

ACUTE AND CHRONIC TOXICITY OF MERCURY TO EARLY LIFE STAGES OF THE RAINBOW MUSSEL, *VILLOSA IRIS* (BIVALVIA: UNIONIDAE)

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**Abstract**—Mercury (Hg) contamination is receiving increased attention globally because of human health and environmental concerns. Few laboratory studies have examined the toxicity of Hg on early life stages of freshwater mussels, despite evidence that glochidia and juvenile life stages are more sensitive to contaminants than adults. Three bioassays (72-h acute glochidia, 96-h acute juvenile, and 21-d chronic juvenile toxicity tests) were conducted by exposing *Villosa iris* to mercuric chloride salt (HgCl<sub>2</sub>). Glochidia were more sensitive to acute exposure than were juvenile mussels, as 24-, 48-, and 72-h median lethal concentration values (LC50) for glochidia were >107, 39, and 14 µg Hg/L, respectively. The 24-, 48-, 72-, and 96-h values for juveniles were 162, 135, 114, and 99 µg Hg/L, respectively. In the chronic test, juveniles exposed to Hg treatments ≥8 µg/L grew significantly less than did control organisms. The substantial difference in juvenile test endpoints emphasizes the importance of assessing chronic exposure and sublethal effects. Overall, our study supports the use of glochidia as a surrogate life stage for juveniles in acute toxicity tests. However, as glochidia may be used only in short-term tests, it is imperative that an integrated approach be taken when assessing risk to freshwater mussels, as their unique life history is atypical of standard test organisms. Therefore, we strongly advocate the use of both glochidia and juvenile life stages for risk assessment.

**Keywords**—Freshwater mussel    Mercury    Glochidia    Juvenile    Chronic

## INTRODUCTION

As scientists become more aware of risks that mercury (Hg) poses to humans and wildlife, concerns for its effects on aquatic ecosystems continue to heighten. Freshwater mussels are currently one of the fastest declining faunal groups in North America and may be more susceptible to Hg pollution than other aquatic organisms. Mussel assemblages are often congregated in depositional zones [1], and these areas likely have higher Hg concentration because of its affinity for binding with fine particulate matter [2–4]. Several in situ studies have shown that bivalves have a propensity to bioaccumulate Hg [2,4], which may be caused by their close association with the water column–sediment boundary and feeding behavior [5,6]. The U.S. Environmental Protection Agency (U.S. EPA) is currently reviewing the water quality criteria for Hg, but only limited toxicological data are available for many aquatic species. Furthermore, few studies have examined the sensitivities of early life stages of freshwater mussels.

Conducting bioassays with freshwater mussels in the laboratory is critical to their conservation because it will enable researchers to determine toxicity under controlled conditions that are not achievable in the field. Previous studies report that early life stages of freshwater mussels are more sensitive to contaminants than are adults [1,6,7]. This finding is also supported by field observations, as alarmingly few young mussels have been found in assemblages with diverse adult populations [7,8]. Furthermore, immature stages of unionids have been documented to be more sensitive than other aquatic species [9,10], including standard regulatory test organisms [11,12]. However, with standard test protocols yet established for fresh-

water mussels, regulatory agencies remain hesitant to apply test results to policy decisions.

Procedures for acute bioassays are better established for glochidia than juveniles, as the former have been more available for testing because they are obtained from gravid adults collected from the field rather than cultured. However, limitations with using glochidia as test organisms have become evident, with researchers reporting substantial declines in viability during laboratory studies after only short periods, ranging from hours to days, depending on the species [7,13,14]. This may be attributable to their limited energy reserves, and therefore, glochidia are only effective as test organisms for assessing acute toxicity. Although test duration is less limited for juvenile bioassays, there have been problems associated with their use as test organisms in chronic tests. Researchers have had difficulty trying to determine test approaches that meet the unique living requirements of juvenile mussels, and as a result, there is little published literature documenting their chronic sensitivities [15].

