant has been pass through the spin filter.
- (i) Place the spin filter basket into a new labeled MoBio 2mL collection tube and add 600µL of Solution PW4.
- (j) Centrifuge the tubes at 13,000 x *g* for 1 min and discard flow through. Place spin filter in a new, labeled MoBio 2mL tube.
- (k) Add 550µL of Solution PW5 and centrifuge at 13,000 x *g* for 1 min. Discard flow through, place spin filter into a new MoBio 2mL labeled tube and centrifuge again at 13,000 for **2 min**. Be sure all traces of EtOH are gone.
- (l) Place the spin filter into a new labeled 1.7mL lab supplied MCT labeled with the sample

identification number.

(m) Add 100µL of sterile water (autoclaved, de-ionized) to the center of the white filter membrane, let it sit for 1-2 minutes, then centrifuge at 13,000 x *g* for 1 min.

(n) Discard the spin filter and store the eluted DNA samples at -20°C. If more than one extraction tube was required for a single sample, combine all replicates into one final extraction tube.

DNA extraction elutes should be placed into a designated freezer for overnight or longer storage, or, if used for PCR within 1-4 hours, stored in the refrigerator. Make note of sample addition to freezer log if necessary (see Exhibit NA). Note completion of extraction on sample log.

****Old Appendix D: Sample Bottle Management Plan**

The WGL study testing used sampling demonstrated that sample bottles can be reused for eDNA sampling, but recommendations were for implementation of a decontamination specialist to oversee and confirm that decontamination was conducted according to the QAPP. The Regional Office has decided that WGL will serve as the single decontamination facility for the Asian carp eDNA monitoring program.

WGL will clean and prepare bottles according to the QAPP and store them in the lab in a clean room. WGL will package bottles according to the following procedure, and send them as needed to the field offices.

Empty bottles for field samples:

- (1) New card board boxes and plastic liners will be purchased by WGL. Boxes will be assembled as needed and lined with the plastic bags.
- (2) Cleaned and autoclaved bottles will be securely closed and placed in the bag.
- (3) Bags will be sealed with tamper evident tape, boxes sealed and shipped to field stations. Water-filled field blanks may need to be sent separately, but all efforts will be made to get field blanks distributed at the annual training meeting each spring (if it is held in La Crosse).
- (4) Field stations will receive boxes, store sealed until they are ready for labeling.
- (5) Field stations will prepare coolers according to the QAPP, in as clean of an area as possible at the station.
 - a. Anytime boxes are opened, staff should be in clean clothes and use a new pair of clean gloves for actually handling the bottles. For example, you may open the box with un-gloved hands. Then, put on gloves to open the bag and touch the bottles.
- (6) Once clean coolers are ready, boxes may be opened, bottles labeled, and placed into clean coolers.
- (7) After use, field staff can use the same bag and box to return the bottles to WGL.

Cooler (field) blanks:

- (8) Plastic bags fit to hold one container will be purchased by WGL
- (9) Cleaned bottles will be filled with reverse osmosis tap water at WGL and autoclaved.
- (10) After cooling, bottles will be securely closed, and placed in the bag.
- (11) Bags will be twisted closed with wire closures.
- (12) Filled bottles will be distributed at the annual field training meeting held at WGL so that each field station will start out the season with the total estimated number of field blanks needed for the sampling season.
- (13) Field stations can store these prepared field blanks as long as needed, because as each bottle is wrapped individually.
- (14) Field stations will prepare coolers according to the QAPP, in as clean of an area as possible at the station.
 - a. Anytime boxes are opened, staff should be in clean clothes and use a new pair of clean gloves for actually handling the bottles. For example, you may open the box with un-gloved hands. Then, put on gloves to open the bag and touch the bottles.
- (15) Once clean coolers are ready, boxes may be opened, bottles labeled, and placed into clean coolers.
- (16) After use, field staff can use the same bag and box to return the bottles to WGL. WGL will have

designated shelves in the garage for boxes of dirty bottles.

Appendix H: Internal report comparing extraction kits

Validation of IBI Scientific and Qiagen DNA extraction kits for use in the early detection and monitoring of invasive carp using environmental DNA

Kyle M. Von Ruden, Nicholas M. Berndt, Emy M. Monroe: US Fish and Wildlife Service, Whitney Genetics Laboratory

Introduction

Environmental DNA (eDNA) has become increasingly used as a surveillance tool to monitor for the genetic presence of aquatic invasive species or threatened and endangered species (Jerde 2011). Environmental samples generally have low levels of the target species DNA (Dejean et al. 2011), thus it is critical to have minimal loss of DNA during processing in the lab.

Once an eDNA water sample is collected, the organic matter is typically concentrated by filtering or centrifugation. Extraction of total DNA from filters or pellet is the first step in workflow. Detection of target DNA in the sample depends on the quantity and quality of DNA which is extracted from the field sample. Commercially available extraction kits may vary in the quantity and quality of target DNA copies available at the end of extraction, so it is critical to be able to maximize both aspects of extraction.

Extraction is a time-consuming step in the lab, especially when there are thousands of eDNA samples to be processed, and this step is often the “bottleneck” in processing. In addition to labor time, other considerations in choice of extraction kits are cost and the ability to purchase individual reagents or pieces of each kit as needed to avoid waste of kit components. Current protocols used in the Whitney Genetics Lab for the processing of eDNA samples in the early detection and surveillance of bigheaded carps is outlined in great detail in the Quality Assurance Project Plan (QAPP). Originally, the QAPP called for use of MoBio PowerWater kits for extraction, but in 2013 after a rigorous comparison study conducted with two other labs, Qiagen DNeasy Blood and Tissue kits were approved for extraction (Amberg et al. in press). The patent on Qiagen kits recently expired, and IBI Scientific created a “copycat” kit called the gMAx mini kit, which uses the same chemical buffers and DNA-binding membranes in a spin kit as the Qiagen version. The IBI kit is less costly at 2.1 cents per sample compared to 2.7 cents per sample, and IBI will sell the spin columns separately unlike Qiagen. This is helpful to reduce reagent waste because there are often left-over reagents that can't be used because the spin columns are used up first.

The objective of this study was to determine whether the IBI kit is as good as or better than the Qiagen kit by comparing copies of target DNA found in three different types of samples. In order to avoid unnecessary contamination of the eDNA lab with Asian carp DNA, bluegill DNA was assayed with real-time quantitative markers (qPCR) (Takahara et al. 2003) after extraction.

Methods

Three sets of 25 samples (24 for the Mississippi River set) were collected or created, filtered according to the QAPP, and then half of the total number of filters were extracted with each kit. To homogenize samples between filters, each 2L sample was inverted multiple times and split into 500ml per filter two times creating a total of one filter per kit per sample. Three different sample types consisted field-collected water, aquarium water, and tap water spiked with bluegill cells collected from the virology lab at the center. Field water was taken from a backwater of the Mississippi River, the aquarium water came from a holding tank of bluegills and largemouth bass, and tap water was spiked with 1ml of cultured bluegill cell line. Bluegill was chosen as a surrogate to invasive carp because they are active in winter,

don't carry contamination concerns, are locally available, and can be detected with a high-quality qPCR assay (Takahara et al 2003).

Once samples were filtered, each set of 25 was extracted following the manufacturer's protocol for their respective kit. Extracts were then analyzed in eight replicate qPCR reactions following methods in the QAPP. Plates were loaded on an Eppendorf epMotion 5075 and cycled on Bio-Rad CFX384 Touch™ Real-Time PCR Detection Systems. Results were based off average starting quantities (SQ) of DNA copy numbers and extraction kits were compared to each other by sample using a Paired T-test.

