Approaches for assessing toxicity of selected contaminants to freshwater mussels (*Bivalvia: Unionidae*)

Theodore Walter Valenti Jr.

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements
for the degree

Master of Science
in the field of
Aquatic Ecotoxicology
Department of Biology

Committee members:
Dr. Donald Cherry (chair)
Dr. Richard Neves
Dr. Rebecca Currie

Defense date:
12-14-04
Blacksburg, Virginia

Key words: freshwater mussel, glochidia, juveniles, toxicity, mercury, chlorine

Approaches for assessing toxicity of selected contaminants to freshwater mussels (*Bivalvia: Unionidae*)

Theodore Walter Valenti Jr.

Abstract

Laboratory bioassays results suggest that early life stages of freshwater mussels are sensitivity to toxicants. However, toxicological databases for unionids are rather limited because standard test methods are yet developed, and no published studies report endpoints for chronic test that are >9 days. The primary goals of my thesis research were to assess acute and chronic toxicities of chlorine and mercury to early life stages.

Inter- and intra-specific species variability in the tolerances of glochidia was observed during acute laboratory bioassays as endpoints were between 8 - 43 µg/L for Hg tests, 1.0 - 2.5 mg/L for NaCl tests, and 70 - 260 µg/L for chlorine (TRC) tests. Glochidia of several species had equal or greater sensitivities to Hg and NaCl than test organisms commonly used to assess environmental risk (i.e. *Ceridaphnia dubia*, *Daphnia magna*, *Pimephales promelas*), whereas they were far more tolerant to TRC than many species.

Twenty-one day chronic test endpoints for juveniles were substantially lower than those calculated during acute bioassays with glochidia. *Villosa iris* 3-mo old juveniles were found to be quite sensitive to Hg as growth was significantly impaired at 8 µg Hg/L. Chronic bioassays with TRC revealed a distinct decrease in susceptibility with increased aged for *V. iris* (relative sensitivities 3-mo > 6-mo > 12-mo), and that 2-mo old *Epioblasma capsaeformis* were more sensitive than comparable age classes of *V. iris*. However, both species were tolerant compared to other aquatic organisms, as the lowest endpoint was 20 µg TRC/L.

Table of contents

List of Tables

List of Figures

Background

Native freshwater mussels (Mollusca: Bivalvia: Unionidae) are one of the most imperiled faunas of North America. Currently, less than one quarter of the 297 identified species have stable populations, whereas 72% of the species are listed as possibly extinct, endangered, threatened, or of special concern (Williams et al. 1993). Declines are attributed to a number of stressors, including the introduction of exotic species, overharvest, and habitat disturbance and/or loss (Neves 1987, Keller and Zam 1991, Goudreau et al. 1993, Jacobson et al. 1993, Williams et al. 1993, Keller and Ruessler 1997,Yeager et al. 1999a). Several federal environmental policies issued in the United States since the 1970's have been essential to the conservation of remaining mussel assemblages. The Clean Water Act (1977) provided the groundwork for regulating the discharge of pollution into waterways, and led to substantial improvements in water quality. The Endangered Species Act (1973) established policies that promoted the conservation of ecosystems inhabited by threatened and endangered species. The ESA also mandated that recovery plans be drafted for "listed" species, which spurred extensive research examining the ecology of these freshwater mussels. Consequently, mussel propagation facilities have been established, and juveniles from several species have been successfully reared for reintroduction programs (Ahlstedt 1979, Sheehan et al. 1989, Michaelson and Neves 1995, Jones and Neves 2002). However, despite apparent progress, experts remain concerned about the prolonged conservation of native freshwater mussels.

By comparing results of historic mussel surveys with those more recent, substantial declines in taxa richness of freshwater mussels in the United States become evident. More importantly, accumulation of long-term monitoring data for mussel assemblages has enabled researchers to make inferences concerning population changes over time (Ahlstedt and Stansbury

1972, Tuberville 1997). One striking trend is the increasing rarity of some species in mussel assemblages (Sheehan et al. 1989, Diamond et al. 2002). Less apparent, yet more disconcerting, is the lack of recruitment in assemblages where adults are found (Jacobson et al. 1997, Henley and Neves 1999). Conservationists are alarmed with current population trends, especially the lack of young mussels, since it takes several years for most juveniles to reach sexual maturity. However, since unionids have multiple immature life stages during reproduction (Fig. 1[xix]), often including a larval parasitic stage that requires a specific fish host, it has proven challenging for researchers to evaluate the severity to which potential factors are impacting recruitment. Although in situ studies and surveys are useful for identifying impairment at specific sites, it is extremely difficult for researchers to isolate variables and distinguish direct cause-effect relationships because of the complexity of river systems[1].

A hypothesis currently proposed by scientists is that recruitment failure is due to early life stages of freshwater mussels being more sensitive to contaminants than adults. The aforementioned population trends, and perhaps more decisively laboratory toxicity test results, provide substantial evidence supporting this hypothesis. Laboratory testing enabled researchers to assess the relative tolerances of different life stages of unionids (i.e, glochidia, juvenile, adult) far easier than could be achieved in the field. Furthermore, it provided scientists a better means to examine cause-effect relationships between impairment and contaminant exposure since treatment concentrations could be manipulated, while all other variables remained constant. Perhaps the most comprehensive study to date is Jacobson et al. (1997), which compared the sensitivity of brooded glochidia (those held with the marsupium of adults), released glochidia (those released into the water column), encysted glochidia (those attached to host fish),

[1] Interaction between multiple non-point and point-source stressors, competitive interaction among organisms, host-fish demographics, varying abiotic and biotic components of ecosystems.

