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Contaminant Sensitivity of Freshwater Mussels

CHRONIC TOXICITY OF COPPER AND AMMONIA TO JUVENILE FRESHWATER MUSSELS (UNIONIDAE)

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Abstract—The objectives of the present study were to develop methods for conducting chronic toxicity tests with juvenile mussels under flow-through conditions and to determine the chronic toxicity of copper and ammonia to juvenile mussels using these methods. In two feeding tests, two-month-old fatmucket (*Lampsilis siliquoidea*) and rainbow mussel (*Villosa iris*) were fed various live algae or nonviable algal mixture for 28 d. The algal mixture was the best food resulting in high survival (≥90%) and growth. Multiple copper and ammonia toxicity tests were conducted for 28 d starting with two-month-old mussels. Six toxicity tests using the algal mixture were successfully completed with a control survival of 88 to 100%. Among copper tests with rainbow mussel, fatmucket, and oyster mussel (*Epioblasma capsaeformis*), chronic value ([ChV], geometric mean of the no-observed-effect concentration and the lowest-observed-effect concentration) ranged from 8.5 to 9.8 μg Cu/L for survival and from 4.6 to 8.5 μg Cu/L for growth. Among ammonia tests with rainbow mussel, fatmucket, and wavy-rayed lampmussel (*L. fasciola*), the ChV ranged from 0.37 to 1.2 mg total ammonia N/L for survival and from 0.37 to 0.67 mg N/L for growth. These ChVs were below the U.S. Environmental Protection Agency 1996 chronic water quality criterion (WQC) for copper (15 μg/L; hardness 170 mg/L) and 1999 WQC for total ammonia (1.26 mg N/L; pH 8.2 and 20°C). Results indicate that toxicity tests with two-month-old mussels can be conducted for 28 d with >80% control survival; growth was frequently a more sensitive endpoint compared to survival; and the 1996 chronic WQC for copper and the 1999 chronic WQC for total ammonia might not be adequately protective of the mussel species tested. However, a recently revised 2007 chronic WQC for copper based on the biotic ligand model may be more protective in the water tested.

Keywords—Freshwater mussels Juvenile mussels Chronic toxicity Copper Ammonia

INTRODUCTION

The early life stages of freshwater mussels are acutely more sensitive to some chemicals when compared to commonly tested aquatic organisms [1–4]. However, only limited data are available to evaluate the chronic toxicity of chemicals to mussels and to compare the acute and chronic effect of these chemicals on mussels [5–7]. A primary limitation for conducting long-term toxicity tests in the laboratory with juvenile mussels is the high mortality of newly transformed juvenile mussels frequently observed a few weeks after released from fish host [7]. As a result of this problem, Valenti et al. [5,6] used older, more developed juvenile mussels and reported high control survival in 21-d water-only toxicity tests.

Little is known about the quantity or quality of food source that provide conditions for sustaining populations in the wild or in the laboratory [8,9]. Laboratory-cultured green algae have been used to propagate juvenile mussels in the laboratory [10–12]. Commercially available nonviable algae (Instant Algae® products *Nannochloropsis*, and Shellfish Diet, Reed Mariculture, Campbell, CA, USA) also have been used for the laboratory culture of juvenile and adult mussels [7,13]. The nonviable algal products are pure marine microalgae that are concentrated for easy storage. The single alga *Nannochloropsis* is used extensively in the aquaculture industry for growing small zooplankton such as rotifers. Shellfish Diet is a mixture of four marine microalgae (*Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira*) that have been used successfully for culturing a variety of marine shellfish including oysters, clams, mussels, and scallops. In our preliminary feeding test, a combination of the single alga and Shellfish diet was found to support >80% juvenile mussel survival for 50 d. Additional studies were needed to evaluate the effects of foods and feeding rates on the survival and growth of juvenile mussels over a 28-d period to optimize conditions for conducting long-term toxicity tests.

A series of studies were undertaken to refine methods for conducting acute and chronic toxicity tests with early life stages of mussels [3,7,13–15]. Based on these studies and previous literature, American Society for Testing and Materials (ASTM) recently published an international *Standard Guide For Conducting Laboratory Toxicity Tests With Freshwater Mussels* [16]. As one of a series of papers developed to assess con-

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Table 1. Food types and feeding levels in feeding test one starting with two-month-old fatmucket (*Lampsilis siliquoidea*)

Food type	Sources	Feeding level (low, medium, high)
Live alga (*Neochloris oleoabundans*)	MSU[a], Springfield, Missouri, USA	20,000; 40,000; 60,000 cells/ml
Live alga (*N. oleoabundans*) + sediment[b]	MSU	As above but with sediment
Live alga (*Selenastrum capricornutum*)	ABS[c], Fort Collins, Colorado, USA	20,000; 40,000; 60,000 cells/ml
Live alga (*Nannochloropsis oculata*)	Virginia Tech[d], Blacksburg, Virginia, USA	30,000; 60,000; 90,000 cells/ml
Nanno: Nonviable alga (*Nannochloropsis*)	Reed Mariculture, Campbell, California, USA	1, 2, 3 ml
Algal mixture: Nanno + Shellfish Diet	Reed Mariculture	1, 2, 3 ml

[a] MSU = Missouri State University.
[b] Fine sediment was added as a thin layer in the bottom of the test chambers at the beginning of the test.
[c] ABS = Aquatic Biosystems.
[d] Polytechnic Institute and State University.

taminant sensitivity of early life stages of freshwater mussels, the present paper summarizes the results of 28-d feeding studies and toxicity tests with two-month-old juvenile mussels during the process of developing the ASTM standard. Specifically, the objectives of the present study were to evaluate survival and growth of juvenile mussels fed various foods at different feeding levels over a 28-d period in intermittent flow-through systems, routinely used for acute and chronic toxicity tests with fish and aquatic invertebrates, and to determine chronic toxicity of copper and total ammonia to juvenile mussels in 28-d tests following standard methods [16]. The two toxicants were selected because of their wide occurrence in contaminated aquatic environments, because acute toxicity data have indicated the early life stages of mussels were sensitive to these toxicants [1–3], and because no (total ammonia) or limited (copper) chronic toxicity data were available for freshwater mussels.

