Pollutant Sensitivity of the Endangered Tar River Spinymussel as Assessed by Single Chemical and Effluent Toxicity Tests

December 2014

U.S. Fish and Wildlife Service
Ecological Services
Raleigh, North Carolina

Preface

The U.S. Fish and Wildlife Service (USFWS), U.S. Geological Survey Columbia Environmental Research Center (USGS CERC), North Carolina State University (NCSU) College of Veterinary Medicine, and North Carolina Division of Water Resources (NCDWR) conducted an evaluation of the habitat conditions for, and pollutant sensitivity of, the endangered Tar River spinymussel (*Elliptio steinstansana*). The work was coordinated by the USFWS's Raleigh Field Office and was funded by the USFWS Division of Environmental Quality (study identifiers 4F42 and 200940001.1).

Toxicity tests were performed by the USGS CERC under the direction of Chris Ingersoll through an intra-agency agreement with the USFWS. Other CERC scientists were involved in the toxicity testing component of the project and included Ning Wang and James Kunz. Carol Hollenkamp of NCDWR assisted with sample site selection and effluent collections. Mussel propagation was coordinated by Jay Levine and Chris Eads of NCSU's Aquatic Epidemiology and Conservation Laboratory through an intra-agency agreement between the USFWS and the USGS North Carolina Cooperative Fish and Wildlife Research Unit. Additional mussel propagation and culture assistance was provided by Rachel Mair of the USFWS White Sulfur Springs National Fish Hatchery. The overall project also included an inventory of nonpoint sources of pollution, the results of which are provided elsewhere. Chris Mebane of USGS in Boise, Idaho performed the biotic ligand model normalizations of copper toxicity test results. This report incorporates comments received on a draft circulated for peer review. Peer reviews were provided by Greg Cope of NCSU's Department of Applied Ecology and Robert Bringolf of the University of Georgia's Warnell School of Forestry and Natural Resources; the co-authors thank them for their time and expertise. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

Questions, comments, and suggestions related to this report can be directed to the USFWS at:

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Suggested citation: Augspurger TP, Wang N, Kunz JL, Ingersoll CG. 2014. Pollutant Sensitivity of the Endangered Tar River Spinymussel as Assessed by Single Chemical and Effluent Toxicity Tests. U.S. Fish and Wildlife Service, Ecological Services, Raleigh, NC.

Keywords: Tar River spinymussel, *Elliptio steinstansana*, Tar River, North Carolina, whole effluent toxicity, 4F42, 200940001.1, NC-1, NC-7, NC-13

Cover photo: Juvenile Tar River spinymussels propagated at the North Carolina State University Aquatic Epidemiology and Conservation Laboratory (photo by Chris Eads, Aquatic Epidemiology and Conservation Laboratory, Raleigh, NC)

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Executive Summary: The federally endangered Tar River spinymussel (*Elliptio steinstansana*) is endemic to the Tar River and Neuse River systems in North Carolina. The extent to which water quality limits Tar River spinymussels' recovery is important to establish, and one aspect of that is understanding the species' pollutant sensitivity. The primary objectives of this study were to 1) develop captive propagation and culture methods for Tar River spinymussels; 2) determine the pollutant sensitivity of captively propagated Tar River spinymussels; 3) examine the utility of the non-endangered yellow lance (*Elliptio lanceolata*), yellow lampmussel (*Lampsilis cariosa*) and notched rainbow (*Villosa constricta*) as surrogates for the Tar River spinymussels' chemical sensitivity; 4) develop a 7-d method for conducting effluent toxicity tests starting with newly transformed mussels; 5) assess the toxicity of municipal wastewater effluents discharged into the Tar River spinymussels' current and historic habitat; and, 6) evaluate the protection afforded by existing effluent toxicity test requirements.

From 2010 to 2012, propagation work for this project produced 23,714 juvenile Tar River spinymussels, and this project demonstrated the utility of the white shiner (*Luxilus albeolus*) and mountain redbelly dace (*Phoxinus oreas*) as highly effective host fish. In addition to supplying Tar River spinymussels for the toxicity tests, thousands of juveniles were reared for potential population augmentation / reintroduction efforts as a result of this project.

Field-collected effluents and laboratory-prepared mock effluents were used to conduct 7-d toxicity tests with newly transformed juvenile mussels (notched rainbow and yellow lance) and two commonly tested organisms (cladoceran, *Ceriodaphnia dubia* and fathead minnow, *Pimephales promelas*). Acute (96-h) reference toxicant tests were also conducted with ammonia, copper, or sodium chloride with four species of mussels, including Tar River spinymussels.

The Tar River spinymussel was between the median and bottom quartile of copper and ammonia species sensitivity distributions for freshwater mussels based on 96-h toxicity tests. As such, the Tar River spinymussel is a sensitive species among the mussels which, as a group, are known to be sensitive to ammonia, chlorine, chloride, copper, nickel, lead, potassium, sulfate, and zinc. While hazard is a function of sensitivity and exposure, the Tar River spinymussels' sensitivity indicates that pollutants are important factors to consider in its management.

The Tar River spinymussel, notched rainbow, and yellow lance were of similar (within a factor of 2) sensitivity to ammonia and copper. The yellow lance had poor control survival in two of two effluent tests and one of five acute tests. In contrast, notched rainbow performed well with 91 to 100% survival of controls in the seven effluent tests and 95 to 100% survival of controls in the five acute tests conducted over three years. Although not as closely aligned with Tar River spinymussel in taxonomy and distribution as is yellow lance, notched rainbow has the most promise of the species we evaluated as a surrogate in Tar River spinymussel toxicity testing based on availability, sensitivity, and control survival.

Three of five municipal wastewater treatment plant effluents were toxic to notched rainbow mussels (used as a surrogate for the Tar River spinymussel) at concentrations approximately

equal to, or less than the effluents' instream waste concentration (the percent of stream flow as effluent under the maximum permitted discharge during the estimated 7Q10 flow, or the 7-d low flow with an expected recurrence interval of 10 years). Wastewater regulation using the instream waste concentration is designed to be protective because facilities rarely discharge at maximum permitted flow. However, there is the potential for toxicity to mussels at extreme low flow and further evaluation in the lab and field is warranted.

Mock effluents were mixtures of ammonia, cadmium, copper, nickel, lead, and zinc. Mock effluent 1 was expected to be toxic between the 12.5 and 25% concentrations as the 12.5% concentration was mixed at the chronic water quality criteria of individual components. Mock effluent 2 was expected to be toxic near the 12.5% concentration which was mixed at the components' individual thresholds of effect based on 28-d EC20s of other mussel species. The IC20s for mussel length were 5.6 and 8.3% effluent indicating that no effect concentration estimates based on previous mussel toxicity tests of individual compounds or based on water quality criteria for individual compounds were similar, but those estimates are not protective of the notched rainbow for the mixtures. The *C. dubia* were more sensitive than the mussels with IC20s for reproduction of less than 3.2% for each effluent indicating adverse effects at concentrations expected to be safe based on chronic water quality criteria for single chemicals.

In five of seven side-by-side comparisons (five wastewater treatment plant effluents and two mock effluents), fathead minnow survival and reproduction were under-protective of mussel endpoints. The fathead minnow does not consistently represent mussel sensitivity and would not be an effective surrogate.

The *C. dubia* control survival was less than acceptable for three effluent tests and those results were not evaluated further. In three of four side-by-side comparisons (two wastewater treatment plant effluents and two mock effluents), *C. dubia* reproduction was protective of mussel endpoints. Notched rainbow survival was more sensitive than *C. dubia* endpoints in one of the two municipal effluents for which both species were tested. While *C. dubia* effluent toxicity testing did not consistently protect mussel endpoints, the test should remain the main regulatory tool for effluent evaluation in Tar River spinymussel habitat at this time because the limited seasonal availability of these species of mussels would not support routine effluent testing. Tar River spinymussel and notched rainbow can be tested directly when a mussel-specific test is warranted based on the magnitude, composition, or location of an effluent. Both Tar River spinymussel acute tests had acceptable control survival, and all seven effluent tests, conducted over three years with different batches of notched rainbow had control survival ≥ 91% and good growth. Hence the method can be used to monitor effluent toxicity in instances when a mussel specific test is desired.

Growth (estimated as shell length) of mussels was a more sensitive endpoint compared to survival with exposure to both of the mock effluents. In contrast, growth (estimated as shell length) of mussels was not a sensitive endpoint in the exposures conducted with the wastewater effluents. This is not surprising given the 7-d exposures. Length was used to measure growth because of the difficulty in measuring dry weight increase in these small mussels. Future studies should evaluate the utility of measuring dry weight of mussels in effluent testing, but determining weight change of these young mussels would require very careful measurements.

Measuring dry weight would allow for estimating biomass of mussels (i.e., the total mass of mussels in each replicate at the end of the exposures).

These are the first acute toxicity test results for ammonia and mussels in the genus *Elliptio*. Using the same set of data quality objectives and same data synthesis procedures as those in the USEPA's 2013 revised ammonia water quality criteria document, *Elliptio* is the third most sensitive of 70 genera of aquatic animals. As genus mean acute values close to the 5^{th} percentile drive the criteria maximum concentrations (CMC) recommendations, the addition of the data for *Elliptio* to the acute criteria dataset would lower the CMC by 11% (from 17 to 15 mg total ammonia as nitrogen/L at pH 7.0 and 20°C) -- an illustration that even for a well-studied pollutant like ammonia, available data for mussels have limitations in representing the U.S.'s nearly 300 species of mussels in need of protection and that taxon-specific approaches to deriving estimates of safe concentrations have merit in certain circumstances.

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Introduction

The federally endangered Tar River spinymussel (*Elliptio steinstansana*; Figure 1) is one of the most imperiled species in North America. One of only three known freshwater mussel species with spines in the world, the species is considered critically imperiled globally (G1 ranking by NatureServe 2013). Listed as endangered by the U.S Fish and Wildlife Service (USFWS) in 1985, the species is known from just five locations in North Carolina. The Tar River spinymussel recovery plan notes the role of water quality in its decline, stating that "degradation resulting from siltation and the runoff and discharge of agricultural, municipal, and industrial pollutants appear to be major factors in the reduction of the species' range" (USFWS 1992).

To assist recovery efforts, the USFWS coordinated an evaluation of the pollutant sensitivity of the Tar River spinymussel. With the assistance of the U.S. Geological Survey Columbia Environmental Research Center (USGS CERC), North Carolina State University (NCSU) College of Veterinary Medicine, and North Carolina Division of Water Resources (NCDWR), this study was initiated with the following objectives:

Figure 1. Juvenile Tar River spinymussels propagated at the Aquatic Epidemiology and Conservation Laboratory (photo by Chris Eads, NC State University)

1) Develop captive propagation and culture methods for Tar River spinymussels;

2) Determine the pollutant sensitivity of captively propagated Tar River spinymussels;

3) Validate the utility of the non-endangered co-occurring and congeneric yellow lance (*Elliptio lanceolata*), yellow lampmussel (*Lampsilis cariosa*), and notched rainbow (*Villosa constricta*) as surrogates for the Tar River spinymussels' chemical sensitivity;

4) Develop a 7-d method for conducting effluent toxicity tests with newly transformed mussels;

5) Assess the toxicity of municipal wastewater effluents discharged into the Tar River spinymussels' current and historic habitat; and,

6) Evaluate protection afforded to mussels by standard 7-d whole effluent toxicity tests (USEPA 2002) by conducting side-by-side effluent tests with mussels, the cladoceran (*Ceriodaphnia dubia*) and fathead minnow (*Pimephales promelas*).

The approach of testing captively reared rare species has been successfully applied in the conservation of other threatened and endangered mussels and fishes (Hamilton 1995, Buhl and

Hamilton 1996, Keller and Augspurger 2005, Dwyer et al. 2005a, b, Besser et al. 2005, 2012, Hewitt et al. 2006, Wang et al. 2007b). This project expands on that model and productive partnerships (Augspurger et al. 1999, Noguchi et al. 2007). This report presents the results of the captive propagation and testing of Tar River spinymussel and surrogate species including testing methods, results, and an interpretation of the findings.

Methods

Overall design

The initial approach for the project was to test captively propagated Tar River spinymussels and non-endangered yellow lance side-by-side with cladocerans and fathead minnows -- two species commonly used to estimate acute and chronic toxicity of effluents and receiving waters (USEPA 2002). All species were to be tested with five field-collected effluents from North Carolina in 7-d, static-renewal laboratory toxicity tests. While thousands of Tar River spinymussels were eventually produced by project partners (NCSU 2013), there were none available in the first two years of the project when effluents were collected for testing. The project was hence separated into two phases: 1) an assessment of the sensitivity of mussels (notched rainbow and yellow lance), cladocerans and fathead minnows to the five field-collected effluents (in 2010 and 2011) or to two laboratory-prepared mock effluents (in 2012) in short-term 7-d exposures (to determine the toxicity of the effluents to mussels and the relative sensitivity of the mussels to typical effluent toxicity testing organisms), and 2) an assessment of the pollutant sensitivity of the Tar River spinymussel relative to three other mussel species (yellow lance, yellow lampmussel, and notched rainbow) in acute 96-h reference toxicant tests with ammonia, copper, or sodium chloride. Overall objectives were still met, albeit indirectly.

