SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 26, No. 10, pp. 2029–2035, 2007
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Printed in the USA
0730-7268/07 $12.00 + .00

Contaminant Sensitivity of Freshwater Mussels

INTRA- AND INTERLABORATORY VARIABILITY IN ACUTE TOXICITY TESTS WITH GLOCHIDIA AND JUVENILES OF FRESHWATER MUSSELS (UNIONIDAE)

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(*Received* 16 *October* 2006; *Accepted* 18 *April* 2007)

Abstract—The present study evaluated the performance and variability in acute toxicity tests with glochidia and newly transformed juvenile mussels using the standard methods outlined in American Society for Testing and Materials (ASTM). Multiple 48-h toxicity tests with glochidia and 96-h tests with juvenile mussels were conducted within a single laboratory and among five laboratories. All tests met the test acceptability requirements (e.g., ≥90% control survival). Intralaboratory tests were conducted over two consecutive mussel-spawning seasons with mucket (*Actinonaias ligamentina*) or fatmucket (*Lampsilis siliquoidea*) using copper, ammonia, or chlorine as a toxicant. For the glochidia of both species, the variability of intralaboratory median effective concentrations (EC50s) for the three toxicants, expressed as the coefficient of variation (CV), ranged from 14 to 27% in 24-h exposures and from 13 to 36% in 48-h exposures. The intralaboratory CV of copper EC50s for juvenile fatmucket was 24% in 48-h exposures and 13% in 96-h exposures. Interlaboratory tests were conducted with fatmucket glochidia and juveniles by five laboratories using copper as a toxicant. The interlaboratory CV of copper EC50s for glochidia was 13% in 24-h exposures and 24% in 48-h exposures, and the interlaboratory CV for juveniles was 22% in 48-h exposures and 42% in 96-h exposures. The high completion success and the overall low variability in test results indicate that the test methods have acceptable precision and can be performed routinely.

Keywords—Freshwater mussels Glochidia Juvenile mussels Toxicity test Variability

INTRODUCTION

Most freshwater mussels have a complex reproductive cycle involving a parasitic stage on fish. Sperm released by a male enters a female through the incurrent siphon, and fertilized eggs develop to larvae called glochidia that mature in specialized chambers (marsupia) of the female's gills. Glochidia are released into the water and must attach to the gills or fins of a suitable host fish. After one to several weeks of the parasitic stage, glochidia transform to juvenile mussels, detach from the fish, and drop to the stream or lake bottom to begin the free-living juvenile stage.

Approximately 70% of North American freshwater mussel species are considered to be endangered, threatened, or of special concern [1,2]. Contamination is considered one of the causal or contributing factors to the declines of freshwater mussel populations [2,3]. The effects of contaminants on freshwater mussels have been studied increasingly over the past 20 years [4]. Glochidia and juvenile mussels are reportedly more sensitive to some chemicals when compared to commonly tested aquatic organisms [5,6]. However, the U.S. Environmental Protection Agency (U.S. EPA) generally has not used toxicity data generated from freshwater mussels in the derivation of water quality criteria, mainly due to a lack of standardized guidance for conducting toxicity tests with freshwater mussels [4].

A joint research project was conducted to develop standardized guidance for conducting toxicity tests with mussels [4], and an American Society for Testing and Materials (ASTM) International Standard guide for conducting laboratory toxicity tests with freshwater mussels recently was published [7]. As one of a series of papers evaluating contaminant sensitivity of early life stages of freshwater mussels [8], the present paper summarizes the results of toxicity tests conducted repeatedly within a single laboratory and among five laboratories to evaluate the test performance and variability within and among laboratories. The intralaboratory test results primarily were used to evaluate temporal variability within a year and between years. The interlaboratory tests were designed to evaluate whether multiple laboratories could conduct the methods successfully and how experience with mussel toxicity testing, shipping, and handling of test organisms influenced test results. By using the same source of dilution water, laboratory supplies, and test organisms at all laboratories we intentionally reduced some potential sources of interlaboratory

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variability. The participating laboratories included two federal and three university facilities with considerable experience in conducting freshwater toxicity tests and with varying experience in conducting toxicity tests with mussels.