MATERIALS AND METHODS

Culture of juvenile mussels

Gravid females of five mussel species were collected from streams and rivers in Virginia (rainbow mussel, *Villosa iris*; wavy-rayed lampmussel, *Lampsilis fasciola*), Tennessee (oyster mussel, *Epioblasma capsaeformis*), and Missouri, USA (fatmucket, *L. siliquoidea*; pink mucket, *L. abrupta*) between March and June of 2004 and 2005. Adult mussels were collected from relatively uncontaminated locations where these species were abundant and apparently healthy, and reproduction and recent recruitment were evident. Glochidia isolated from at least three female mussels were pooled for the production of juvenile mussels from host fish in laboratories at Virginia Polytechnic Institute and State University (Virginia Tech, Blacksburg, VA, USA) or Missouri State University (Springfield, MO, USA; see Wang et al. [13] for a description of the methods used to transform juvenile mussels). Transformed juveniles were reared with laboratory-cultured algae (*Neochloris oleoabundans*) for two months either in a recirculating aquaculture system containing fine sediment [11] or in a compact recirculating system [12] before testing.

Feeding tests

Feeding test one. The first feeding test was conducted for 28 d starting with two-month-old (two months after transformation) fatmucket and various algal sources. The juvenile mussels were shipped overnight to the Columbia Environmental Research Center (CERC, U.S. Geological Survey, Columbia, MO) for testing. Once juvenile mussels were received, 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 test water (ASTM reconstituted hard water [17]) at least three times over a 2- or 3-d acclimation period, during which juveniles were fed live algae (*N. oleoabundans*) twice daily. At the beginning of the feeding test, 10 juveniles with foot movement were impartially transferred into each of thirty 300-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 [16]. The test was conducted in a water-renewal system [18], which delivered 60 ml of additional water into each beaker every 4 h. Each beaker had a 2.5-cm hole in the side covered with 50 mesh (279 micron opening) stainless-steel screen and contained 200 ml of water. At each cycle of water addition, excess water in the beakers flowed out through the screen, with no water exchange among replicate beakers (in contrast to previous studies conducted by Valenti et al. [5,6], where there was water exchange between replicate exposure chambers). Beakers were placed in temperature-controlled water baths at 20 ± 1°C. Juvenile mussels were fed three species of laboratory-cultured live algae or two nonviable algae concentrates at three feeding levels (Table 1): Low level, which approximated the feeding level used for the mussel culture in continuous feeding and calculated from information (e.g., the density of algae) on algae concentrate containers provided by the manufacturers; medium level (two times the low level); and high level (three times the low level). Each treatment had two replicates. Juvenile mussels were fed twice daily in the morning and afternoon immediately after an addition of water. In addition, juveniles in one treatment were not fed algae but received only a thin layer of fine sediment at the start of the test (Table 1; see Jones et al. [11] for the description of the sediment characteristics), and juveniles in another control treatment received neither food nor sediment during the test.

Fresh live algae were provided by Virginia Tech and Missouri State University and were delivered twice to our laboratory at the beginning and middle of the 28-d feeding test. Each batch of algae was used for two weeks and kept in a refrigerator at approximately 3°C. The single alga food (Nanno; Table 1) was prepared by adding 1 ml of *Nannochloropsis* concentrate into 1.8 L of well water, and the combination of Nanno and Shellfish Diet (algal mixture; Table 1) was prepared by adding 1 ml of *Nannochloropsis* concentrate and 2 ml of Shellfish Diet concentrate into 1.8 L of water. Each batch of

Table 2. Summary of test conditions for conducting chronic toxicity tests with juvenile mussels in accordance with methods outlined in American Society for Testing and Materials (ASTM) [16]

Test chemicals	Copper sulfate or ammonium chloride
Test type	Flow-through
Test duration	28 d
Temperature	20 ± 1°C
Light quality	Ambient laboratory light
Light intensity	200 lux
Photoperiod	16:8 h light:dark
Test chamber	300-ml glass beaker
Test solution volume	200 ml
Renewal of solution	Additional 120 ml of solution to each beaker once every 4 h
Age of test organism	2 months
Number of organisms per test chamber	10
Number of replicate chambers per concentration	4 (except the test with wavy-rayed lampmussel, $n = 3$)
Feeding	Twice daily with live algae or with nonviable algal mixture
Chamber cleaning	None
Aeration	None
Dilution water	Reconstituted ASTM hard water (hardness 160–180 mg/L as $CaCO_3$, alkalinity 110–120 mg/L as $CaCO_3$ [17])
Dilution factor	0.5
Test concentration	Copper: 0, 3.125, 6.25, 12.5, 25, and 50 μg/L Total ammonia: 0, 0.5, 1, 2, 4, and 8 mg N/L or 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg N/L
Chemical residues	Copper weekly and total ammonia once every 3 to 5 d
Water quality	Dissolved oxygen, pH, conductivity, hardness, and alkalinity at the control, medium-, and high-exposure concentrations weekly
Endpoint	Survival (foot movement) and growth (shell length)
Test acceptability criterion	≥80% control survival on day 28

algal foods was used for one week and kept in a refrigerator at approximately 3°C.

