14 Case Study: Sensitivity of Mussel Glochidia and Regulatory Test Organisms to Mercury and a Reference Toxicant

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INTRODUCTION

Freshwater mussel populations have declined substantially in North America, and more than two-thirds of the identified species (*Unionidae*) are classified as extinct, endangered, threatened, or of special concern (Williams et al. 1993; Naimo 1995; Jacobson et al. 1997). Although exploitation from commercial over-harvest and the introduction of nonnative species have had substantial impacts (Williams et al. 1993; Yeager, Neves, and Cherry 1999), many declines are attributed to anthropogenic stresses that have eliminated or degraded the natural habitat of mussels (Keller and Zam 1991; Williams et al. 1993; Naimo 1995; Milam and Farris 1998; Henley and Neves 1999; Diamond, Bressler, and Serveiss 2002; Weinstein 2002). Scientists have addressed these potential risks by improving agricultural practices, waste management, and pollution monitoring in the United States, and consequently, water quality has substantially improved. Furthermore, the implementation of regulatory policies that are focused on preserving wildlife and the environment, such as the Endangered Species Act of 1973 and Clean Water Act of 1977, promotes the protection of not only native unionids, but also their habitat. However, despite clear progress, there is still concern about the future conservation of native mussels, as survey efforts have shown little recruitment (Neves and Widlak 1987; Breunderman and Neves 1993; Henley and Neves 1999).

Researchers have observed that, of the remaining diverse mussel assemblages, many are comprised primarily of older, adult mussels, and few have an abundance of young mussels present (Henley and Neves 1999; Weinstein 2001). These trends indicate that populations are unstable and declining. Conservationists are especially concerned because it may take years for young mussels currently residing in rivers to reach peak sexual maturity. The complex life history of unionids has made it difficult for researchers to determine the causes of reproductive failure. However, there is substantial evidence that pollution is a contributing factor, as several laboratory studies have documented that freshwater mussels, like most aquatic organisms, are more sensitive to contaminants during their early life stages than as adults (Naimo 1995; Jacobson et al. 1997; Keller and Ruessler 1997; Yeager, Neves, and Cherry 1999; Weinstein 2001).

Jacobson et al. (1997) conducted a comprehensive study that examined the effects of copper exposure on the various life stages of freshwater mussels. Their study compared the sensitivities of

Villosa iris glochidia that were brooded (still in the gills of a gravid adult), released (in the water column), and encysted (attached to a fish host). Released glochidia were impacted at lower copper concentrations (36–80 µg Cu/L) than encysted glochidia (greater than 400 µg Cu/L). No adverse effects were observed for any treatments in the brooded glochidia test; however, the highest concentration tested was only 19 µg Cu/L. Interestingly, released glochidia and juveniles had very similar tolerances, as 24-hour LC50 values for glochidia of *V. iris* and *Pyganodon grandis* were 36–80 and 46–347 µg Cu/L, respectively, while those for juveniles were 83 and 44 µg Cu/L. More important, the study provided clear evidence that early life stages of freshwater mussels have far lower acute contaminant exposure thresholds than adults, as the 96-hour LC50 for adults was greater than 1000 µg Cu/L.

Only a few other studies have examined the acute tolerances of glochidia and juvenile mussels of the same species, but most concur with Jacobson et al. (1997) and report that glochidia are as sensitive or more sensitive than juveniles in acute exposures. In a study examining the toxicity of ammonia, Augspurger et al. (2003) recorded higher tolerances for juveniles than glochidia, despite a longer exposure duration. The 96-LC50 values for juvenile pheasantshell mussels (*Actinonaias pectorosa*) and paper pondshell mussels (*Utterbackia imbecillis*) were 14.05 and 10.60 mg total ammonia as N/L, respectively, while the corresponding 48-hour value for glochidia were 3.76 and 5.85 mg total ammonia/L. Similarly, the mean 96-hour LC50 for the rainbow mussel (*V. iris*) was 6.75 mg total ammonia/L, and the 24-hour value for glochidia was 3.79 mg total ammonia/L. Keller and Ruessler (1997) examined the toxicity of malathion to early life stages of the pondshell (*U. imbecillis*), little spectaclecase (*Villosa lienosa*), and downy rainbow mussel (*Villosa villosa*), and also recorded substantially lower tolerances for glochidia than for juveniles.

Additional studies have also documented that glochidia are more acutely sensitive to contaminants than standard regulatory organisms used for Whole Effluent Toxicity (WET) testing, and US Environmental Protection Agency (USEPA) Water Quality Criteria (WQC). Cherry et al. (2002) compared the acute sensitivities of 17 species of freshwater organisms to copper. Four of the five most sensitive test organisms were freshwater mussel glochidia, while standard regulatory test organisms *Ceriodaphnia dubia* and *Pimephales promelas* ranked sixth (88µg Cu/L), and fourteenth (310 µg Cu/L), respectively. The Genus Mean Acute Values (GMAV) for glochidia of the four most sensitive mussels species ranged from 37 to 60 µg Cu/L. Studies that examined the toxicity of ammonia to early life stages of freshwater mussels also reported LC50 values that are within the ranges described for standard test organisms *C. dubia*, *P. promelas*, *Daphnia magna*, and *Oncorhynchus mykiss* (rainbow trout) (Goudreau, Neves, and Sheehan 1993; Mummert et al. 2003). Milam and Farris (1998) noted that glochidia of *Leptodea fragilis* were more sensitive than *P. promelas* to partially treated mine water but less sensitive than *D. magna* and *C. dubia*. However, their study contrasted the 24-hour acute glochidia LC50s with 48-hour acute LC50s for *D. magna* and 7-day fecundity EC_{50}s for *C. dubia*. Although the results of the aforementioned studies may influence freshwater regulatory policy, agencies are hesitant to accept test results because there is concern about the effectiveness of glochidia as test organisms in the laboratory.