MATERIALS AND METHODS

Toxicity testing with glochidia

Obtaining glochidia. Gravid female mucket (*Actinonaias ligamentina*) were collected from the Big River (St. Louis, MO, USA), and fatmucket (*Lampsilis siliquoidea*) were collected from Silver Fork of Perche Creek (Boone County, MO, USA) between March and April of 2003 and 2004. Both species are long-term brooders, which spawn in late summer and brood glochidia over winter for release the following spring [9]. Over 90% of glochidia isolated from both species can remain viable for more than 6 d at 20°C [4]. Female mussels were brought to the Columbia Environmental Research Center (CERC), U.S. Geological Survey ([USGS], Columbia, MO), and held in a 600-L flow-through fiberglass tank with well water (hardness 280 mg/L as $CaCO_3$, alkalinity 250 mg/L as $CaCO_3$, pH 7.8) at a flow rate of approximately 2 L/min. Water was aerated and maintained at 10 to 13°C to prevent the mussels from releasing glochidia. Plastic containers (35 × 24 × 23 cm) with a 10-cm layer of creek gravel (0.2–1.5 cm diameter) were submerged in the tank. Five adult mussels were placed in each container. Approximately 20 ml of a commercial nonviable microalgal *Nannochloropsis* concentrate (Reed Mariculture, Campbell, CA, USA) and 20 ml of Shellfish Diet (a mix of four marine microalgae, i.e., *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira* [Reed Mariculture]) were added to the tank every morning. The food remained in the water column for approximately 6 h. In late September of each year, the adult mussels were returned to the sites of collection.

Toxicity tests with glochidia. Test conditions and procedures for conducting toxicity tests were in accordance with the recommendations outlined by ASTM [7]. Before starting a toxicity test, the viability of glochidia isolated from each mussel was determined. Glochidia were flushed gently from the marsupium of a mussel into a 300-ml crystallizing dish using a large bore (~1 mm) needle and 35-ml syringe filled with culture water. Three subsamples of 100 to 200 glochidia were impartially taken from the dish using a 2-mm wide-bore pipette and transferred to each of three wells of a 24-well polystyrene tissue-culture plate filled with approximately 2 ml of well water. Glochidia in each well were examined with a dissecting microscope at ×10 magnification, and the number of closed glochidia was recorded. After adding one drop of saturated NaCl solution (~12 g of reagent-grade NaCl in 50 ml of deionized water) to each well, open and closed glochidia were counted within 1 min. Valve closure is an ecologically relevant endpoint that is critical for glochidia to successfully transform onto the host [7]. Glochidia that closed their valves in response to NaCl were categorized as alive (i.e., viable), and glochidia that were closed before the addition of NaCl or that remained open after the addition of NaCl were categorized as dead (i.e., nonviable). Survival (viability) of glochidia was calculated as

$$\text{Survival (\%)} = 100 \times (\text{Number of closed glochidia after adding NaCl solution} - \text{Number of closed glochidia before adding NaCl solution}) \div (\text{Total number of open and closed glochidia after adding NaCl solution})$$

If the survival of glochidia from an individual mussel was >90%, the remaining glochidia isolated from that mussel were used for toxicity testing. Glochidia isolated from five or six mussels were pooled and mixed in a Pyrex® glass baking dish (29 × 19 × 24 cm) for a test. Glochidia were acclimated to a mixture of 50% well water and 50% dilution water, and the water gradually was adjusted to the test temperature over a period of 2 h before the start of a toxicity test.

Test conditions for conducting the toxicity tests with glochidia were summarized in Table 1. The reconstituted ASTM hard water [10] was used as dilution water in all tests. Copper sulfate ($CuSO_4$, 99.9% purity; JT Baker, Phillipsburg, NJ, USA), ammonium chloride (NH_4Cl, 99.5% purity; Fisher Scientific, Houston, TX, USA), and sodium hypochlorite (NaOCl, available chlorine 10–13%; Aldrich, Milwaukee, WI, USA) were used as toxicants. The three toxicants were selected because of their wide occurrence in contaminated aquatic environments and limited toxicity data of these toxicants for freshwater mussels [6,11,12]. Each test consisted of three replicates at each of five exposure concentrations in a 50% serial dilution, plus a control. Copper or ammonia toxicity tests were conducted in 200-ml glass crystallizing dishes (75 mm diameter, 35 mm high) containing approximately 100 ml of dilution water. Because chlorine is highly volatile, chlorine toxicity tests were conducted in a flow-through diluter system [13]. The diluter system provided approximately 120 ml of solution to each 300-ml beaker every 20 min. An inline flow splitter was attached to each delivery line to partition the water flow evenly to each of replicate beakers. Each beaker had a 2.5-cm hole in the side covered with 50 mesh (279-μm opening) stainless steel screen and contained 200 ml of water. The diluter system delivered five concentrations with a dilution factor of 0.5, plus a control. Chlorine stock solution was held in a 600-ml plastic blood pack (Baxter Healthcare, Deerfield, IL, USA) to reduce volatilization of chlorine from the stock solution and was delivered with each cycle of the diluter by a Hamilton syringe pump (Hamilton, Reno, NV, USA).