Water quality was determined weekly on composite samples of each treatment using standard methods [19]. Water hardness ranged from 160 to180 mg/L as $CaCO_3$, alkalinity ranged from 110 to120 mg/L as $CaCO_3$, pH ranged from 8.4 to 8.6, dissolved oxygen was >8.0 mg/L, and total ammonia was <0.07 mg N/L across all treatments. Ambient laboratory light was approximately 200 lux, with a 16:8 h light:dark photoperiod. Survival (foot movement within 5 min [16]) was determined at the end of each test using a dissecting microscope. Surviving mussels in the two replicates of each treatment were pooled and preserved in 70% ethanol for subsequent growth measurements. The maximum shell length of each mussel was measured to the nearest 0.001 mm using a digitizing system with video micrometer software (Image Caliper, Resolution Technology, Dublin, OH, USA). Because of the limited replication in this feeding test, the differences in survival and growth were not statistically compared among treatments.

Feeding test two. The second feeding test was conducted for 28 d with two-month-old rainbow mussels and the best food found in feeding test one. The test was conducted in an intermittent flow-through diluter system [20]. The system provided approximately 120 ml of water to each glass beaker every 4 h. An in-line flow splitter was attached to each delivery line to partition the water flow evenly to each of replicate beakers. Test chambers, acclimation of test organisms, water temperature, light quality, photoperiod, and test water were as described above for feeding test one. Ten juveniles were transferred impartially into each of four replicate beakers per feeding level. A sample with approximately 10 juveniles also was impartially collected at the beginning of the feeding test, and preserved in 70% ethanol to record initial shell length. Juveniles were fed the algal mixture as described in feeding test one at four feeding levels: Manually adding 2 or 4 ml of the food into a beaker twice daily, or automatic delivery of 1 or 2 ml of the food using a Hamilton syringe pump (Hamilton Company, Reno, NV, USA) six times daily, with each water addition. In addition, juvenile mussels in two beakers were not fed during the test.

Water quality was determined weekly on composite samples of each treatment. The hardness ranged from 160 to180 mg/L as $CaCO_3$, alkalinity ranged from 110 to120 mg/L as $CaCO_3$, and pH ranged from 8.2 to 8.4. Dissolved oxygen was >7.9 mg/L and total ammonia was <0.05 mg N/L. Survival was determined at the end of the feeding test. Surviving mussels in each replicate beaker were preserved in 70% ethanol for subsequent growth measurement as described above. Differences in mean survival and growth among feeding treatments were analyzed using analysis of variance and Tukey's test [21]. The level of statistical significance was set at $p \leq 0.05$.

Toxicity test

Test conditions used to conduct the chronic toxicity tests with juvenile mussels were summarized in Table 2. Copper sulfate ($CuSO_4$, 99.9% purity; JT Baker, Phillipsburg, NJ, USA) and ammonium chloride (NH_4Cl, 99.5% purity; Fisher Scientific, Houston, TX, USA) were used as toxicants. Tests were conducted in a flow-through diluter system as described in feeding test two, which delivered five exposure concentrations with a dilution factor of 0.5 plus a control, and provided approximately 120 ml of test solution to each replicate beaker per concentration every 4 h. Beakers were placed in temperature-controlled water baths. Toxicant stock solution was delivered with each cycle of the diluter by the Hamilton syringe pump. Test chambers, acclimation of test organisms, light quality, photoperiod, and dilution water were as described above for the feeding tests.

At the beginning of a toxicity test, 10 juveniles exhibiting foot movement were impartially transferred into each beaker. Approximately 20 juveniles also were impartially sampled and

Table 3. Mean shell length of juvenile mussels at the beginning of the toxicity test (n = 10 to 20) and mean water quality characteristics over a 28-d period of each test (n = 5 to 7 sampling times). Standard deviation is in parentheses

Test	Species	Mean length (mm)	pH	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (μS/cm)
Cu1[a]	Fatmucket	0.76 (0.08)	8.3 (0.1)	171 (3.0)	122 (6.4)	577 (12)
Cu2[a]	Pink mucket	0.59 (0.08)				
Cu3	Rainbow mussel	0.90 (0.08)	8.3 (0.1)	171 (8.4)	119 (1.5)	589 (8.3)
Cu4	Fatmucket	0.63 (0.12)	8.7 (0.2)	165 (4.1)	124 (1.8)	563 (21)
Cu5	Oyster mussel	0.84 (0.27)	8.2 (0.1)	162 (2.5)	88 (11)	589 (11)
TN1[a]	Fatmucket	0.76 (0.08)	8.4 (0.2)	168 (5.2)	118 (5.6)	586 (7.2)
TN2[a]	Pink mucket	0.59 (0.08)				
TN3	Rainbow mussel	0.90 (0.08)	8.2 (0.1)	175 (4.2)	121 (3.3)	610 (9.5)
TN4	Fatmucket	0.62 (0.13)	8.2 (0.1)	161 (4.4)	92 (16)	556 (45)
TN5	Wavy-rayed lampmussel	0.66 (0.07)	8.2 (0.1)	163 (3.6)	92 (12)	579 (24)

[a] Water quality data were the same in copper test one (Cu1) and two (Cu2) or in total ammonia test one (TN1) and two (TN2), which were conducted concurrently in one diluter system with the same source of dilution water.

preserved in 70% ethanol to record initial shell lengths. In the first four tests with fatmucket (copper test one [Cu1] and total ammonia nitrogen test one [TN1]; Table 3) and pink mucket (Cu2 and TN2), juveniles were fed live algae (*N. oleoabundans*), previously used for the culture of these juveniles and provided by Missouri State University. A sample of fresh algae was kept in a refrigerator at 3°C and used for approximately two weeks. The fresh algae were added into beakers twice daily, in early morning and in late afternoon, at a feeding rate of approximately 40,000 cells/ml, which was double the amount of food used to culture these juveniles in continuous feeding systems [12]. Because of the high control survival and growth of juveniles fed the algal mixture in feeding tests, the algal mixture was used in six subsequent toxicity tests (Cu3, Cu4, Cu5 and TN3, TN4, and TN5; Table 3), where juveniles were fed 2 ml of the algal mixture twice daily.