Participating facilities and effluent collection

The NCDWR Aquatic Toxicology Unit coordinated a review of all the National Pollutant Discharge Elimination System (NPDES) permitted wastewater treatment plant (WWTP) discharges into occupied and historic habitat for the Tar River spinymussel. Facility type, effluent composition, discharge volume, receiving stream, dilution at low flow, proximity to Tar River spinymussel occurrences, compliance history with State toxicity testing requirements, and other data were reviewed for effluent prioritization. Five facilities were selected for the study, and NCDWR coordinated their voluntary participation (Table 1).

Two effluents were evaluated June 14-23, 2010 and three effluents were evaluated June 6-15, 2011. At each facility, effluent samples were collected directly into new 4-L certified clean CUBITAINERS® containers following North Carolina Whole Effluent Toxicity Testing procedures (NCDENR 1998). A 24-h composite of 20 liters of effluent was collected on Tuesday (initiated Monday), chilled immediately, maintained at 1 to 4°C, and shipped overnight to the USGS Columbia Environmental Research Center (USGS CERC) on Tuesday. Effluents were received by USGS on Wednesday when testing commenced. A second 24-h effluent composite sample was collected at each facility on Thursday (initiated Wednesday) and shipped to USGS CERC on Thursday for effluent renewal in test chambers. Tests ended the following Wednesday.

Table 1. Wastewater treatment plant effluents evaluated and their maximum permitted flow (in million gallons per day, MGD). The instream waste concentration (IWC) is the percent of the stream flow that is comprised by a particular facility's maximum permitted effluent during 7Q10 flows (the predicted seven-day low flow with an average recurrence interval of once every ten years). Test results in this report are presented without attribution to particular facilities.

Facility	**County**	**Receiving stream**	**Permitted flow** (MGD)	**IWC** (%)
Kenly WWTP	Johnston	Little River	0.63	22
Louisburg Water Reclamation Facility	Franklin	Tar River	1.37	13
Tar River Regional WWTP	Nash	Tar River	21	35
Tarboro WWTP	Edgecombe	Tar River	5	8
Warren County WWTP	Warren	Fishing Creek	2	76

Test organism culture

Neonate *C. dubia* (less than 24-h old and all within 8 h of the same age) were cultured at the USGS CERC in control water (dilution water) prepared by: 1) diluting well water with deionized water to a hardness about 100 mg/L as $CaCO_3$, pH 8.2, and dissolved organic carbon about 0.5 mg/L (Wang et al. 2011b) and 2) preparing ASTM reconstituted hard water (160-180 mg/L as $CaCO_3$, pH 8.3, and dissolved organic carbon about 0.3 mg/L; ASTM 2013a). Fathead minnows (<24-h old) were obtained from Aquatic Bio Systems Inc., Fort Collins, Colorado.

Juvenile notched rainbow were supplied by White Sulfur Spring National Fish Hatchery. In April each year, gravid female mussels to produce juvenile notched rainbow were collected from Johns Creek, Maggie, Virginia. Fish used to produce juvenile mussels were sculpin (*Cottus* sp.) and were collected from Howards Creek, Greenbrier County, West Virginia. Fish were held in AHAB-style systems with sieves to collect the juvenile mussels once they metamorphosed from the fish. Fish were held in these systems at about 20^oC and about 2 to 3 weeks later, juveniles excysted from the fish. Juveniles were then counted and placed in Barnhart Bucket (Barnhart 2006) juvenile mussel culture systems until they were shipped 1 to 2 d later to the USGS CERC for toxicity testing.

Juvenile Tar River spinymussel, yellow lance, and yellow lampmussel were supplied by the NCSU Aquatic Epidemiology and Conservation Lab (AECL) in Raleigh, North Carolina (NCSU 2013). Tar River spinymussel brood stock was collected from Little Fishing Creek in the Tar River Basin over multiple years. Tar River spinymussels were maintained at the Marion Conservation Aquaculture Center (Marion, North Carolina) where they spawned and became gravid. Gravid females were transported to the AECL for production of juveniles by host fish transformation of glochidia. Between 2010-2012, the AECL infected 818 fish across 42 infestation events, producing 23,714 juvenile Tar River spinymussels. White shiner (*Luxilus albeolus*) was the primary host used for all propagation efforts, but the mountain redbelly dace (*Phoxinus oreas*) was identified as another highly effective host. For all species, the newly transformed juveniles obtained from 1 to 3 collection days during the peak drop-off of juveniles from the host fish were shipped overnight to USGS CERC for testing (NCSU 2013).

WWTP effluent toxicity testing

Once received by USGS CERC, juvenile mussels were acclimated to control water (dilution water) and test temperature (25°C) for 2 d before the start of the toxicity testing (ASTM 2013b). The juvenile mussels were fed an algal mixture (*Nannochloropsis* concentrate and Shellfish Diet, Reed Mariculture, Campbell, California; Wang et al. 2007a) during the acclimation period twice daily in the morning and afternoon. Algal density in the acclimation containers was about 5 to 10 nl cell volume/ml after each feeding. At the beginning of each WWTP effluent test (Table 2), ten juvenile mussels exhibiting foot movement were impartially transferred into each of four replicate 300-ml glass beakers containing about 200 ml of 100%, 50%, 25%, 12.5%, 6.25% test effluent, and dilution water (control water). In addition, about 20 juveniles were also impartially sampled and preserved in 80% ethanol for initial length measurement (see initial lengths in Tables A2 and A4). About 75% water in each replicate was removed and renewed daily. Mussels were fed 2 ml of the algal mixture once daily after water renewal.

Survival of juvenile mussels was determined at the end of the test. Juvenile mussels were classified as alive if they exhibited foot movement within a 5 min observation period using a dissecting microscope (ASTM 2013b). The test acceptability criterion was $\geq$ 80% control survival. Surviving mussels at the end of the tests were preserved for shell length determinations. The maximum shell length of each surviving mussel was measured to the nearest 0.001 mm using a digitizing system with video micrometer software (Image Caliper, Resolution Technology, Dublin, Ohio).

Neonate *C. dubia* were assigned impartially to test chambers by placing one organism in one of ten replicates per effluent concentration (Table 3). Test chambers were placed in a water bath at 25ºC. Each day before water renewal, each first-generation *C. dubia* was recorded as alive or dead (immobility as lack of movement within 5 seconds in response to gentle prodding) and was transferred to a new test chamber containing fresh exposure water. The number of young released from females over each 24-h period was recorded. The *C. dubia* were fed 0.1 ml each yeast-cerophyll-trout chow (YCT; 1800 mg/L stock solution) and algal (*Pseudokirchnerella subcapitata*) suspension (3.0 to 3.5 X 10^7 cell/ml) per chamber daily. Exposures were conducted until 60% of the control cladocerans produced three broods (a 7-d exposure). The test acceptability criterion was ≥80% control survival and ≥15 young/female in controls.

Fathead minnows were acclimated to control water and test temperature (25°C) for 24 h before testing (Table 4). The fish were fed newly hatched (less than 24-h old) brine shrimp (*Artemia*) nauplii twice daily at a rate of adding 1 ml of a concentrated suspension of the nauplii into 2 L of water during the acclimation period. At the beginning of a test, ten fish (<48-h old) were impartially transferred into each of four replicate 500-ml glass beakers containing about 250 ml of water. About 80% of the water was renewed daily. The fish were fed 0.15 ml of a concentrated suspension of less than 24-h-old brine shrimp nauplii twice daily on test day 0 to 6. Sufficient numbers of nauplii were provided to assure that some nauplii remain alive in the test chamber for several hours after each feeding. Fish survival was determined at the end of the test. Surviving fish per replicate were dried at 60ºC for 24 h for dry weight measurement. Biomass was then determined as total dry weight of surviving fish in a replicate. The test acceptability criterion was ≥80% control survival and average dry weight per surviving individual in control chambers equals or exceeds 0.25 mg.

Table 2. Summary of conditions for conducting effluent toxicity tests with juvenile mussels (notched rainbow, *Villosa constricta*; yellow lance, *Elliptio lanceolata*) in basic accordance with ASTM (2013a, b) and USEPA (2002).

Test species:	Notched rainbow and yellow lance
Test chemicals:	Five field-collected effluent samples from North Carolina
Test type:	Static renewal
Test Duration:	7 d
Temperature:	25±1°C
Light quality:	Ambient laboratory light
Light intensity:	200 lux
Photoperiod:	16L:8D
Test chamber size:	300 ml
Test solution volume:	200 ml
Renewal of solution:	Daily (about 75% replacement of water)
Age of test organism:	About 7 days after transformation
Number of organisms per test chamber:	10
Number replicate chambers per concentration:	4
Feeding:	2 ml of algal mixture once daily after water renewal
Chamber cleaning:	None
Aeration:	None
Dilution water:	Diluted well water (100 mg/L as $CaCO_3$)
Dilution factor:	0.5
Test concentration:	0, 6.25, 12.5, 25, 50, 100% effluent
Chemical residues:	Major cations and anions in 100% effluents at start of test
Water quality:	pH, conductivity, hardness, alkalinity measured in the control, medium and high exposure concentrations on Days 0 and 7; dissolved oxygen measured every other day.
Endpoint:	Survival and growth (shell length)
Test acceptability criterion:	≥80% control survival (no test acceptability criteria have been established for 7-d test with newly transformed mussels)

Table 3. Summary of test conditions for conducting chronic effluent tests with the cladoceran (*Ceriodaphnia dubia*) in basic accordance with USEPA (2002) and ASTM (2013c).

Test species:	*Ceriodaphnia dubia*
Test chemicals:	Five field-collected effluent samples from North Carolina
Test type:	Static renewal
Test Duration:	7 d, when 60% of control animals produce 3 broods
Temperature:	25±1°C
Light quality:	Ambient laboratory light
Light intensity:	200 lux
Photoperiod:	16L:8D
Test chamber size:	30 ml (disposable polystyrene cup)
Test solution volume:	15 ml
Renewal of solution:	Daily
Age of test organism:	<24 h (within 8 hours of age)
No. organisms per test chamber:	1
No. replicate chambers per concentration:	10
Feeding:	0.1 ml each yeast-cerophyll-trout chow (YCT; 1800 mg/L stock solution) and algal (*Raphidocelis subcapitata*) suspension (3.0 to 3.5 X 10^7 cell/ml) per chamber daily
Chamber cleaning:	New plastic cups daily
Aeration:	None
Dilution water:	Diluted well water (100 mg/L as $CaCO_3$)
Dilution factor:	0.5
Test concentration:	0, 6.25, 12.5, 25, 50, 100% effluent
Chemical residues:	Major cations and anions in 100% effluents at start of test
Water quality:	pH, conductivity, hardness, alkalinity measured in the control, medium and high exposure concentrations on days 0 and 7; dissolved oxygen measured every other day.
Endpoint:	Survival and reproduction
Test acceptability criterion:	≥80% control survival and ≥15 young/female in controls and ≥60% of surviving control females had three broods.

Table 4. Summary of test conditions for conducting toxicity tests with fathead minnow (*Pimephales promelas*) in basic accordance with USEPA (2002).

Test species:	Fathead minnow
Test chemicals:	Five field-collected effluent samples from North Carolina
Test type:	Static renewal
Test Duration:	7 d
Temperature:	25±1°C
Light quality:	Ambient laboratory light
Light intensity:	200 lux
Photoperiod:	16L:8D
Test chamber size:	500 ml
Test solution volume:	250 ml
Renewal of solution:	Daily (about 80% replacement of water)
Age of test organism:	<48 h
Number of organisms per test chamber:	10
Number replicate chambers per concentration:	4
Feeding:	On days 0-6, feed 0.15 ml of concentrated suspension of 24-h-old brine shrimp nauplii twice daily (early morning and afternoon after water renewal).
Chamber cleaning:	Siphon daily, immediately before water renewal
Aeration:	None, unless dissolved oxygen concentration <4.0 mg/L
Dilution water:	Diluted well water (100 mg/L as $CaCO_3$)
Dilution factor:	0.5
Test concentration:	0, 6.25, 12.5, 25, 50, 100% effluent
Chemical residues:	Major cations and anions in 100% effluents at start of test
Water quality:	pH, conductivity, hardness, alkalinity measured in the control, medium and high exposure concentrations on days 0 and 7; dissolved oxygen measured every other day.
Endpoint:	Survival and biomass (based on dry weight)
Test acceptability criterion:	≥80% control survival and average dry weight per surviving organism in control chambers > 0.25 mg/individual.

Mock effluent preparation

The sensitivity of test organisms to mock effluents was evaluated in 2012. Mock effluent 1 was a mixture of ammonia (NH_4Cl), cadmium ($CdCl_2$), copper ($CuSO_4$), nickel ($NiCl_2$), lead ($PbNO_3$), and zinc ($ZnCl_2$). The USEPA chronic ambient water quality criteria for these chemicals were selected as the concentrations of equitoxic proportion for each toxicant (Table 5). The 12.5% dilution was set at the criteria continuous concentration (red text in Table 5). Mock effluent 2 was a mixture of the same chemicals as in mock effluent 1, but the concentrations of equitoxic proportion were selected based on EC20s or chronic values (geometric mean of NOEC and LOEC) for mussels (Table 6). The 12.5% dilution was set at the EC20 or chronic value for mussels (red text in Table 6) except the 25% dilution was used for lead due to low solubility. Because mussels and *C. dubia* are generally more sensitive to metals than fathead minnows, the mussels and *C. dubia* were tested in six dilutions of 0, 3.2, 6.25, 12.5, 25, and 50% of each mock effluent while fathead minnows were tested in six dilutions of 0, 6.25, 12.5, 25, 50, and 100%.