transformed juveniles, and adult mussels to copper. This study demonstrated that brooded and encysted glochidia are very tolerant, and seemingly impervious to copper exposure since they are sequestered from their external environment by either marsupial or fish host tissue[2]. Hoggath and Gaunt (1988) report that encysted glochidia are completely encapsulated by the host tissue once attached, which may explain their tolerance. Conversely, released *Villosa iris* glochidia and juveniles were extremely sensitive to copper, as 24-h LC50 values were 36-80 µg/L and 83 µg/L, respectively. Adult mussels were found to be more tolerant than either sensitive life stage by more than 10-fold. During 96 h stream exposures, no adult *V. iris* mortalities were recorded despite exposure to copper concentrations as high as 1000 µg/L**.** Additional studies also report that early life stages of freshwater mussels are more sensitive than adults (Harrison et al. 1984, Huebner and Pynnonen 1992), which is typical for most aquatic organisms. Lasee et al. (1991) reported LC50 values of 141 and 345 µg/L for newly transformed and 14-d-old juvenile *Lampsilis ventricosa* exposed to cadmium, respectively, while Naimo et al. (1992) did not observe any mortalities for adults of the same species exposed to 305 µg Cd/L for 28 d. Keller and Ruessler (1997) reported no mortality for adults after 96 h for concentrations as high as 350 mg malathion/L, whereas 24- and/or 48-h LC50s for juvenile and glochidia were between 7-374 mg/L.

Studies comparing the acute tolerances of glochidia and juvenile mussels of the same species are sparse, but of those that do exist, most conclude that glochidia are more sensitive. Jacobson et al. (1997) examined the copper sensitivities 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, while those for *Pyganodon grandis* were 46-347 µg/L and 33-44 µg/L,

[2] Other studies have concluded that brooded glochidia are at risk to contaminant exposure (Huebner and Pynnonen 1992), and this may only apply to heavy metals and/or be species specific.

respectively. Augspurger et al. (2002) reported that glochidia were more sensitive to ammonia than juveniles despite shorter exposure times. In their study, 24-h L50 values for glochidia of *V. iris*, *Utterbackia imbecillis,* and *Actinonaias pectorosa* were 3.8, 5.9, and 3.8 mg NH_3/L, respectively, while 96-h LC50 values for juveniles those species were 6.8, 10.6, and 14.0 mg NH_3/L, respectively. Keller and Ruessler (1997) documented that glochidia from several species, *U. imbecillis*, *V. lienosa*, and *V. villosa*, were more sensitive than juveniles during acute studies assessing the toxicity of the insecticide malathion. However, additional experiments assessing the sensitivity of early life stages of the same species are warranted since test methods employed in former studies were not always consistent, and little is known of intra- and inter-specific variation in tolerances. Susceptible of different life stages is also probably dependent of the species and specific toxicant of concern, and statistical interactions between these two variables are not likely to be uniform.

More importantly, several studies have reported that these early life stages of unionids are more sensitive to toxicants than test organisms typically used by regulatory agencies to establish water quality standards and assess environmental risk. In a study comparing Cd, Cr, Cu, Hg, Ni, and Zn toxicity to *U. imbecillis* juveniles, and larval stages of *Daphnia magna* (zooplankton), *Chironomus* (midge), and *Pimephales promelas* (fathead minnow), and *Lepomis* (bluegill), Keller and Zam (1991) reported that juvenile mussels were either the most, or second most sensitive to all the metals except Hg. Furthermore, LC50 values for juveniles were in general similar to those for *D. magna* and substantially lower than those for either fish species. Cherry et al. (2002) conducted acute bioassays with 18 aquatic species to establish an acute site-specific Criterion Maximum Concentration (CMC) for copper to evaluate the impact of a coal-burning power plant's effluent on mussel populations in the Clinch River, VA. Five of the six most

sensitive test organisms were freshwater mussel glochidia, while the other was the mayfly *Isonychia bicolor*. Commonly used test organisms, *Ceriodaphnia dubia* (zooplankton) and *P. promelas*, ranked 7th and 15th, respectively. The Genus Mean Acute Values (GMAV) for glochidia of the four most sensitive mussels species were between 37-60 µg/L, while those for *C. dubia* and *P. pimephales* were 88 and 310 µg/L, respectively. In another study examining copper toxicity, Jacobson et al. (1993) reported 24-h LC50 values of 83 and 44 µg/L for *V. iris* and *A. grandis* juveniles, respectively, which are again lower than comparable values for *C. dubia* and *P. pimephales* in the Cherry et al. (2002) study. Weinstein and Polk (2001) stated that glochidia of *U. imbecillis* are relatively sensitive to photo-activated anthracene compared to other species (LC50 value=1.93 µg/L), and 6.3 fold more sensitive than oligochaetes to photoactivated pyrene. Milam and Farris (1998) reported lower mean toxicological endpoints for *C. dubia* (1.12 mg ferrous iron/L) and *D. magna* (2.7 mg Fe/L), than for *Leptodea fragilis* glochidia (3.6 mg Fe/L). *Pimephales promelas* was the most tolerant tested species, as its mean endpoint was 10.8 mg Fe/L. However, it is important to note that these researchers contrasted 7-day EC50 values for *C. dubia*, 48-h LC50 values for *D. magna* and *P. promelas*, and 24-h LC50 values for glochidia. Other researchers have generated data that are contrary in that species typically used in bioassays were more sensitive to contaminants than early life stages of freshwater mussels (Keller 1993, Masnado et al. 1995); but as with most, these studies only examined a single species of mussel, *U. imbecilis*, which is widespread and is assumed to be tolerant relative to other species.

As adverse effects are observed at low concentrations of toxicant(s) in laboratory studies with early life stages, researchers have inferred that native freshwater mussels are perhaps one of the most at-risk aquatic faunas to pollution. However, toxicological data for unionids are not

included in current water quality standards developed by the United State Environmental Protection Agency (EPA) for the protection of aquatic life. Regulatory agencies excluded freshwater mussel toxicity data when calculating water quality criteria (WQC) because they questioned the validity of test results. Skepticism stemmed primarily from the lack of standardized test protocols, and quality concerns surrounding the health of test organisms. Although these concerns are warranted, it seems contradictory for the EPA to exclude freshwater mussel toxicity data from WQC, while at the same time incorporating results of studies with other species, such as aquatic insects, that also do not have standardized test methods or quality assurances established. Regardless, hesitancy to apply laboratory test results to policy makes the development of standardized test methods for freshwater mussels especially important since current WQC may not be stringent enough to ensure their protection.