At the start of a toxicity test, approximately 1,000 glochidia were transferred impartially from the pooled sample of glochidia into each of 18 test chambers held in water baths at 20 ± 1°C. Dissolved oxygen, pH, conductivity, hardness, and alkalinity were measured on composite samples of initial and final test solutions at the control, medium, and high concentrations using standard methods [14].

During a 48-h exposure, survival was determined at 24 h and 48 h. A subsample of approximately 100 glochidia with 2 ml of dilution water was taken from each replicate test chamber using a 2-mm wide-bore pipette and placed into one well of a clean 24-well tissue-culture plate. One drop of the NaCl solution was added into the well, and the response of glochidia (valve closure) within 1 min was recorded. Glochidia added into test chambers generally were not 100% viable at the beginning of a test. Hence, initial survival for a test was estimated by determining the viability of three replicate controls at the beginning of the test. The mean estimated viability value was used to adjust the survival of glochidia after 24- or 48-h exposures. For example, if the mean viability in the control was 90% at 0 h, and an observed viability of glochidia in a replicate test chamber at 24 h was 85%, then the adjusted survival at 24-h exposure was 94% (85/90). Adjusted survival values were used for the calculation of acute effect concentrations and control survival over the exposure periods.

Table 1. Summary of test conditions and test acceptability criteria for conducting toxicity tests with glochidia of freshwater mussels in basic accordance with American Society for Testing and Materials (ASTM) [7]

Test species:	Mucket (*Actinonaias ligamentina*) or fatmucket (*Lampsilis siliquoidea*)
Glochidia collection:	Flush mussel gills with water from syringe
Test chemicals:	Copper sulfate, ammonium chloride, or sodium hypochlorite
Test type:	Static for copper or ammonia tests; flow-through with a diluter system for chlorine tests
Test duration:	24 and 48 h
Temperature:	20±1°C
Light quality:	Ambient laboratory light
Light intensity:	100 to 200 lux
Photoperiod:	16:8 h light:dark
Test chamber:	200-ml crystallizing dish for static tests; 300-ml glass beaker for flow-through tests
Test solution volume:	100 ml in a dish; 200 ml in a beaker
Renewal of solution:	None for static tests; additional 120-ml solution to each beaker once every 20 min for flow-through tests
Age of test organism:	<2 h after releasing from marsupium
Organisms/chamber:	About 1,000
Replicates/concentration:	3
Feeding:	None
Aeration:	None
Dilution water:	Reconstituted ASTM hard water (hardness 160 to 180 mg/L as $CaCO_3$, alkalinity 110 to 120 mg/L as $CaCO_3$; ASTM [10])
Dilution factor:	0.5
Test concentration:	Copper: 0, 6.25, 12.5, 25, 50, and 100 μg/L
	Total ammonia: 0, 1, 2, 4, 8, and 16 mg N/L
	Chlorine: 0, 6.25, 12.5, 25, 50, and 100 μg/L
Chemical residues:	Collect water samples from each concentration at 0 and 48 h
Water quality:	Measure dissolved oxygen, pH, conductivity, hardness, and alkalinity at the control, medium, and highest exposure concentrations at 0 and 48 h
Endpoint:	Survival (valve closure with the addition of NaCl)
Test acceptability:	≥90% survival in controls