Mock effluent toxicity testing

Mock effluent tests were conducted with notched rainbow mussels, cladocerans and fathead minnows using protocols described previously for the WWTP effluent tests (Tables 2 to 4) with the following modifications. The control water (dilution water) in mock effluent tests was reconstituted ASTM hard water (160-180 mg/L as $CaCO_3$; ASTM 2013a) and light intensity was 500 lux. To improve the process of daily water renewal and recovery of mussels at the end of the test, newly designed mussel exposure units were used in the mock effluent tests. The exposure units consisted of a 160-ml inner chamber and a 200-ml outer beaker (modified from the exposure unit described in Miao et al. 2010). The inner chamber was a glass tube with stainless-steel screen (120-µm opening) at the bottom. Each of four replicate 200-ml glass beakers contained about 150 ml of water. Feeding was 1 ml of the algal mixture twice daily after water renewal. Water in each replicate exposure unit was removed and renewed (100%) daily. Testing the mussels in these exposure units made it easier to renew water daily during the exposures and to recover the mussels at the end of the exposures. Survival of juvenile mussels was determined at the end of the test. Surviving mussels were preserved in ethanol (80%) for subsequent shell length measurement.

Table 5. Mock effluent 1 for 7-d test with *Ceriodaphnia dubia*, fathead minnow (*Pimephales promelas*) and the notched rainbow (*Villosa constricta*) freshwater mussel in diluted well water (hardness 100 mg/L as $CaCO_3$, dissolved organic carbon 0.5 mg/L). The 12.5% dilution (red text) was set at the USEPA criteria continuous concentration at hardness 100 mg/L, DOC 0.5 mg/L, pH 8.3, and 20 or 22°C.

Conc. (µg/L)	Control	3.13%	6.25%	12.5%	25%	50%	100%
Ammonia	Control	250	500	1000	2000	4000	8000
Cadmium	Control	0.06	0.13	0.25	0.5	1	2
Copper	Control	0.38	0.75	1.5	3	6	12
Nickel	Control	13	26	52	104	208	416
Lead	Control	1.25	1.25	2.5	5	10	10
Zinc	Control	30	60	120	240	480	960
Acute toxic unit to mussels, fatmucket or paper pondshell[a]							
Ammonia	Control	0.04	0.08	0.15	0.31	0.62	1.23
Cadmium	Control	0.00	0.00	0.01	0.02	0.04	0.08
Copper	Control	0.02	0.03	0.06	0.12	0.24	0.48
Nickel	Control	0.05	0.10	0.21	0.41	0.83	1.65
Lead	Control	0.00	0.00	0.00	0.01	0.01	0.01
Zinc	Control	0.10	0.19	0.39	0.77	1.55	3.10
Sum		0.21	0.41	0.82	1.64	3.28	6.55

[a] The toxic units are determined based on hardness or BLM-normalized EC50s from previous 96-h tests with newly transformed mussels:

	EC50 (µg/L)	Reference
Ammonia	6500	Fatmucket (*Lampsilis siliquoidea*); Wang et al. 2008
Cadmium	26	Fatmucket; Wang et al. 2010
Copper	25	Fatmucket; Wang et al. 2009
Nickel	252	Paper pondshell (*Utterbackia imbecillis*); Keller et al. 1991
Lead	670	Fatmucket; Wang et al. 2010
Zinc	310	Fatmucket; Wang et al. 2010

Table 6. Mock effluent 2 for 7-d test with *Ceriodaphnia dubia*, fathead minnow (*Pimephales promelas*) and notched rainbow (*Villosa constricta*) mussel in diluted well water (hardness 100 mg/L as $CaCO_3$, dissolved organic carbon 0.5 mg/L). The 12.5% dilution (red text) was set close to the EC20 or geometric mean of the NOEC and LOEC for fatmucket (*Lampsilis siliquoidea*) and rainbow (*Villosa iris*) mussels in previous 28-d water-only tests at hardness 100 mg/L, DOC 0.5 mg/L, pH 8.3, and 20 or 22°C (except for lead in 25% dilution due to low solubility).

Conc. (µg/L)	Control	3.13%	6.25%	12.5%	25%	50%	100%
Ammonia	Control	100	200	400	800	1600	3200
Cadmium	Control	2	4	8	16	32	64
Copper	Control	2	4	8	16	32	64
Nickel	Control	11.5	23	46	92	184	368
Lead	Control	6	11	22	44	88	176
Zinc	Control	31	62	124	248	496	992
Acute toxic unit to fatmucket or paper pondshell [a]							
Ammonia	Control	0.02	0.03	0.06	0.12	0.25	0.49
Cadmium	Control	0.08	0.15	0.31	0.62	1.23	2.46
Copper	Control	0.08	0.16	0.32	0.64	1.28	2.56
Nickel	Control	0.05	0.09	0.18	0.37	0.73	1.46
Lead	Control	0.01	0.02	0.03	0.07	0.13	0.26
Zinc	Control	0.10	0.20	0.40	0.80	1.60	3.20
Sum		0.33	0.65	1.30	2.61	5.22	10.44

[a] The toxic units are determined based on hardness or BLM-normalized EC50s from previous 96-hour tests with newly transformed mussels:

	EC50 (µg/L)	Reference
Ammonia	6500	Fatmucket (*Lampsilis siliquoidea*); Wang et al. 2008
Cadmium	26	Fatmucket; Wang et al. 2010
Copper	25	Fatmucket; Wang et al. 2009
Nickel	252	Paper pondshell (*Utterbackia imbecillis*); Keller et al. 1991
Lead	670	Fatmucket; Wang et al. 2010
Zinc	310	Fatmucket; Wang et al. 2010

Reference toxicant tests

Juvenile mussels were acclimated to the control water (the ASTM reconstituted hard water; ASTM 2013a) and test temperature (20°C) for 2 d before testing. The juvenile mussels were fed algal mixture (Wang et al. 2007a) at a rate of 2 ml of the algal mixture into 200 ml of water twice daily during the acclimation period. Test conditions are summarized in Table 7. At the beginning of the 4-d static-renewal reference toxicant test with copper or sodium chloride, five mussels were impartially transferred into each of four replicate 50-ml glass beakers containing about 30 ml of test solution. Mussels were not fed during the exposure. Test solution was renewed at 48 h.

Ammonia reference toxicity tests were conducted in a flow-through diluter system to maintain constant ammonia concentrations during the 4-d exposures. Ten mussels were impartially transferred into each exposure unit which consisted of a 160-ml inner chamber and a 300-ml outer beaker (Miao et al. 2010). The inner chamber was a glass tube with stainless-steel screen (120-µm opening) at the bottom. The diluter system delivered about 120 ml of test solution into each inner chamber once every hour. Mussels were not fed during the exposure.

Survival of mussels was determined at the end of the tests. The test acceptability was ≥90% control survival in the reference toxicant tests.

Water quality and chemical analysis

Dissolved oxygen, pH, conductivity, hardness, alkalinity, and total ammonia nitrogen were measured using standard methods (Eaton et al. 2005) on composite water samples collected from the replicates in the control, medium, and high concentrations at the beginning and end of each test. Dissolved oxygen was measured every other day on composite water samples collected before daily water renewal. Water samples (filtered through a 0.45-µm pore size membrane) for major cations (calcium, potassium, magnesium, and sodium) and major anions (chloride and sulfate) were collected in the 100% effluents at the start of WWTP effluent exposures. The cation samples were stabilized within 24 h by adding 16 M nitric acid to each sample at a volume proportion of 1:100 (1% v/v). Major cations were analyzed by Laboratory and Environmental Testing (Columbia, Missouri), using inductively coupled plasma atomic emission spectroscopy (ICPAES) according to the U.S. Environmental Protection Agency method 200.7 (USEPA 1994). Major anions were analyzed at the USGS CERC using ion chromatography in basic accordance with USEPA (2007a) method 9056A.

For the mock effluent exposures, water samples (20 ml) for analyses of the five metals were collected in each exposure concentration at the beginning and end of the test with a polypropylene syringe, filtered through a 0.45-µm pore size polyethersulfone membrane into a polyethylene bottle, and stabilized within 24 h by adding 16 M nitric acid to each sample at a volume proportion of 1:100. Concentrations of the five metals were determined by inductively coupled plasma-mass spectrometry (ICP-MS), (PE/SCIEX ELAN DRCe, PerkinElmer, Norwalk, Connecticut) in accordance with U.S. Environmental Protection Agency method 6020A (USEPA 2007a). Ammonia was also analyzed daily in each of the exposure concentrations. The concentration of ammonia was determined with an Orion ammonia electrode and Orion EA940 meter (Thermo Electron, Beverly, Massachusetts).

Table 7. Summary of test conditions for conducting 4-day acute copper, ammonia, and sodium chloride reference toxicant tests with juvenile Tar River spinymussel (*Elliptio steinstansana*), yellow lance (*Elliptio lanceolata*), notched rainbow (*Villosa constricta*), yellow lampmussel (*Lampsilis cariosa*), fathead minnows (*Pimephales promelas*) and cladocerans (*Ceriodaphnia dubia*) in basic accordance with ASTM (2013a, b).

Test chemicals:	$CuSO_4$, NH_4Cl, NaCl
Test type:	Static renewal (copper, sodium chloride), flow through (ammonia)
Test Duration:	96 h
Temperature:	20°C
Light quality:	Ambient laboratory light
Light intensity:	200 lux
Photoperiod:	16L:8D
Test chamber size:	Static renewal: 50 ml for mussel and cladoceran, 500 ml for minnow; Flow through: 300-ml outer beaker with 160-ml inner chamber (Miao et al 2010)
Test solution volume:	Static renewal: 30 ml for mussel and cladoceran, 250 ml for minnow; Flow through: 200 ml (outer beaker) and 100 ml (inner chamber)
Renewal of solution:	Static renewal: After 48 h Flow through: Additional 120-ml to each beaker once every 4 h
Age of test organism:	Mussel: about 7 after transformation Cladoceran: <24 h Fathead minnow: 48 h
No. organisms per test chamber:	5 or 10 (mussel), 1 (cladoceran), or 10 (minnow)
No. replicate chambers per concentration:	4 (mussels),10 (cladoceran), or 2 (minnow)
Feeding:	No feeding (except 0.2 ml *Artemia* nauplii concentrate 2 h before water renewal at 48 h for minnow)
Chamber cleaning:	None
Aeration:	None
Dilution water:	Reconstituted ASTM hard water (160-180 mg/L as $CaCO_3$)
Dilution factor:	0.5
Test concentration:	0, 1, 2, 4, 8, 16 g NaCl/L 0, 1.0, 2.0, 4.0, 8.0 and 16 mg total ammonia nitrogen/L 0, 6.25, 12.5, 25, 50, 100 µg copper/L
Chemical residues:	Ammonia in each concentration measured at least every other day. Water samples for copper and sodium chloride analysis collected at the beginning and the end of test
Water quality:	Dissolved oxygen, pH, conductivity, hardness, and alkalinity determined at the control, medium, and high exposure concentrations at the beginning and the end of test
Endpoint:	Survival
Test acceptability criterion:	≥90% control survival

The meter was calibrated before measuring samples with 1.0 and 10 mg N/L calibration standards. The method detection limit was 0.03 mg N/L. Water samples for dissolved organic carbon (DOC) analysis were collected from the control beakers and shipped overnight in a cooler with ice packs to Huffman Laboratories (Golden, Colorado). Water samples were filtered at 0.45 µm before analysis. The Huffman laboratory used a TOC Analyzer (OI Analytical Model 700) following persulfate-ultraviolet oxidation with infrared detection (method 5310C; Eaton et al. 2005) and reported a method detection limit of 0.05 mg C/L.

For the reference toxicant tests, ammonia was measured at each exposure concentration at the beginning and the end of tests, and copper was measured at each concentration at the beginning of the test. The NaCl concentrations were not measured. Salinity and conductivity were measured at the beginning and the end of each test to confirm the target NaCl concentrations.

Data analysis

No-observed-effect concentration (NOEC), lowest-observed-effect concentration (LOEC), and 20% inhibition concentration (IC20) for survival, length (mussels), biomass (minnows), or reproduction (*C. dubia*) in the short-term 7-d effluent tests, and 50% effect concentration (EC50) in the acute reference toxicant tests were estimated using TOXSTAT software (version 3.5, Western EcoSystems Technology, Cheyenne, Wyoming) following the methods outlined in USEPA (2002). The EC50s in the reference toxicant tests for copper or ammonia were calculated based on measured concentrations, and the EC50s in the reference toxicant tests for NaCl were calculated based on nominal concentrations.

Results and Discussion

Whole effluent toxicity tests

Table 8 summarizes the WWTP effluent toxicity test results; the appendix Tables A1 to A4 provide individual test results and supporting water chemistry. Control survival of notched rainbow and fathead minnow was acceptable in all effluent tests (notched rainbow control survival ranged from 91 to 100%, and fathead minnow control survival ranged from 85 to 100%). The *C. dubia* control survival was 90% and control reproduction was 21 and 22 young per female in effluents 1 and 2 (tested in 2010 with the same batch of *C. dubia*) but was only 30, 60, and 100% in effluents 3, 4, and 5 (tested in 2011 with the same batch of *C. dubia*). The *C. dubia* control reproduction was also low in the three effluents tested in 2011, ranging from 9 to 15. Therefore, *C. dubia* results for effluents 3, 4, and 5 are not included in Table 8. In addition, yellow lance was tested in effluents 1 and 2 in 2010. The control survival was 51% (Table A2) and did not meet the test acceptability requirement of ≥80% control survival (Table 2). Therefore, the effect concentrations for yellow lance were not included in Table 8.