For years, several independent scientific organizations have been involved with research that has advanced the development of standardized test procedures for early life stage of freshwater mussels. Johnston et al. drafted a document entitled "Proposed guide for conducting acute toxicity tests with the early-life stages of freshwater mussels" for the EPA (EPA 1990). The document played an important role by triggering interaction among laboratories affiliated with freshwater mussel toxicology and propagation, although it failed to provide sufficient resolve to many questions pertinent to the development of standardized test methods. Subsequent studies focused efforts on addressing areas of criticism, and researchers are now close to drafting a new set of standardized test guidelines for acute bioassays with glochidia (Wang et al. 2003). Recent efforts show great promise, as experimenters have attempted to structure test criteria based on protocols established for commonly used aquatic test organisms

(EPA 1993). The major area of concern still remaining is the lack of QA/QC assurances for verifying the health and maturity of glochidia used in bioassays.

Juvenile mussels are continually becoming more available for toxicological experiments because of higher yield from culturing facilities due to improved techniques and greater knowledge of fish host relationships (Zale and Neves 1982, Michaelson and Neves 1995, Uthaniwan et al. 2001). As with glochidia bioassays, many components of protocols for acute laboratory tests with common test organisms are compatible for juvenile mussel experiments (EPA 1993). Difficulties with obtaining sufficient numbers of individuals for experiments, and perplexities with culturing some species in the laboratory have slowed development of a standardized juvenile mussel test protocol. Of the published studies assessing toxicity of contaminants to juvenile mussels in the laboratory, nearly all include experiments that are conducted for only short durations (i.e., <96 h), and in most instances, with only one species. Examples of species typically used are *U. imbecillis* and *V. iris*, both of which are relatively common, adaptive to laboratory conditions, and considered to be quite tolerant compared to other mussel species.

One of the only published works that examined chronic exposure is "Effect of test conditions on the toxicity of copper to juvenile unionid mussels" by Keller et al. (1999). The researchers reported 9-d LC50 values for *Lampsilis straminea* that were between 11-36 µg Cu/L, which are substantially lower than comparable values for most other aquatic species. This study also attempted to determine whether silt, algae, and photoperiod affects toxicity, but reported no influences, except for the presence of silt that caused a reduction in total Cu in the water column. However, effects of any of these factors may not have become evident because the period of exposure was of short duration.

Aquaculture of freshwater mussels has provided toxicologists with valuable information about living requirements of juveniles; however, it is often not ideal to employ the same techniques for conducting toxicity experiments. The primary objective of culturing facilities is to produce a maximum yield of healthy individuals, and consequently, this is often achieved by incorporating large, high-volume systems. Such designs are not conducive for toxicological experiments because it is difficult, costly, and space-consuming to maintain multiple replicates for each treatment. Handling of wastewater from large systems is also troublesome because of high costs associated with safe disposal due to human health and environmental risks. Collecting and transporting large volumes of water from impacted river systems for bioassays is also not practical. The aforementioned limitations have led scientists to begin experimentation with smaller microcosms; however, determining and incorporating integral components of large-scale systems to these has proven rather difficult.

Conducting tests in the laboratory for longer durations requires that scientists address additional components of experimental design, such as feeding, artificial habitat, and test conditions, that are pertinent to acute tests. Depriving juveniles of food during long-term experiments will likely cause them to be more susceptible to toxicants. In addition, starvation will alter toxicokinetics (i.e., absorption, distribution, biotransformation, and excretion of toxic substances in the body) as filtration rates and metabolic processes (Naimo 1995). Patterson et al. (1999) observed differences in the glycogen levels in the mantle tissue of adults mussels fed a controlled diet, versus those starved in quarantine. Irrevocably, feeding juveniles during chronic tests is pertinent for determining accurate toxicological endpoints, as demonstrated by numerous studies that established test procedures for other aquatic organisms currently used to assess risk (EPA 2002). Several researchers have suggested ca. 3×10^7 algae cells/ml as an appropriate

daily dose for feeding (Gatenby et al. 1997, Yeager et al. 1994); however, concentrations may fluctuate depending on the specific test designs. In addition, tri-algal suspensions, containing *Chlamydomonas*, *Ankistrodesmus*, and *Chlorella*, seem ideal (Gatenby et al. 1997), although single species suspensions of these strains have also been used with success.

Habitat requirements of juvenile mussels, such as the presence of substrate and aeration/flow, are also important to consider in long-term tests (Gatenby et a, 1997), as their neglect can ultimately lead to declines in fitness as severe as those for starvation. Hudson and Isom (1984) found convincing evidence that substrate improves the health and survivorship of juvenile mussels in laboratory culturing experiments, which seems quite plausible because they are often found in depositional zones in rivers (Neves and Widlak 1987) and rely more heavily on pedal feeding than adults (Yeager et al. 1994). Aeration and/or flow in test chambers is also important to consider when using freshwater mussels as test organisms, as previous studies document that lotic organisms become stressed in the laboratory experiments if maintained under static conditions (Kennedy et al. 2003). This is a concern since test organisms in control treatments must remain healthy throughout the duration of an experiment for results to be valid, and survivorship must typically be 80% or above in chronic bioassays (EPA 2002). Individuals will likely alter their normal behavior if habitat requirements are not sufficiently met, which may affect their susceptibility. Chen et al. (2001) observed that freshwater mussels alter their behavior (i.e. filtration rate, metabolism) depending on the dissolved oxygen concentration. Therefore maintaining test organisms in the laboratory during bioassays under conditions that are similar to those in environmental scenarios will allow researchers to more accurately infer relative risk in river systems.