Results

The average amount of DNA measured in eight replicate qPCRS from each extract is presented in (Figure 1; field samples top panel, aquarium samples lower panel). Extractions using both kits for the cell line samples were overwhelmed with DNA and are not shown. Paired t-tests comparing the total copies of bluegill DNA in each sample type indicate that there was a significant difference between the two kits ($p=0.0003$ field, and $p=0.0004$ aquarium) and in 22 of the 24 comparisons, the IBI kit extracted more target DNA copies than the Qiagen kit.

Discussion

The IBI gMAX kit extracted significantly more target DNA than the Qiagen kit. The field samples had lower overall copies for both kits, but this was expected because diluted environmental samples typically yield less target DNA. The aquarium samples yielded high amounts of naturally sloughed DNA, and IBI performed better than Qiagen here as well.

Results for our cell spiked samples appeared to overload each kit with Qiagen being maxed at 5×10^6 cells and IBI at 1×10^7 cells (www.ibisci.com, www.qiagen.com). Yield and quality of DNA can be affected when overloading an extraction column with cells. Thus, these results were not considered in our analysis.

Extraction time using the IBI kit was approximately one hour less and each filter cost approximately \$0.60 less to extract. This time savings adds up to hours during the week that can now be used by WGL staff to move monitoring cases along more quickly, significantly reducing turnaround time for reporting results. Further efficiency can be realized by completely using all kit reagents because IBI will sell the spin columns separately, but Qiagen will not. Because the IBI kit is advertised as a replica of the Qiagen kit, switching from one kit to the other does not require a large change in protocol in the lab, thus it can be seamlessly integrated into our normal workflow and added as an extraction option in the QAPP. Since the IBI kit performed at least as well as the Qiagen kit, data generated by the lab will remain of the highest quality.

Our results show that IBI Scientific gMAX Mini Kit extracts more copies of the target DNA compared to Qiagen's DNeasy® Blood & Tissue Kit. When considering other aspects of extracting DNA samples in a high through-put facility, the IBI kit also appears to be more efficient and cost friendly. Based on this study, we recommend using IBI Scientific gMAX Mini Kit for extraction of eDNA samples in WGL.

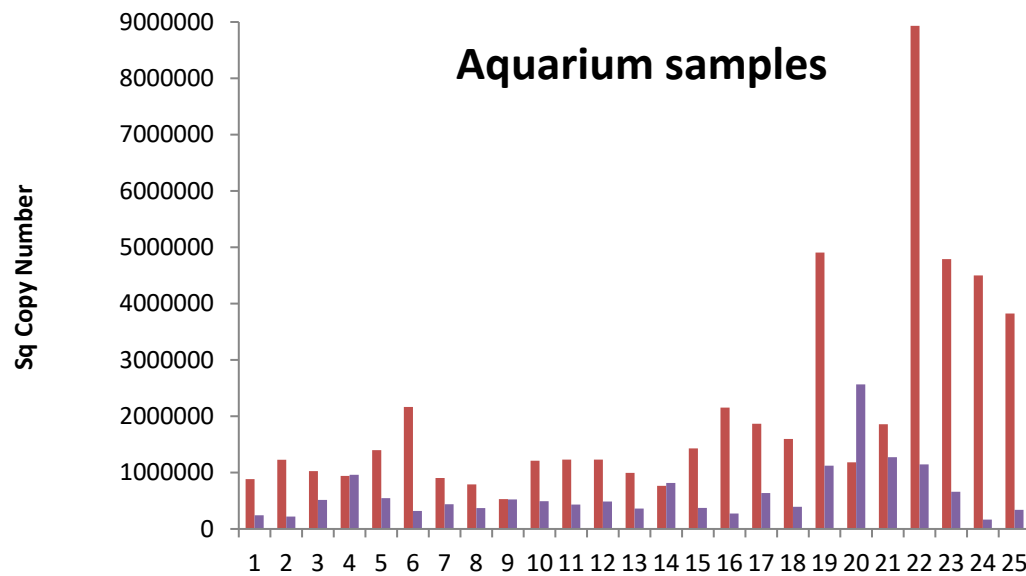
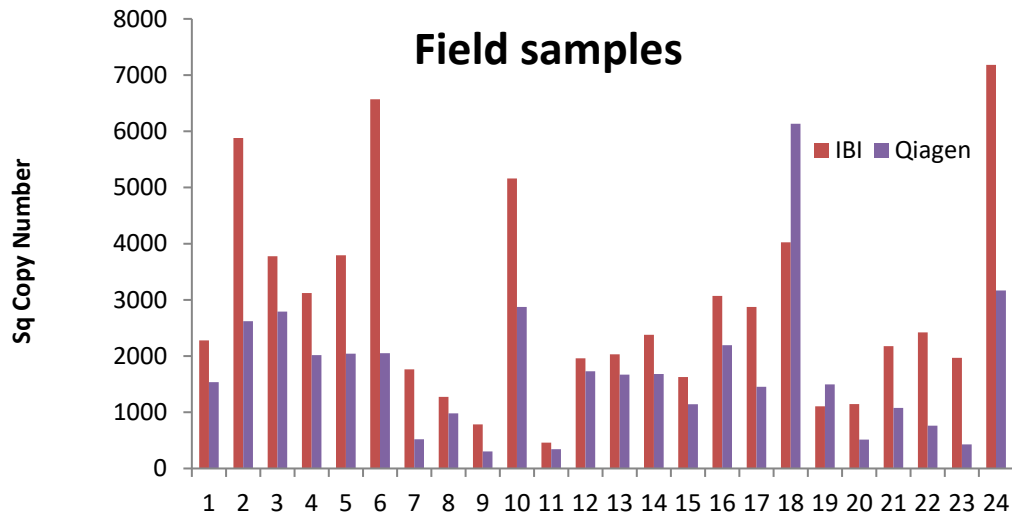


Figure 1. Average copies of bluegill DNA per octet for each sample using IBI and Qiagen extraction kits. Sample number is on the x-axis, and Sg copy number is on the y axis. IBI extraction kit is labeled in red, and Qiagen is purple.

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Appendix I: Internal Reports by Whitney Genetics Lab

HemT internal positive control for determining inhibition in eDNA monitoring samples

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Introduction

Quantitative PCR (qPCR) is used to detect and quantify very small amounts of genetic material in a water sample. Genetic material in an environmental sample is called environmental DNA or eDNA, and the ability to detect the presence of the genetic material is of interest to resource managers. However, surface waters are likely to contain several known inhibitors such as humic and tannic acids and algal cells which can prevent the amplification of target DNA in a sample by blocking the action of DNA *taq* polymerase, and thus create false negative results. In order to overcome this potential problem of PCR inhibitors, WGL tested and will adopt an Internal Positive Control (IPC) for the 2015 monitoring season. One procedure used to account for the presence of inhibition is to amplify synthetic DNA or a reference gene at the same time as the target gene and compare the copy number (Nolan et al. 2006, Randall et al. 2010). This procedure is the most commonly used method to detect inhibition in laboratories spanning from clinical diagnostic settings, forensic settings to environmental research (Hoorfar et al. 2004, Kontanis and Reed 2006).

The US Geological Survey Columbia Environmental Research Center (USGS-CERC) developed an IPC (Table 1) made up of a novel mouse gene, HemT, which encodes for a hematopoietic cell-specific transcript (Xue et al. 1999). Known numbers of the HemT will be spiked into each well of the plate containing field samples, which will then be quantified using standard curves for that target. If fewer copies of the spike are measured in a sample, inhibition is assumed, and the extract will be cleaned up with a PCR clean-up kit (OneStep Inhibitor Removal kit by Zymo Research, Irvine, CA), and re-amplified (Turner et al. 2015). Samples will then continue through the eDNA workflow. This method will not determine what particular inhibitor may be present, and will only test for inhibition due to blockage of *taq* activity. The objective of our study was to re-analyze three cases from the 2014 sampling season to develop the methodology of the IPC and determine how to precisely define inhibition, how to overcome inhibition, and develop data processing pipelines for the upcoming 2015 eDNA season.