Based on IC20s, four of the five effluents were toxic to at least one test species (all except for effluent 5) (Table 8). In effluent 1, *C. dubia* reproduction was a more sensitive endpoint than any endpoints of the notched rainbow or fathead minnows (Table 8). However, in effluent 2 notched rainbow survival and growth were both more sensitive endpoints than any endpoints of *C. dubia* and fathead minnows. In effluents 3 and 4, there were no acceptable test results for *C. dubia* for comparison to mussels, but the mussels were always more sensitive than fathead minnows.

Table 8. Effect concentrations of cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constricta*) in 7-d exposures to five permitted effluent samples. The no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and 20% inhibition concentrations (IC20) with 95% confidence interval (CI) are presented for each endpoint. See Table A2 and Table A4 for additional detail.

	Cladoceran		Fathead minnow		Notched rainbow	
Treatment	Survival	Reproduction	Survival	Biomass	Survival	Length
Effluent 1						
NOEC (%)	100	100	100	100	100	100
LOEC (%)	>100	>100	>100	>100	>100	>100
IC20 (CI; %)	>100	<6.25	>100	>100	>100	>100
Effluent 2						
NOEC (%)	100	100	100	50	50	25
LOEC (%)	>100	>100	>100	100	100	50
IC20 (CI; %)	>100	>100	>100	>100	24 (12-67)	>100
Effluent 3						
NOEC (%)	NR[a]	NR	100	100	50	50
LOEC (%)	NR	NR	>100	>100	100	>50
IC20 (CI; %)	NR	NR	>100	>100	<6.25	>100
Effluent 4						
NOEC (%)	NR	NR	100	100	12.5	12.5
LOEC (%)	NR	NR	>100	>100	25	>12.5
IC20 (CI; %)	NR	NR	>100	>100	69 (23-120)	>100
Effluent 5						
NOEC (%)	NR	NR	100	100	100	100
LOEC (%)	NR	NR	>100	>100	>100	>100
IC20 (CI; %)	NR	NR	>100	>100	>100	>100

[a] Not reported because of low control survival.

The IC20s were used to evaluate effluent toxicity and to compare sensitivity of the test species. While informative in a weight of evidence approach, the NOEC and LOEC are generated by post-analysis analysis of variance (ANOVA) multiple comparison tests and have important limitations (e.g., NOEC and LOEC can only take values of a tested concentration, failure to reject a null hypothesis of no difference does not mean there was no effect because a biologically significant effect may occur and not be detected by the ANOVA). For example, in the toxicity test with effluent 1, the NOEC was 100% but the IC20 was <6.25% and the biological effect observed was 23 to 37% reduction in growth (length) relative to the control. Others have observed between 10 and 34% effects occurring at the reported NOEC when test data are re-evaluated by a regression-derived estimate rather than hypothesis testing (Crane and Newman 2000).

Mussels are known to be sensitive to potassium, sulfate, and chloride (Soucek 2006, USEPA 2010, Gillis 2011, Wang et al. 2012, 2013, Ivey et al. 2013) and relatively tolerant of nitrate

(USEPA 2010, Soucek and Dickinson 2012) which were all among the anions and cations analyzed in the whole effluents (Tables A1 and A3). Potassium 96-h EC50s for six mussel species range from about 31 to 52 mg/L (Wang et al. 2013); an approximate no to low effect concentration of 15 mg/L (from taking the low EC50 and dividing by two) is not exceeded in any effluent but is approached by effluents 3, 4 and 5 which had potassium concentrations between 10.5 and 13.9 mg/L (Tables A1 and A3). There were no concentrations of the other major ions in the effluents exceeding those known to be harmful to mussels based on the references cited above. Mussels are also known to be sensitive to ammonia (USEPA 2013), and effluent 2 contained 0.91 to 3.41 mg/L total ammonia as nitrogen (TAN) over the course of the 7-d exposures (Table A1). While no follow-up toxicity identification evaluation was performed for the effluents, it is plausible that ammonia was the source of toxicity. Ammonia concentrations exceeded those known to be lethal to freshwater mussels (Wang et al. 2007b, 2008) and exceeded acute and chronic water quality criteria for ammonia (normalized to pH 7 and 20^{o}C; USEPA 2013).

The percent of the stream flow that is comprised by a particular facility's maximum permitted effluent during 7Q10 flows (the predicted seven-day low flow with an average recurrence interval of once every ten years) is known as the instream waste concentration (IWC). The IWC's for effluents 2, 3, and 4 were approximately equal to, or greater than concentrations that impacted mussel survival. While results are presented without attribution to particular facilities, effluents 2, 3, and 4 were toxic to mussels at concentrations that potentially may occur in the environment during situations of maximum permitted discharge under low flow conditions.

Mock effluent toxicity tests

Table 9 summarizes the mock effluent toxicity test results; appendix Tables B1 to B3 provide the individual test results and supporting water chemistry. The measured concentrations of toxicants (ammonia and the five metals) typically ranged from 80 to 120% of the nominal concentrations (Table B1). Control survival for each of three test species was 100% and met test acceptability criteria for each mock effluent, and reproduction of *C. dubia* and weight of fathead minnows also met test acceptability criteria. The two mock effluents were toxic to all test species, and the sublethal endpoints (*C. dubia* reproduction, fathead minnow biomass, and notched rainbow mussel length) were generally more sensitive than the lethal endpoint (Table 9). Based on the IC20s for the sublethal endpoints, the species sensitivity to the two mock effluents was *C. dubia* > notched rainbow > fathead minnow. However, the LOECs based on mussel length were lower than the LOECs based on *C. dubia* reproduction (Table 9).

The 12.5% dilution in mock effluent 1 consisted of toxicants mixed at the USEPA chronic water quality criteria (those in effect in 2012) for the hardness, pH, and DOC of the dilution water and test temperature. Accordingly, the threshold for toxicity in mock effluent 1 was expected to be between the 12.5 and 25% concentrations. However, mock effluent 1 was more toxic to mussels and *C. dubia* than expected; the IC20 for *C. dubia* survival (7%), *C. dubia* reproduction (<3.1%) and mussel length (5.6%) indicated effects at concentrations less than those expected to be safe based on 2012 chronic water quality criteria of individual compounds.

Table 9. Effect concentrations of cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constricta*) in 7-d exposures to two mock effluents. The no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and 20% inhibition concentrations (IC20) with 95% confidence interval (CI) are presented for each endpoint. See Table B3 for additional detail.

	Cladoceran		Fathead minnow		Notched rainbow	
Treatment	Survival	Reproduction	Survival	Biomass	Survival	Length
Mock Effluent 1						
NOEC (%)	6.25	3.125	25	25	25	<3.125
LOEC (%)	12.5	6.25	50	50	50	3.125
IC20 (CI; %)	7.0 (4.3-7.8)	<3.1	34 (21-41)	33 (20-40)	25 (15-28)	5.6 (4.2-10)
Mock Effluent 2						
NOEC (%)	6.25	3.125	25	12.5	25	<3.125
LOEC (%)	12.5	6.25	50	25	50	3.125
IC20 (CI; %)	6.9 (4.3-7.5)	<3.2	29 (19-36)	20 (16-28)	26 (21-29)	8.3 (5.7-11)

The 12.5% treatment in mock effluent 2 consisted of toxicants mixed near their individual thresholds of effect based on EC20s of other mussel species in 28-d exposures. Accordingly, the threshold for toxicity to mussels in mock effluent 2 was expected to be near the 12.5% concentration. However, the IC20 for mussel length (8.3%) indicates an effect at concentrations less than those expected to be safe based on published mussel chronic toxicity test results of individual compounds used in the effluents.

Based on mortality, the effluents exhibited similar toxicity to notched rainbow with survival IC20s at about 25%. The IC20s for mussel length were also similar (5.6 and 8.3%, but with overlapping confidence intervals). This indicates that no effect concentration estimates based on previous mussel toxicity tests of individual compounds or based on water quality criteria for individual compounds were similar, but those estimates are not protective of the notched rainbow for the mixture of metals and ammonia in these tests.

Reference toxicant tests

The EC50s for acute 4-d reference toxicant (sodium chloride, copper, or ammonia) tests with the four mussel species are provided in Table 10 (individual test results and supporting water chemistry are in the appendix Tables C1 to C8). Control survival in all acute tests was >90%, except one ammonia test with yellow lance (76% control survival, the EC50 for which is not included in Table 10). The three table entries with more than one value (notched rainbow copper and sodium chloride, and yellow lance copper) are results from reference toxicant tests conducted in different years of the project and generally show consistency of results between years. The notched rainbow 3-fold difference in copper sensitivity between years is within the range of historical intra-species, intra-lab test variation (e.g., Wang et al. 2007b, c), but at the high end of the range for intra-lab test variation. Tar River spinymussel, yellow lance and notched rainbow were of similar sensitivity to ammonia, with overlapping EC50 95% confidence intervals (Table 10). Yellow lance was the most sensitive species to copper among the four tested species, and was also more sensitive than notched rainbow to sodium chloride (Table 10).

Table 10. EC50s (95% confidence interval) in acute 4-d reference toxicant tests with the endangered Tar River spinymussel (*Elliptio steinstansana*) and three potential surrogate mussel species (notched rainbow, *Villosa constricta*; yellow lance, *Elliptio lanceolata*; and yellow lampmussel, *Lampsilis cariosa)* in ASTM reconstituted hard water (ASTM 2013a).

	Tar River spinymussel	Notched rainbow	Yellow lance	Yellow lampmussel
Copper (µg Cu/L)	26 (22-31)	12 (9.6-15)[a] 39 (32-48)[b]	16 (13-20)[a] 16 (13-19)[b]	46 (32-68)
Total ammonia (mg N/L)	3.6 (3.1-4.3)	3.5 (2.9-4.3)	3.1 (2.6-3.7)	8.2 (7.2-9.2)
Sodium chloride (g/L)	NT[d]	3.9 (3.3-4.7)[c] 4.1 (3.5-4.8)[a]	2.1 (1.8-2.5)	NT

[a] test conducted in 2011
[b] test conducted in 2012
[c] test conducted 2010
[d] Not tested.

To compare relative species sensitivity with other mussels tested in previous studies, species mean acute values (SMAVs) were calculated and ranked in Table 11 for ammonia and Table 12 for copper. An SMAV is the geometric mean of 24 to 96-h EC50s from all acceptable tests for a species. Because no toxicity tests were conducted with sodium chloride and the endangered Tar River spinymussel, no species sensitivity comparison with other mussels was conducted. The aquatic toxicity of ammonia varies by temperature and pH, and the toxicity of copper is influenced by several water quality characteristics, including pH, concentrations of cations and anions, and dissolved organic carbon. We used the ammonia toxicity temperature-pH normalization equations of the revised USEPA water quality criteria for ammonia (USEPA 2013) to normalize acute values for ammonia SMAV calculations. The Tar River spinymussel, notched rainbow, and yellow lance, were of similar sensitivity within or near the bottom quartile of the ammonia sensitivity distribution for mussels (Table 11), indicating notched rainbow and yellow lance would be suitable surrogates for Tar River spinymussel ammonia sensitivity. However, the yellow lampmussel was more tolerant than these species and would not appear to be a good surrogate for the Tar River spinymussel based on ammonia sensitivity.

These are the first acute toxicity test results for ammonia and mussels in the genus *Elliptio*. Using the same set of data quality objectives and same data synthesis procedures as those in the revised ammonia water quality criteria document (USEPA 2013), the genus *Elliptio* can be added to the data used to derive the criteria. *Elliptio* is the third most sensitive of 70 genera of aquatic animals in the expanded dataset with a genus mean acute value (GMAV, the geometric mean of the SMAVs by genus) of 31.05 mg/L total ammonia as N at pH 7 and 20^{o}C (there is some uncertainty about the phylogenetic classification of Tar River spinymussel, but because the SMAV for the yellow lance is less than that of the Tar River spinymussel, the GMAV for *Elliptio* would still be the third most sensitive if Tar River spinymussel was assigned to another genus). As genus mean acute values close to the 5th percentile drive the criteria maximum concentration (CMC) recommendations, the addition of the data for *Elliptio* to the acute criteria

Table 11. Ranked ammonia genus mean acute values (GMAVs) with associated species mean acute values (SMAVs). An SMAV is the geometric mean of 24 to 96-h EC50s from all acceptable tests and a GMAV is the geometric mean of the SMAVs by genus. Data are from Table 3 of the revised ambient water quality criteria for ammonia (USEPA 2013) with newly tested North Carolina mussel species from this study added (in red). All values are normalized to mg-N/L at pH 7 and 20°C (USEPA 2013). The 2013 and 1999 criteria maximum concentrations and final acute values are provided for reference.