Test conditions, such as temperature, lighting, and water chemistry, can also have substantial impacts on test results. Temperature extremes can adversely affect organisms and/or alter respiration and metabolism, which will again influence the susceptibility of an organism to a particular toxicant. Keller and Ruessler (1997) reported substantial differences in the sensitivities of juveniles acutely exposed to malathion at temperatures of 25 and 32˚C, though a similar comparison were less striking in glochidia experiments. Effects of photoperiod is also important to consider during test design, as recent studies show changes in light intensity and UV-radiation influence toxicity (Weinstein 2001,Weinstein and Polk 2001); however, behavioral and physiological effects are not yet well studied. It is critical for researchers to be aware that environmental conditions affect the steriochemsitry of a toxicant, and only slight variations in test conditions can have profound effects on toxicity. Furthermore, incorporating uniform test criteria and conditions will enable researchers to better compare laboratory results from different locations and times, or with different species and toxicants.

Overall, laboratory toxicity testing with early life stages is vital to conservation of freshwater mussels because it enables researchers to evaluate risk more accurately. The ability to not only reduce the complexity, but also manipulate variables in the laboratory that are uncontrollable in lakes and rivers allow specific stressors to be examined with greater detail. As a result, scientists are able to more effectively establish cause-effect relationships between stressors and impairment. Although conducting short-term bioassays in the laboratory are useful for assessing the toxicity of effluents[3] or other highly toxic environmental samples[4], it is seldom that contaminant concentrations reach such elevated levels in aquatic systems. The most substantial toxicological threat to mussel assemblages, nearly universally among waterways, is

[3] Referring to those associated with NYPDS, or equivelant state and local agencies.
[4] Examples would included mine drainage, chemical spills, and zones of spray drift.

the stress induced by chronic, sub-lethal exposure to contaminants. Therefore, conducting long-term bioassays in the laboratory with juvenile mussels provides a more realistic environmentally scenario than acute tests. Just as substantial, chronic tests also allow researchers to quantifiably measure sub-lethal endpoints that are often more sensitive than examining survivorship alone.

Sub-lethal effects are often far more sensitive indicators of impairment than survivorship, and are especially important to consider for freshwater mussels because of their long life spans. Foot immobilization (Doherty and Cherry 1988), filtration rate (Doherty and Cherry 1988, Salanki and V.-Balogh 1989), oxygen consumption (Naimo et al. 1992, Sivaramakrishna et al. 1992), ion levels (Sivaramakrishna et al. 1992) and valve activity (Jacobson et al. 1993) have all been used as endpoints. However, reduced growth is perhaps a more definitive illustration of reduced fitness, as juvenile mussels can neither propagate nor survive if they do not grow. Studies with marine bivalves have observed substantial growth impairments for individuals exposed to varying levels of heavy metals (Stromgren 1982). Reduced growth also suggests that individuals are unable to obtain sufficient energy reserves that may lead to latent mortality, especially for young juveniles if they are unable to develop sufficiently during summer-fall months to endure winter[5].

[5] Notably, summer months are typically periods when concentrations of contaminants are highest due to lower flow and less dilution, in addition, metabolic rates are often higher for juveniles because of temperatures are high; therefore risk of toxicity is greatest.