Toxicity testing with juvenile mussels

Transformation and culture of juvenile mussels. Gravid fatmucket were collected from Silverfork Creek (Boone County, MO, USA) between March and April of 2004 and 2005 and were brought to Missouri State University (Springfield, MO, USA) during the spawning season. Glochidia were isolated and pooled from six female mussels as described above. Host fish were hatchery-reared largemouth bass (*Micropterus salmoides*, approximately 8 cm total body length) obtained from the Missouri Department of Conservation, Chesapeake Fish Hatchery (Chesapeake, MO, USA). The host fish were inoculated with glochidia to obtain an infesting level of approximately 200 encysted glochidia per fish. The fish were maintained in a hatchery raceway for the first week after inoculation. Afterwards, the fish were moved to a recirculating system designed to recover excysting juvenile mussels and were not fed during the next two weeks (see details in a final report for the project entitled *Culture and Restoration of Special Concern Mussel Species*, prepared for Missouri Department of Conservation by C. Barnhart, Missouri State University, Springfield, MO, USA, unpublished data). Juvenile mussels were collected daily from 150-μm plankton nets and isolated from debris using Nitex® nylon screens (225 and 150 μm; TETKO, Elmsford, NY, USA). The newly transformed juveniles were held in the reconstituted ASTM hard water and fed laboratory-cultured algae (*Neochloris oleoabundans*; obtained from the Missouri State University) ad libitum. The daily catch and time-course of recovery of juveniles were monitored and recorded to determine the peak excysting period. Juvenile mussels derived from a single collection day during the peak of drop-off (excystment) from the host fish were shipped overnight to laboratories for toxicity tests. Either plastic bags or square, 250-ml wide-mouth polyethylene bottles containing clean culture water were used to hold juvenile mussels. Containers were placed into coolers for shipping. Teflon® tape was wound around the threads of the bottle to help seal the lid. Bubble wrap, newspaper, or foam peanuts was added to reduce jostling and keep the bottles or bags more secure in the coolers. To stabilize the temperature of shipping, ice packs were placed into the coolers but not in direct contact with the containers holding mussels [7].

Toxicity test with juvenile mussels. Test conditions and procedures for conducting toxicity tests with newly transformed juvenile mussels (Table 2) were in accordance with the recommendations outlined in ASTM [7]. When 1-d-old juvenile mussels were received in a laboratory, the temperature of the water was adjusted to the test temperature by placing the containers containing mussels into a water bath at 20°C. Approximately 50% of the water in the containers then was replaced gradually with dilution water at least three times over a 24- or 48-h acclimation period. Gentle aeration of water was provided to the containers through a glass pipette. During the acclimation period, the juveniles were fed live algae (*N. oleoabundans*; obtained from the Missouri State University) at a feeding rate of approximately 20,000 cell/ml once daily. Dilution water, dilution factor, and the collection and measurement of water quality characteristics were the same as those described above for the glochidia tests. However, test duration of the juvenile mussel tests was 96 h and copper sulfate ($CuSO_4$, 99.9% purity; JT Baker) was used as a toxicant. At the start of a toxicity test, juvenile mussels in a stock dish were observed with a dissecting microscope. Five juveniles exhibiting foot movement were transferred impartially into each of twenty-four 50-ml glass beakers using a 1-ml syringe with a 2.5-cm-long, 16-gauge needle connected to a 60-cm-long Tygon® tubing (1.0-mm inner diameter; Saint-Gobain Performance Plastics, Akron, OH, USA) with a glass capillary tube (1.17-mm inner diameter) at the end [7]. Test solution was renewed at 48 h by replacing approximately 75% of the test solution volume. Survival of juvenile mussels was deter-

Table 2. Summary of test conditions and test acceptability criteria for conducting toxicity tests with juvenile mussels in basic accordance with American Society for Testing and Materials (ASTM) [7]

Test species	Fatmucket (*Lampsilis siliquoidea*)
Test chemicals	Copper sulfate
Test type	Static renewal
Test duration	96 h (also check survival at 48 h)
Temperature	20 ± 1°C
Light quality	Ambient laboratory light
Light intensity	100 to 200 lux
Photoperiod	16:8 h light:dark
Test chamber	50-ml glass beaker
Test solution volume	30 ml
Renewal of solution	After 48 h
Age of test organism	<5 d old (after release from the gill of host fish)
Organisms/chamber	5
Replicates/concentration	4
Feeding	None
Aeration	None
Dilution water	Reconstituted ASTM hard water (hardness 160 to 180 mg/L as $CaCO_3$, alkalinity 110 to 120 mg/L as $CaCO_3$; ASTM [10])
Dilution factor	0.5
Test concentration	Copper: 0, 6.25, 12.5, 25, 50, and 100 μg/L
Chemical residues	Collect water samples from each concentration at 0 and 96 h
Water quality	Measure dissolved oxygen, pH, conductivity, hardness, and alkalinity at the control, medium, and highest exposure concentrations at 0 and 96 h
Endpoint	Survival (foot movement)
Test acceptability	≥90% survival in control

mined at 48 and 96 h using dissecting microscopes. In order to observe the juveniles under the microscope during a test, it was necessary to remove some of the dilution water from the beaker. Gently swirling the beaker created a slight vortex in the water and concentrated the juveniles in a small area, making it easier to see all of the organisms simultaneously in the field of view under the microscope. Juvenile mussels that exhibited foot movement within a 5-min observation period were classified as alive [7].