GMAV (mg N/L)	Species	SMAV (mg N/L)
109.0	Dwarf wedgemussel, *Alasmidonta heterodon*	109.0
109.0	Pink papershell, *Potamilus ohiensis*	109.0
72	1999 FAV[a]	
71.25	Mucket, *Actinonaias ligamentina*	63.89
	Pheasantshell, *Actinonaias pectorosa*	79.46
70.73	Giant floater mussel, *Pyganodon grandis*	70.73
50.01	Pink mucket, *Lampsilis abrupta*	26.03
	Plain pocketbook, *Lampsilis cardium*	50.51
	Yellow lampmussel, *Lampsilis cariosa*	76.22
	Wavy-rayed lampmussel, *Lampsilis fasciola*	48.11
	Higgin's eye, *Lampsilis higginsii*	41.90
	Neosho mucket, *Lampsilis rafinesqueana*	69.97
	Fatmucket, *Lampsilis siliquoidea*	55.42
47.40	Atlantic pigtoe, *Fusconaia masoni*	47.40
46.93	Pondshell mussel, *Utterbackia imbecillis*	46.93
36	1999 CMC[b]	
33.52	2013 FAV[c]	
33.37	Notched rainbow, *Villosa constricta*	32.53
	Rainbow mussel, *Villosa iris*	34.23
31.14	Oyster mussel, *Epioblasma capsaeformis*	31.14
31.05	Yellow lance, *Elliptio lanceolata*	28.81
	Tar River spinymussel, *Elliptio steinstansana*	33.46
23.41	Green floater, *Lasmigona subviridus*	23.41
23.12	Ellipse, *Venustaconcha ellipsiformis*	23.12
17	2013 CMC[d]	

[a] Final Acute Value, Salmonids absent (USEPA 1999).
[b] Criterion Maximum Concentration, Salmonids absent - estimate of the highest concentration to which an aquatic community can be exposed briefly without resulting in an unacceptable effect (USEPA 1999). The CMC is the FAV divided by two.
[c] Final Acute Value (USEPA 2013).
[d] Criterion Maximum Concentration (USEPA 2013).

dataset would lower the CMC by 11%. It is also interesting that adding the data for two additional species of *Lampsilis* and *Villosa*, genera already represented in the dataset, changed the value of their GMAVs but had little influence on their ranks (i.e., the new SMAVs were similar to those already available for other species in those genera). While it is also possible to look at the *Elliptio* GMAV as similar (within a factor of two) of the of those already in the database and, therefore, of not much value, it should serve as a reminder that even for a well-studied pollutant like ammonia, the data for 17 mussel species have limitations in representing the U.S.'s nearly 300 species of mussels in need of protection and that alternate approaches to deriving estimates of safe concentrations have merit in certain circumstances (Augspurger 2013).

Biotic ligand models have been developed to enable mechanistic modeling of copper bioavailability and acute toxicity as a function of metal speciation and the protective effects of competing cations. We used the biotic ligand model normalization equations of HydroQual Inc. (2007) to normalize acute values for copper SMAV calculations. The yellow lance was the second most sensitive mussel species to copper; the Tar River spinymussel and notched rainbow were of similar sensitivity in the median of the mussel species sensitivity distribution for copper (Table 12). However, the yellow lampmussel was more tolerant than these species. Similar to the conclusion based on the comparisons of ammonia sensitivity, the notched rainbow and yellow lance, but not the yellow lampmussel, would be suitable surrogates for the Tar River spinymussel based on the difference in copper sensitivity.

Table 12. Ranked freshwater mussel genus mean acute values (GMAVs) for copper with associated species mean acute values (SMAVs). An SMAV is the geometric mean of 24 to 96-h EC50s from all acceptable tests and a GMAV is the geometric mean of the SMAVs by genus. Data are biotic ligand model (BLM)-normalized from Table S3 of Wang et al. 2009 with species from this study added (in red). The USEPA (2007b) criteria maximum concentration (CMC) and final acute value (FAV) are provided for reference.

GMAV (µg/L)	Species	SMAV (µg/L)
55.3	Green floater, *Lasmigona subviridis*	55.3
15.8	Pondshell, Utterbackia imbecillis	15.8
12.7	Yellow lampmussel, *Lampsilis cariosa*	21.1
	Neosho mucket, *Lampsilis rafinesqueana*	15.1
	Wavy-rayed lampmussel, Lampsilis fasciola	9.43
	Fatmucket, *Lampsilis siliquoidea*	8.58
11.5	Rainbow, *Villosa iris*	13.0
	Notched rainbow, *Villosa constricta*	10.1
10.3	Tar River spinymussel, *Elliptio steinstansana*	12.9
	Yellow lance, *Elliptio lanceolata*	8.24
9.90	Scaleshell, *Leptodea leptodon*	9.90
4.79	Oyster mussel, *Epioblasma capsaeformis*	4.79
	FAV[a]	4.7
	CMC[b]	2.3

[a] Final Acute Value (USEPA 2007b).
[b] Criterion Maximum Concentration (USEPA 2007b).

Management Implications

Freshwater mussels are one of North America's most imperiled faunal groups (Lydeard et al. 2006). Thirty-five species of freshwater mussels are extinct (Turgeon et al. 1998) and 86 species are federally listed as threatened or endangered. Widespread and chronic impacts such as water pollution and physical habitat alteration are considered the most important current impairments (Richter et al. 1997, Strayer et al. 2004). Toxic effects are reasonable hypotheses to test as limiting factors for mussels because aspects of their life history make them vulnerable to degraded water or sediment quality. Juvenile and adult mussels are benthic suspension feeders exposed to pollutants in surface water, sediment, and pore water and through ingestion of filtered particles with sorbed contaminants (Cope et al. 2008). Chemical aspects of water quality have been historically cited as detrimental to mussels; associations between mussel decline and pollutant sources or impaired water quality have been documented for decades (Fuller 1974).

The Tar River spinymussel recovery plan speaks to water quality overtly, and this project has helped address components of the action plan (USFWS 2009) and recovery plan (USFWS 1992), particularly Recovery Task 2.2 - Identify and eliminate current and future threats to the species' survival, including water quality and habitat degradation. Each of the study objectives outlined on page 1 is revisited here with regard to management implications.

1) The project further developed captive propagation and culture methods for Tar River spinymussels.

The project expanded knowledge of Tar River spinymussel life history and advanced its propagation and culture (NCSU 2013). Fish host identification is a high priority action in the Tar spinymussel recovery plan (USFWS 1992), and this project demonstrated the utility of the white shiner (*Luxilus albeolus*) and mountain redbelly dace (*Phoxinus oreas*) as highly effective host fish (NCSU 2013). The white shiner is commonly collected from the same streams as Tar River spinymussel and was the most effective host fish in the laboratory transformations. From 2010 to 2012, Tar River spinymussel propagation work for this project produced 23,714 juvenile Tar River spinymussels. In 2013, a total of 57 individuals from the 2010-year class remained alive at the North Carolina Wildlife Resources Commission hatchery in Marion, North Carolina and had grown to 30-37 mm in length. There were 1,074 individuals produced from the 2011-year class at the Marion facility that were approximately 12 to 22 mm in length. As of spring 2013, there were thousands of surviving juveniles from the 2012-year class ranging in length from 700-1500 μm at NCSU's Aquatic Epidemiology and Conservation Laboratory. Those are being reared for potential population augmentation or reintroduction efforts as a result of this project (NCSU 2013).

2) The project determined that Tar River spinymussels appear to be sensitive to contaminants.

Freshwater mussels are among the most sensitive forms of aquatic life to toxicity from ammonia, chlorine, chloride, copper, nickel, lead, potassium, sulfate, and zinc (Augspurger et al. 2003, Soucek 2006; Wang et al. 2007a, b, c, 2008, 2009, 2010, 2011a, b, 2012, 2013; March et al. 2007; Besser et al. 2011, 2013; Gillis 2011; Ivey et al. 2013) which are common pollutants of surface waters. We tested the acute sensitivity of the Tar River spinymussel to ammonia and copper (Table 11 and 12); the Tar River spinymussel was between the median and bottom quartile of the species sensitivity distributions for mussels. As such, the Tar River spinymussel is a sensitive species among the mussels which, as a group, are known to be sensitive to ammonia and copper. It is reasonable to expect that the species may also be sensitive to other pollutants known to be of concern to mussels, including chloride, chlorine, lead, nickel, potassium, sulfate, and zinc (Wang et al. 2013). While hazard is a function of sensitivity and exposure, the species' sensitivity indicates pollutants are important factors to consider in management of the Tar River spinymussel.

Three effluents were toxic to notched rainbow mussels (used as a surrogate for the Tar River spinymussel) at concentrations approximately equal to or less than the effluents' instream waste concentration, or IWC – concentrations that potentially may occur in the environment during situations of maximum permitted discharge under low flow conditions. Wastewater regulation using the IWC is designed to be protective in this scenario that is unlikely to occur because facilities rarely discharge at maximum permitted flow. However, there is the potential for toxicity to mussels at extreme low flow from some of the facilities' maximum permitted discharge, and some existing effluents should be further evaluated in the lab and field.

Mock effluents were expected to be toxic between the 12.5 and 25% concentrations as the 12.5% concentration was mixed at the chronic water quality criteria of individual components (mock effluent 1) or their individual thresholds of effect based on EC20s of other mussel species in 28-d exposures (mock effluent 2). The IC20s for mussel length were 5.6 and 8.3% effluent which are about 2-times lower than concentrations expected to be safe. This indicates that no effect concentration estimates based on previous mussel toxicity tests of individual compounds or based on water quality criteria for individual compounds were similar, but those estimates are not protective of the notched rainbow for the mixture of metals and ammonia in these tests.

3) The non-endangered co-occurring and congeneric yellow lance and the notched rainbow have promise as surrogates for Tar River spinymussels' chemical sensitivity.

The Tar River spinymussel, notched rainbow, and yellow lance were of similar (within a factor of 2) sensitivity to ammonia and copper. The yellow lampmussel was more tolerant than these species and would not appear to be a good surrogate based on the difference in chemical sensitivity. The yellow lance had poor control survival in the effluent tests (appendix Table A2). While one bad test performance does not make a bad surrogate, notched rainbow performed well with 91 to 100% survival of experimental controls in all tests conducted over the three year study. The notched rainbow is also native to Tar and Neuse River drainages (as well as the Catawba, Pee Dee, Cape Fear, Roanoke and Chowan river basins). Although not as closely aligned with Tar River spinymussel in its taxonomy and distribution as is yellow lance, notched rainbow has the most promise of the species that we evaluated as a surrogate in Tar River spinymussel toxicity testing based on its availability, sensitivity, and control survival.

4) In seven side-by-side comparisons (five WWTP effluents and two mock effluents), fathead minnow survival and reproduction were frequently under-protective of mussel endpoints.

The objective of a whole effluent toxicity test is to determine whether an effluent concentration equal to the wastewater's maximum permitted concentration in the receiving stream during low stream flow conditions has significant detrimental impact upon reproduction and survival, or growth of test organisms (NCDWQ 2010a, b). Two WWTP effluents were not toxic to mussels or fathead minnows. In the other three WWTP effluents and both mock effluents, the mussels were more sensitive than fathead minnows. The fathead minnow does not consistently represent mussel sensitivity.

5) There were no C. dubia to Tar River spinymussel effluent test comparisons and insufficient C. dubia to notched rainbow effluent test comparisons to definitively determine the protectiveness of the C. dubia test for freshwater mussels. In side-by-side comparisons (two WWTP effluents and two mock effluents), C. dubia reproduction was protective of mussel endpoints in three of four tests. While C. dubia effluent toxicity testing did not consistently protect mussel endpoints, the test should remain the main regulatory tool for effluent evaluation in Tar River spinymussel habitat at this time. Tar River spinymussel and notched rainbow can be tested directly when a mussel-specific test is warranted based on the magnitude, composition, or location of an effluent.

Notched rainbow survival and growth (determined as shell length) were more sensitive than *C. dubia* endpoints in one of the two municipal effluents for which both species were tested. While mussel growth was a sensitive endpoint, it has limitations. For example, the toxicant may selectively kill small mussels and increase the mean length of surviving mussels, or no meaningful length data may be obtainable in high exposure concentrations when all mussels die (Table B3). Also, the lab reported that it was very difficult to obtain dry weight for the newly transformed juveniles (and, therefore, not able to calculate biomass). Future studies should evaluate the utility of measuring dry weight of mussels in effluent testing, but weight determination of these young mussels would require very careful measurements. Measuring dry weight would allow for estimating biomass of mussels (i.e., the total mass of mussels in each replicate at the end of the exposures). Also, producing newly transformed juvenile mussels for effluent testing is not routine, with seasonal constraints on the availability of juveniles for most species making it more difficult to obtain test organisms on a regular basis compared to *C. dubia*.

Considering these challenges to estimating sublethal effect concentrations in effluent testing with mussels and that the 7-d *C. dubia* chronic test was protective of mussel endpoints in three of four tests, the *C. dubia* survival and reproduction test should remain the main regulatory tool for effluent evaluation in Tar River spinymussel habitat at this time. Side-by-side tests of effluents with *C. dubia* and Tar River spinymussels, as was originally envisioned, remains a research need that may be accommodated as propagation methods and capacity improve.

There will be cases when a location, composition, or scale of a discharge makes the additional effort to conduct a mussel toxicity test warranted. Just as notched rainbow were more sensitive than *C. dubia* in one of our four side-by-side comparisons, others evaluating surrogates for mussels in whole effluent toxicity testing note that *C. dubia* tests are typically, but not always protective of mussels (Table 13). The successful propagation of Tar River spinymussels and surrogates as part of this project (NCSU 2013) provides the opportunity for additional testing of these species directly when a water quality issue merits that level of scrutiny.