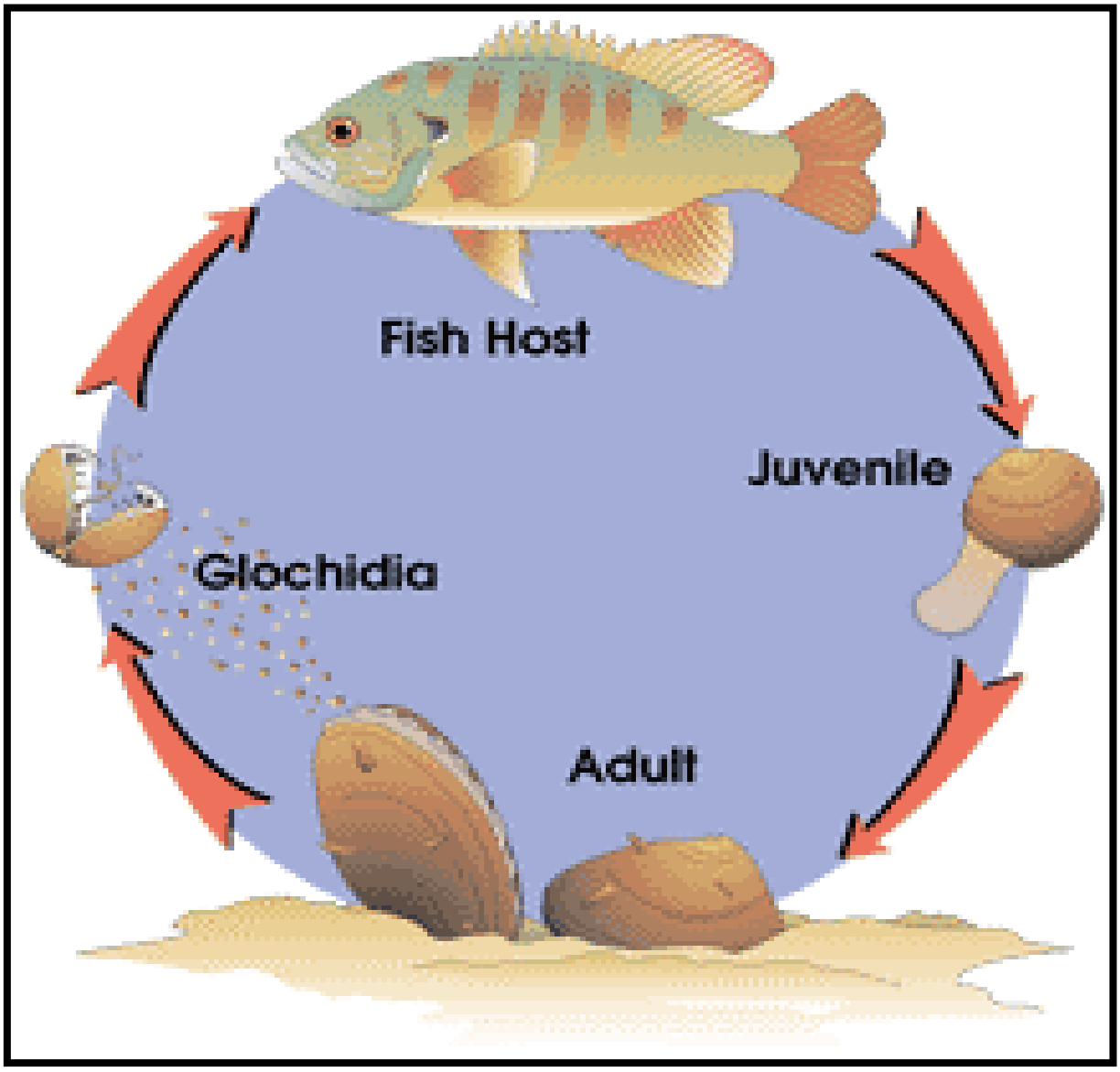


Figure 1. Basic life history of freshwater mussels.

Research Objective

The primary goal of my Master's thesis (MS) was to assess the toxicity of different contaminants to early life stages of freshwater mussels (family: Unionidae) under laboratory conditions. Test designs used in experiments needed to be defendable in court, since standardized test methods do not yet exist for freshwater mussels. Guidelines for acute glochidia bioassays were established by integrating methods described in published works; knowledge of standardized test protocols for commonly used test organisms *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimphales promelas; c*ommunication with experts in the field; and personal observation/experimentation. Furthermore, as several researchers have voiced concern for verifying the health of glochidia used in bioassays, I explored the potential of employing sodium chloride as a reference toxicant. It was more difficult to develop test procedures for examining the effect of chronic contaminant exposure to juveniles since little published data exist, and sufficient numbers of test organisms have only recently become available with improvements in culturing techniques. Therefore, most of the work was pioneering research, and several pilot studies to explore different test conditions were conducted before completion of chronic experiments described in this thesis.

Structure of Work

There are four distinct chapters presented in this thesis, each was prepared as a manuscript for submission to journals/books for publication. It is important for readers to realize that manuscripts in this work may be altered prior to publication in order to address reviewers' comments or meet a journal's specific formatting requirements. The decision to structure the work in this way was made early during my Master's education, as substantial competition among laboratories for funding made it important to publish study results. Data collected for many experiments were conducive for publication because more incorporated pioneer test designs/concepts for toxicity tests with early life stages of freshwater mussels, and/or addressed voids in toxicological databases for contaminants.

Chapter 1, entitled "Sensitivity of mussel glochidia and regulatory test organisms to mercury and a reference toxicant", was inspired by the results of Cherry et al. (2002), as their study showed that glochidia from several freshwater mussel species were more sensitive to copper than commonly used test organisms. However, experiments described in this chapter examine the sensitivities of glochidia from widespread and threatened mussel species, and typical test organisms *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* to inorganic and organic mercury salts. Initially, regulatory agencies were not overly concerned of the release of Hg because most is emitted as elemental or inorganic forms not highly toxic to wildlife. However, researchers have since discovered that several abiotic and biotic factors convert these forms of Hg into methyl-mercury (MeHg), which is 100 times more toxic, assimilates quickly into biota, and bioaccumulates in food webs. The consumption of contaminated fish is a substantial Hg exposure risk to humans (French et al. 1999, Mason et al. 2000), and has resulted in the issuing of Fish Consumption Advisories (FCA) in most U.S. states.

Currently, more than 60% of the FCA issued nationwide are due to Hg (Webber and Haines 2002). Alarm for Hg contamination also has risen because studies suggest that it may be transported great distances from sources through atmospheric deposition (Slemr et al. 2003, Walcek et al. 2003), as elevated concentrations have been recorded at remote sites (Huckabee and Hildebrand 1974, Mason et al. 2000). Furthermore, Hg is highly persistent in aquatic systems because it may be trapped in the sediment (Cattani et al. 1999, French et al. 1999, Wiener and Shield 2000), which may be a source of pollution for decades (Wiener and Shield 2000). Several freshwater systems polluted in the 1970's still have concentrations in the sediment that are a risk to wildlife (Stansbery 1972, Carter 1977, Cattani et al. 1999, Beckvar et al. 2000). Since freshwater mussels a threatened aquatic fauna, it is important for researchers to assess their sensitivity to contaminants of concern. Furthermore, it is of the utmost importance for researchers to determine the relative sensitivities of early life stages of unionids and organisms typically used assessing environmental risk if the latter are going to be used as surrogate test species. Chapter 1 was presented as part of a special platform session at the Society of Environmental Toxicology and Chemistry (SETAC) 25th annual meeting, Austin, TX, 2002. The data will also be published in the SETAC book "Bivalve Toxicology" (eds. Farris and Van Hassel). Date of release is unknown. (Peer reviewed by authors, resubmitted, and now waiting for comments from independent reviewers editing entire book).

Data for Chapter Two was in a manuscript submitted to the journal Environmental Toxicology and Chemistry (ET&C) entitled "The use of sodium chloride as a reference toxicant for assessing the health of freshwater mussel glochidia". However, the work was rejected for publication because of insufficient evidence supporting the use of NaCl as a reference toxicant, despite its recommended use by the EPA for other freshwater organisms. However, reviewers

agreed that conceptually the approach seems valid. Since it will likely take collaborative effort among several laboratories to generate sufficient quantitative evidence to verify NaCl as an acceptable reference toxicant for glochidia, authors on the paper are considering either re-submitting a revised copy of the version to another journal, or alternatively, presenting it in a manuscript that focuses on intra-specific variation in tolerances of glochidia from different adults, as few studies on subject have been published. Concerns were also expressed for statistical analysis by reviewers, which will be addressed before re-submission of the data. Jumpin 5.0® was originally used and limitations of the program may have flawed statistical analysis, therefore SAS will be used.