Table 1. Sequences for the HemT primers, probe, and ultramer used to access inhibition.

Product	Sequence 5'-3'
HemT Forward Primer	TCTGAGTGTCCCTCGAATCT
HemT Reverse Primer	GCAGTCCTTGAGAACATAGAGC
HemT Probe	TGA CAG TCT CCT TTC GTG TGA ACA TTC G
HemT Ultramer	CTA CAT AAG TAA CAC CTT CTC ATG TCC AAA GCT CTC TGA GTG TCC CTC GAA TCT CAG ACG CTG TAT GAC AGT CTC CTT TCG TGT GAA CAT TCG GCT GCT CTA TGT TCT CAA GGA CTG CAC

Methods

Preliminary tests - An IPC protocol was obtained from CERC for further method development at WGL. First, we had to determine if the addition of the IPC target and primers and probes reduced the efficiency of the ACTM 1 and 3 carp markers. This was done by adding the IPC components to four replicate standard curves and comparing qPCR performance to several standard curve results from the 2014 eDNA monitoring season (Table 2). The ACTM 1 and 3 markers performed equal to results in the 2014 monitoring season (Table 3). After this initial test, 12 replicate standard curves with the IPC and ACTM 1

and 3 were also tested to confirm repeatability and accuracy of the IPC when competing with ACTM 1 and 3 standards (Table 3).

2014 monitoring season re-runs - Three cases (10089, 10103, and 10104) from the 2014 monitoring season were tested to compare detection results with the IPC. Because standard curve wells have varied amounts of the HemT target, and the rest of the plate has 100 copies of the HemT target, each plate is made with two master mixes. The master mix for all unknown field samples plus the 4 negative control samples had 10^2 (100) copies of the HemT target in each reaction, whereas the mix for standard samples did not have any target HemT included, so that the proper dilution could be used to create the standard curve. Samples were run in replicates of eight with 2 replicate standard curves per plate according the QAPP. All reactions were set up for qPCR on an Eppendorf epMotion 5075 and analyzed using Bio-Rad CFX384 Touch™ Real-Time PCR Detection Systems.

Each 20µl qPCR reaction for the standards consisted of 10.0µl of Taqman® Environmental Master Mix, 1.0µl of HemT forward and reverse primer mix at 5.0µM, and 0.75µl of HemT probe at 2.5µM, 1.0µl of ACTM1/3 forward and reverse primer mix at 10µM, 1.0µl of ACTM1/3 Probe at 2.5µM, 4.25µl of water along with 1.0µl of the appropriate gBlock standard dilution and 1.0µl of the appropriate HemT standard dilution. Cycling conditions were as follows: 95°C for 10min, followed by 40 cycles of (95°C for 15 sec, 60°C for 1 min, followed by a plate read).

Table 2. Paired HemT IPC and gBlock standards run in duplicate on every plate.

Standard dilution copy number of HemT IPC	Standard dilution copy number of ACTM 1 and ACTM 3 gBlock
10^5	6250
10^4	1250
10^3	250
10^2	50
10^1	10

Results

Preliminary tests - Both the paired standard tests of four and twelve replicate standard curves resulted in R^2 and Efficiencies that performed within the WGL reporting range without any standard points being removed (Table 3 and Figure 1), indicating that the reactions had enough reagents to amplify all three targets without negatively affecting efficiency of the reactions. One target failed to amplify in the lowest ACTM 1 standard (10 copies), but the rest of the standards allowed for generation of standard curves with R^2 and efficiency values within WGL limits. The Limit of Detection (LOD) for ACTM 1 and 3 remained at 10 copies, which is important when detecting low copy numbers in eDNA samples.

Table 3. Comparisons of mean efficiencies, R² and slopes from the 2014 monitoring season and preliminary tests of the HemT IPC spike.

	FAM			HEX		
	Efficiency	R ²	Slope	Efficiency	R ²	Slope
2014* season	93.9 (7.62)	0.99 (0.01)	-3.49 (0.21)	96.4 (9.08)	0.98 (0.01)	-3.43 (0.24)
Four replicate standard curves	91.9 (14.4)	0.98 (0.01)	-3.53 (0.36)	100.6 (12.5)	0.98 (0.01)	-3.31 (0.28)
Twelve replicate standard curves	100.6 (11.06)	0.97 (0.01)	-3.31 (0.26)	103.4 (19.9)	0.95 (0.01)	-3.24 (0.43)

*mean values from 6 standard curves off of 3 plates from 6 cases analyzed in July 2014 to January 2015.

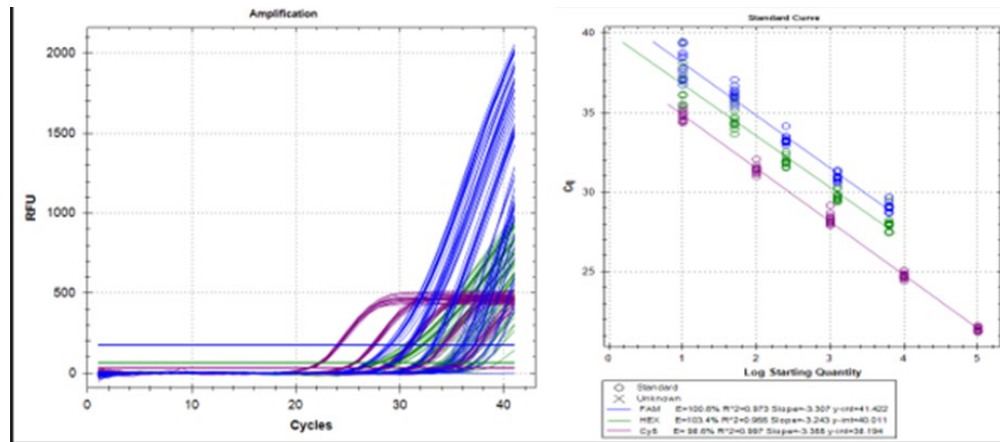


Figure 1. CFX Manager Software screen capture of the twelve replicate standard curve preliminary test (Left panel). ACTM 1 is represented by the blue lines (FAM), ACTM 3 represented by the green lines (HEX) and the IPC spike is represented by the purple line (Cy5). Right panel shows standard curves with data on each curve in the lower box.

2014 monitoring season re-runs - Case 10089 was chosen for reanalysis because it had the highest average number of filters (10) per sample from the 2014 monitoring season. An uninhibited average C_q, or the reference C_q, was calculated for each plate using the mean C_q for the duplicate 10² copy standard wells and the four Negative/NTC wells with the 10² copies of the IPC. This reference C_q was used to determine the threshold for inhibition in all other unknown samples. Initially, inhibition was defined as any sample with a C_q 2 cycles or more above the reference C_q. This resulted in zero samples being inhibited for case 10089. After further review of literature (Hartman et al. 2005, Turner et al. 2015) we changed how potential inhibition would be determined. Inhibition is now defined as any single replicate of the eight replicate PCRs run per field sample which has a C_q greater than the reference C_q by more than the range of C_qs used in the reference calculation, or at least 1 cycle if the range is less than one. Any sample well that had no C_q for the IPC is considered completely inhibited. Using these guidelines, 10 samples were determined as inhibited, and were re-analyzed after using a OneStep™ PCR Inhibitor Removal Kit by Zymo Research (Turner et al. 2015). After clean-up, nine of the samples were no longer inhibited, while the other sample had only one replicate of its eight that still showed signs of inhibition. There were no further presumptive positives for Asian Carp once the samples were purified.

Case 10103 consisted of 37 samples and was found to have four samples positive for either bighead or silver carp, as well as nine inhibited samples (Figure 2). Of those nine inhibited samples, eight were re-amplified using a 1:1 dilution. Four of these samples were cleared of inhibition after the dilution and one of the samples was then also confirmed positive for silver carp. Further dilutions of 2:1 and 3:1 finally alleviated inhibition in all but one of the remaining samples, and no further carp DNA was detected. One

sample remained mildly inhibited, but no further steps were taken to try and eliminate inhibition in that particular sample, because in the future, all inhibited samples will be cleaned with OneStep Inhibitor Removal kits to expedite processing and allow for rapid confirmation and case completion.