Table 13. Effluent toxicity tests comparing the sensitivity of freshwater mussels to standard toxicity test organisms including *Ceriodaphnia dubia* (CD) and fathead minnow (FHM).

Reference	**Species evaluated**	**Type of effluent**	**Results**
This study	*Villosa constricta*, CD, FHM	Municipal wastewater	*Villosa constricta* more sensitive than CD in 1 of 2 effluents. Both species more sensitive than FHM.
This study	*Villosa constricta*, CD, FHM	Mixtures of ammonia, cadmium, copper, nickel, lead, and zinc	CD and *Villosa constricta* of similar sensitivity. Both species more sensitive than FHM.
EPRI 2011	*Anodonta imbecilis*, CD	Coal-fired power plant	*Anodonta imbecilis* and CD of comparable sensitivity
McKinney et al. 1996	*Anodonta imbecilis*, CD	Pulp and paper mill	*Anodonta imbecilis* substantially more sensitive than CD
Masnado et al. 1995	*Anodonta imbecilis*, CD, FHM	Synthetic mine effluent (mixture of Cd, Cr, Cu, Ni and Zn)	CD always more sensitive than *Anodonta imbecilis*
Keller 1993	*Anodonta imbecilis*, *CD, FM*	Municipal wastewater	CD more sensitive than *Anodonta imbecilis*

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Appendix

Individual toxicity test results and supporting test water chemistry for whole effluent tests conducted in 2010 and 2011 (Tables A1 to A4), mock effluent tests conducted in 2012 (Tables B1 to B3), and reference toxicant tests conducted between 2010 and 2012 (Tables C1 to C8)

Table A1. Water quality characteristics of two North Carolina permitted effluent samples measured at the beginning and the end of 7-d tests conducted in 2010 with cladoceran (*Ceriodaphnia dubia* [CD]), fathead minnow (*Pimephales promelas* [FHM]), and freshwater mussels (notched rainbow [NR], *Villosa constricta* and yellow lance [YL], *Elliptio lanceolata*).

Effluent	Test day	Dilution	Test species	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (mg N/L)	Major cations and anions (mg/L) Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	SO_4^{2-}	Cl^{-}
Effluent 1	0	0%	All	8.3	8.27	247	100	86	0.04						
	0	6.25%	All	8.2	8.26	257	96	84	0.05						
	0	12.5%	All	8.3	8.22	278	92	80	0.06						
	0	25%	All	8.3	8.17	303	86	80	0.06						
	0	50%	All	8.5	8.00	363	70	68	0.01						
	0	100%	All	8.6	7.65	496	**40**	50	0.16	13.2	3.4	87.8	9.4	74.8	51.0
	2	100%[a]	All	9.0	7.84	485	**40**	50	0.43						
	7	0%	NR	7.1	8.20	265	106	94	0.19						
	7	25%	NR	6.9	8.08	315	90	84	0.11						
	7	100%	NR	6.9	7.90	502	44	64	0.30						
	7	0%	YL	7.1	8.16	269	110	100	0.16						
	7	25%	YL	6.9	8.06	324	102	100	0.16						
	7	100%	YL	7.0	7.90	503	50	64	0.26						
	7	0%	FHM	7.1	8.12	336	114	94	0.19						
	7	25%	FHM	7.5	7.90	366	100	86	0.05						
	7	100%	FHM	7.1	7.89	572	48	62	0.20						
	7	0%	CD	7.6	8.44	270	112	100	0.19						
	7	25%	CD	7.7	8.31	340	100	90	0.21						
	7	100%	CD	7.6	8.10	544	50	64	0.55						

Calculated hardness of 100% effluent sample based on measured Mg and Ca:

399 (mg/L as $CaCO_3$)

The colorometric measure of hardness is prone to interferences (e.g., elevated Fe can cause an artifact).

Continued on next page

[a] The second batch of effluent samples were also measured when receiving on test Day 2.

[b] Red numbers indicate ammonia concentrations above EPA chronic water quality criterion (USEPA 2013).

[c] Bold red numbers indicates ammonia concentrations above EPA acute water quality criterion (USEPA 2013).

Table A1 (concluded). Water quality characteristics of two North Carolina permitted effluent samples measured at the beginning and the end of 7-d tests conducted in 2010 with cladoceran (*Ceriodaphnia dubia* [CD]), fathead minnow (*Pimephales promelas* [FHM]), and freshwater mussels (notched rainbow [NR], *Villosa constricta* and yellow lance [YL], *Elliptio lanceolata*).

Effluent	Test day	Dilution	Test species	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (mg N/L)	Major cations and anions (mg/L) Ca^{2+}	K^+	Mg^{2+}	Na^+	SO_4^{2-}	Cl^-
Effluent 2	0	0%	(the same control for Effluent 1 test)												
	0	6.25%	All	8.1	8.39	248	98	74	0.22						
	0	12.5%	All	8.3	8.32	246	90	80	0.41[b]						
	0	25%	All	8.2	8.26	248	78	80	0.72						
	0	50%	All	8.4	8.12	254	60	80	1.78						
	0	100%	All	8.4	7.87	273	**18**	56	2.71	5.4	1.2	65.4	5.6	13.6	40.8
	2	100%[a]	All	9.0	7.99	374	**20**	84	**3.41**[c]	Calculated hardness of 100% effluent					
	7	25%	NR	7.1	8.12	279	84	90	0.65	sample based on measured Mg and Ca:					
	7	100%	NR	6.0	7.68	338	18	68	0.91	**286** (mg/L as $CaCO_3$)					
	7	25%	YL	7.0	8.07	278	80	88	0.63						
	7	100%	YL	6.0	7.58	338	16	70	1.33						
	7	0%	FHM	7.1	8.12	360	114	94	0.14						
	7	25%	FHM	7.1	8.11	332	88	90	0.63						
	7	100%	FHM	7.0	8.00	415	18	80	**2.78**						
	7	0%	CD	7.8	8.46	297	112	92	0.29						
	7	25%	CD	7.6	8.39	303	90	90	0.61						
	7	100%	CD	7.5	8.20	367	20	78	**1.91**						

[a] The second batch of effluent samples were also measured when receiving on test Day 2.

[b] Red numbers indicate ammonia concentrations above EPA chronic water quality criterion (USEPA 2013).

[c] Bold red numbers indicates ammonia concentrations above EPA acute water quality criterion (USEPA 2013).

Table A2. Responses of cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and two freshwater mussels (notched rainbow, *Villosa constricta*; yellow lance, *Elliptio lanceolata*) in 7-d exposures to two effluent samples. Values are means with standard deviation in parentheses (n = 10 for the cladoceran and 4 for other three species). Value in the shaded cells indicate a significant reduction in an endpoint relative to control (Dunnett's test or Steel's many-one rank test, $p< 0.05$; USEPA 2002). No-observed-effect concentration [NOEC], lowest-observed-effect concentration [LOEC]), and 20% inhibition concentrations (IC20) with 95% confidence interval (CI) are presented for each endpoint. Mussel length data at the concentration above the NOEC for survival (means below dashed lines) were excluded from hypothesis testing when calculating the NOEC and LOEC

	Cladoceran		Fathead minnow		Notched rainbow		Yellow lance	
Treatment	Survival (%)	No. of young	Survival (%)	Biomass (mg)	Survival (%)	Length (mm)[a]	Survival (%)	Length (mm)
Effluent 1								
0%	90	22 (8.9)	100 (0)	3.83 (0.37)	92 (10)	0.40 (0.03)	51 (30)	NM[b]
6.25%	80	14 (8.1)	100 (0)	3.30 (0.40)	100 (0)	0.47 (0.03)	45 (52)	NM
12.5%	90	15 (7.0)	100 (0)	3.78 (0.82)	93 (5.0)	0.37 (0.04)	20 (23)	NM
25%	100	16 (4.9)	95 (5.8)	3.15 (0.62)	70 (41)	0.41 (0.03)	40 (32)	NM
50%	100	16 (6.2)	98 (5.0)	3.48 (0.52)	93 (15)	0.42 (0.02)	93 (5.0)	NM
100%	100	17 (5.9)	98 (5.0)	3.63 (0.42)	93 (15)	0.46 (0.04)	73 (35)	NM
NOEC (%)	100	100	100	100	100	100	100[c]	
LOEC (%)	>100	>100	>100	>100	>100	>100	>100[c]	
IC20 (CI; %)	>100	<6.25	>100	>100	>100	>100	>100[c]	
Effluent 2								
0%	90	21 (8.6)	100 (0)	4.38 (0.28)	92 (10)	0.40 (0.03)	51 (30)	NM
6.25%	90	20 (7.1)	100 (0)	4.10 (0.50)	98 (5.0)	0.43 (0.02)	48 (36)	NM
12.5%	100	26 (9.3)	98 (5.0)	4.30 (0.25)	90 (14)	0.39 (0.05)	57 (40)	NM
25%	100	28 (4.1)	100 (0)	4.78 (0.34)	65 (36)	0.41 (0.02)	67 (38)	NM
50%	100	30 (3.9)	100 (0)	3.93 (0.39)	85 (13)	0.34 (0.01)	48 (37)	NM
100%	90	31 (8.5)	100 (0)	3.60 (0.22)	38 (43)	0.35 (0.03)[d]	2.5 (5.0)	NM
NOEC (%)	100	100	100	50	50	25	50[c]	
LOEC (%)	>100	>100	>100	100	100	50	100[c]	
IC20 (CI; %)	>100	>100	>100	>100	24 (12-67)	>100	53 (42-67)[c]	

[a] Mean initial shell length and standard deviation at the beginning of the test: 0.284 ± 0.029 mm (n = 14).
[b] Not measured because of low control survival.
[c] The value should be used with caution due to low control survival.
[d] Based on only three replicates (n=3) due to 100% mortality in one replicate at this exposure concentration.

Table A3 Water quality characteristics of three North Carolina effluent samples measured at the beginning and the end of 7-d test conducted in 2011 with cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas* [FHM]), and freshwater mussel (notched rainbow [NR], *Villosa constricta*).

Effluent	Test day	Dilution	Test species	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Hardness (as $CaCO_3$)	Alkalinity (as $CaCO_3$)	Ammonia (mg N/L)	Major cations and anions (mg/L)						
										Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	NO_3^{-}	SO_4^{2-}	Cl^{-}
Effluent 3	0	0%	All	8.4	7.99	257	100	90	0.07							
	0	25%	All	9.5	7.95	400	100	92	0.02							
	0	100%	All	10.5	7.53	417	50	82	0.04	15.0	11.5	3.5	69.1	2.6	18.3	66.9
	2	100%[a]	All	10.3	7.69	485	50	100	0.03							
	7	0%	NR	7.3	8.09	247	100	90	0.05							
	7	25%	NR	7.4	8.04	301	90	90	0.03							
	7	100%	NR	7.4	8.07	466	60	88	0.02							
	7	0%	FHM	7.0	8.03	384	112	100	0.55							
	7	25%	FHM	6.8	7.96	395	100	92	0.19							
	7	100%	FHM	7.0	8.06	599	62	100	0.17							
Effluent 4	0	0%	(the same control for Effluent 3 test)													
	0	25%	All	9.5	8.05	358	104	90	0.04							
	0	100%	All	11.5	7.33	657	120	94	0.08	45.3	13.9	5.2	80.4	98.9	41	60.5
	2	100%[a]	All	10.3	7.69	485	112	100	0.06							
	7	0%	NR	7.2	8.12	250	100	90	0.05							
	7	25%	NR	6.9	8.07	348	104	90	0.02							
	7	100%	NR	6.8	8.10	649	120	106	0.02							
	7	0%	FHM	7.0	8.03	389	118	100	0.90							
	7	25%	FHM	6.8	8.02	467	116	100	0.22							
	7	100%	FHM	6.6	8.02	765	118	110	0.15							
Effluent 5	0	0%	(the same control for Effluent 3 test)													
	0	25%	All	9.6	8.16	485	76	82	0.09							
	0	100%	All	10.7	7.27	594	38	70	0.16	12.2	10.5	3.4	105	53.7	58.9	67.7
	2	100%[a]	All	9.9	7.35	572	46	64	0.13							
	7	0%	NR	6.9	8.12	244	92	90	0.07							
	7	25%	NR	6.9	8.04	333	82	82	0.07							
	7	100%	NR	6.9	7.90	606	56	76								
	7	0%	FHM	7.3	8.20	362	102	92	0.55							
	7	25%	FHM	7.0	8.26	490	104	100	0.13							
	7	100%	FHM	6.7	7.87	681	60	70	0.05							

[a] The second batch of effluent samples were also measured when receiving on test Day 2.

Table A4. Responses of cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constricta*) in 7-d exposures to three permitted effluent samples. Values are means with standard deviation in parentheses (n=10 for the cladoceran and n=4 for other two species). Values in the shaded cell indicates a significant reduction in an endpoint relative to control (p< 0.05). No-observed-effect concentration [NOEC] and the lowest-observed-effect concentration [LOEC]), and 20% inhibition concentrations (IC20) with 95% confidence interval (CI) are presented for each endpoint. Mussel length data at the concentration above the NOEC for survival (means below dashed lines) were excluded from hypothesis testing when calculating the NOEC and LOEC.