The third chapter, "Acute and chronic sensitivity of early life stages of freshwater mussels (Bivalvia: Unionidae) to total residual chorine relative to water quality criteria- 1984" contains data from acute bioassays with glochidia from several species, and chronic bioassays with juveniles of two species. Although results for acute bioassays with glochidia are valuable since several threaten/endangered species were tested, results for chronic tests are far more intriguing. Three age-classes of *V. iris* (2-, 6-, and 12-mo old), and one age-class of *Epioblasma capsaeformis* (2-mo old) juveniles were exposed to chlorine for 21-d. To our knowledge, these data are the first to compare the chronic sensitivity of different age-class and species. Chronic test were conducted in large re-circulating systems that were similar to, at the time of experiments, the systems used by the Virginia Tech Aquaculture Center to rear juveniles. Peristalsis pumps fed different concentrations of Cl stock solutions into systems to account for the high dissipation rate of chlorine so that constant treatment concentrations could be maintained. An innovative test chamber that enabled researchers to measure growth of an

individual juvenile, and allowed flow of test solution through a fine mesh screen, was used successfully.

Data for Chapter Four, "Acute and chronic toxicity of mercury to early life stages of the rainbow mussel, *Villosa iris* (Bivalvia: Unionidae) -Short Communication" were first presented in a poster at the 25th annual SETAC meeting, Austin, TX, 2002. The principle goals of this research were to compare the sensitivity of *V. iris* glochidia and juvenile to acute Hg exposure, and to determine how chronic Hg exposure affects juvenile survivorship and growth. An innovative test design that met the unique living requirements of juveniles allowed researchers to assess the effects of 21-d exposure. Small-scale, aerated microcosms were selected as the test apparatus because they required small volumes of test solution, which minimized human health risks, and the production of Hg wastewater. Furthermore, this design improved statistical power by making it easier to have multiple replicates for different treatments. Tests require only a small amount of space (i.e., standard incubator, 2 x 3 m flat space), few laboratory requirements (i.e., air line, temperature controlled environment), and minimal materials that are affordable. Similar test designs are most applicable for use when attempting to establish standardized chronic test protocols since bioassays can be conducted at nearly any research facility. Data in this chapter were originally prepared for submission to ET&C in a full manuscript, but at the request of the editor, was revised and re-submitted as a Short-Communication. This version has been accepted by the journal, and is now under re-review with the initial round of peer reviews completed.

Work Cited

1) Ahlstedt SA. 1979. Recent mollusk transplants into the North Fork Holston River in southwestern Virginia. *Bull Am Malacol Union* pp 21- 23.

2) Ahlstedt SA, JD Tuberville. April 1997. Proceedings of a UMRCC symposium- Conservation & management of freshwater mussels II: Initiatives for the future. pp 72-81.

3) Beckvar N, S Salazar, M Salazar, K Finkelstein. 2000. An in situ assessment of mercury contamination in the Sudbury River, Massachusetts, using transplanted freshwater mussels (*Elliptio complanata*). *Can J Fish Aquat Sci* 57: 1103-1112.

4) Carter LJ. 1977. Chemical plants leave unexpected legacy for two Virginia Rivers. *Science*: 198: 1015-1020.

5) Cattani O, D Fabbri, M Salvati, C Trombini, I Vassura. 1999. Biomonitoring of mercury pollution in wetland near Ravenna, Italy by translocated bivalves (*Mytilus galloprovincialis*). *Environ Tox Chem* 18: 1801-1805.

6) Chen LY, AG Heath, RJ Neves. 2001. Comparison of oxygen consumption in freshwater mussels (Unionidae) from different habitats during declined dissolved oxygen concentrations. *Hydrobiologia* 450: 200-214.

7) Cherry DS, JH Van Hassel, JL Farris, DJ Soucek, RJ Neves. 2002. Site-specific derivation of the acute copper criteria for the Clinch River, Virginia. *Human Ecol Risk Assess* 8: 591-601.

8) Diamond JM, DW Bressler, VB Serveiss. 2002. Assessing relationships between human land uses and the decline of native mussels, fish, and macroinvertebrates in the Clinch and Powell River Watershed, USA. *Environ Tox Chem* 21: 1147-1155.

9) Doherty FG, Cherry DS. 1988. Tolerance of the Asiatic clam *Corbicula* sp. to lethal levels of toxic stressors-a review. *Environ Pollut* 51: 269-313.

10) French KJ, DA Scruton, MR Anderson, DC Schneider. 1999. Influence of physical and chemical characteristics on mercury in aquatic sediment. *Water, Air, Soil Pollut* 110: 347-362.

11) Gatenby CM, BC Parker, RJ Neves. 1997. Growth and survival of juvenile rainbow mussels, *Villosa iris* (Lea, 1829) (Bivalvia : Unionidae), reared on algal diets and sediments. *Amer Malacol Bull* 14: 57-66.

12) Goudrea SE, Neves RJ, Sheehan RJ. 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. *Hydrobiologia* 252: 211-230.

13) Hagg WR, ML Warren. 1999. Mantle display of freshwater mussels elicit attack from fish. *Freshwater Biol* 42: 35-40.

14) Harrison FL, JP Knezovich, DW Rice. 1984. The toxicity of copper to the adult and early life stages of the freshwater clam, *Corbicula manilensis*. *Arch Environ Contam Toxicol* 13: 85-92.

15) Henley WF, Neves RJ. 1999. Recovery status of freshwater mussels (Bivalvia: Unionidae) in the North Fork Holston River, Virginia. *Am Malacol Bull* 15:65-73.