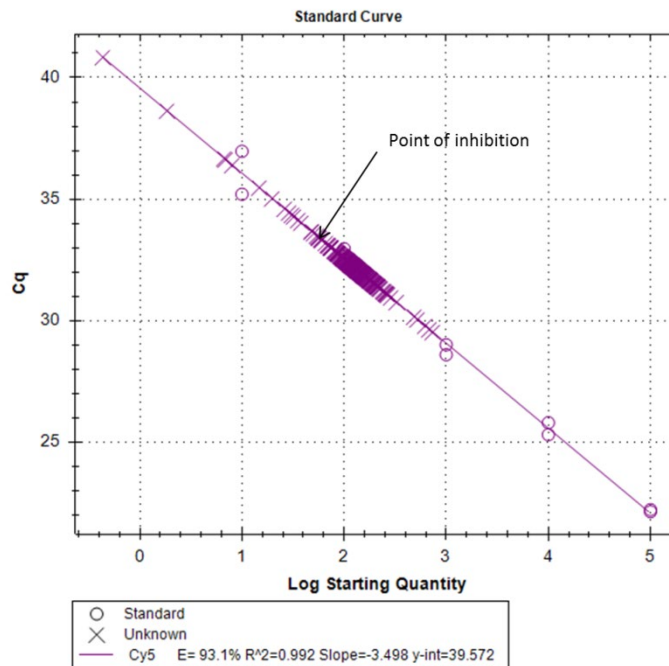


Figure 2. IPC standard curve showing the point of inhibition based on our guidelines for reference Cq in case 10103. Samples with Cq greater than this point are considered inhibited and will be re-amplified after clean-up with a PCR clean up spin kit.

Case 10104 consisted of 31 samples and was also re-analyzed with the IPC. None of these samples were inhibited, thus all samples remained negative for silver and bighead carp (data not shown).

Conclusion

Our results show the addition of the HemT IPC primers, probe, and template have no negative effects on the performance of our previously validated Asian Carp Markers and also the HemT IPC can be used to indicate inhibition in the activity of *taq* polymerase. Based on these results we recommend amending the QAPP for the 2015 eDNA monitoring of Asian Carp at WGL to include the HemT Internal Positive Control in all cases in an effort to quantify the rate of inhibition, and then remove inhibitors with a simple clean up kit, which will eliminate this source of false negative results.

To this end, WGL has incorporated the HemT IPC in the initial assay screening all samples for the ACTM 1 & 3 markers. Samples shown to be inhibited will be cleaned with the PCR inhibitor clean up kit (see above), and then re-assayed with the ACTM 1 & 3 markers. Presumptive positive samples will be moved through the confirmation assays, using the now inhibitor-free extract. The total number of inhibited samples will be tracked by case so that trends at particular sites or at particular times of year can be monitored.

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Appendix I. Internal Reports by Whitney Genetics Lab

Validation of centrifugation for eDNA sample collection and processing

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Introduction

Environmental DNA (eDNA) has been applied as an early detection and surveillance tool for Asian carps in tributaries of the Great Lakes and the Upper Mississippi and Ohio Rivers by resource agencies. The current protocol in use, the Quality Assurance Project Plan (QAPP), was initially developed by the U.S. Army Corps of Engineers, based on the methods of Jerde et al. (2011). The QAPP requires 2-L water samples be collected and then filtered within 16 hours, which is problematic for some surface waters that contain large amounts of filter-clogging particulate matter (e.g., tributaries of Lake Erie). Various methods of sample collection for eDNA analysis are found in the literature including smaller volume grab samples (i.e., 15 and 50 mL) that are concentrated by centrifugation (Ficetola et al. 2008, Dejean et al. 2011, Minamoto et al. 2012, Foote et al. 2012, and Thomsen et al. 2012) and larger volumes that are filtered, such as those outlined in the QAPP. Both centrifugation and filtration methods have been field validated and were successful at identifying organismal DNA at low levels in their respective studies. It may be more time and cost-effective for the QAPP to allow the use of smaller water volumes that concentrate DNA by centrifugation. Prior to implementation of centrifugation methodology for early detection and monitoring programs, a study must be conducted to determine if the techniques yield comparable detection rates of eDNA samples collected in the same location at the same time.

Project Overview

The current QAPP requires collection of a 2-L water sample which must be filtered within 16 hours to avoid DNA degradation. While this technique may be useful to concentrate DNA from the water sample, it also concentrates suspended materials, resulting in the need for several filters for a single sample. For example, in the Chicago Area Waterways (CAWS), on average one 2-L sample requires 4 filters (Whitney Genetics Lab (WGL) case log 2013 and 2014); however in the Maumee and Sandusky Bays of Lake Erie, one 2-L sample required upwards of 20-45 filters (C. Olds, pers. comm.), and the maximum for 2014 for a single 2L water sample was 65 filters (WGL case log 2014). Additionally, one case of 200 samples in 2014 averaged 10 filters per sample. The total number of filters processed in 2014 at WGL was over 23000, but total samples reported was only 6330 (WGL case log 2014). This large number of filters per sample pushed sample processing up against the 16-hr time limit for field staff, cost \$2.70 per filter to extract in the lab, and increased the time necessary for laboratory personnel to extract a single case of samples. According to the QAPP, the extraction kits (Qiagen DNeasy) can only handle one filter per extraction resulting in a large number of extractions in the laboratory. Filtering water also creates multiple opportunities for contamination among samples to occur. The 2-L sample containers are challenging for field staff to store, move, and rapidly cool due to size and weight. Large coolers are required to move and store samples with sufficient ice to prevent DNA degradation. Boat size and number of sample coolers limit the number of samples that can be taken per trip.

The ability to collect smaller volumes of water would increase field sampling efficiency, because all sampling containers needed for a collection event could be carried in a boat at one time. The smaller volumes would also require less ice to rapidly cool and prevent further DNA degradation. The use of

sterile centrifuge tubes and centrifugation reduces contamination risk since exposure is limited to water decanting after pellet centrifugation. Centrifuged samples also reduce potential DNA degradation or loss during shipping because they are preserved in 95% ethanol or 70% isopropyl, which will last indefinitely, unlike the filters which are shipped in dry ice. Laboratory time and cost savings are also a result because the entire pellet from one to five centrifuge tubes can be collected on a single sterile buccal swab and processed in a single extraction.