Guidelines for conducting acute toxicity tests with early life stages of freshwater mussels were submitted to the USEPA in 1990 (USEPA 1990). The effort brought laboratory toxicity testing with freshwater mussels to the foreground of aquatic toxicology but failed to address several aspects essential for the development of a standard protocol. The primary criticism was the use of glochidia in toxicity tests that were obtained from gravid adults collected from rivers. There is concern that environmental variables, such as pollution or nutrient availability, may affect the ability of gravid females to produce fit offspring. The maturity of glochidia collected from different adults of the same species will likely vary, as not all individuals from a species have synchronized reproductive cycles. The time of season that mussels are obtained from the field may also influence maturity of glochidia, as unionids can be categorized into long- and short-term brooders (Jacobson et al. 1997). Unhealthy or immature glochidia are likely to be more susceptible to contaminant exposure

(Huebner and Pynnonen 1992; Goudreau, Neves, and Sheehan 1993; Jacobson et al. 1997), and their use in tests may lead to biased, false-positive results. Although verifying test organism health is a universal concern for all toxicological studies, it is especially problematic for research with glochidia because researchers are still unsure of appropriate methods. There have been substantial strides towards establishing acceptable test parameters and methodologies for glochidia tests (Chapter 5), but efforts will go unheeded unless better techniques for assessing the health of glochidia are developed.

Study Goals

The primary purpose of this study was to compare the sensitivities of glochidia from different species of freshwater mussels to mercury (Hg) by conducting laboratory tests with organic and inorganic mercury salts. Many freshwater systems are contaminated by mercury pollution, as anthropogenic sources, such as the incineration of medical wastes, disposal of mercury-laden material, industrial processing, pesticide use, and the burning of fossil fuels, have made it more available in ecosystems. Although most mercury is emitted in elemental or inorganic forms that are not highly toxic, several abiotic and biotic factors may facilitate the conversion of these forms into methylmercury (MeHg) in water (Barkay, Gillman, and Turner 1997; Wiener and Shields 2000; Mauro, Guimaraes, and Hintelmann 2002). This organic form of mercury is highly toxic to aquatic life and has been documented to bio-accumulate in food webs (Barkay, Gillman, and Turner 1997; French et al. 1999; Mason, Laporte, and Andres 2000; Wiener and Shields 2000; Mauro, Guimaraes, and Hintelmann 2002). The USEPA is currently reassessing the WQC for mercury, as researchers have become more aware of the threat it poses to humans and wildlife (Moore, Teed, and Richardson 2003). Fish Consumption Advisories (FCA) for mercury have been issued in nearly every US state (French et al. 1999; Mason, Laporte, and Andres 2000; Webber and Haines 2003). However, recent studies examining the sensitivities of freshwater organisms are sparse, and results from older studies may be flawed because technology for measuring a low concentration of mercury did not exist. Furthermore, there is little known about the sensitivity of freshwater mussels to mercury, despite documented declines in polluted water (Henley and Neves 1999; Beckvar et al. 2000). It is pertinent to address these voids because a more comprehensive species database will be needed to establish appropriate water standards.

Another objective of this study was to compare the mercury sensitivities of glochidia to those of standard regulatory organisms, *C. dubia*, *D. magna*, and *P. promelas*. Several studies have noted that glochidia are extremely sensitive compared to the larvae stages of other aquatic biota (Jacobson et al. 1997; Weinstein 2001; Weinstein and Polk 2001; Cherry et al. 2002). We wanted to determine if standard, freshwater, regulatory test organisms are adequate surrogate test organisms for assessing mercury exposure risks to glochidia. Environmental risk is often inferred by conducting toxicity tests with standard monitoring organisms that are sensitive to most toxicants. This approach should not be implemented for assessing risk to freshwater mussels until the relative tolerances of the respective genera are discerned.

The final goal of this study was to expose glochidia to sodium chloride (NaCl) to determine if it is an appropriate reference toxicant. Tests were conducted based on methods described in protocol for standard freshwater test organisms (USEPA 1993). Reference toxicity test measures are useful QA/QC assurances for standard test organisms because they enable researchers to evaluate the relative health of the test organisms, verify the acceptability of test conditions or procedures, and validate toxicity tests results. Reference toxicant tests are supposed to be conducted monthly at culturing facilities, and concurrently with acute and chronic WET testing with standard test organisms. Similar approaches have not been applied to glochidia, and the inadequacy of current methods for assessing the health of glochidia must be addressed for regulatory agencies to be willing to incorporate test results into environmental policy.