16) Hoggarth MA, AS Gaunt. 1988. Mechanics of glochidial attachment (Mollusca: Bivalvia: Unionidae). *J Morph* 198: 71-81.

17) Huckabee JW and SG Hildebrand. 1974. Background concentrations of mercury and methylmercury in unpolluted freshwater environments. *Barcelona*: 6 –10.

18) Hudson RG, BG Isom. 1984. Rearing juveniles of the freshwater mussels (Unionidae) in a laboratory setting. *Nautilus* 98: 879-883.

19) Huebner JD, KS Pynnonen. 1992. Viability of two species of Anodonta exposed to low pH and selected metals. *Can J Zool* 70: 2348-2355.

20) Jacobson PJ, RJ Neves, DS Cherry, JL Farris. 1997. Sensitivity of glochidial stages of freshwater mussels (Bivalvia: Unionidae) to copper. *Environ Tox Chem* 16: 2384-2392.

21) Jacobson PJ, JL Farris, DS Cherry, RJ Neves. 1993. Juvenile freshwater mussel (Bivalvia: Unionidae) responses to acute toxicity testing with copper. *Environ Tox Chem* 12: 879-883.

22) Jones J, RJ Neves. 2002. Life history and propagation of the endangered fanshell pearlymussel, *Cyprogenia stegaria* Rafinewque (Bivalvia: Unionidae). *J N Am Benthol* Soc 21: 76-88.

23) Keller A. 1993. Acute toxicity of several pesticides, organic compounds, and a wasterwater effluent to the freshwater mussel, *Anodonta imbecilis*, *Ceriodaphnia dubia*, and *Pimephales promelas*. *Bull Environ Contam Toxicol* 51: 696-702.

24) Keller AE, Ruessler DS, Kernaghan NJ. 1999. "Effect of test conditions on the toxicity of copper to juvenile unionid mussels" in Environmental Toxicology and Risk Assessment: Standardization of biomarkers for endocrine disruption, ASTM STP 1364.

25) Keller AE, Ruessler DS. 1997. The toxicity of malathion to unionid mussels: Relationship to expected environmental concentration. *Environ Toxicol Chem* 16: 1028-1033.

26) Keller AE, Zam SG. 1991. The acute toxicity of selected metals to the freshwater mussel, *Anodonta imbecillis*. *Environ Toxicol Chem* 10:539-546.

27) Kennedy AJ, Cherry DS, Currie RJ. 2003. Evaluation of ecologically relevant bioassays for a lotic system impacted by a coal-mining effluent using *Isonychia biocolor*. *Environ Monitor Assess* 44: 324-331.

28) Masnado RG, SW Geis, WC Sonzogni. 1995. Comparative acute toxicity of a synthetic mine effluent to *Ceriodaphnia dubia*, Larval fathead minnow, and the freshwater mussel *Anodonta imbecilis*. *Environ Tox Chem* 14: 1913-1920.

29) Mason RP, JM Laporte, S Andres. 2000. Factors controlling the bioaccumulation of mercury, methylmercury, arsenic, selenium, and cadmium by freshwater invertebrates and fish. *Arch Environ Contam Toxicol* 38: 283-297.

30) Michaelson DL, RJ Neves. 1995. Life history and habit of the endangered dwarf wedgemussel *Alasmidonta heterodon* (Bivalvia: Unionidae). *J N Am Benthol Soc* 14: 324-330.

31) Milam C, JL Farris. 1998. Risk identification associated with iron-dominated mine discharges and their effect upon freshwater bivalves. *Environ Tox Chem* 17: 1611-1619.

32) Naimo TJ. 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4: 341-362.

33) Naimo TJ, GJ Atchison, LE Hooand-Bartels. 1992. Sublethal effects of cadmium on physiological responses in the pocketbook mussel, Lampsilis ventricosa. Environ Toxicol Chem 11: 1013-1021.

34) Neves, RJ. 1987. Recent die-offs of freshwater mussels in the United States: An overview. Proceedings, Workshop, Die-Offs of Freshwater Mussels in the United States, US Fish and Wildlife and Virginia Polytechnic Institute and State University, Blacksburg, VA, pp. 7-18.

35) Patterson MA, BC Parker, RJ Neves. 1999. Glycogen concentrations in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. *Am Malacol Bull* 15: 47-50.

36) Salanki J, K, V.-Balogh. 1989. Physiological background for using freshwater mussels in monitoring copper and lead pollution. *Hydrobiologia* 188/189: 445-454.

37) Sheehan RJ, RJ Neves, HE Kitchel. 1989. Fate of freshwater mussels transplanted to formerly polluted reaches of the Clinch and North Fork Holston Rivers, Virginia. *J Fresh Ecol* 5: 139-149.

38) Sivaramakrishna B, K Radhakrishnaiah, A Suresh. 1991. Assessment of mercury toxicity by the changes in oxygen consumption and ion levels in the freshwater snail, *Pila globosa*, and the mussel, *Lamellidens marginalis*. *Bull Environ Contam Toxicol* 46: 913-920.

39) Slemr F, Brunke EG, Ebinghaus R, Temme C, Munthe J, Waengberg I, Schroeder W, Steffen A, Berg T. 2003. Worldwide trend of atmospheric mercury since 1977. *GeoPhys Res* Let 30: [np].

40) Stansbery DH. 1972. The mollusk fauna of the North Fork Holston River at Saltville, VA. *Bull Amer Malacol Union*, Inc. 45-46.

41) Stromgren T. 1982. Effect of heavy metals (Zn, Hg, Cu, Cd, Pb, Ni) on the length growth of *Mytilus edulis*. *Mar Biol* 72: 69-72.

42) U.S. Environmental Protection Agency. 2002. Short-term methods for estimation the chronic toxicity of effluents receiving water to freshwater organisms- 4th Ed. EPA/821-R-02-013. Washington, DC.