Preliminary studies and results

Preliminary studies comparing centrifugation and filtration have been conducted by federal labs as part of the environmental DNA calibration study (ECALS (<http://www.asiancarp.us/ecals.htm>) labs), but contamination in the trials precluded any interpretation of the data (ACRCC 2014). WGL conducted two preliminary studies to examine the feasibility of using the centrifugation method to concentrate eDNA in water samples. The first study conducted by ECALS laboratories occurred simultaneously with validation of an alternate extraction method in 2013. For this study, samples were collected from the Black River (La Crosse County, WI) and spiked with a slurry of silver carp slime and feces with a known amount of DNA (Amberg et al. in press). Two-liter water samples were collected and spiked with 0, 200, 750, 1000, and 2000 ng of DNA (5 treatment classes) and immediately extracted. At the same time, 10 replicates of these same treatments were being filtered for use in the Upper Midwest Environmental Sciences Center (UMESC) bridging study (Amberg et al. in press) testing the two extraction kits. Each 2-L sample from the five treatments was mixed by inversion, and twenty 50-ml sterile centrifuge tubes were filled. The samples were then centrifuged at ~4500 rcf (max speed on Sorvall Legend STR) at 4°C for 30 minutes. Water was carefully decanted; making sure the pellet remained intact in the bottom of the tube, and the pellet was covered with 5-15 ml of isopropanol, and spun again at ~4500 rcf for 5 minutes at 4°C. Excess isopropanol was decanted carefully to remove as much liquid as possible without disturbing the pellet. Samples were stored at room temperature on open shelves until processing. The day before extracting, samples were prepared by evaporating all residual isopropanol in a laminar flow hood. Samples were placed in centrifuge tube racks, moved into a sterile hood area, had caps removed, and were left to dry. Each batch of tubes included a hood negative control to test for contamination during the drying process. Samples were then moved into the extraction room where they were extracted with the Qiagen DNeasy Blood and Tissue Kit per the QAPP. Each sample was processed according to Amberg et al. (in press) for both conventional (cPCR) markers (Jerde et al. 2011) and quantitative PCR markers (qPCR; Merkes et al. 2014). Results from the centrifuged samples were compared to 10 replicate filtered samples run in WGL in the Amberg et al (in press) study. Overall, the centrifuged samples had comparable results to the filtered samples in both assays (Tables 1 and 2). Although these results seemed promising, it is important to note the 20 centrifuged samples are pseudo replicates from one 2-L spiked sample. Thus, further confirmation was required.

The second preliminary study was executed in fall of 2013, when 120 paired samples (N = 240) were collected in four sites (60 samples per site) in the Chicago Area Waterway System (CAWS). Filtered samples (N = 120) were collected as part of the formal early detection and monitoring program (MWRG Plan 2013, <http://www.asiancarp.us/documents/MRP2014.pdf>), but an additional five 50-ml samples (centrifuged) were collected side-by-side (N = 120) with each 2-L sample (filtered). These were extracted with the Qiagen DNeasy kit according to the QAPP. Since eDNA of Asian carp is not consistently detected in the CAWS, markers for detecting eDNA of bluegill (Takahara et al. 2003) and common carp (Barnes et al. 2014) were used to compare detection of DNA between the two processing methods within each site. It should be noted that Takahara's bluegill marker will also detect other *Lepomis* species, but it should do so equally in both sample types. Results from this second preliminary study based on field samples indicated that the processes do not differ (Paired Chi-square test by sample site, $p=0.435$ bluegill and $p=0.947$ common carp) (Figure 1).

Both preliminary studies indicated equal rates of DNA detection between the two methods to process field samples, but neither study used early detection markers for Asian carp in field samples. Thus the goal of this study was to compare results of bigheaded carp eDNA amplification from 2-L water samples that were filtered and 50-ml samples that were centrifuged from surface waters containing known abundances of bigheaded carps. This study used samples from the Illinois River in areas that have low, medium, or high levels of Asian carp (details below). Field work for the study was carried out by the Green Bay Fish and Wildlife Conservation Office (FWCO) with assistance from the Carterville, Columbia, Alpena, and La Crosse FWCOs. Laboratory analyses were carried out at the U.S. Army Corps of Engineers Engineering Research and Development Center (ERDC), the U.S. Fish and Wildlife Whitney Genetics Lab (WGL), and the U.S. Geological Survey Upper Midwest Environmental Sciences Center (UMESC).

Methods

Paired water samples were collected and processed by centrifuging and filtering from three reaches with low, medium, and high carp densities (Figure 2, Table 3). Each reach was sampled in a 2.5-km long section encompassing the area of interest. Water samples within each selected section were taken at 23 sites following a probabilistic sampling design targeting areas where eDNA had the highest probability of accumulation (QAPP). At the selected site, surface water (< 4 cm) was collected in a sterilized container approximately 10 L in volume. The surface water was homogenized and distributed into three 2-L bottles and fifteen 50-ml tubes designated for that site. Each 2-L bottle and set of five 50-ml tubes is a replicate (n=3) for that site. A new 10-L container was used for each eDNA accumulation site and not reused during this study. Two of the 23 sampling sites were randomly selected for cooler blanks filled with DI water (i.e., three 2-L bottles and fifteen 50-ml centrifuge tubes per site). The negative controls did not replace the collection of water samples at the site, but served as negative controls. All samples were filtered or centrifuged according to the QAPP in mobile eDNA trailers and sent to the laboratories. One set was shipped directly to ERDC, and the other two were hand-delivered to WGL personnel who then distributed the final set to UMESC for analysis. Three study reaches in the Illinois River (Figure 2) were identified based on Asian carp densities (Table 3): Brandon Road Pool (low), Marseilles Pool (medium), and La Grange Pool (high; Figure 2; see also Baerwaldt et al. 2014). Samples were collected within a 2.5 km stretch immediately below the upstream barrier (lock and dam structure) within each reach (Figure 3).

All preparation activities followed those specified in the QAPP and other guiding documents/protocols (e.g., eDNA Trailer SOP). Water samples within each randomly selected section were taken at 23 sites following a probabilistic sampling approach described above. Targeted areas included backwaters, island side channels, pooled areas, below and around structures, confluences of tributaries, and any areas where floating debris accumulates. Actual sample collection locations were determined by the Collection Crew Leader from *in situ* observations of wind direction, accumulation areas, and water levels. No eDNA sampling occurred within 5 days after a significant rainfall event (more than 1.5 inches in a 24-hr period) or on the rising limb of a hydrograph of the river as it exceeded flood stage.

Samples were collected according to the QAPP, and a total of 225 2-L samples and 1125 50-ml samples were collected. All samples were processed according to the QAPP, including centrifuge samples (QAPP already contains method for centrifugation which is allowed for research purposes), and DNA was extracted using the Qiagen DNeasy kits. At the time this study was planned, the QAPP used conventional PCR markers from Jerde et al. (2011); however, after samples had been collected in Spring of 2014 but before they were analyzed (June at ERDC and winter at UMESC and WGL), new qPCR markers had been validated and thus adapted in the QAPP (Farrington et al. 2015), and these new markers were used to analyze samples. The validation study provided limits of detection (3-9 copies) and limits of quantification (10 copies) for these new markers, therefore a sample is detected as positive for a marker

if starting copies of template DNA are between 3-9, and a sample can be detected as positive, and starting copies of template DNA can be reported as a numerical value for 10 copies or greater.

Data from each lab was reported in two ways: samples confirmed positive for Bighead, Silver, or both species, and copy number. The overall probability of detection for positive samples was calculated for each reach by each processing method to assess detection rate. The detection rate for both filtration and centrifugation was then used to calculate the number of samples needed to achieve a specified detection probability using equations modified for binomial variables (McArdle 1990, Reed 1996, and Kery 2002). All eDNA samples were assumed to be comparable and independent, therefore the probability of not detecting Asian carps in N eDNA samples is:

$$\text{Probability } (N \text{ unsuccessful samples}) = \alpha(1 - p)^N$$

where α is the probability of making a Type I error under the hypothesis that the eDNA is actually present (i.e., concluding the eDNA is absent when it is actually present), p is probability of detection, and N is the total number of eDNA samples. Solving for N gives the number of samples required at a desired Type I error rate.

$$N = \frac{\log(\alpha)}{\log(1 - p)}$$

As more samples are collected, the probability of detecting presence of Asian carp eDNA (p^*) increases and can be calculated as

$$p^* = 1 - (1 - p)^N$$

Samples that had detections with any of the 6 markers were used to test for differences in copy number between the two processing methods using a permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distance matrix in Primer V6 (Anderson et al. 2008). The PERMANOVA is a non-parametric multivariate approach which does not make any assumption of distribution, is insensitive to correlation among response variables and zeros, and allows the use of copy numbers from all the markers in one test (Anderson 2001). Specific contrasts were used to test for the difference between centrifuging and filtration within each of the reaches using Type III sum of squares and 9999 permutations.