METHODS

Test Organisms

Gravid specimens of *Lampsilis fasciola* (Wavyrayed lampmussel), *V. iris* (Rainbow mussel), *Epioblasma capsaeformis* (Oyster mussel), and *Epioblasma brevidens* (Cumberland combshell) were obtained from the Virginia Polytechnic Institute & State University (VPI&SU) Aquaculture Center in Blacksburg, VA. Gravid adults of the various species were collected from the Clinch River, VA, and stored at the Buller Fish Hatchery in Marion, VA. Adult mussels were acclimated to laboratory conditions for at least 48 hours before the glochidia were harvested. Glochidia were extracted by gently prying open the valves of a gravid female, puncturing the gill tissue with a sterile, water-filled syringe, and then injecting water to flush individuals out. Glochidia were loaded into test chambers less than 2 hours after extraction.

Daphnids, *C. dubia* and *D. magna* (less than 24 hours old), were cultured at the VPI&SU Aquatic Toxicology Laboratory according to standard procedure (APHA, AWWA, and WEF 1998). Organisms were cultured in an 80:20 mixture of moderately hard, synthetic water (EPA^{100}) (USEPA 1993) and filtered reference water at 25 ± 1°C under a 16:8, light:dark photoperiod and were fed a diet of unicellular algae (*Selenastrum capricornutum*) and YCT (yeast/cereal leaves/trout chow). Fathead minnows were obtained from a commercial supplier (Aquatox, Inc., Hot Springs, AR).

Preparation of Mercury Test Solutions

Mercuric chloride (MC) and methylmercuric chloride (MMC) salts were used to create the inorganic and organic test solutions, respectively. Test concentrations were 8, 15, 30, 60, and 120 µg/L total Hg, plus a control, in all bioassays, except for some *C. dubia* and *D. magna* tests when the highest concentration, 120 µg/L, was replaced with the lower concentration of 4 µg/L total Hg.

Toxicity Tests

Glochidia. Because a protocol has yet to be established for glochidia bioassays, we attempted to adhere to the test design described in USEPA protocol (1993) for standard freshwater test organisms. The main modification was an increase in the number of test organisms per replicate. The small size of glochidia makes them difficult to monitor individually; therefore, researchers assessed viability for a sub-sample of individuals from each replicate. This approach provided a more accurate estimate of viability per replicate and also minimized problems from potential handling stress.

Glochidia were randomly distributed to 50-mL glass beakers filled with ~35 mL of test solution. There were eight replicates of 50–100 glochidia for each treatment. Viability was assessed in four randomly selected replicates after 24 hours, and the remaining four replicates were assessed after 48 hours. Tests were conducted at 20 ± 1°C under a 12:12, light:dark photoperiod.

Glochidia viability was assessed through a sodium chloride response test, similar to that described by Huebner and Pynnonen (1992), Goudreau, Neves, and Sheehan (1993), Jacobson et al. (1997), and Keller and Ruessler (1997). A sample of glochidia from a replicate was transferred with a fine-tip glass to a petri dish for observation using a dissecting scope. The total number of open and closed glochidia was recorded, and after which, a concentrated sodium chloride solution was added. Any glochidia closed prior to, or remaining open after, the addition of the salt solution were documented as functionally dead.

EPA test organisms. Acute 48-hour toxicity tests were conducted with *C. dubia*, *D. magna*, and *P. promelas* according to USEPA standard protocol (1993). Cladoceran bioassays were conducted in 50-mL glass beakers with approximately 35 mL of test solution. There were four replicates of

five individuals for each treatment. *Pimephales* bioassays were conducted in 300-mL glass beakers filled with ~250 mL of test solution. There were two replicates of ten individuals for each concentration. Mortality was assessed after 24 and 48 hours. All tests were conducted at 20±1°C under a 12:12, light:dark photoperiod, and organisms were not fed during the tests.

Reference Toxicant Tests

Reference toxicity tests were conducted with glochidia of *L. fasciola*, *E. capsaeformis*, and *E. brevidens*. Sodium chloride was used as the toxicant because it is the suggested contaminant for reference bioassays with standard freshwater regulatory test organisms (USEPA 1993). A 0.5 serial dilution was used to create treatments, which include a control, 0.5, 1.0, 2.0, 4.0, and 8.0 g NaCl/L diluent water; these are the same concentrations for *C. dubia* reference tests. Certified reference-grade sodium chloride was used as the toxicant, and EPA^{100} was used as the diluent and control treatment. Viability of glochidia was assessed after 24 and 48 hours of exposure. Bioassays were conducted at 20±1°C under a 12:12, light:dark photoperiod.

Results of monthly acute sodium chloride reference toxicant tests at the VPI&SU Aquatic Toxicology Laboratory for NPDES permit tests with *C. dubia*, *D. magna*, and *P. promelas* were compiled for comparative purposes. Tests were conducted according to standard protocol (USEPA 1993) between January 2001 and August 2003.