Intralaboratory testing

Four toxicity tests with mucket glochidia and six or seven toxicity tests with fatmucket glochidia were conducted with copper, ammonia, or chlorine at CERC between April and September of 2003 and 2004. Five copper toxicity tests with juvenile fatmuckets also were conducted at CERC between May and August of 2004 and 2005. Test organisms used over one calendar year originated from the same female mussels collected in that year.

Interlaboratory testing

Toxicity tests with fatmucket glochidia or juveniles were conducted by the five laboratories using copper as a toxicant in July and August 2005. Detailed toxicity test procedures, test organisms, and test materials including test chambers, the saturated NaCl solution for determining the viability of glochidia, dilution water, and data forms were supplied by CERC to each laboratory. The reconstituted ASTM hard water, the test solution of the highest copper concentration, and the isolated glochidia or newly transformed juvenile mussels were shipped overnight in a cooler with ice packs to each laboratory. All tests began when glochidia were approximately 24 h old and juvenile mussels were 4 d old (after release from the gills of host fish). Previous studies indicated that median effective concentrations (EC50s) for copper were similar when using glochidia isolated from a female fatmucket after 2 or 24 h [4]. Water samples collected during toxicity tests for dissolved copper analysis were shipped overnight in a cooler with ice packs to CERC. Data from all tests were sent to CERC for analysis.

Chemical analysis

Water samples for analysis of toxicants were collected at the beginning and the end of each toxicity test. A 30-ml sample was collected by compositing approximately 10 ml of test solution from three or four replicates of each exposure concentration. Total ammonia nitrogen of each concentration in ammonia tests was measured within 1 h of sample collection using an Orion Ammonia Electrode and Orion EA940 meter (Thermo Electron, Beverly, MA, USA). The detection limit was 0.1 mg/L. Water samples for chlorine measurement were analyzed immediately after sample collection. Total residual chlorine was measured at the highest chlorine concentrations (50 or 100 μg/L) with a Hach spectrophotometer (model DR/2000, Hach, Loveland, CO, USA) using *N,N*-diethyl-*p*-phenylenediamine method 8370 for clean water (http://www.hach.com). The lower concentrations (<50 μg/L) could not be detected consistently using this meter and therefore were not measured. The meters for total ammonia and chlorine analyses were calibrated each time before measuring samples following procedures recommended by the manufacturers.

Water samples for copper analysis were acidified to 1% (v/v) ultrapure nitric acid. Water samples collected at the beginning of glochidia tests and at the beginning and the end of juvenile mussel tests were analyzed at all or at the control, low-, medium-, and high-copper concentrations. Additionally, samples collected at the end of four glochidia tests were analyzed to determine the consistency of waterborne copper concentrations over the 48-h exposure period. Dissolved copper concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS, PE Sciex Elan 6000, PerkinElmer, Norwalk, CT, USA). Samples were delivered automatically to the ICP-MS by means of a software-controlled CETAC ASX-500/ADX-100 autosampler/autodiluter system (CETAC Technologies, Omaha, NE, USA). A calibration blank and an independent calibration verification standard were an-

Table 3. Ranges of water quality characteristics and percent nominal concentrations for measured test concentrations in intra- and interlaboratory toxicity tests with glochidia and juvenile mucket (*Actinonaias ligamentina*) or fatmucket (*Lampsilis siliquoidea*). Values of pH, alkalinity, hardness, and conductivity are means of measurements of control, medium-, and high-exposure concentrations at the beginning and the end of each test. Values of percent nominal concentrations are mean percentages of nominal concentrations for measured concentrations at the beginning of each 48-h static copper or ammonia tests with glochidia, or for average measured concentrations at the beginning and the end of each 48-h flow-through chlorine tests with glochidia or 96-h static-renewal copper test with juvenile mussels