Water quality was determined weekly on composite water samples collected from the control, medium-, and high-exposure concentrations. Additionally, total ammonia was measured periodically during copper tests and never exceeded 0.05 mg N/L. Measured dissolved oxygen was above 7.0 mg/L during all tests. Mean values of hardness and alkalinity over the 28-d period of each test (Table 3) were generally within the range recommended for ASTM hard water (hardness 160–180 mg/L as $CaCO_3$, alkalinity 110–120 mg/L as $CaCO_3$ [17]). Mean pH values ranged from 8.2 to 8.7 for copper tests and from 8.2 to 8.4 for ammonia tests (Table 3), which were above the listed range of 7.8 to 8.0 in ASTM [17]. To maintain a more stable pH value in three tests (Cu5, TN4, and TN5; Table 3), the dilution water was initially adjusted between 8.0 and 8.2 in a 700-L polypropylene tank before the water was delivered to the flow-through diluter. The pH was adjusted by injecting dilute hydrochloric acid (HCl) into the tank using a pH pump control system with proportional output (Barnant HD PH-P1, Barrington, IL, USA). However, the addition of HCl to the dilution water lowered the alkalinity in these three tests (Table 3).

During the 28-d exposure period, survival was determined on test days 4, 10, 21, and 28. In order to observe the juveniles under the microscope during a test, it was necessary to remove some of the test 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 exhibiting foot movement within a 5-min observation period were classified as alive. The acceptability criterion was ≥90% control survival in 4-d exposures and ≥80% control survival in 10-, 21-, and 28-d exposures [16]. Surviving juveniles at the end of 28-d tests were preserved in 70% ethanol for growth measurement as described above.

For dissolved copper analysis, water was collected weekly from composite samples of each of the six exposure concentrations. Each water sample was acidified to 1% (v/v) ultrapure nitric acid. Copper concentrations were determined by inductively coupled plasma-mass spectrometry (PE/SCIEX ELAN 6000, PerkinElmer, Norwalk, CT, USA). Samples were automatically delivered to the inductively coupled plasma-mass spectrometry by means of a software-controlled CETAC ASX-500/ADX-100 autosampler/autodiluter system (CETAC Technologies, Omaha, NE, USA; see Wang et al. [13] for additional detail). Analytical precision for quantitative inductively coupled plasma-mass spectrometry was determined by analyzing samples in duplicate during the instrumental run and determining the relative percent differences, which were <2.7% for all analysis duplicates. Recoveries of copper spiked into water samples and analyzed by quantitative inductively coupled plasma-mass spectrometry ranged from 98 to 103%. Instrumental detection limit was <0.016 μg/L, and the method detection limit was <0.2 μg/L. During ammonia tests, total ammonia nitrogen of each exposure concentration was measured every 3 to 5 d using an Orion Ammonia Electrode and Orion EA940 meter (Thermo Electron, Beverly, MA, USA). The meter for total ammonia analyses was calibrated each time before measuring samples with 0.1 and 1 mg/L or 1 and 10 mg/L independent calibration verification standards, depending on the range of total ammonia concentrations to be measured. The percent recovery of the standards ranged from 90 to 100%. For ammonia nitrogen concentrations in water samples, a minimum reporting limit of 0.1 mg N/L was selected based on the method detection limit of 0.02 mg N/L and method quantitation limits of 0.06 mg N/L.

Mean measured copper and total ammonia concentrations (Table 4) were similar to nominal concentrations (Table 2) throughout the 28-d tests. The mean measured concentrations for each test were used to calculate the acute and chronic effect concentrations. The median effective concentrations (EC50s) for survival on test days 4, 10, 21, and 28 were calculated with Toxstat software [21], using a Probit model when appropriate, and either a Spearman-Karber or trimmed Spearman-

Table 4. Mean measured concentrations of copper (Cu, μg/L) and total ammonia (TN, mg N/L) over 28-d exposure periods in toxicity tests with juvenile mussels. Standard deviation is in parentheses

Test	Species	*n*	Mean measured concentration					
			Control	Low	Med-low	Medium	Med-high	High
Cu1/2[a]	Fatmucket/pink mucket	4	0.8 (0.2)	3.4 (0.3)	5.8 (0.5)	13 (2.6)	25 (4.5)	56 (11)
Cu3	Rainbow mussel	4	0.9 (0.2)	4.1 (0.9)	6.4 (2.2)	15 (7.3)	21 (2.1)	51 (3.0)
Cu4	Fatmucket	4	1.0 (0.4)	3.1 (0.9)	6.6 (2.8)	11 (2.1)	21 (1.6)	45 (2.6)
Cu5	Oyster mussel	4	1.3 (0.2)	3.1 (0.7)	6.7 (1.1)	13 (2.0)	23 (4.6)	50 (6.1)
TN1/2[a]	Fatmucket/pink mucket	10	0.12	0.44 (0.09)	0.79 (0.14)	1.6 (0.34)	3.3 (0.45)	7.4 (0.82)
TN3	Rainbow mussel	9	<0.1	0.40 (0.07)	0.81 (0.10)	1.7 (0.22)	3.5 (0.29)	7.6 (0.44)
TN4	Fatmucket	7	<0.1	0.13 (0.02)	0.28 (0.10)	0.49 (0.03)	1.0 (0.07)	2.0 (0.16)
TN5	Wavy-rayed lampmussel	6	<0.1	0.13 (0.02)	0.34 (0.16)	0.44 (0.11)	1.0 (0.13)	2.0 (0.12)

[a] Copper test one (Cu1) and two (Cu2) or total ammonia test one (TN1) and two (TN2) were conducted concurrently in a diluter system with the same dilution water.