Results

Samples were successfully collected and two sets shipped to WGL and the third to ERDC. Samples were checked in, and held until processing (WGL held both UMESC and WGL samples until processing). Filters were stored at -80°C and centrifuged samples preserved with isopropyl were held at ambient lab temperature. At WGL this ambient storage was in open shelves. Samples were processed at ERDC in June. Samples were processed at WGL in December and at UMESC, in January. All WGL samples passed quality control checks, and thus are included in analysis. However, there were some control samples (centrifuge control 13, hood control 7, and extraction negative 7) that at first appeared to be contaminated in the set analyzed at ERDC. Upon closer inspection, only the centrifuge control was presumptive positive with one of 8 reactions amplifying both ACTM markers, but it was overall negative since the confirmation markers for both species failed to detect. The other two control samples had just one of the ACTM markers detected, so they were not presumptively positive, thus all of ERDCs data were used in the final analysis. Unfortunately, UMESC data had to be discarded from analysis for two reasons. 1) Centrifuged samples 1-25 completely failed amplification compared to WGL and ERDC. To further investigate potential reasons for this failure, 10 μl of each extract was electrophoresed in 2% agarose gels

with 5 WGL samples from the same sample set for comparison. The UMESC extracts did not have as much DNA (much fainter streaks) or as wide a range of DNA lengths (all less than 100 base pairs) compared to WGL extracts (data not shown). The lack of large strands indicates samples may have degraded during storage at room temperature exposed to lab lights, or that the extraction kit reagents failed. Kit failure is possible, since it was noted by the technician that some of the buffers had crystallized and were used without re-dissolving the buffer. 2) Filtered samples 51-75 from the high-abundance carp reach of the river also completely failed amplification. Again, extracts were electrophoresed and compared to 5 extracts from WGL samples from the same set of samples. The gel revealed orders of magnitude less DNA than those extracted by WGL and DNA all less than 100 base pairs (data not shown), which indicates failure of extraction kits since the filters were stored in the dark in ultra-cold freezers until they were processed. Thus, results will only be based on two replicates (WGL & ERDC) instead of three.

Overall, results between ERDC and WGL had similar rates of detection for both species in all three reaches (Table 4). Mean copy numbers (8 replicate reactions x 25 samples) varied between labs, but were similar among reaches for each processing method. Mean copy numbers also reflected the Asian carp density from their respective reaches (i.e., high density reach had higher copy number), and for the most part, centrifuged samples had higher copies than filtered samples (Table 5). Detection probability was calculated by reach for each method using the results from both labs for each individual site. For example if WGL (+) and ERDC (-) then the site was considered (+). Detection probabilities were similar for the medium and high reaches, but centrifuging had a 26% detection rate compared to 0% for filtration in the low density reach (Table 6). The total length of the study reach was 2.50 km with an average width of 0.12 km and a total area of 0.30 km². Therefore, using centrifugation a 95% detection probability would require 10 samples (i.e., 10 sets of five 50 ml tubes) to be collected per 2.5 km reach or 4 samples per river km. To achieve a 99% detection probability at total of 15 samples per 2.5 km or 6 samples per river km would be collected.

For the statistical analyses, the copy numbers were averaged for the replicate samples between the two labs. Therefore, every site had an average copy number for each of the six markers. The average copy numbers were then log₁₀ transformed, and a dissimilarity matrix was then generated based on Euclidean distance for the PERMANOVA. Results from the PERMANOVA showed significant differences between centrifuging and filtration for all three reaches ($P < 0.001$; Table 7). Centrifuging had significantly higher copy numbers than filtration for high and low density reaches, but filtration had significantly higher numbers in the medium density reach (Tables 5 and 7). Reasons for higher numbers in the filtered samples from the medium reach could be related to differences in inhibitors between reaches, or other confounding environmental variables, but that is beyond the scope of this study. However, a statistical difference in the medium reach where filtering produced higher copy numbers is not as relevant in a detection program, since the overall detection rate is a difference of 96% versus 100%, and both methods would provide positive results for this particular reach. Since early detection programs focus on detection in low-density reaches, it is important that the centrifugation method was able to detect DNA when the filtering method was not.

Conclusions and Recommendations

This study strongly supports the use of centrifuging as a method to process eDNA field-collected samples in early detection and monitoring programs, most notably due to its performance in the low-abundance carp reaches of the Illinois River where centrifuged samples were able to detect carp DNA, but *filtered* samples were *not*. One potential explanation for why centrifuging outperformed filtering is that smaller volumes of water will have smaller amounts of inhibitors (Gibson et al. 2012), which would improve detection over heavily inhibited filtered samples. This study also confirms centrifugation as a method for

processing eDNA samples in other applications, such as confirmation of spawning events or detection of over-wintering habitats. There are several benefits to centrifuging over filtering, but the most important is preventing contamination in the field. The reduced handling in the field creates far fewer chances for cross-sample contamination, and single-use, sterile tubes eliminate contamination between sampling events. In other agencies, field staff could fill both field blanks and centrifuge control samples in the lab or processing trailer, but WGL will fill all field blanks and distribute as needed. In addition, there are large improvements in efficiency in the field and lab which will allow for the collection and processing of many more samples within the sampling season. Thus, based on the same outcome in three different studies comparing centrifugation to filtration as a water processing method, we recommend that centrifugation be approved for use in the early detection and surveillance of bigheaded carps, specifically in the Quality Assurance Project Plan.

Tables and Figures

Table 1. Qiagen extraction kit conventional PCR results of twenty 50-mL samples processed by centrifugation from one 2-L sample container, compared to ten 2-L replicates processed by filtration.

Quantity of DNA (ng)	*Centrifuged Number positive bands counted (80)	Centrifuged Number samples positive (20)	*Filtered Number positive bands counted (20)	Filtered Number samples positive (10)
(0)	0	0	0	0
(200)	32	14	16	9
(750)	67	20	20	10
(2000)	77	20	20	10
(4000)	77	20	20	10
Extraction Negative	Na	0	0	0
Extraction Positive	Na	All +	All +	All +
PCR Negative	Na	0	0	0
PCR Positive	Na	All +	All +	All +

* For centrifuged samples, one swab represents one sample for a total of 20 samples per treatment. Four replicate reactions for cPCR for centrifuged samples, two replicate PCRs for filtered samples.

Table 2. Qiagen extraction kit quantitative PCR results of twenty 50-mL samples processed by centrifugation taken from one 2-L sample container, compared to ten 2-L replicates processed by filtration.

Quantity of DNA (ng)	*Centrifuged Number positive curves counted (40)	Centrifuged Number samples positive (20)	Centrifuged Ave Cq value ¹ (SE)	*Filtered Number positive curves counted (20)	Filtered Number samples positive (10)	Filtered Ave Cq value ¹ (SE)
(0)	0	0	0 (0)	0	0	0 (0)
(200)	25	15	36.83 (0.21)	19	10	23.50 (0.20)
(750)	40	20	35.58 (0.18)	19	10	29.44 (0.23)
(2000)	38	20	34.03 (0.22)	20	10	27.71 (0.11)
(4000)	36	20	31.84 (0.20)	20	10	27.28 (0.13)
Ext Negative	Na	0	0	0	0	0
Ext Positive	na	All +	33.18 (0.31)	All+	All+	36.29 (0.15)

* For centrifuged samples, one swab represents one sample for a total of 20 samples per treatment. Two replicate reactions for filtered samples and 4 replicate reactions for centrifuged samples.

¹Cq value calculated on positive samples, zero readings were not included in summary numbers.

Table 3. Catch per unit effort of bighead and silver carp in selected pools of the Illinois Waterway in 2013. Data were acquired from the IDNR, SIU, and INHS from projects outlined in the Monitoring and Response Plan for Asian Carp in the Upper Illinois River and Chicago Area Waterway System (2013). All of the sites used within each project were fixed and chosen as a likely area of inhabitation by Asian carp within that pool.