Water Chemistry and Mercury Analysis

Temperature was monitored twice daily. Dissolved oxygen, conductivity, and pH were measured for all in-water and out-water in the bioassays. Alkalinity and hardness were measured for the control and highest concentration for in-water. An Accumet® (Fisher Scientific, Pittsburgh, PA, USA) pH meter with an Accumet gel-filled combination electrode (accuracy less than ±0.05 pH at 25°C) was used to measure pH. Dissolved oxygen and conductivity were measured with a 54A meter® and model 30 conductivity meter®, respectively, from Yellow Springs (Yellow Springs, OH, USA). Total hardness and alkalinity (as mg/L $CaCO_3$) were measured in accordance with APHA, AWWA, and WEF (1998) through colorimetric titrations.

Samples of in- and out-water from several replicates were combined for each treatment and prepared for Inductively Coupled Plasma (ICP) spectrometry according to USEPA (1991) standard methods. Trace metal-grade pure hydrochloric acid was used to reduce the sample pH to less than or equal to two. The prepared samples were refrigerated until analysis at the VA Tech Soil Laboratory (Blacksburg, VA).

Data Analysis

Toxicity test results were presented as LC50 values and were calculated by Spearman Karber analysis on computer software (Gulley 1993). All calculations based on nominal total mercury concentrations as treatments less than 15 µg Hg/L were below detection limits (BDL).

RESULTS

Control Survivorship

The combined mean glochidia viability in control treatments for all of the bioassays was greater than 89% for the species tested after 24 hours (Figure 14.1). Mean control survivorship remained greater than 80% after 48 hours for all species except *L. fasciola*, which declined to 78%. Overall, viability did substantially decrease with increased exposure time for all species except *V. iris*.

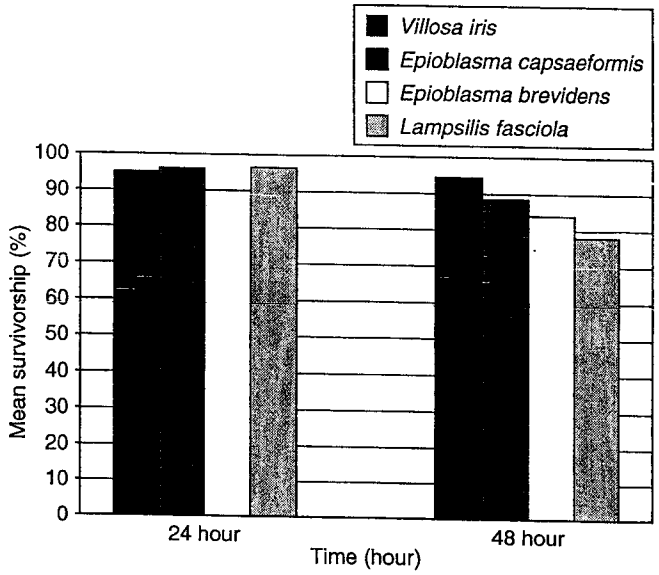

FIGURE 14.1 Glochidia control survivorship.

Mercury Salt Results

Mercuric chloride. Glochidia from the different species of freshwater mussels had similar tolerances to MC, as 24-hour and 48-hour LC values for *L. fasciola*, *E. capsaeformis*, and *E. brevidens* ranged from 25–54 and 27–40 μg Hg/L, respectively (Table 14.1). Although not evident by the LC50 values, viability decreased with increased exposure time in nearly every treatment. Survivorship remained high in the control (24 hours = greater than 89% and 48 hours = greater than 81%) but was substantially reduced in treatments containing elevated concentrations of mercury. After 48 hours, 100% mortality was observed in treatments greater than or equal to 120 μg Hg/L.

Ceriodaphnia was far more sensitive to MC than *D. magna*, as the respective 48-hour LC50 values were 7 and 19 μg Hg/L (Table 14.1). Sensitivity increased with exposure time in both tests, and the largest contrast in 24- and 48-hour LC50 values (90 and 15 μg Hg/L, respectively) was observed with *D. magna*. Survivorship in the control remained 100% but was substantially reduced in treatments with measurable concentrations of mercury for both species.

Methylmercuric Chloride

The LC50 values for glochidia of *E. capsaeformis* and *E. brevidens* exposed to MMC were substantially lower than those documented in MC tests. The LC50 values after 24 hours ranged from 21 to 26 μg Hg/L for the two species (Table 14.2). However, 48-hour LC50 values could not be calculated because mortality was more than 50% in the lowest test treatment, 8 μg Hg/L. Therefore, these values were reported conservatively as less than 8 μg Hg/L. *Villosa iris* glochidia were far more tolerant than the two other species. A 24-hour LC50 could not be calculated because only 38% of the individuals exposed to 120 μg Hg/L died; however, the value was reported as more than 120 μg Hg/L for comparative purposes. After 48 hours, the LC50 for *V. iris* declined substantially to 43 μg Hg/L, but was still five times higher compared to the values found for glochidia from the other species.