The purpose of this study was to conduct several toxicity tests in the laboratory with early life stages of the rainbow mussel (*Villosa iris*) to determine their sensitivity to Hg. *Villosa iris* was selected as the test organism because it is a widespread species in the Southern Appalachians that has been successfully reared in the laboratory. We conducted acute bioassays with glochidia and two-month-old juveniles and a 21-d chronic test with juveniles. Younger juveniles are likely more susceptible to contaminants, but we chose not to use them because of the population bottleneck exhibited by many species. Researchers have documented extremely low juvenile survivorship two weeks after successful transformation (<50%) [16,17], which may be attributable to high predation

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Table 1. Summary of test parameters for acute tests with glochidia and juveniles, and the chronic test with juvenile freshwater mussels

Parameters	Test type/life stage		
	Acute glochidia	Acute juvenile	Chronic juvenile
Test type	Static/nonrenewal	Static/nonrenewal	Dynamic/renewal
Temperature (°C)	20 ± 1	20 ± 1°C	20 ± 1°C
Test duration	72 h	96 h	21 d
Toxicant	MC <sup>a</sup>	MC	MC
Test concn. (µg/L) <sup>b</sup>	0, 8, 15, 30, 60, 120	0, 15, 30, 60, 120, 250	0, 4, 8, 15, 30, 60, 120
Test chamber	50-ml glass beaker	50-ml glass beaker	1-L glass beaker
Amount of test solution (ml)	40	40	950
Diluent water <sup>c</sup>	U.S. EPA <sup>100</sup>	U.S. EPA <sup>100</sup>	U.S. EPA <sup>100</sup>
Sediment	none	none	2 ml sieved to 200 microns
Age test organisms	<2 h after extraction	2 months old	2 months old
Organisms/replicate	50–75	5	5
No. of replicates	4 per time interval	4 total	4 total
Feeding	none	none	30,000 cells daily
Aeration	none	none	yes
Endpoints <sup>d</sup>	LC50	LC50	Survivorship and growth NOAEC + LOAEC

<sup>a</sup> MC = mercuric chloride.

<sup>b</sup> Nominal concentrations.

<sup>c</sup> Moderate hard synthetic water, prepared according to standard U.S. Environmental Protection Agency (U.S. EPA) protocol [23].

<sup>d</sup> NOAEC = no-observable-adverse effects concentration; LOAEC = lowest-observable-adverse effects concentration; LC50 = median lethal concentration.

[18]. Therefore, we decided to conduct bioassays with older, more developed juveniles that are past the phase when high mortality is still anticipated in the laboratory, because concerns about control survivorship have brought the validity of previous unionid tests into question.

## METHODS

### Test organisms

Gravid *V. iris* females were collected from the Clinch River (VA, USA) and transported in water-filled coolers to the laboratory where they were acclimated for 48 h in recirculating troughs (20°C). To extract glochidia, the valves of an adult female were pried open, and a syringe filled with water from the troughs was inserted into the marsupial gill. The water was then slowly injected, thus causing the glochidia in the swollen gill tissue to be flushed and released. After glochidia were extracted, four samples from each adult were assessed for viability using a concentrated NaCl solution, as described by Jacobson et al. [7] and Goudreau et al. [9]. Glochidia from three adult mussels with >90% initial viability were combined and used in the toxicity test.

Juvenile mussels were produced in vivo using rock bass (*Ambloplites rupestris*) as fish hosts at the Virginia Tech Aquaculture Center (Blacksburg, VA, USA). Fish hosts were infested according to the procedure described by Zale and Neves [19]. After juvenile mussels excysted from fish hosts, they were reared for approximately two months before being used as test organisms. Over this period, mussels were housed in sediment (150 µm) within recirculating troughs and fed a daily diet of  $3 \times 10^7$  cells *Neochloris* algae/L.

### Acute toxicity tests

**Glochidia.** Test conditions are summarized in Table 1. Viability was determined by transferring a subsample (~50) of glochidia from a replicate to a glass Petri dish for observation under magnification (×40). The total number of glochidia and number of closed glochidia were tallied, after which a concentrated salt solution (20 g NaCl/L) was added. Then the

number of glochidia not responding to NaCl by contracting their valves was recorded. Any glochidia closed prior to, or open but not responding to, NaCl were classified as functionally dead based on the premise that they would be unable to attach to host fish [7,9].

**Juveniles.** Test conditions are summarized in Table 1. Juvenile mussels were randomly appropriated to replicates by transferring them with a fine-tip glass pipette. To determine survival, mussels in each replicate were observed under magnification (×40) for movement (defined as pedal feeding, active filtering, valve contractions, or visceral mass movement observed through the shell). Individuals showing no movement for 3 min were recorded as dead.