Karber method otherwise [22]. The no-observed-effect concentration and the lowest-observed-effect concentration for survival and growth at the end of 28-d toxicity tests were determined by analysis of variance, with mean comparison made by one-tailed Dunnett's test or by Steel's many-one rank test [21,23]. To focus on effects on growth that occurred at concentrations less than those affecting survival, exposure concentrations above the lowest-observed-effect concentration for survival were excluded from statistical analysis for growth [23]. The level of statistical significance was set at $p \leq 0.05$. Chronic value (ChV) was calculated as the geometric mean of the no-observed-effect concentration and lowest-observed-effect concentration. A linear interpolation method [23] also was used to estimate 10 and 25% inhibition concentrations for survival and growth at the end of 28-d toxicity tests [21]. When calculating 10% inhibition concentration and 25% inhibition concentration, survival and growth data from all concentrations were included in the analysis [23].

RESULTS AND DISCUSSION

Feeding test one with live and instant nonviable algae

Mean 28-d survival of juvenile fatmucket fed various foods at the low feeding levels ranged from 70 to 90% (Table 5), whereas the mean survival in no-feeding treatments was 35% (water-only) and 25% (with sediment). Higher feeding levels generally did not increase the survival or growth of juvenile mussels (Table 5). The best survival (≥90%) and growth were observed in feeding treatments with the algal mixture (Table 5).

Lower survival and growth of juvenile mussels in treatments with live algae might have resulted from underfeeding. Although the feeding levels were one to three times those used for the culture of juvenile mussels in continuous feeding, underfeeding may have occurred because the algae were added twice daily and some of the algae would have been flushed from the beakers with each cycle of water addition into the beaker every 4 h. In addition, using algae held for up to two weeks might have influenced the quality of the algae. Because the live algae have been used to successfully culture juvenile mussels [11,12], it is likely that live algae can be used as a food source in toxicity tests. However, more studies are needed to evaluate the quantity and quality of live algae for use in flow-through toxicity tests.

Feeding test two with the algal mixture

Results of the first feeding test indicate that the algal mixture was a good food source for the juvenile mussels held under flow-through conditions. Further study on the feeding levels of the algal mixture was conducted in the second feeding test. Mean survival of juvenile rainbow mussels at each of the feeding levels ranged from 85 to 100%, and mean shell length increased 47 to 71% over the 28-d test (Table 6). In contrast, mean survival in no-feeding treatment was 50% and mean shell length increased only 16% (Table 6). Among the four manual and automatic feeding treatments, mean survival and growth were not significantly different. The results indicate that the algal mixture can be used to maintain high control survival and growth of juvenile mussels in flow-through conditions and that a lower manual feeding level (i.e., adding 2 ml of the food mixture twice daily) can be used in subsequent chronic toxicity tests. Advantages of using the algal mixture include low cost, long storage time (three months in refrigerator), and easy preparation compared to maintaining laboratory cultures of live algae.

Table 5. Mean 28-d survival and growth of juvenile fatmucket (*Lampsilis siliquoidea*) fed various foods at low, medium (2 times the low level), and high (3 times the low level) feeding levels in feeding test one. Standard deviation is in parentheses

Treatment	Mean survival (%; $n = 2$)			Mean shell length (mm)[a]		
	Low	Medium	High	Low	Medium	High
Live alga (*Neochloris oleoabundans*)	75	80	75	0.81 (0.09)	0.82 (0.09)	0.83 (0.10)
Live alga (*N. oleoabundans*) + sediment	70	55	60	0.75 (0.14)	0.74 (0.13)	0.75 (0.15)
Live alga (*Selenastrum capricornutum*)	70	50	30	0.83 (0.17)	0.87 (0.10)	NR[b]
Live alga (*Nannochloropsis oculata*)	80	45	55	0.82 (0.08)	NR	0.81 (0.11)
Nanno	85	80	65	0.82 (0.11)	0.79 (0.18)	0.75 (0.15)
Algal mixture	90	95	100	0.87 (0.07)	0.83 (0.09)	0.84 (0.19)

[a] Surviving juveniles of two replicates per food were pooled for growth measurement ($n = 10$ to 20).
[b] Growth data were not reported when mean survival was below 50% in a feeding treatment.

Table 6. Mean 28-d survival and growth of juvenile rainbow mussels (*Villosa iris*) fed the algal mixture at various feeding levels in feeding test two. Standard deviation is in parentheses

Treatment	*n*	Survival (%)	Growth	
			Length (mm)[a]	% Increase[b]
Manual feeding: 2 × 2 ml	4	100 (0) X	1.54 (0.07) X	71
Manual feeding: 2 × 4 ml	4	85 (24) X	1.50 (0.06) X	67
Auto feeding: 6 × 1 ml	4	95 (10) X	1.37 (0.16) X	52
Auto feeding: 6 × 2 ml	4	98 (5.0) X	1.32 (0.13) X	47
No food	2	50 (42)	1.04 (0.09)	16

[a] Means with a same letter in a column are not significantly different (the data from no-food treatment were not included in this analysis).
[b] Mean shell length of juveniles at the beginning of the feeding test was 0.90 ± 0.08 mm (n = 10).