Ceriodaphnia was the most sensitive organism tested to MMC, as 100% mortality occurred in treatments greater than or equal to 8 μg Hg/L, despite 100% survivorship in the control (Table 14.2). The 48-hour LC50 could not be calculated in either *C. dubia* test because of high mortality in low concentrations. Subsequently, these values were reported conservatively as less

TABLE 14.1
Comparative Acute Toxicity of Glochidia from Three Mussel Species and Two Daphnids to Mercuric Chloride

Organisms	Species	Concentration (µg Hg/L)	*n*	24-hour % Mortality	24-hour LC50 (95% CI)	*n*	48-hour % Mortality	48-hour LC50 (95% CI)
Glochidia	*L. fasciola*	Control	200	6	40 µg Hg/L (40–50)	200	19	40 µg Hg/L (30–40)
		5	200	4		200	17	
		10	200	4		200	15	
		15	200	6		200	16	
		30	200	7		200	10	
		60	200	9		200	30	
		120	200	85		200	100	
		250	200	100		200	100	
Glochidia	*L. fasciola*	Control	200	3	40 µg Hg/L (30–40)	n/a	n/a	n/a
		8	200	4				
		15	200	13				
		30	200	60				
		60	200	100				
		120	200	100				
Glochidia	*E. capsaeformis*	Control	50	4	25 µg Hg/L (22–25)	50	18	27 µg Hg/L (n/a)
		8	50	6		50	10	
		15	50	16		50	36	
		30	50	64		50	68	
		60	50	100		50	100	
		120	50	100		50	100	
Glochidia	*E. capsaeformis*	Control	100	3	54 µg Hg/L (49–60)	100	10	36 µg Hg/L (33–38)
		8	100	6		100	7	
		15	100	8		100	6	
		30	100	14		100	28	
		60	100	50		100	95	
		120	100	100		100	100	

(continued)

TABLE 14.1 ***(Continued)***

Organisms	Species	Concentration (μg Hg/L)	*n*	24-hour % Mortality	24-hour LC50 (95% CI)	*n*	48-hour % Mortality	48-hour LC50 (95% CI)
Glochidia	*E. brevidens*	Control	100	11	47 μg Hg/L (42–53)	100	17	27 μg Hg/L (24–30)
		8	100	8		100	21	
		15	100	12		100	16	
		30	100	17		100	53	
		60	100	62		100	100	
		120	100	100		100	100	
Cladoceran	*C. dubia*	Control	20	0	11 μg Hg/L (10–12)	20	0	7 mg Hg/L (5–9)
		4	20	5		20	15	
		8	20	30		20	60	
		15	20	60		20	85	
		30	20	100		20	100	
		60	20	100		20	100	
Cladoceran	*D. magna*	Control	20	0	90 μg Hg/L (80–100)	20	0	19 μg Hg/L (17–22)
		8	20	0		20	5	
		15	20	5		20	40	
		30	20	5		20	80	
		60	20	15		20	100	
		120	20	80		20	100	

TABLE 14.2
Comparative Acute Toxicity of Glochidia from Three Mussels Species and Three Standard USEPA Test Organisms to Methylmercuric Chloride

Organisms	Species	Concentration (µg Hg/L)	N	24-hour % Mortality	24-hour LC50 (95% CI)	n	48-hour % Mortality	48-hour LC50 (95% CI)
Glochidia	*E. capsaeformis*	Control	50	4	21 µg Hg/L (17–24)	50	18	8 µg Hg/L (4–9)
		8	50	10		50	70	
		15	50	36		50	80	
		30	50	68		50	100	
		60	50	100		50	100	
		120	50	100		50	100	
Glochidia	*E. capsaeformis*	Control	100	3	26 µg Hg/L (23–28)	100	10	<8 µg Hg/L (n/a)
		8	100	4		100	49	
		15	100	13		100	100	
		30	100	60		100	100	
		60	100	100		100	100	
		120	100	100		100	100	
Glochidia	*E. brevidens*	Control	100	11	25 µg Hg/L (22–28)	100	17	<8 µg Hg/L (n/a)
		8	100	10		100	56	
		15	100	26		100	100	
		30	100	51		100	100	
		60	100	100		100	100	
		120	100	100		100	100	
Glochidia	*V. iris*	Control	326	6	>120 µg Hg/L	305	5	43 µg Hg/L (41–45)
		8	246	4		316	5	
		15	257	6		309	8	
		30	316	6		325	15	
		60	276	8		314	90	
		120	255	38		336	100	
Cladoceran	*C. dubia*	Control	20	0	30 µg Hg/L (20–30)	20	5.0	<8 µg Hg/L (n/a)
		8	20	10		20	100	

(continued)

TABLE 14.2 *(Continued)*

Organisms	Species	Concentration (µg Hg/L)	N	24-hour % Mortality	24-hour LC50 (95% CI)	n	48-hour % Mortality	48-hour LC50 (95% CI)
		15	20	15		20	100	
		30	20	60		20	100	
		60	20	90		20	100	
		120	20	100		20	100	
Cladoceran	*C. dubia*	Control	20	0	25 µg Hg/L (20–30)	20	0	<4 µg Hg/L (n/a)
		4	20	5		20	85	
		8	20	15		20	100	
		15	20	15		20	100	
		30	20	30		20	100	
		60	20	100		20	100	
Cladoceran	*D. magna*	Control	20	0	20 µg Hg/L (20–22)	20	0	18 µg Hg/L (15–21)
		8	20	0		20	5.0	
		15	20	0		20	15	
		30	20	95		20	100	
		60	20	100		20	100	
		120	20	100		20	100	
Cladoceran	*D. magna*	Control	20	0	>60 µg Hg/L	20	0	15 µg Hg/L (11–19)
		4	20	0		20	0	
		8	20	0		20	5	
		15	20	5		20	45	
		30	20	15		20	100	
		60	20	35		20	100	
Fish	*P. promelas*	Control	20	0	120 µg Hg/L (n/a)	20	0	67 µg Hg/L (57–77)
		0.008	20	0		20	0	
		0.015	20	0		20	0	
		0.03	20	0		20	0	
		0.06	20	0		20	35	
		0.12	20	15		20	100	