### Juvenile chronic toxicity test

Test conditions are summarized in Table 1. The test apparatus was a modified version of the self-contained, simulated lotic microcosm described by Kennedy et al. [20] that provides flow for lotic organisms. Each simulated lotic microcosm consisted of five small glass vials (outside diameter × height = 28 × 15 mm) placed in a glass Petri dish housed in a 1-L beaker filled with 950 ml of test solution. The Petri dish rested on top of two inverted 50-ml glass beakers. Each vial was filled with 2 ml of sediment sieved to <200 µm and held one juvenile ( $n = 20$ ). A 1-ml glass pipette connected to an air source was placed into the test apparatus.

Juveniles were randomly selected and measured under magnification (×40) using an ocular lens before being transferred to test chambers. Ocular shell length was converted into millimeters. Treatment water was renewed every third day by siphoning and replacing 50% with fresh test solution. The test chambers were supplied daily with  $3 \times 10^7$  *Neochloris* cells/L as food. Test organisms were removed after 21 d, assessed for survivorship, and measured for length, as previously described. Mussels were found by rinsing the contents of a vial into a 250-µm sieve, which caught the juveniles but allowed sediment to pass through.

Table 2. Mean survivorship and median lethal concentration values (LC50) after 24, 48, and 72 h for *Villosa iris* glochidia exposed to different concentrations of mercuric chloride (HgCl<sub>2</sub>)

Total [Hg] (μg/L)	Survivorship (%)		
	24 h	48 h	72 h
Control <sup>a</sup>	94	95	91
8 <sup>a</sup>	96	95	85
12 <sup>b</sup>	94	92	31
25.5	94	86	0
62	92	10	0
107	62	0	0
LC50 value	>107 <sup>c</sup>	39	14
95% confidence limits	NA	37–41	12–15

<sup>a</sup> Below detection limit (<8.4 μg/L) and expressed as nominal value.

<sup>b</sup> To calculate mean concentration, 8.4 μg/L was used as the 72-h value because actual concentration was below detection limit.

<sup>c</sup> Insufficient mortality to generate a median lethal concentration value (LC50).

### Mercury analysis

Total Hg was measured in samples by inductively coupled plasma spectrometry according to U.S. EPA standard methods [21]. Samples were acidified with 50% trace-metal-grade nitric acid (2 ml acid/250 ml of sample). For the glochidia bioassay, initial treatment Hg concentrations were measured at time 0 by preparing a sample of water used to fill test replicates. At 24, 48, and 72 h, samples of out-water from replicates for each treatment were combined and analyzed. Mercury concentrations for the acute juvenile bioassay were measured at time 0 and 96 h, except for the highest treatment, which was measured at 24 h because of complete mortality. During the chronic juvenile test, treatment concentrations were measured for in-water (d 1, 4, 7, 10, 13, 17, 20) and out-water (d 4, 7, 10, 13, 17, 20, 21). The same in-water was used to fill all replicates of a given treatment, and out-water samples from each replicate were combined before being analyzed.

### Statistical analysis

The Toxstat<sup>®</sup> Version 3.5 (West, Laramie, WY, USA) [22] computer program was used to calculate trimmed Spearman-Kärber median lethal concentration values (LC50) for the acute bioassays and no-observable-adverse-effects concentration and lowest-observable-adverse-effects concentration for the chronic test ( $p = 0.05$ ). Data analysis for survivorship and growth followed the U.S. EPA protocol for chronic bioassays with *Pimephales promelas* [23]. Growth (in mm) was calculated by subtracting the initial length from the final length. Measured Hg concentrations were used when values were not below the detection limit.

## RESULTS AND DISCUSSION

### Acute glochidia and juvenile toxicity tests

*Villosa iris* glochidia were more sensitive than two-month-old juveniles to acute Hg exposure; there was nearly a 10-fold difference in 72-h LC50 values. In both tests, there was a dose-dependent response, and toxicity increased with exposure time. After 24 h, glochidia viability remained high and an LC50 value could not be calculated because survivorship was >50% in the highest test treatment (107 μg Hg/L; Table 2). The viability of glochidia decreased substantially after 72 h and was ≤31% for treatments with ≥12 μg Hg/L. Viability in the control and lowest test treatment remained high throughout

Table 3. Mean survivorship and median lethal concentration values (LC50) after 24, 48, 72, and 96 h for *Villosa iris* juveniles exposed to different concentrations of mercuric chloride (HgCl<sub>2</sub>)

Total [Hg] (μg/L)	Survivorship (%)			
	24 h	48 h	72 h	96 h
0 <sup>a</sup>	100	100	100	95
13 <sup>b</sup>	100	100	95	95
26	100	100	100	95
59	100	90	85	75
129	90	75	55	40
234	0	0	0	0
LC50 value	162	135	114	99
95% confidence limits	148–178	114–160	92–141	80–122

<sup>a</sup> Below detection limit (<8.4 μg/L) and expressed as nominal value.