Pool	Electrofishing (fish/hr)		Gill Netting (fish/100 yds)		Hoop Netting (fish/set)	
	Bighead	Silver	Bighead	Silver	Bighead	Silver
Brandon Road	0	0	0	0	0	0
Marseilles	0	1.73, 5.88	0.38	0.87	0.07, 0.04	0.96
La Grange	0	16.78, 46.53	1.07*	3.47*	0.71	na

*2012 data.

Table 4. Confirmed detections for Bighead and Silver carp DNA in water samples collected from river reaches with varying Asian carp densities and processed in the field with filtering and centrifuging.

Asian Carp Density	Processing Lab	Filtered Water (2-L Bottles)			Centrifuged Water (50-mL tubes)		
		Silver Positive	Bighead Positive	Both Positive	Silver Positive	Bighead Positive	Both Positive
High	ERDC	23	23	23	23	23	23
	WGL	19	19	19	23	23	23
Medium	ERDC	23	21	21	18	9	8
	WGL	23	21	21	19	14	12
Low	ERDC	0	0	0	2	1	1
	WGL	0	0	0	1	3	0

*total sample number less cooler blanks, 69 for each lab.

Table 5. Mean copy number for 25 samples (from 8 replicate reactions) for each of six Asian carp qPCR markers amplified in water samples collected from river reaches with varying Asian carp densities and processed in the field with filtering and centrifuging. Limit of detection is 3-9 copies, limit of quantification (LOQ) is 10 copies. Samples with copy numbers above the LOQ are reported, but those that detected but were below the LOQ are designated (+).

Fish Density	Lab	ACTM1		ACTM3		BHTM1		BHTM2		SCTM4		SCTM5	
		Filter	Cent.	Filter	Cent.	Filter	Cent.	Filter	Cent.	Filter	Cent.	Filter	Cent.
High	ERDC	128	272	58	165	64	17	168	24	70	163	243	156
	WGL	234	654	93	510	12	22	46	32	54	357	295	644
Medium	ERDC	33	16	17	10	+	+	+	+	43	17	39	+
	WGL	54	27	43	22	23	+	16	+	21	21	32	33
Low	ERDC	0	20	+	21	0	50	0	107	0	11	0	+
	WGL	+	23	+	25	0	27	0	55	0	17	0	24

Table 6. Detection probabilities for centrifuging and filtration processing methods were calculated for each of the reaches. A site was considered (+) for Asian carp eDNA if either or both WGL and EDRC had a confirmed (+) for that site.

Asian Carp Density	Filtered water (2-L bottles)	Centrifuged water (50-ml tubes)
High	100%	100%
Medium	100%	96%
Low	0%	26%

Table 7. PERMANOVA results table contrasting the differences of centrifuging and filtration in different reaches of density of Asian carp based on average copy number from WGL and EDRC for six markers at each site. Table reports degrees of freedom (df), Sum-of-squares (SS), Mean square (MS), the F-statistic (Pseudo-F), and the p-value (P).

PERMANOVA						
Source		df	SS	MS	Pseudo-F	P (perm)
Overall		5	2486.3	497.25	122.44	0.0001
	Low	1	26.359	26.359	7.7438	0.0001
	Medium	1	85.825	85.825	24.404	0.0001
	High	1	118.9	118.9	22.593	0.0001
Res		132	536.07	4.0612		
Total		137	3022.3			

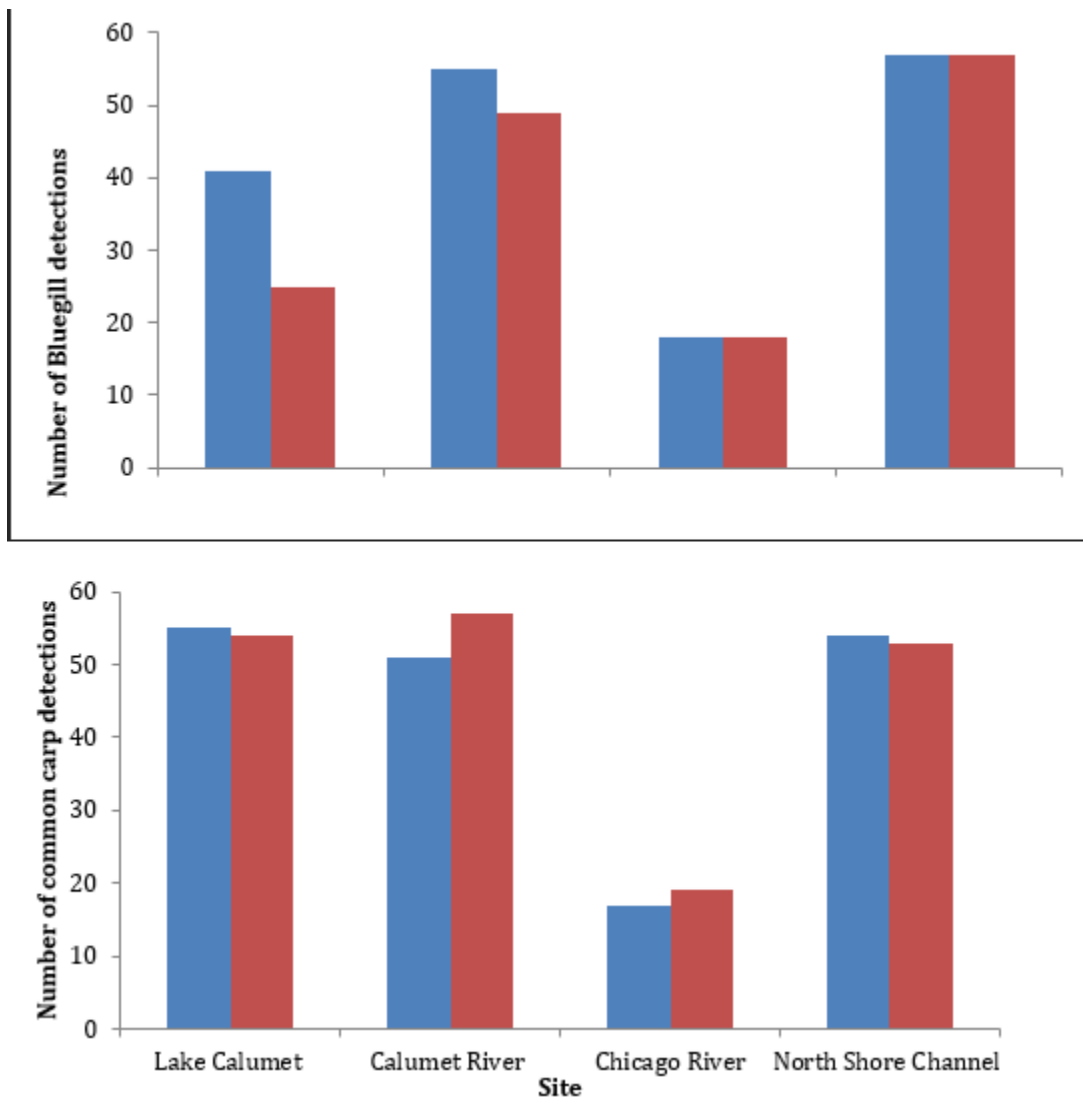


Figure 1. Detection of bluegill (top) and common carp (bottom) eDNA in four sites in the Chicago Area Waterway System. Detection of eDNA in centrifuged samples in blue, filtered samples in red. Due to a combined sewage overflow (CSO), there were only 20 samples collected in the Chicago River instead of 60.