than 4 and less than 8 µg Hg/L. *Daphnia* were also quite sensitive to MMC, as 48-hour LC50 values for the two trials were 18 and 15 µg Hg/L. *Pimephales promelas* was extremely tolerant to MMC exposure, as a 24-hour LC50 value could not be calculated due to only 15% mortality in the highest treatment; this value was expressed as more than 120 µg Hg/L. A 48-hour LC50 value was calculated for *P. promelas* that was considerably lower, 67 µg Hg/L, but remained markedly higher than values for the other species.

Reference Toxicant Results

Glochidia. Glochidia of *L. fasciola*, *E. capsaeformis*, and *E. brevidens* had similar tolerances to sodium chloride, as the upper and lower 95% confidence limits for the 24- and 48-hour LC50 values nearly overlapped (Table 14.3). After 48 hours, control survivorship was extremely low in the *L. fasciola* bioassay (68%); therefore, the reported LC50 value of 2.25 g NaCl/L is considered unreliable. Control survivorship was more stable in the bioassays with the other species. As in the mercury tests, viability decreased with increased exposure times in most treatments. The average 48-hour LC50 for glochidia from all three species combined was 2.46 g NaCl/L (Figure 14.2).

Standard Regulatory Test Organisms

The three different standard regulatory organisms, *C. dubia*, *D. magna*, and *P. promelas*, had very distinct sodium chloride tolerances. The most sensitive species was *C. dubia*, as the average 48-hour LC50 was 2.33 g NaCl/L. Similar values for *D. magna* and *P. promelas* were 4.96 and 9.84 g NaCl/L (Figure 14.2).

Water Chemistry and Mercury Concentrations

Water chemistry parameters and mercury concentration analysis results for the different test treatments are summarized in Table 14.4. Dissolved oxygen remained more than 5.0 mg/L in all bioassays. Other water parameters for in- and out-water did not differ substantially, except for total mercury concentration, which was substantially lower in out-water. Treatments less than or equal to 15 µg Hg/L were below detection limit. The in-water for treatments greater than or equal to 30 µg Hg/L were very close to nominal concentrations.

DISCUSSION

Mercury Tests

Glochidia sensitivities. Researchers have noted that glochidia may sporadically clasp or completely seal their valves when exposed to contaminants during laboratory toxicity tests. Effects induced by toxicants may also be less apparent if glochidia remain open. However, researchers can infer the viability of these individuals by exposing them to a noxious substance, such as sodium chloride, that is known to elicit this avoidance behavior (Huebner and Pynnonen 1992; Goudreau, Neves, and Sheehan 1993; Jacobson et al. 1997; Keller and Ruessler 1997). Several laboratory studies have reported that released glochidia have substantially lower viability in treatments containing elevated concentrations of contaminants (Huebner and Pynnonen 1992; Goudreau, Neves, and Sheehan 1993; Jacobson et al. 1997; Keller and Ruessler 1997; Cherry et al. 2002). These observations have incited speculation that contaminants may be attributing to the lack of recruitment in the water column by reducing the ability of glochidia to successfully attach to host fish. A decrease in this ability would inevitably lower the reproductive potential of impacted individuals. In our study, very low concentrations of mercury drastically affected the viability of glochidia. However, additional research is needed to more accurately determine species tolerances because impairment was observed in treatments with mercury concentrations BDL in this study. Regardless, this study

TABLE 14.3
Mussel Glochidia Acute Response to a Reference Toxicant, NaCl

Species	Concentration (g NaCl/L)	Temperature	*N*	24-hour % Mortality	24-LC50 (95% CI)	*n*	48-hour % Mortality	48-hour LC50 (95% CI)
L. fasciola	Control	22	200	3.5	3.08 g NaCl/L (2.91–3.26)	200	32	2.25 g NaCl/L (2.14–2.35)
	0.5	22	200	3.0		200	18	
	1.0	22	200	2.0		200	23	
	2.0	22	200	17.5		200	49.5	
	4.0	22	200	73.5		200	100	
	8.0	22	200	100		200	100	
E. capsaeformis	Control	20	50	6.0	2.71 g NaCl/L (2.54–2.88)	50	8.0	2.45 g NaCl/L (2.21–2.70)
	0.5	20	50	4.0		50	6.0	
	1.0	20	50	4.0		50	8.0	
	2.0	20	50	8.0		50	22	
	4.0	20	50	100		50	100	
	8.0	20	50	100		50	100	
E. brevidens	Control	20	50	8.0	2.68 g NaCl/L (2.50–2.88)	100	14	2.67 g NaCl/L (2.54–2.79)
	0.5	20	50	6.0		100	12	
	1.0	20	50	6.0		100	12	
	2.0	20	50	10		100	10	
	4.0	20	50	100		100	100	