<sup>b</sup> To calculate mean concentration, 8.4 μg/L was used as the 72-h value since actual concentration was below detection limit.

both tests, >90% and ≥85%, respectively. During the juvenile acute test, all individuals died in the highest test concentration after 24 h (234 μg Hg/L), but survivorship remained high for the other treatments (≥90%; Table 3). Survivorship decreased slightly at each time interval the test was monitored but remained >95% after 96 h in treatments of >26 μg Hg/L.

There have been few studies that have examined the toxicity of Hg to early life stages of freshwater mussels. Valenti et al. [12] reported 48-h LC50 values spanning from <8–43 μg/L Hg/L for glochidia of several species, with *V. iris* being the most tolerant species. Keller and Zam [24] conducted bioassays with newly transformed *Anodonta imbecillis* juveniles (1–2 d old), and reported 48- and 96-h LC50 values of 233 μg/L and 171 μg/L, respectively. In experiments similar to those of this study with three-month-old endangered juvenile oyster mussels (*Epioblasma capsaeformis*), we recorded 24-h and 48-h LC50 values of 160 and 140 μg/L total Hg, respectively. Toxicological endpoints similar to those calculated in our study have been generated in studies with juvenile marine bivalves, as LC50 values for different species were between 125 and 161 μg Hg/L [25,26].

Although laboratory toxicity testing with early life stages of freshwater mussels has become a more often utilized approach for assessing environmental risk, few researchers have compared the toxicity of contaminant(s) to glochidia and juveniles of the same species. Jacobson et al. [7] compared the copper sensitivity for different life stages of two species of freshwater mussels. In their study, acute endpoints for *V. iris*-released glochidia and juvenile mussels were 36–80 μg/L and 83 μg/L, respectively, whereas those for *Pyganodon grandis* were 46–347 μg/L and 33–44 μg/L, respectively. Augspurger et al. [27] documented lower mean LC50 values for glochidia than juveniles of three species (*Actinonaias pectorosa*, *Utterbackia imbecillis*, and *V. iris*), despite shorter exposure times to ammonia. Glochidia from three species of freshwater mussels (*U. imbecillis*, *V. lienosa*, and *V. villosa*) were also found to be more sensitive than juveniles in studies conducted by Keller and Ruessler [28] that examined the toxicity of malathion.

The results of previous studies, in addition to those of our experiments, suggest that glochidia may be a more appropriate life stage for assessing acute toxicity based on the regulatory approach of deriving risk from the most sensitive life stage of a species. Glochidia are advantageous as test organisms because they are more easily obtained and are more cost-effective

Table 4. Mean survivorship and growth of 2-month-old juvenile *Villosa iris* exposed to different concentrations of mercuric chloride for 21 d

Total [Hg] <sup>a</sup> ( $\mu\text{g/L}$ )	Survivorship (%)	Growth $\pm$ SD <sup>b</sup> (mm)
0 <sup>b</sup>	90	0.51 $\pm$ 0.17
4 <sup>b</sup>	95	0.53 $\pm$ 0.11
8 <sup>b</sup>	100	0.37 $\pm$ 0.13*
15	95	0.26 $\pm$ 0.13*
32	100	0.06 $\pm$ 0.05*
62	100	0.01 $\pm$ 0.02*
114	100	0 $\pm$ 0*

<sup>a</sup> Mean in-water concentrations; out-water for all concentrations was below detection limit ( $<8.4 \mu\text{g/L}$ ).

<sup>b</sup> SD = standard deviation.

<sup>c</sup> Below detection limit ( $<8.4 \mu\text{g/L}$ ) and expressed as nominal value.