Toxicity tests

Mean survival of juvenile mussels in the first two copper tests (Cu1 and Cu2) and two ammonia tests (TN1 and TN2) was >90% on test days 4, 10, and 21, but declined to <30% by the end of the 28-d test. The four toxicity tests were conducted before the feeding tests, and live algae were used as food source. It is likely that mussels were underfed in the first four toxicity tests, as discussed above in feeding test one. Low control survival also was observed after 21 d in a long-term toxicity test conducted with four-month-old fatmucket fed live algae (Robert Bringolf, North Carolina State University, Raleigh, NC, USA, unpublished data).

In contrast, all of the six subsequent toxicity tests using the algal mixture were successfully completed over 28-d exposures, with control survival ranging from 88 to 100% (Tables 7 and 8). In addition, compared to the initial length (Table 3), the mean length of juveniles in the controls at the end of tests (Tables 7 and 8) increased substantially, ranging from a 32 to 83% increase. An exception was the wavy-rayed lampmussel, with only 9.1% increase. The ChVs for growth in three of the six tests were lower than the ChVs for survival (Tables 7 and 8), indicating that the growth endpoint was more sensitive than survival and can be a useful sublethal endpoint for chronic toxicity tests with juvenile mussels. The copper ChV for growth ranged from 4.6 to 8.5 μg/L for rainbow mussel, fatmucket, and oyster mussel (Table 7), although the total ammonia ChV for growth ranged from 0.37 to 0.67 mg N/L for rainbow mussel, fatmucket, and wavy-rayed lampmussel (Table 8). The 10% inhibition concentration for growth in each copper or ammonia test was generally similar to the ChV for growth, and was 16 to 48% lower than the 25% inhibition concentration (Tables 7 and 8). All of these chronic effect concentrations (ChVs, 10% inhibition concentrations, and 25% inhibition concentrations) were below the U.S. Environmental Protection Agency's (U.S. EPA) 1996 hardness-dependent chronic water quality criterion (WQC) for copper (15 μg/L at a hardness of 170 mg/L [24]) or the 1999 temperature- and pH-dependent chronic WQC for ammonia (1.26 total ammonia N/L at pH 8.2 and 20°C [25]). Results of these toxicity tests indicate that the national WQC might not adequately protect the early life stages of freshwater mussels tested in the present study from chronic exposure to copper or total ammonia. The U.S. EPA published the 2007 revised WQC for copper [26], which is based on the biotic ligand model and dependent on a number of water quality parameters (e.g., dissolved organic carbon, pH, temperature, major cations and anions). The biotic ligand model–based chronic copper criterion is 3.9 μg/L for the reconstituted ASTM hard water used in the present study (20°C, pH 8.3, and the estimated water quality parameters based on the specified formulas [26]), is approximately four times lower than hardness-based copper criterion [24], and may be more protective of the mussel species tested. However, additional studies are needed to determine how dissolved organic carbon, other water quality parameters, and dietary exposure to copper influence the chronic toxicity of copper to mussels.

The toxicity data used in the derivation of the WQC have not routinely included data generated for freshwater mussels. Recent studies have demonstrated that use of acute toxicity data for early life stages of mussels would lower the acute WQC for copper [2] and total ammonia [1]. The chronic toxicity data in the present study should be considered to re-evaluate the chronic WQC for copper and total ammonia. Mussels may not be more sensitive to other chemicals. Chronic

Table 7. Mean survival and growth (shell length, n = 4) of juveniles of three mussel species at the end of the 28-d copper toxicity tests. Chronic value (ChV) and 10% and 25% inhibition concentrations (IC10, IC25 with 95% confidence interval [CI]) are presented for each endpoint. Standard deviation is in parentheses. An asterisk (*) indicates a significant reduction relative to control. Concentrations above the concentration causing significant reduction in survival were excluded from the hypothesis test for growth effect

Rainbow mussel			Fatmucket			Oyster mussel		
Mean concn.	Survival (%)	Length (mm)	Mean concn.	Survival (%)	Length (mm)	Mean concn.	Survival (%)	Length (mm)
0.9	88 (10)	1.31 (0.11)	1.0	98 (5)	1.15 (0.11)	1.3	100 (0)	1.11 (0.09)
4.1	96 (7.5)	1.34 (0.11)	3.1	80 (14)	1.30 (0.19)	3.1	88 (14)	1.05 (0.08)
6.4	68 (13)	1.13 (0.05)*	6.6	80 (12)	1.09 (0.12)	6.7	88 (14)	0.97 (0.05)*
15	48 (29)*	—	11	58 (17)*	—	13	6.3 (7.2)*	—
21	13 (19)*	—	21	5 (10)*	—	23	0*	—
51	0*	—	45	0*	—	50	0*	—
ChV	9.8	5.1	ChV	8.5	8.5	ChV	9.3	4.6
IC10 (CI)	4.9 (4.6–5.6)	5.7 (4.8–6.4)	IC10 (CI)	<3.1	6.3 (4.8–12)	IC10 (CI)	<3.1	5.5 (3.3–6.9)
IC25 (CI)	6.3 (5.5–14)	7.5 (6.9–7.9)	IC25 (CI)	8.0 (5.5–11)	12 (10–15)	IC25 (CI)	7.6 (6.7–8.1)	7.6 (7.2–7.9)