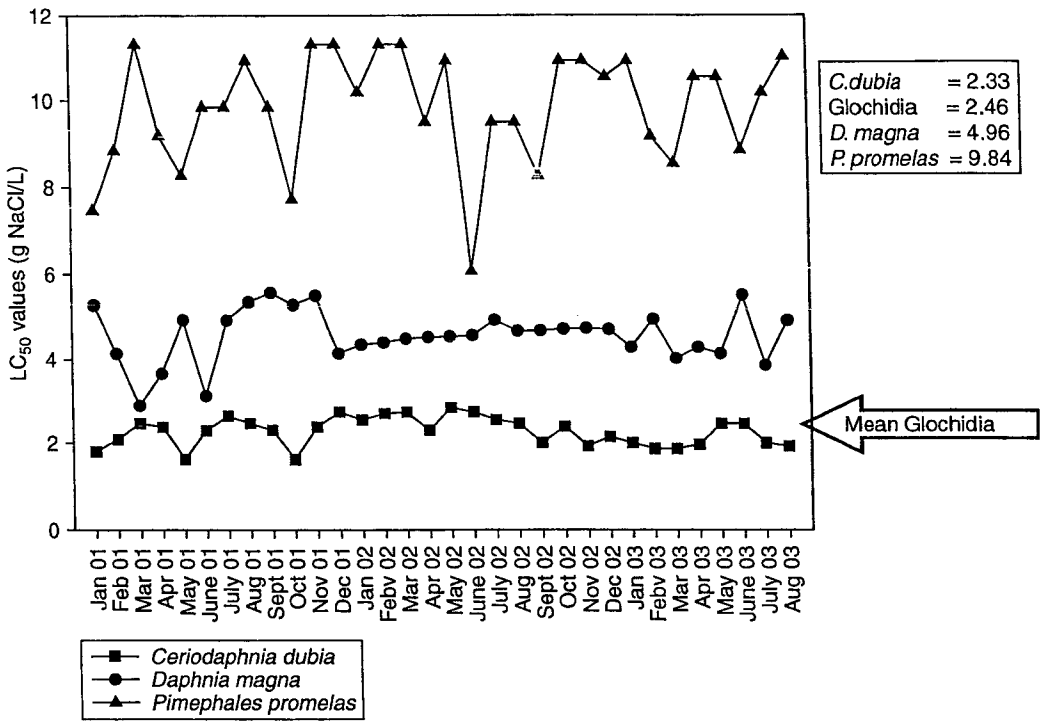

FIGURE 14.2 Reference data.

provided substantial evidence that released glochidia from some species of freshwater mussels are sensitive to mercury at concentrations that may be measured in the environment.

Interestingly, we also noted substantial interspecific variability in mercury tolerances among glochidia of different species, as individuals from *L. fasciola*, *E. capsaeformis*, and *E. brevidens* were highly sensitive to acute exposure but those from *V. iris* were not. Only a few other studies have conducted experiments with glochidia from numerous species of freshwater mussels; however, those that have often report substantial variability in species tolerances. Cherry et al. (2002) examined the effect of copper on glochidia from eight species of mussels, and reported mean LC50 values that ranged from 37 to 137 µg Cu/L. Keller and Ruessler (1997) conducted experiments on glochidia of six mussel species with the pesticide malathion and reported an even greater range, as the 48-hour LC50 value for the most sensitive species, *Lampsilis siliquoidea*, was 7 mg/L compared to 324 mg/L for the most tolerant species tested, *U. imbecillis*.

There was also a distinct difference in the toxicity of the different mercury salt forms, as glochidia from *L. fasciola*, *E. capsaeformis*, and *E. brevidens* were far more sensitive to MMC than to MC. Although the same total mercury concentrations were tested for both salt treatments, the 48-hour LC50 values were approximately three times lower for glochidia exposed to MMC. Several other studies have documented similar differences in the toxicity of mercury salts with test organisms other than glochidia. Baby and Menon (1987) observed that juvenile marine bivalves were more sensitive to mercury if exposed to the organic salt $(CH_3COO)_2$ Hg, than when exposed to the inorganic salt $HgCl_2$. Similarly, Wobeser (1975) reported that MMC is more toxic and accumulates faster than MC in the tissues of young age classes of rainbow trout. Biesinger, Anderson, and Eaton (1982) cited that *D. magna* in a chronic study excreted mercury slower when exposed to MMC than MC.