Species	Life stage	Toxicant	No. of tests	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (μS/cm)	Percent nominal exposure concn.
Intralaboratory tests								
Mucket	Glochidia	Copper	4	8.4–8.5	118–128	173–187	537–620	97–124
	Glochidia	Ammonia	4	8.3–8.6	109–122	160–188	571–669	98–119
	Glochidia	Chlorine	3	8.4–8.6	114–122	169–186	541–594	89–104
Fatmucket	Glochidia	Copper	7	8.3–8.7	114–125	152–184	570–652	105–118
	Glochidia	Ammonia	6	8.2–8.5	115–124	159–183	605–677	95–118
	Glochidia	Chlorine	6	8.2–8.5	112–119	160–170	553–563	101–112
	Juvenile	Copper	5	8.5–8.7	119–132	164–193	566–603	99–114
Interlaboratory tests								
Fatmucket	Glochidia	Copper	5	8.0–8.6	117–133	157–185	491–600	85–111
	Juvenile	Copper	5	8.0–8.6	119–126	172–193	438–610	90–109

alyzed every 10 samples to confirm the calibration status of the ICP-MS during instrumental analyses of all water samples. Results from the analysis of a reference solution used as a laboratory control sample in ICP-MS quantitative analysis indicated that copper recoveries ranged from 93 to 101%. Analytical precision for quantitative ICP-MS was determined by analyzing samples in duplicate during the instrumental run and determining the relative percent differences, which were $<2.2\%$ for all analysis duplicates. Recoveries of copper spiked into water samples and analyzed by quantitative ICP-MS ranged from 85 to 107%. Instrumental detection limit was <0.016 μg/L, and the method detection limit was <0.2 μg/L.

Data analysis

Mean values of water quality for each toxicity test were calculated based on measurements of the control, medium-, and high-exposure concentrations at the beginning and the end of each test. The EC50s were calculated using a Probit model when appropriate and either a Spearman-Karber or trimmed Spearman-Karber method otherwise [15] with Toxstat® software [16]. Nominal concentrations were used for the EC50 calculations. Intra- and interlaboratory variability was described by the mean, standard deviation (SD), and coefficient of variation (CV) of the calculated EC50s from the replicated toxicity tests. The CV was calculated by dividing the SD by the mean and then multiplying by 100. A few EC50s from tests with total ammonia and chlorine were greater than the highest exposure concentrations. These values were considered as estimated EC50s for the calculation of the mean EC50 and CV. Although this approach biases the mean by using a number lower than the actual EC50, not including this high number would provide an even greater bias in the analysis.

RESULTS AND DISCUSSION

Water quality

Hardness and alkalinity of the dilution water (Table 3) were typically within the ranges of the reconstituted hard water listed in ASTM [10]. The pH ranged from 8.2 to 8.7 for intralaboratory tests and ranged from 8.0 to 8.6 for interlaboratory tests (Table 3), which were higher than the listed range of 7.8 to 8.0 in ASTM [10]. Higher pH values of the reconstituted hard water have been reported in previous studies [17–19]). The ranges of pH, alkalinity, hardness, and conductivity within each intra- or interlaboratory test were generally low, except for pH and conductivity in the interlaboratory tests (Table 3). Because the same dilution water was used at the five laboratories for the interlaboratory tests, variation of measured pH and conductivity may have been due to the difference in the methods used to measure pH by the individual laboratories. Dissolved oxygen was above 7.4 mg/L during all tests.

Measured exposure concentrations

Mean percent change of copper concentrations between the beginning and the end of the exposure was 9.3% (SD = 9.0%, n = 12) in four 48-h glochidia toxicity tests, and was 14% (SD = 18%, n = 39) in ten 96-h juvenile tests (individual measurements for each test are reported in a USGS project summary, unpublished data). Average percentage of measured copper concentrations ranged from 97 to 124% of the nominal concentrations in the intralaboratory tests and from 85 to 111% of the nominal concentrations in the interlaboratory tests (Table 3).

Average percentage of measured ammonia concentrations at the beginning of tests ranged from 98 to 119% of the nominal concentrations in the mucket glochidia tests and from 95 to 118% of the nominal concentrations in the fatmucket glochidia tests (Table 3). However, total ammonia concentrations decreased over exposure periods. The average decrease in total ammonia concentrations from 10 ammonia tests with glochidia was 42% (SD = 17%, n = 54; USGS, unpublished data) over 48-h exposure periods. The decrease in ammonia concentration over time should not affect the interpretation of the results of repeated ammonia tests in the single laboratory because conditions (e.g., temperature, beaker size, and dilution water) were consistent across tests.