Table 8. Mean survival and growth (shell length) of juveniles of three mussel species at the end of 28-d ammonia toxicity tests (n = 4, except for test with wavy-rayed lampmussel, n = 3). Chronic value (ChV) and 10% and 25% inhibition concentrations (IC10, IC25 with 95% confidence interval [CI]) are presented for each endpoint. Standard deviation is in parenthesis. An asterisk (*) indicates a significant reduction relative to control. Concentrations above the concentration causing significant reduction in survival were excluded from the hypothesis test for growth effect

	Rainbow mussel		Fatmucket		Wavy-rayed lampmussel		
Mean concn.	Survival (%)	Length (mm)	Mean concn.	Survival (%)	Length (mm)	Mean concn.	Survival (%)	Length (mm)
0.08	100 (0)	1.52 (0.11)	0.04	98 (5.0)	0.95 (0.18)	0.04	100 (0)	0.72 (0.03)
0.40	98 (5.0)	1.32 (0.05)*	0.13	83 (15)	0.84 (0.07)	0.13	83 (15)	0.77 (0.04)
0.81	98 (5.0)	1.10 (0.08)*	0.28	88 (10)	0.95 (0.11)	0.34	77 (5.8)	0.74 (0.04)
1.67	15 (24)*	—	0.49	18 (21)*	—	0.44	73 (15)	0.72 (0.09)
3.45	0*	—	1.00	45 (39)*	—	1.02	30 (36)*	—
7.56	0*	—	1.99	13 (25)*	—	1.98	0*	—
ChV	1.16		ChV	0.37	0.37	ChV	0.67	0.67
IC10 (CI)	0.89 (0.84-0.92)		IC10 (CI)	<0.13	0.32 (0.28–1.1)	IC10 (CI)	<0.13	0.48 (0.40–0.50)
IC25 (CI)	1.0 (0.99–1.1)	0.73 (0.56–0.84)	IC25 (CI)	0.32 (0.30–0.36)	0.44 (0.31–1.4)	IC25 (CI)	0.39 (0.23–0.55)	0.57 (0.52–0.59)

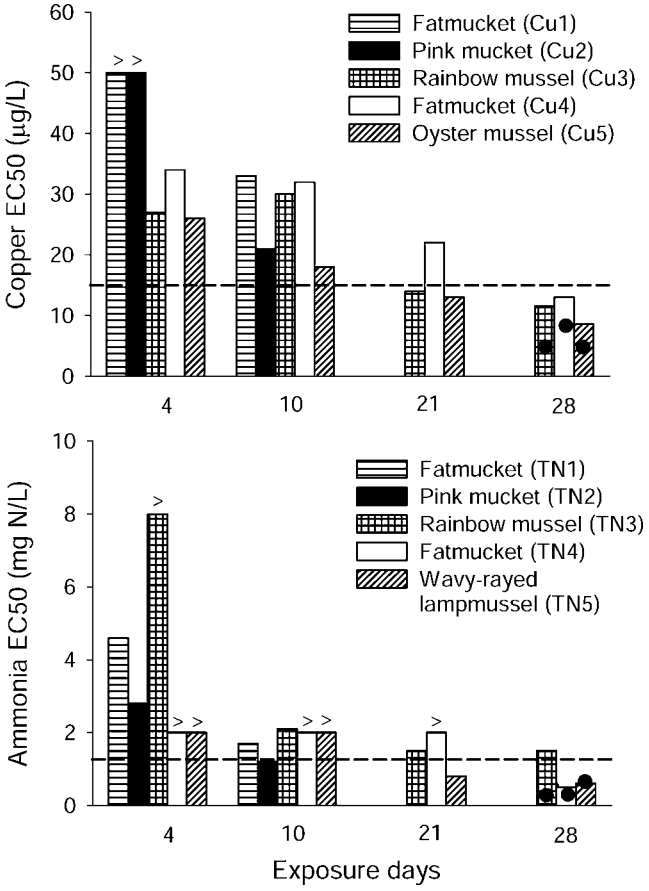

Fig. 1. Median effective concentrations (EC50s) for copper and total ammonia over 4-, 10-, 21-, or 28-d exposures in tests starting with two-month-old juvenile mussels. Black circles represent chronic values for survival and growth of juvenile mussels on test day 28. Dashed line represents the U.S. Environmental Protection Agency's 1996 hardness-dependent chronic water quality criterion (WQC) for copper (hardness 170 mg/L [24]) or 1999 temperature- and pH-dependent chronic WQC for ammonia (pH 8.2, 20°C [25]). The symbol (>) above a bar indicates that the EC50 value was greater than the highest test concentration.

tests conducted by others for chlorine [6] and chlorpyrifos (insecticide) [15] indicate the mussels are much less sensitive to these compounds and likely would be protected by current chronic WQC for chlorine and chlorpyrifos.