	Cladoceran		Fathead minnow		Notched rainbow	
Treatment	Survival (%)	No. of young	Survival (%)	Biomass (mg)	Survival (%)	Length (mm)[a]
Effluent 3						
0%	30	9 (8.7)	88 (13)	5.00 (1.22)	93 (9.6)	0.40 (0.03)
6.25%	40	3 (4.0)	93 (10)	5.40 (1.15)	69 (21)	0.38 (0.02)
12.5%	20	2 (2.9)	95 (5.7)	5.20 (0.67)	63 (15)	0.33 (0.04)
25%	30	2 (3.7)	95 (10)	5.05 (0.70)	59 (22)	0.37 (0.04)
50%	50	0.5 (1.6)	85 (10)	4.85 (0.58)	72 (13)	0.36 (0.09)
100%	40	0.8 (2.5)	95 (5.8)	5.90 (0.54)	65 (5.8)	0.40 (0.05)
NOEC (%)	NR[b]	NR	100	100	50	50
LOEC (%)	NR	NR	>100	>100	100	>50
IC20 (CI; %)	NR	NR	>100	>100	<6.25	>100
Effluent 4						
0%	60	12 (11)	85 (10)	4.62 (0.97)	93 (9.6)	0.40 (0.01)
6.25%	70	6 (8.8)	95 (10)	6.32 (0.38)	85 (10)	0.39 (0.03)
12.5%	70	8 (11)	95 (5.8)	6.50 (0.82)	85 (10)	0.37 (0.04)
25%	70	14 (11)	100 (0)	6.40 (0.36)	37 (20)	0.40 (0.03)
50%	60	15 (17)	98 (5.0)	6.73 (0.42)	41 (7.0)	0.36 (0.06)
100%	50	15 (16)	95 (5.8)	7.40 (0.62)	18 (24)	0.38 (0.01)[c]
NOEC	NR	NR	100	100	12.5	12.5
LOEC	NR	NR	>100	>100	25	>12.5
IC20 (CI)	NR	NR	>100	>100	69 (23-120)	>100
Effluent 5						
0%	100	15 (5.6)	85 (10)	6.98 (0.17)	91 (11)	0.42 (0.03)
6.25%	30	3 (5.5)	98 (5.0)	8.02 (0.61)	77 (16)	0.43 (0.05)
12.5%	40	1 (4.4)	98 (5.0)	6.30 (1.60)	73 (15)	0.42 (0.01)
25%	40	0.8 (1.6)	100 (0)	7.27 (0.36)	83 (21)	0.39 (0.02)
50%	70	4 (5.0)	98 (5.0)	7.77 (0.43)	85 (5.8)	0.40 (0.01)
100%	40	1 (3.0)	95 (5.8)	8.12 (0.42)	88 (19)	0.37 (0.05)
NOEC	NR	NR	100	100	100	100
LOEC	NR	NR	>100	>100	>100	>100
IC20 (CI)	NR	NR	>100	>100	>100	>100

[a] Mean initial shell length and standard deviation at the beginning of the test: 0.330 ± 0.059 mm (n = 19).

[b] Not reported due to low control survival in two of the three effluent tests (possible due to low quality of test organisms used).

[c] Based on only two replicates (n=2) due to 100% mortality in two replicates at this exposure concentration.

Table B1. Concentrations of ammonia and five metals in two mock effluents used in 7-d toxicity tests with cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constrict*). Standard deviation in parentheses (n = 1 to 2 for metals and 7 for ammonia)

Species	Mock effluent	Dilution	Total ammonia (mg N/L)		Nickel (µg/L)		Copper (µg/L)		Zinc (µg/L)		Cadmium (µg/L)		Lead (µg/L)	
			Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured
Cladoceran	1	0%	0	0.30 (0.32)	0	0.9 (0.2)	0	0.4 (0.2)	0	7.6 (1.6)	0	0.02 (0.01)	0	0.05 (0.04)
		3.12%	0.25	0.32 (0.15)	13	13 (0.8)	0.4	0.7 (0.1)	30	26 (2.9)	0.06	0.06 (0.02)	0.3	0.14 (0.0)
		6.25%	0.5	0.54 (0.14)	26	26 (1)	0.8	1.1 (0.1)	60	47 (6.5)	0.13	0.11 (0.04)	0.6	0.25 (0.1)
		12.5%	1.0	0.99 (0.10)	52	55	1.5	1.6	120	96	0.25	0.23	1.3	0.5
		25%	2.0	1.98 (0.14)	104	109	3.0	2.5	240	195	0.5	0.46	2.5	1.0
		50%	4.0	4.01 (0.15)	208	217	6.0	5.4	480	464	1.0	1.03	5	3.2
	2	0%	0	0.24 (0.22)	0	0.9 (0.2)	0	0.4 (0.2)	0	7.6 (1.6)	0	0.02 (0.01)	0	0.05 (0.04)
		3.12%	0.1	0.16 (0.08)	12	12 (0.6)	2	2.0 (0.3)	31	27 (2.3)	2	1.7 (0.2)	6	2.7 (0.2)
		6.25%	0.2	0.26 (0.13)	23	24 (2)	4	3.0 (0.5)	62	41 (11)	4	3.1 (0.9)	11	4.4 (1.0)
		12.5%	0.4	0.42 (0.04)	46	49	8	6.0	124	95	8	8.0	22	10
		25%	0.8	0.80 (0.06)	92	97	16	13	248	232	16	16	44	28
		50%	1.6	1.63 (0.17)	184	193	32	26	496	483	32	33	88	62
Fathead minnow	1	0%	0	0.14 (0.09)	0	0.9 (0.2)	0	0.3 (0.03)	0	7.0 (0.6)	0	0.01 (0.0)	0	0.05 (0.4)
		6.25%	0.5	0.35 (0.48)	26	31 (5.3)	0.8	1.0 (0.02)	60	59 (24)	0.13	0.15 (0.02)	0.6	0.28 (0.1)
		12.5%	1.0	0.60 (0.91)	52	60 (7.2)	1.5	1.7 (0.1)	120	119 (33)	0.25	0.25 (0.03)	1.3	0.63 (0.2)
		25%	2.0	1.88 (0.34)	104	120 (16)	3.0	2.7 (0.4)	240	239 (62)	0.5	0.53 (0.09)	2.5	1.4 (0.6)
		50%	4.0	3.81 (0.60)	208	239 (31)	6.0	5.5 (0.2)	480	520 (79)	1.0	1.1 (0.12)	5	3.7 (0.7)
		100%	8.0	8.29 (0.27)	416	444	12	10	960	951	2.0	2.0	10	8
	2	0%	0	0.18 (0.14)	0	0.8 (0.0)	0	0.2 (0)	0	6.5 (0.0)	0	0.02 (0.01)	0	0.1 (0.0)
		6.25%	0.2	0.22 (0.04)	23	27 (2.6)	4	3.3 (1)	62	53 (27)	4	4.1 (0.5)	11	5.6 (2.9)
		12.5%	0.4	0.42 (0.04)	46	53 (6.5)	8	6.9 (1)	124	122 (38)	8	8.6 (0.9)	22	14 (5.7)
		25%	0.8	0.77 (0.09)	92	109 (17)	16	14 (1)	248	274 (59)	16	18 (2.6)	44	36 (11)
		50%	1.6	1.53 (0.32)	184	214 (30)	32	27 (3)	496	547 (90)	32	36 (4.8)	88	76 (20)
		100%	3.2	3.26 (0.12)	368	383	64	54	992	1040	64	66	176	158
Notched rainbow	1	0%	0	0.07 (0.01)	0	0.9 (0.1)	0	0.2 (0.1)	0	6.8 (0.4)	0	0.014 (0.0)	0	0.05 (0.03)
		3.12%	0.25	0.27 (0.04)	13	14 (0.4)	0.4	0.5 (0.2)	30	30 (8.7)	0.06	0.07 (0.0)	0.3	0.2 (0.0)
		6.25%	0.5	0.48 (0.07)	26	27 (0.0)	0.8	0.8 (0.3)	60	53 (15)	0.13	0.14 (0.01)	0.6	0.3 (0.1)
		12.5%	1.0	0.93 (0.09)	52	55 (0.5)	1.5	1.4 (0.4)	120	111 (21)	0.25	0.25 (0.03)	1.3	0.6 (0.1)
		25%	2.0	1.93 (0.21)	104	110 (1.4)	3.0	2.4 (0.1)	240	223 (38)	0.5	0.49 (0.04)	2.5	1.3 (0.4)
		50%	4.0	3.91 (0.32)	208	219 (2.1)	6.0	4.9 (0.6)	480	478 (20)	1.0	1.0 (0.02)	5	3.4 (0.3)
		100%	8.0	8.57 (0.27)	416	444 (0.7)	12	10 (0.1)	960	947 (6.4)	2.0	2.0 (0.06)	10	8.0 (0.1)
	2	0%	0	0.07 (0.01)	0	0.88 (0.2)	0	0.2 (0.1)	0	6.8 (0.4)	0	0.01 (0.01)	0	0.05 (0.04)
		3.12%	0.1	0.13 (0.02)	12	13 (0.1)	2	1.6 (0.1)	31	30 (6.0)	2	1.9 (0.1)	6	2.8 (0.1)
		6.25%	0.2	0.21 (0.02)	23	25 (0.1)	4	2.8 (0.01)	62	47 (20)	4	3.9 (0.2)	11	4.5 (1.4)
		12.5%	0.4	0.40 (0.06)	46	49 (0.4)	8	5.8 (0.2)	124	114 (28)	8	8.2 (0.3)	22	13 (4.1)
		25%	0.8	0.77 (0.10)	92	97 (0.1)	16	13 (0.1)	248	250 (25)	16	17 (0.4)	44	33 (7.1)
		50%	1.6	1.57 (0.22)	184	194 (0.7)	32	25 (0.5)	496	508 (35)	32	33 (0.9)	88	69 (10)
		100%	3.2	3.26 (0.12)	368	383 (0.0)	64	53 (1.3)	992	1030 (14)	64	66 (0.8)	176	155 (4.9)

Table B2. Water quality characteristics of two mock effluents used in 7-d toxicity tests with cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constrict*). Standard deviation in parentheses if n=2.

Species	Mock effluent	Dilution	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Hardness	Alkalinity
						(mg/L as $CaCO_3$)	
Cladoceran	1	0%	8.5 (0.3)	8.4 (0.02)	297 (47)	103 (1)	103 (4)
		3.12%	8.6 (0.3)	8.4 (0.04)	273 (9)	103 (1)	100 (0)
		6.25%	8.4	8.4	280	102	100
		12.5%	NM[a]	NM	NM	NM	NM
		25%	9.2	8.3	285	104	104
		50%	NM	NM	NM	NM	NM
	2	0%	8.7 (0.1)	8.4 (0.03)	272 (11)	104 (3)	103 (4)
		3.12%	8.6 (0)	8.4 (0.03)	276 (6)	105 (1)	99 (1)
		6.25%	8.4	8.4	284	106	96
		12.5%	NM	NM	NM	NM	NM
		25%	9.0	8.3	276	106	100
		50%	NM	NM	NM	NM	NM
Fathead minnow	1	0%	8.6 (0.2)	8.4 (0.02)	301 (52)	117 (21)	108 (3)
		6.25%	8.6	NM	345	NM	NM
		12.5%	8.5	8.4	328	134	112
		25%	8.9 (0.4)	8.3	313 (39)	104	104
		50%	8.7	8.3	352	106	100
		100%	NM	NM	NM	NM	NM
	2	0%	8.7 (0.2)	8.4 (0.02)	295 (43)	118 (22)	111 (7)
		6.25%	8.4	NM	325	NM	NM
		12.5%	8.5	8.4	329	134	116
		25%	8.7 (0.4)	8.3	300 (33)	106	100
		50%	8.5	8.4	341	134	110
		100%	10	8.3	286	106	100
Notched rainbow	1	0%	8.7 (0.1)	8.4 (0.1)	273 (12)	104 (3)	103 (4)
		3.12%	8.7 (0.1)	8.3	274 (11)	102	100
		6.25%	8.7	NM	284	NM	NM
		12.5%	8.8	8.4	288	106	102
		25%	8.9 (0.2)	NM	292 (9)	104	104
		50%	9	8.3	318	106	100
		100%	10.7	8.2	334	108	106
	2	0%	9.0 (0.3)	8.4 (0.1)	273 (13)	103 (4)	103 (1)
		3.12%	8.7 (0.2)	8.4	277 (6)	100	106
		6.25%	9	NM	283	NM	NM
		12.5%	8.8	8.4	284	106	98
		25%	8.9 (0.1)	NM	283 (10)	NM	NM
		50%	8.5	8.4	298	112	100
		100%	10.8	8.3	288	106	100

[a] Not measured.

Table B3. Responses of cladoceran (*Ceriodaphnia dubia*), fathead minnow (*Pimephales promelas*), and notched rainbow (*Villosa constricta*) in 7-d exposures to two mock effluents. Values are means with standard deviation in parentheses (n=10 for the cladoceran and n=4 for other two species). Value in shaded cells indicate a significant reduction in an endpoint relative to control (p< 0.05). No-observed-effect concentration [NOEC], lowest-observed-effect concentration [LOEC]), and 20% inhibition concentrations (IC20) with 95% confidence interval (CI) are presented for each endpoint.