Relatively constant concentrations of chlorine were maintained in the diluter system during the exposure periods. Mean percent change of measured chlorine concentrations from the beginning to the end of nine 48-h glochidia tests was 7.4% (SD = 9.5%, n = 9; USGS, unpublished data). Average measured concentrations of chlorine during 48-h exposure ranged

Table 4. Intralaboratory variability of median effective concentrations (EC50s) for copper, ammonia, or chlorine in 48-h tests with glochidia and in 96-h tests with newly transformed juvenile mucket (*Actinonaias ligamentina*) or fatmucket (*Lampsilis siliquoidea*)

			Copper EC50 (μg/L)		Total ammonia EC50 (mg N/L)		Chlorine EC50 (μg/L)	
Test	Test date	Species (life stage)	24 h	48 h	24 h	48 h	24 h	48 h
1	April 2003	Mucket (glochidia)	59 (57–62)[a]	23 (22–24)	6.9 (6.7–7.2)	5.9 (5.7–6.1)	74 (71–77)	40 (38–42)
2	July 2003		66 (62–69)	32 (31–33)	5.7 (5.4–6.0)	3.4 (3.3–3.6)	>100	48 (46–51)
3	March 2004		53 (51–55)	31 (30–32)	10 (10–11)	7.6 (7.3–8.0)	NT[b]	NT
4	May 2004		35 (34–36)	20 (19–21)	9.1 (8.7–9.6)	4.7 (4.5–4.9)	>100	52 (48–55)
Mean			53	26	7.9	5.5	91	47
SD[c]			13	5.9	2.0	2.0	15	6.1
CV[d] (%)			25	22	25	36	17	13
1	April 2003	Fatmucket (glochidia)	36 (34–38)	23 (21–24)	11 (11–12)	9.1 (8.8–9.5)	47 (45–49)	34 (32–35)
2	June 2003		42 (40–45)	28 (27–30)	15 (14–16)	12 (12–13)	90 (82–99)	79 (75–84)
3	June 2003		29 (27–30)	15 (14–15)	9.8 (9.5–10)	8.0 (7.7–8.3)	81 (78–85)	60 (57–63)
4	April 2004		31 (29–32)	20 (19–21)	13 (13–14)	12 (11–12)	67 (65–69)	57 (54–60)
5	June 2004		38 (36–40)	31 (30–32)	>16	13 (12–13)	>100	>100
6	July 2004		41 (39–43)	23 (22–24)	NT	NT	NT	NT
7	September 2004		33 (32–35)	17 (16–18)	7.3 (7.0–7.7)	5.2 (4.9–5.4)	82 (78–86)	70 (66–74)
Mean			36	22	12	9.9	78	67
SD			5.1	5.9	3.3	3.0	19	22
CV (%)			14	26	27	30	24	34
			48 h	96 h				
1	August 2004	Fatmucket (juveniles)	29 (23–36)	18 (15–22)	NT	NT	NT	NT
2	May 2005		35 (30–42)	20 (18–24)	NT	NT	NT	NT
3	June 2005		45 (37–55)	25 (20–30)	NT	NT	NT	NT
4	June 2005		52 (37–72)	23 (19–27)	NT	NT	NT	NT
5	July 2005		34 (27–42)	21 (18–25)	NT	NT	NT	NT
Mean			39	21	NT	NT	NT	NT
SD			9.3	2.7	NT	NT	NT	NT
CV (%)			24	13	NT	NT	NT	NT

[a] Numbers in parentheses represent 95% confidence interval.
[b] NT = not tested.
[c] SD = standard deviation.
[d] CV = coefficient of variation.

from 89 to 104% of nominal concentrations in the mucket glochidia tests and from 101 to 112% of nominal concentrations in the fatmucket glochidia tests (Table 3).

These results indicate that copper and chlorine concentrations were maintained constantly and close to the nominal concentrations throughout the exposure periods. In contrast, total ammonia concentrations decreased during the exposures.

Intralaboratory testing

Control survival in all tests with glochidia and juvenile mussels was above 90%. The EC50s in most cases were similar among repeated tests with a same species, toxicant, and test duration during or between years (Table 4). Intralaboratory variability of EC50s of copper, total ammonia, and chlorine for mucket and fatmucket glochidia, expressed as CV, ranged between 14 and 27% in 24-h exposures and between 13 and 36% in 48-h exposures (Table 4). The intralaboratory CV of copper EC50s for juvenile fatmucket was 24% in 48-h exposure and 13% in 96-h exposure (Table 4). The variability in test results did not increase with exposure durations (i.e., 24 h vs. 48 h for glochidia tests; 48 h vs. 96 h for juvenile tests). These CVs were within the range of CVs reported in previous intralaboratory acute toxicity tests using commonly tested aquatic organisms. For example, CVs of 21 to 58% for 48-h tests with cladoceran (*Daphnia magna*) and 20 to 120% for 96-h tests with fathead minnows (*Pimephales promelas*) have been reported for three toxicants (sodium dodecyl sulfate, sodium pentachlorophenate, and cadmium) [15,20]. The results indicate that intralaboratory variability in toxicity tests with glochidia and juvenile mussels was similar to the variability reported for commonly tested cladoceran and fish species, and acute toxicity tests conducted with glochidia and juvenile mussels were repeatable.