The EC50s for copper or total ammonia generally decreased over the exposure periods of 4, 10, 21, and 28 d (Fig. 1). The 28-d ChVs for survival and growth typically were lower than 28-d EC50s for survival and were lower than the 1996 chronic WQC for copper or the 1999 chronic WQC for ammonia (Fig. 1). Toxicity tests evaluating juvenile mussel survival and sublethal responses have been conducted for 10 to 21 d [3,5,6, 15,27,28]. The 10-d EC50s in our previous static-renewal toxicity tests (without feeding) with two-month-old fatmucket and rainbow mussels (8.6–32 µg Cu/L, 0.8–1.5 mg total ammonia N/L [3]) were similar to those in the present flow-through tests (Fig. 1), but were two to three times higher than the 28-d ChVs (Tables 7 and 8; Fig. 1). These results indicate that a prolonged test period and sublethal endpoint are needed to estimate chronic toxicity of a toxicant to juvenile mussels. Because freshwater mussels are long-lived organisms with some species often living over 30 years, a concern has been expressed that a 28-d chronic test with early life stages of mussels may not

Table 9. Acute-chronic ratio (ACR) for copper or total ammonia calculated from 4-d mean median effective concentrations (EC50s) in static-renewal or flow-through tests with newly transformed or two-month-old juvenile mussels, and 28-d chronic values (ChVs) for survival and growth in flow-through tests with two-month-old juvenile mussels

Chemical	Species	EC50				ChV Older/flow	ACR
		Young/static[a]	Older/static[a]	Older/flow[b]	Mean		
Copper (μg/L)	Rainbow mussel	17	29[c]	27	24	5.1	4.7
	Fatmucket	21[c]	46[c]	34	34	8.5	4.0
	Oyster mussel	12[c]	NT[d]	26	19	4.6	4.1
Ammonia (mg N/L)	Rainbow mussel	6.3	7.0[c]	>8.0[e]	6.7	0.40	17
	Fatmucket	10	4.9	4.6	6.5	0.37	18
	Wavy-rayed lampmussel	7.4	NT	>2.0[e]	7.4	0.67	11

[a] Value from the previous static-renewal tests with newly transformed (young) or two-month-old (older) juveniles [3].
[b] Value from flow-through tests with two-month-old juveniles (Fig. 1, this study).
[c] Mean value of two to five tests [3].
[d] NT = Not tested.
[e] Value greater than the highest exposure concentration was not included for calculating mean EC50.

represent adequately the concentrations of chemicals elicit harmful effects on the long-lived species [2].

McKim [29] evaluated relationships between full life-cycle toxicity tests and early life stage toxicity tests. Based on 72 life-cycle tests with four freshwater species and 37 organic and inorganic chemicals plus one saltwater species and 11 organic chemicals, McKim [29] concluded that, in 83% of these tests, the early life stage portion of the life cycle gave the same maximum acceptable toxicant concentration (a range of no-observed-effect concentration and lowest-observed-effect concentration) as the full life cycle, although the remaining 17% of the early life stage tests showed more or less sensitivity than the life cycle test by a factor of two. The variation of a factor of two is almost meaningless because the maximum acceptable toxicant concentrations for specific toxicants, species, and water combinations easily can vary by a factor of two [29]. However, given that the evaluation by McKim [29] did not include mussel toxicity data, there is uncertainty as to how well a 28-d toxicity test with juvenile mussels can be used to predict effect of a toxicant in long-term exposures with other life stages of mussels, where effects on survival, growth, or reproduction might occur.

Because total ammonia concentrations could decline up to 40% during 96-h static-renewal tests with juvenile mussels, the EC50s calculated based on nominal ammonia concentrations might underestimate ammonia toxicity [3]. However, similar 96-h EC50s were observed in ammonia tests conducted concurrently with two-month-old juvenile mussels under static-renewal (fluctuating total ammonia concentration; Table 4 in Wang et al. [3]) and flow-through (relatively constant ammonia concentrations; Fig. 1) conditions: The EC50 for fatmucket was 4.9 mg N/L in the static-renewal test and 4.6 mg N/L in the flow-through test; the EC50 for pink mucket was 2.3 mg N/L in the static-renewal test and 2.8 mg N/L in the flow-through test; and the EC50 for rainbow mussel was 11 mg N/L in the static-renewal test and >8.0 mg N/L in the flow-through test. Results of these studies indicate that the ammonia toxicity data generated from static-renewal tests with somewhat variable ammonia concentrations were comparable to those from flow-through tests with more consistent total ammonia concentrations.

Conducting chronic toxicity tests is more expensive and time-consuming compared to acute toxicity tests. In addition, specific methods may not be available for conducting chronic toxicity tests with some mussel species. Therefore, the acute–chronic ratio (ACR) may be useful to estimate chronic effect from acute toxicity data. We calculated ACR for copper or total ammonia based on acute and chronic toxicity data for four mussel species tested in our previous [3] and present studies (i.e., 4-d EC50 for survival divided by the 28-d ChV for survival and growth). The ACR for copper ranged from 4.0 to 4.7 for rainbow mussel, fatmucket, and oyster mussel, whereas the ACR for total ammonia ranged from 11 to 18 for rainbow mussel, fatmucket, and wavy-rayed lampmussel (Table 9). These values were relatively consistent among species, and were within the range of ACR for copper or total ammonia reported in the U.S. EPA WQC [24,25].

CONCLUSION

In summary, standardized chronic toxicity tests with two-month-old juvenile mussels were conducted successfully in flow-through diluter systems, and all chronic effect concentrations of copper and total ammonia for survival and growth were below the U.S. EPA 1999 pH- and temperature-dependant chronic WQC for total ammonia and the 1996 hardness-dependent chronic WQC for copper. Results indicate that the early life-stages of freshwater mussels are chronically sensitive to copper or ammonia, and the U.S. EPA 1996 chronic WQC for copper and 1999 chronic WQC for ammonia may not be adequately protective of the mussel species tested. For the water tested in the present study, the 2007 biotic ligand model–based chronic WQC for copper is lower than the 1996 hardness-based WQC and may be more protective of chronic toxicity to mussels. Additional studies also are needed to test the assumption that 28-d exposures with juvenile mussels can be used to predict the effects of toxicants on other life stages of mussels in long-term tests.

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