	Cladoceran		Fathead minnow		Notched rainbow	
Treatment	Survival (%)	No. of young	Survival (%)	Biomass (mg)	Survival (%)	Length (mm)[a]
Mock Effluent 1						
0%	100	20 (6.3)	100 (0)	5.72 (0.72)	100 (0)	0.478 (0.023)
3.125%	100	15 (5.2)	NT[b]	NT	100 (0)	0.410 (0.007)
6.25%	90	11 (4.4)	100 (0)	5.92 (0.79)	98 (5.0)	0.374 (0.005)
12.5%	10	1.0 (2.1)	100 (0)	6.18 (0.83)	90 (9.0)	0.358 (0.013)
25%	0	0	90 (20)	5.30 (1.16)	80 (18)	0.347 (0.005)
50%	0	0	60 (14)	3.58 (0.81)	0	NA[b]
100%	NT[c]	NT	0	0	NT	NT
NOEC (%)	6.25	3.125	25	25	25	<3.125
LOEC (%)	12.5	6.25	50	50	50	3.125
IC20 (CI; %)	7.0 (4.3-7.8)	<3.1	34 (21-41)	33 (20-40)	25 (15-28)	5.6 (4.2-10)
Mock Effluent 2						
0%	100	21 (9.9)	100 (0)	6.50 (0.49)	100 (0)	0.473 (0.024)
3.125%	100	13 (6.4)	NT	NT	97 (6.0)	0.428 (0.008)
6.25%	90	11 (4.4)	98 (5)	5.75 (0.53)	100 (0)	0.393 (0.011)
12.5%	0	0	93 (10)	6.70 (0.43)	95 (5.8)	0.350 (0.006)
25%	0	0	85 (17)	4.50 (1.52)	85 (13)	0.340 (0.009)
50%	0	0	50 (22)	2.02 (0.90)	0	NA
100%	NT	NT	0	0	NT	NT
NOEC (%)	6.25	3.125	25	12.5	25	<3.125
LOEC (%)	12.5	6.25	50	25	50	3.125
IC20 (CI; %)	6.9 (4.3-7.5)	<3.2	29 (19-36)	20 (16-28)	26 (21-29)	8.3 (5.7-11)

[a] Mean initial shell length and standard deviation at the beginning of the test: 0.349 ± 0.025 mm (n = 20).

[b] Not applicable due to no survival at this exposure concentration; No length value at this concentration was used for calculating LOEC or IC20.

[c] Species was not tested at this exposure concentration.

Table C1. Survival and EC50s (95% confidence interval) in acute 4-d NaCl reference toxicant tests with two mussel species (notched rainbow, *Villosa constricta and* yellow lance, *Elliptio lanceolata*) in ASTM hard water (ASTM 2013a). Tests conducted in 2010.

Nominal concentration (g NaCl/L)	Measured salinity (g/L)	Survival (%)	
		Notched rainbow	Yellow lance
0	0.0	100	100
1	1.2	100	93
2	2.2	100	60
4	4.4	53	0
8	8.6	0	0
16	16.7	0	0
EC50 (CI)		3.9 (3.3-4.7)	2.1 (1.8-2.5)

Note: Only 3 replicates per concentration due to limited mussels.

Table C2. Mean concentrations of copper (n=2; standard deviation in parentheses) and water quality characteristics in acute 4-d copper reference toxicity tests with three mussel species (notched rainbow, *Villosa constricta*; yellow lance, *Elliptio lanceolata*; and yellow lampmussel, *Lampsilis cariosa*). Tests conducted in 2011.

Species	Nominal copper (µg Cu/L)	Measured copper (µg Cu/L)	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Alkalinity	Hardness
						(mg/L as $CaCO_3$)	
Notched rainbow	0	0.8 (0.1)	7.69	8.3	567	120	169
	6.25	5.3 (0.4)	NM[a]	NM	NM	NM	NM
	12.5	10 (0.9)	NM	NM	NM	NM	NM
	25	19 (1.4)	7.66	8.3	564	120	172
	50	37 (4.7)	NM	NM	NM	NM	NM
	100	70 (7.8)	7.74	8.4	560	120	166
Yellow lance	0	0.6 (0.1)	8.20	8.4	578	122	166
	6.25	5.3 (0.5)	NM	NM	NM	NM	NM
	12.5	9.3 (0.9)	NM	NM	NM	NM	NM
	25	19 (1.5)	7.96	8.3	570	120	164
	50	36 (4.0)		NM	NM	NM	NM
	100	70 (8.7)	8.12	8.3	570	120	166
Yellow lampmussel	0	0.7 (0.3)	8.10	8.4	552	114	156
	6.25	5.2 (0.2)	7.97	8.4	522	114	148
	12.5	10 (0.4)	NM	NM	NM	NM	NM
	25	20 (0.6)	7.75	8.4	528	114	156
	50	39 (1.1)	NM	NM	NM	NM	NM
	100	75 (2.8)	8.10	8.4	552	114	156

[a] Not measured.

Table C3. Mean concentrations of total ammonia (n=4; standard deviation in parentheses) and water quality characteristics in acute 4-day ammonia reference toxicity tests with three mussel species (notched rainbow, *Villosa constricta*; yellow lance, *Elliptio lanceolata*; and yellow lampmussel, *Lampsilis cariosa*). Tests conducted in 2011.

Species	Nominal ammonia (mg N/L)	Measured ammonia (mg N/L)	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Notched rainbow	0	0.1 (0.1)	7.88	8.4	569	120	166
	1	1.0 (0.1)	NM[a]	8.4	NM	NM	NM
	2	2.0 (0.1)	NM	8.4	NM	NM	NM
	4	3.9 (0.1)	7.91	8.4	608	120	168
	8	6.8 (0.5)	NM	8.4	NM	NM	NM
	16	18 (2.7)	7.91	8.3	731	120	168
Yellow lance	0	0.1 (0.01)	7.95	8.4	575	122	170
	1	1.0 (0.1)	NM	8.4	586	NM	NM
	2	1.9 (0.1)	NM	8.3	594	NM	NM
	4	3.4 (0.2)	8.04	8.4	608	124	170
	8	6.2 (0.1)	NM	8.3	633	NM	NM
	16	17.4 (0.2)	7.87	8.3	731	128	170
Yellow lampmussel	0	0.1 (0.03)	7.86	8.4	538	110	144
	1	1.1 (0.5)	NM	8.4	NM	NM	NM
	2	2.0 (0.7)	NM	8.5	NM	NM	NM
	4	3.6 (0.9)	7.75	8.5	586	114	136
	8	7.0 (0.5)	NM	8.4	NM	NM	NM
	16	16 (1.6)	7.87	8.4	701	114	136

[a] Not measured.

Table C4. Mean salinity (n=2; standard deviation in parentheses) and water quality characteristics in acute 4-day NaCl reference toxicity test with notched rainbow (*Villosa constricta*). Tests conducted in 2011.

Species	Nominal NaCl (g/L)	Measured Salinity (g/L)	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Notched rainbow	0	0 (0)	8.8	8.4	569	120	166
	1	1.1 (0.1)	NM[a]	8.4	NM	NM	NM
	2	2.1 (0.0)	NM	8.4	NM	NM	NM
	4	4.2 (0.1)	8.81	8.4	608	120	168
	8	8.2 (0.1)	NM	8.4	NM	NM	NM
	16	16.3 (0.1)	8.89	8.3	731	120	168

[a] Not measured.

Table C5. Survival and EC50s (95% confidence interval) in acute 4-d reference toxicant tests with three mussel species (notched rainbow, *Villosa constricta;* yellow lance, *Elliptio lanceolata*; and yellow lampmussel, *Lampsilis cariosa*) in ASTM hard water (ASTM 2013a). Tests conducted in 2011.

Notched rainbow		Yellow lance		Yellow lampmussel	
Measured concentration	Survival (%)	Measured concentration	Survival (%)	Measured concentration	Survival (%)
Copper (µg Cu/L)					
0.78	100	0.56	100	0.67	100
5.34	90	5.26	100	5.24	100
9.97	50	9.32	75	10.45	80
18.80	40	18.55	25	20.35	90
36.85	0	35.75	24	39.30	40
70.30	0	70.15	5	74.50	42
EC50 (CI)	12 (9.6-15)		16 (13-20)		46 (32-68)
Total ammonia (mg N/L)					
0.05	100	0.09	76	0.07	95
1.04	91	0.97	90	1.11	100
1.98	94	1.85	71	2.01	97
3.90	67	3.42	21	3.61	100
6.84	0	6.15	0	6.99	28
18.40	0	17.35	0	16.05	0
EC50 (CI)	3.5 (2.9-4.3)		2.2 (1.9-2.5)		8.2 (7.2-9.2)
Sodium chloride (salinity, g/L)					
0.0	95	NT[a]		NT	
1.1	100	NT		NT	
2.1	100	NT		NT	
4.2	53	NT		NT	
8.2	0	NT		NT	
16.3	0	NT		NT	
EC50 (CI)	4.1 (3.5-4.8)[a]	NT		NT	

[a] Not tested

[b] EC50 for NaCl was estimated based on nominal NaCl concentrations of 0, 1, 2, 4, 8, and 16 g NaCl/L.

Table C6. Mean copper concentrations and water quality characteristics (n=2; standard deviation in parentheses) in acute 4-day copper reference toxicity tests with three mussel species (yellow lance, *Elliptio lanceolata*; notched rainbow, *Villosa constrict*; Tar River spinymussel, *Elliptio steinstansana*). Tests conducted in 2012.

Species	Nominal copper (µg Cu/L)	Measured copper (µg Cu/L)	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Notched rainbow	0	1.1	8.7	8.6	588	124	154
	6.25	7.2	NM[a]	NM	NM	NM	NM
	12.5	8.6	NM	NM	NM	NM	NM
	25	25	8.6	8.6	589	116	148
	50	33	NM	NM	NM	NM	NM
	100	88	8.7	8.6	598	120	160
Yellow lance	0	0.8 (0.01)	8.4 (0.3)	8.5 (0)	602 (37)	119 (4)	167 (4)
	6.25	5.8 (0.4)	8.3 (0.3)	8.3 (0)	610 (3)	120 (0)	163 (1)
	12.5	11 (0.1)	NM	NM	NM	NM	NM
	25	23 (0.4)	8.5 (0.2)	8.4 (0.1)	586 (13)	117 (4)	164 (6)
	50	44 (0.2)	NM	NM	NM	NM	NM
	100	81 (3.8)	8.4 (0.4)	8.4 (0.1)	581 (11)	117 (4)	159 (10)
Spinymussel	0	0.7 (0.04)	8.4 (0.4)	8.5 (0)	582 (8)	118 (3)	167 (4)
	6.25	6.8 (0.3)	8.4 (0.4)	8.4 (0.01)	584 (6)	121 (1)	164 (3)
	12.5	11 (0.6)	NM	NM	NM	NM	NM
	25	22 (1.1)	8.5 (0.3)	8.4 (0.02)	581 (6)	117 (4)	162 (2)
	50	44 (0.4)	NM	NM	NM	NM	NM
	100	82 (7.2)	8.5 (0.4)	8.4 (0.1)	579 (8)	116 (3)	158 (8)

[a] Not measured.

Table C7. Mean concentrations of total ammonia (n=4) and water quality characteristics (n=2) for acute 4-day ammonia toxicity tests with yellow lance (*Elliptio lanceolata*) and Tar River spinymussel (*Elliptio steinstansana*). Standard deviation in parentheses. Tests conducted in 2012.

Nominal ammonia (mg N/L)	Measured ammonia (mg N/L)	Dissolved oxygen (mg/L)	pH	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
0	0.1 (0.02)	8.4 (0.1)	8.5 (0.03)	616 (16)	121 (4)	163 (4)
1	0.8 (0.12)	8.6 (0.1)	8.4 (0.3)	633 (30)	123 (3)	164 (3)
2	1.6 (0.18)	NM[a]	NM	NM	NM	NM
4	3.6 (0.26)	8.4 (0.1)	8.4 (0.01)	659 (18)	122 (3)	164 (6)
8	7.7 (0.40)	NM	NM	NM	NM	NM
16	18 (0.41)	8.2 (0.4)	8.3 (0.01)	729 (66)	123 (4)	166 (3)

[a] Not measured.

Table C8. Survival and EC50s (95% confidence interval, CI) in acute 4-d reference toxicant tests with three mussel species (yellow lance, *Elliptio lanceolata*; notched rainbow, *Villosa constrict*; Tar River spinymussel, *Elliptio steinstansana*). Tests conducted in 2012.

Notched rainbow		Yellow lance		Spinymussel	
Measured concentration	Survival (%)	Measured concentration	Survival (%)	Measured concentration	Survival (%)
Copper (µg Cu/L)					
1.1	100	0.8	100	0.8	100
7.2	100	6.0	100	6.8	100
8.6	100	11	60	11	95
25	90	23	35	22	80
33	75	44	5	44	5
88	0	81	0	82	0
EC50 (CI)	39 (32-48)		16 (13-19)		26 (22-31)
Total ammonia (mg N/L)					
NT[a]		0.1	100	0.1	100
NT		0.8	100	0.8	96
NT		1.6	69	1.6	100
NT		3.6	61	3.6	54
NT		7.7	0	7.7	0
NT		18	0	18	0
EC50 (CI)			3.1 (2.6-3.7)		3.6 (3.1-4.3)

[a] Not tested