TABLE 14.4
Mean Water-Quality Data for the Acute Mercury and Reference Toxicant Tests—Samples from the Different Treatments Were Combined before Analysis

Test	Treatment	Conductivity (μmhos)	pH (su)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	In Hg (μg/L)	Out Hg (μg/L)
All	Control	298±9	7.78±0.13	62.7±4.4	83.5±4.6	BDL	BDL
MC	4	297±8	7.81±0.11	n/a	n/a	BDL	BDL
	8	300±3	7.80±0.09	n/a	n/a	BDL	BDL
	15	294±14	7.77±0.14	n/a	n/a	BDL	BDL
	30	296±8	7.83±0.06	n/a	n/a	31.4±8.0	26.6±9.0
	60	301±5	7.76±0.18	n/a	n/a	63.2±7.1	52.7±18.0
	120	297±11	7.81±0.08	63.1±5.7	86.2±7.8	117.7±21.6	98.4±27.0
MMC	4	297±12	7.78±0.08	n/a	n/a	BDL	BDL
	8	302±18	7.82±0.14	n/a	n/a	BDL	BDL
	15	293±7	7.81±0.12	n/a	n/a	BDL	BDL
	30	298±5	7.79±0.09	n/a	n/a	32.9±7.3	22.5±14.0
	60	299±11	7.81±0.14	n/a	n/a	62.0±18.4	46.7±22.0
	120	294±9	7.83±0.12	61.9±7.2	84.8±5.6	133.6±38.8	86.6±41.0
NaCl	0.5	1218±104	7.84±0.06	n/a	n/a	n/a	n/a
	1	2154±131	7.82±0.04	n/a	n/a	n/a	n/a
	2	3884±248	7.84±0.11	n/a	n/a	n/a	n/a
	4	7160±177	7.83±0.12	n/a	n/a	n/a	n/a
	8	14,570±342	7.81±0.13	62.6±3.9	82.9±5.8	n/a	n/a

Glochidia tolerance compared to standard test organisms. Both *C. dubia* and *D. magna* were more sensitive to MC than glochidia from any species of freshwater mussel tested, as 48-hour LC50 values for standard organisms were 7 and 19, respectively, compared to a range of 27–40 μg Hg/L for glochidia. The GMAV of 28 aquatic organisms exposed to MC are documented in the 1984 WQC for Hg (USEPA 1985), and of them, only four genera had mean LC50 values lower than the GMAV of 30 μg Hg/L for *Epioblasma* in our study. The documented GMAVs for the more sensitive genera are 2.6 for *Daphnia*, 10 for *Gammarus* (amphipod), 20 for *Chironomus* (midge), and 20 μg/L for *Faxonella* (crayfish). The GMAVs for other standard test organisms *Salmo gairdneri* (Rainbow trout) and *P. promelas* were 275 and 159 μg/L, respectively.

Both glochidia and standard regulatory organisms were more sensitive to MMC than MC. Methylmercury is more toxic than other forms because it is more available to biota and accumulates in aquatic food webs. Inorganic mercury is converted into methylmercury through natural microbial respiration in aquatic systems, and we assume that this conversion occurred during the bioassays. Though we did not measure methylmercury concentrations, MMC salt may have been more toxic than MC salt because a greater portion of the total measured mercury in solution already existed in the organic form. It will be important to have lower detection limits, and analyze both total mercury and methylmercury concentrations in future bioassays. Overall, this study suggests that aquatic organisms are highly sensitive to mercury, regardless of which salt is tested.

Sodium Chloride Glochidia Reference Test

A dose-dependent response was evident in all of the glochidia reference tests, as viability was substantially reduced in treatments with higher sodium chloride concentrations. Furthermore,

glochidia were quite sensitive to sodium chloride, as LC50 values were near those recorded for *C. dubia*, which is currently regarded as one of the more sensitive standard test organisms. These observations support the use of sodium chloride as a reference toxicant for glochidia, but additional studies are needed to verify these results and improve to the precision of acceptable tolerance ranges for species.

Standard USEPA methods (USEPA 1991) require that reference toxicant tests be conducted concurrently with WET testing for both acute and chronic *C. dubia* and *P. promelas* bioassays. For results to be valid, endpoints of reference toxicant bioassays must be within an acceptable range for a given species. Researchers are able to infer the relative health of test organisms by comparing reference toxicity endpoints to established databases based on the premise that the healthy individuals of a species will have similar tolerances to a toxicant. Reference test endpoints that are below the acceptable species ranges suggest that organisms used in the test may have inferior health or that test conditions were not acceptable.

Results are only acceptable for standard test organisms if a certain control survivorship is maintained throughout tests; these thresholds are typically greater than or equal to 90% for acute and greater than or equal to 80% for chronic bioassays (USEPA 1994). Additional requirements have also been established for chronic *C. dubia* bioassays and include a minimum control survivorship of greater than or equal to 80%, greater than or equal to 60% of organisms in control treatments that have three broods within eight days, and surviving organisms in controls average greater than or equal to 15 neonates. Chronic *P. promelas* bioassay test results are only acceptable if control organisms survivorship is greater than or equal to 80% and average growth is greater than or equal to 2.5 mg/individual. These thresholds have been established by compiling the results of numerous trials and provide researchers with specific thresholds.

Several researchers have proposed similar validity endpoints for glochidia bioassays, such as greater than or equal to 90% after extraction or greater than or equal to 80% during the duration of the test (Keller 1993; Jacobson et al. 1997). Although these endpoints are useful and may potentially be incorporated into protocols for glochidia bioassays, additional QA/QC measures are essential for validating test results. We advocate conducting reference bioassays concurrently with other glochidia toxicant tests as a means for inferring the relative health of organisms used. Currently, additional research is needed to verify the acceptable sodium chloride tolerances of glochidia from different species of freshwater mussels before reference test endpoints can be used effectively as QA/QC measures. Lastly, it will be important to determine intra-specific variability within several mussel species and how this may vary seasonally.

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