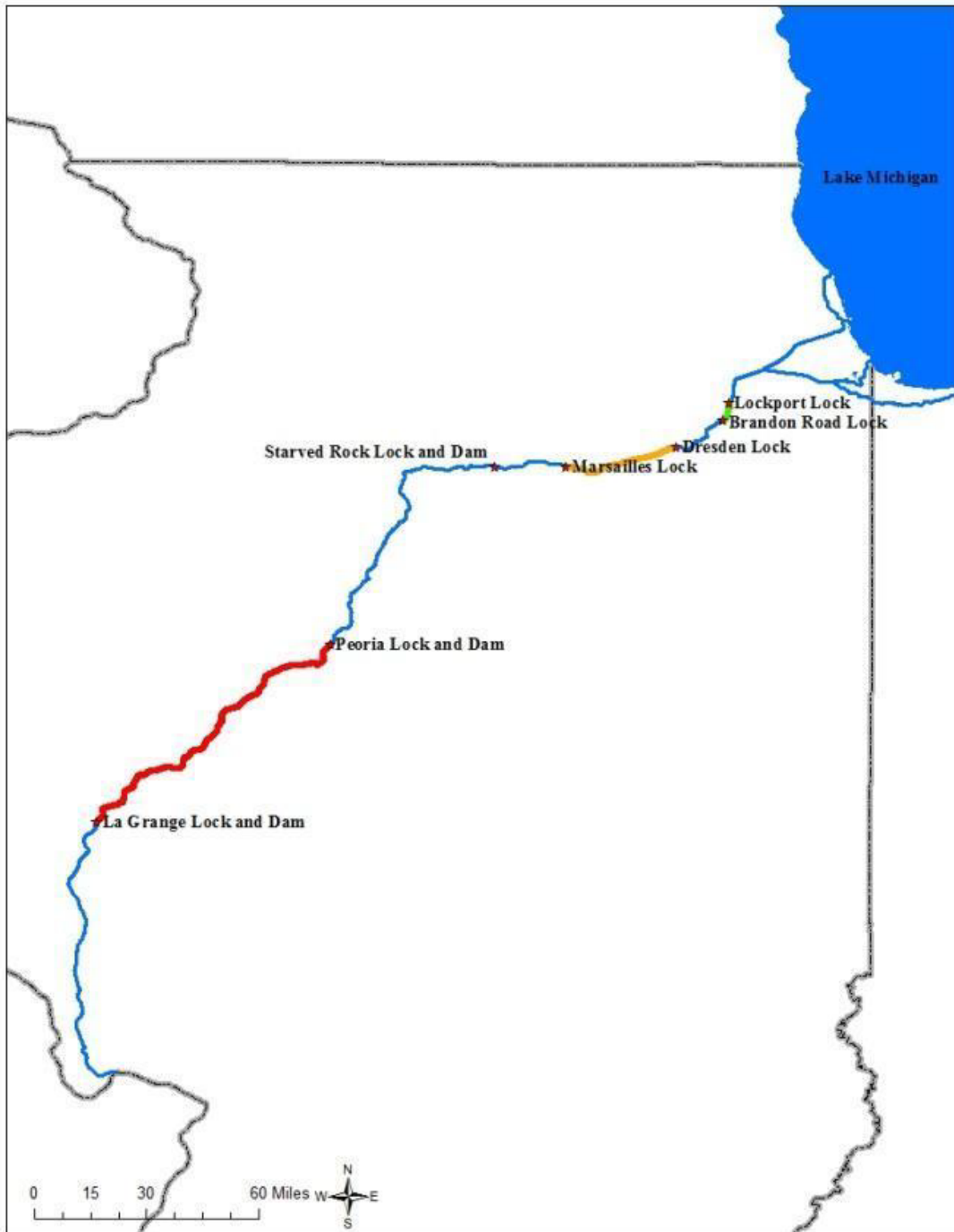


Figure 2. Map of the Illinois Waterway. Highlighted pools indicate sites selected as high (red), medium (yellow), or low (green) concentrations of Asian carp. Brandon Road pool (green), Marseilles pool (yellow), and La Grange pool (red) were selected based on sampling data from the Monitoring and Response Workgroup Fish Surveys. Note: figure misspells 'Marsailles'.

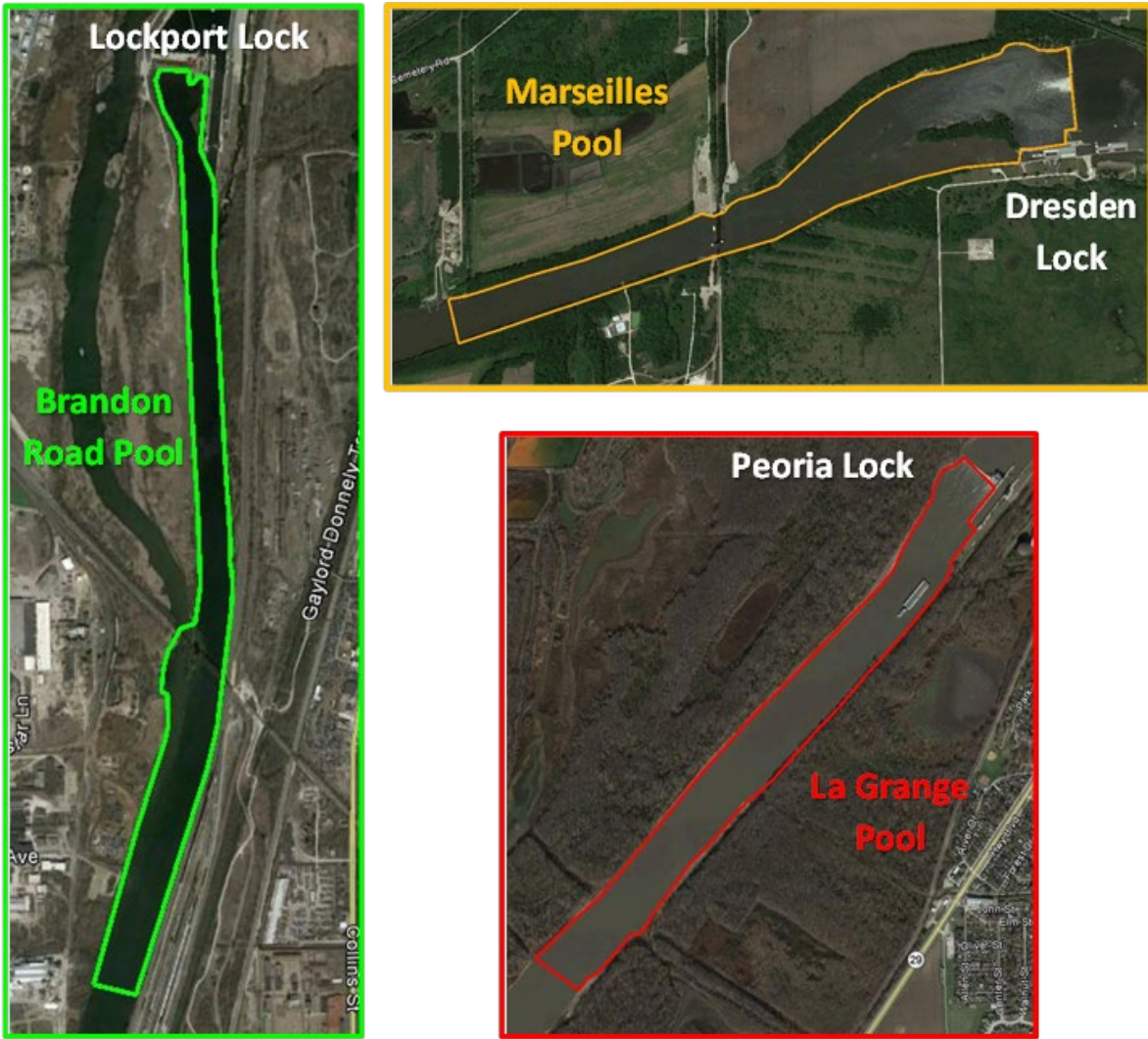


Figure 3. Study reaches in the Illinois River. Each polygon encompasses a study reach 2.5 river km below the upstream lock in the high (red), medium (orange), and low (green) bigheaded carp density pools.

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