Interlaboratory testing

All five laboratories successfully completed copper toxicity tests with fatmucket glochidia and juvenile mussels. Control survival in all tests was above 90%. The interlaboratory CV of EC50s for glochidia was 13% in the 24-h exposure and 24% in the 48-h exposure; the CV of EC50s for juvenile mussels was 22% in the 48-h exposure and 42% in the 96-h exposure (Table 5). These CVs were similar to those for the intralaboratory tests (Table 4). The high test completion success in all five laboratories and similar variability of EC50s between intra- and interlaboratory tests indicate that the methods can be performed routinely by laboratories experienced in conducting freshwater toxicity tests and potential stress caused from handling and shipping of test organisms did not substantially influence test results.

The interlaboratory CVs for the glochidia and juvenile tests were less than or equal to those reported in previous interlaboratory acute toxicity tests using commonly tested aquatic organisms and reference toxicants. For example, the interlaboratory CV in 48-h KCl toxicity tests with cladoceran (*Ceriodaphnia dubia*) is 49% [15] and the inter-laboratory CVs in toxicity tests using silver and endosulfan as toxicants range from 51 to 166% in 48-h toxicity tests with cladoceran (*D. magna*), from 38 to 53% in 96-h toxicity tests with fathead

Table 5. Interlaboratory variability of median effective concentrations (EC50s) for copper (μg Cu/L) in 48-h tests with fatmucket (*Lampsilis siliquoidea*) glochidia and in 96-h tests with newly transformed juvenile fatmucket

	Glochidia		Juvenile mussels	
Laboratory	24-h EC50	48-h EC50	48-h EC50	96-h EC50
1	29 (28–31)[a]	13 (12–14)	29 (23–36)	18 (15–22)
2	33 (32–35)	24 (22–25)	48 (40–59)	18 (16–20)
3	27 (25–29)	26 (24–28)	47 (40–54)	41 (35–47)
4	38 (35–41)	21 (20–23)	34 (26–45)	21 (17–25)
5	32 (31–34)	20 (19–21)	36 (24–54)	19 (12–30)
Mean	32	21	39	23
SD[b]	4.2	5.0	8.3	9.9
CV[c] (%)	13	24	22	42

[a] Numbers in parentheses represent 95% confidence interval.
[b] SD = standard deviation.
[c] CV = coefficient of variation.

minnows and from 33 to 88% in 96-h tests with rainbow trout (*Oncorhyncus mykiss*) [15]. However, the present study used the same batch of test organisms and the same dilution water, which were different from the referenced interlaboratory studies and might have contributed to the lower variability in test results. The present study was designed to determine the inherent variability in the test. Higher variability would be likely if the interlaboratory tests were conducted with different sources of dilution water and organisms (e.g., from different populations or watersheds). Therefore, additional study is needed to further characterize potential variability associated with the newly developed ASTM standard methods for conducting acute toxicity tests with early life stages of freshwater mussels.

CONCLUSION

In summary, standardized acute toxicity tests with glochidia and juvenile mussels following procedures recommended by ASTM [7] were conducted successfully within a single laboratory over a two-year period and among five laboratories. The overall variability in test results was within the range of variability reported for acute toxicity tests with commonly tested organisms. Thus, the procedures for conducting mussel toxicity tests described in ASTM [7] can be used to consistently generate toxicity data with acceptable precision and accuracy.

Acknowledgement—We thank J. Arms, M. Bartsch, R. Bringolf, W. Brumbaugh, E. Brunson, N. Cooper, D. Hardesty, C. Ivey, B. Kaiser, J. Kunz, A. Mager, and R. Mair for technical assistance. Funding for this research was provided in part by U.S. Fish and Wildlife Service (U.S. FWS, Environmental Contaminants Program, project 301812N037) and U.S. EPA (project DW14937809-01-0). We also thank J. Fairchild, D. Papoulias, and two anonymous reviewers for providing review comments on an earlier version of the manuscript. This paper has been reviewed in accordance with U.S. Geological Survey and U.S. FWS peer review policies. Publication does not signify that the contents reflect the views of the U.S. FWS. References to trade names or manufacturers do not imply government endorsements of commercial products.

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