\* Significantly lower than control ( $p < 0.05$ ).

to use in toxicological studies than juveniles. Sufficient numbers can be obtained from only a few females, which is especially important when assessing risk for endangered species or species that have yet been successfully cultured in the laboratory. Furthermore, high viability ( $>90\%$ ) has repeatedly been observed in control treatments after 48 h in experiments with glochidia from *V. iris* and other species [12]. In addition, glochidia, unlike juveniles, are less able to avoid toxicants because they have thinner, more permeable shells [29]. Conversely, juvenile mussels that are several months old may be able to avoid toxicants by altering their normal metabolism to a lipid catabolism. This would enable them to reduce their filtration rates and close their valves for extended periods to avoid contaminants, which would limit their effectiveness as test organisms.

#### Chronic 21-d juvenile toxicity test

The mean growth of individuals in the control and 4  $\mu\text{g}$  Hg/L treatments were  $>0.5$  mm and did not vary significantly (Table 4). Growth was reduced by 25% at 8  $\mu\text{g}$  Hg/L and by  $\geq 50\%$  in the remaining treatments. Individuals exposed to  $\geq 32$   $\mu\text{g}$  Hg/L only grew approximately 10% as much as the controls. No dead juveniles were found, and the only apparent mortalities recorded were for individuals not located. No-observable-adverse-effects concentration and lowest-observable-adverse-effects concentration for growth were 4 and 8  $\mu\text{g}$  Hg/L, respectively.

Several studies have shown that bivalves decrease oxygen consumption, growth, and byssal thread production when exposed to Hg [6,7,25]. Salanki and Balogh [5] reported that Hg affects filtration rates of bivalves; exposed individuals had shorter periods of activity and extended periods of rest. The lower growth observed in treatments containing higher Hg concentrations may be caused by ingesting less food for assimilation into new body tissue. The high survivorship despite the lack of growth observed in the chronic bioassay emphasizes the importance of assessing sublethal effects. Although these types of impairment do not cause immediate mortality, they likely have adverse latent effects on survivorship and may be more appropriate endpoints for assessing environmental impairment.

The absence of mortality in the chronic bioassay is attributable to several differences between the acute and chronic test designs. The addition of sediment in the chronic tests likely reduced toxicity by serving as a physical barrier to exposure

or by binding up some of the Hg. Keller et al. [15] reported that the use of silt in juvenile mussel experiments removed a substantial portion of copper from the water column. Difficulties with maintaining constant Hg concentrations in the water column caused juveniles to be only intermittently rather than chronically exposed to Hg. Further, not feeding the mussels in the acute test also may have expedited their uptake rate; Naimo [6] noted that some bivalves accumulate Hg faster when not fed. Acute bioassay test conditions may have stressed juveniles, causing them to be more susceptible to Hg, because test chambers were not aerated and did not contain sediment. In early attempted experiments, high mortality was observed ( $>50\%$ ) in control treatments without sediment or aeration after 7 d, despite feeding and water renewal.

#### CONCLUSIONS

Our study supports the use of glochidia as a surrogate life stage for juveniles in acute bioassays, because their ability to avoid exposure is limited and because they are more readily available and cost-effective to obtain for toxicity tests. There is concern that conducting acute bioassays with older juveniles could lead researchers to underestimate toxicity, because individuals may be able to avoid toxicants by closing their valves for sustained periods. However, there are limitations with using glochidia as test organisms, because they may be used only in short-term tests. Therefore, it is imperative that a new integrated approach be taken when assessing risk to freshwater mussels, as their unique life history is atypical of standard test organisms. We strongly advocate the use of both glochidia and juvenile life stages in future toxicological studies. In addition to standard test organisms, glochidia may be ideally used during initial phases of risk assessment in acute experiments. These tests will provide researchers with a cost-effective means for determining whether further investigation is needed for specific environmental scenarios. If reasonable threat is apparent, then chronic tests with juvenile mussels may be warranted.

Overall, it is important that researchers still use caution when using findings from laboratory bioassays to infer environmental risk. Glochidia are most susceptible to contaminants in the water column after being released by the gravid adult and before encysting on host fish [7], which is often only a short time. Conversely, juvenile mussels are more likely threatened by chronic exposure to toxicants in sediment or interstitial water; Yeager et al. [1] noted that juvenile *V. iris* burrow into the substrate and rely more heavily on pedal feeding, rather than filter feeding. Additional research exploring new testing techniques is needed before researchers are able to determine workable approaches for assessing pollution risk to mussel assemblages.

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