43) U.S. Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms- 4th ed. EPA/600/4-90/027F, Washington, DC.

44) U.S. Environmental Protection Agency. 1990. Proposed guide for conducting acute toxicity tests with the early life stages of freshwater mussels. EPA/68/02/4278. Washington, DC.

45) Uthaiwan K, N Noparatnaraporn, J Machado. 2001. Culture of glochidia of the freshwater pearl mussel *Hyriopsis myersiana* (Lea, 1856) in artificial media. *Aquaculture* 195: 61-69.

46) Walcek C, De Santis S, Gentile T. 2003. Preparation of mercury emissions inventory for eastern North America. *Environ Pollut* 123: 375-381.

47) Wang N, C Ingersoll, E Greer, D White, A Roberts, J Dwyer, T Augspurger, C Kane, C Tibbott, D Neves, C Barnhart. 2003. Developing standardized guidance for conducting toxicity tests with glochidia of freshwater mussels. Presented at Society of Environmental Toxicology and Chemistry, November 9-13, 2003. Austin, TX, USA. Published abstract.

48) Webber HM, TA Haines. 2002. Mercury effects on predator avoidance behavior of a forage fish, golden shiner (*Notemigonus crysoleucas*). *Environ Tox Chem* 22: 1556-1561.

49) Weinstien JE. 2001. Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel, *Utterbacki imbecillis*. *Environ Tox Chem* 20: 412-419.

50) Weinstein JE, KD Polk. 2001. Photoperiod of anthracene and pyrene to glochidia of the freshwater mussel *Utterbackia imbecillis*. *Environ Tox Chem* 20: 2021-2028.

51) Williams JD, ML Warren, KS Cummings, JL Harris, RJ Neves. 1993. Conservation status of freshwater mussels of United States and Canada. *Fisheries* 18: 6-22.

52) Wiener JG, PJ Sheilds. 2000. Mercury in the Sudbury River (Massachusetts, USA): pollution history and synthesis of recent research. *Can J Fish Aquat Sci*. 57: 1053-1061.

53) Yeager MM, RJ Neves, DS Cherry. 1999. Competitive interactions between early life stages of *Villosa iris* (Bivalvia: Unionidae) and adult Asian clams (*Corbicula fluminea*). Proceedings of the First Freshwater Mollusk Conservation Society Symposium, pp. 253-259.

54) Yeager MM, DS Cherry, RJ Neves. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, *Villosa iris*, (Bivalvia: Unionidae). *J N Am Benthol Soc* 13: 217-222.

55) Zale AV, RJ Neves. 1992. Fish hosts of four species of lampsiline mussels (Mollusca: Unionidae) in Big Moccasin Creek, Virginia. *Can J Zool* 60: 2335-2542.

Acknowledgments

I thank my primary advisor, Dr. Donald S. Cherry, for the opportunity to study under him at Virginia Tech. The wealth of knowledge he taught me from his decades of experience in the field of aquatic toxicology provided me not only a better understanding of the science and policies, but also how to best collaborate with regulatory agencies. I also thank my co-advisors Dr. Richard J. Neves of the U.S. Geological Survey, who drastically improved my writing and provided invaluable insight about freshwater mussels, and Dr. Rebecca Currie of Roanoke College's Biology Department, who aided my development as a research scientist through the teaching of standard culturing/bioassay protocols. Additional professors also provided me with substantial guidance throughout my time at Virginia Tech, including Dr. Maury Vallet and Dr. Madline Schrieber, both of whom were co-authors of a manuscript not in the thesis that examined impacts of an abandoned arsenic mine on benthic communities. I also owe a debt of gratitude both to present, and former Cherry Lab students Branden Locke, Matt Simon, Brandi Echols, Matthew Hull, Alan Kennedy, and Timothy Chad Merricks, Patrick Berry, and Conrad Peters, in addition to Jake Chaffin of the VT Stream Team, for my educational experience, and also for some enjoyable times in B-burg. I also greatly appreciated of members of the VT Aquaculture center, chiefly Rachel Mair, Jess Jones, and Dr. William Henley of the Virginia Tech. Without the test organisms they provided, my research would not have been feasible. I would also like to thank John Schmerfeld and Cindy Kane of the U.S. Fish and Wildlife Service for allowing me to attend meetings discussing declines of freshwater mussels, and test protocols. But most of all, I want to thank my family, and friends from back home. Without you, my success would have been more difficult to achieve, and far less meaningful. Mom, dad, sis, and bro, I love you all very much and will never be able to express my full gratitude.

Chapter 1: Sensitivity of mussel glochidia and regulatory test organisms to mercury and a reference toxicant.

1.1 Abstract

Forty-eight hour acute bioassays were conducted on multiple species of freshwater glochidia and regulatory organisms to contrast their sensitivities to inorganic and organic forms of mercury salt. Currently, debate exists on the use of glochidia in bioassays because of a lack in adequate QA/QC measures. To alleviate this deficiency, I conducted glochidia NaCl reference tests for some species of freshwater mussels. Results of my study indicated that the regulatory test organisms *Ceriodaphnia dubia* and *Daphnia magna* and glochidia from *Epioblasma capsaeformis*, *E. brevidens*, and Lampsilis fasciola are very sensitive to mercury (48-h LC50 values ranged from < 4 – 36 µg total Hg/L). Glochidia from *Villosa iris* and the fathead minnow, *Pimephales promelas*, were tolerant to acute mercury exposure; 48-h LC50 values were 43 and 67 µg total Hg/L, respectively. The studied clearly showed high levels of inter-specific variation in mercury tolerances and emphasizes the need for multi-species level risk assessment.

Keywords: glochidia, toxicity, mercury, lethal concentration

1.2 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 overharvest and the introduction of non-native species have had substantial impacts (Williams et al. 1993, Yeager et al. 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 et al. 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, promote to protect not only native unionids, but also their habitat. However, despite clear progress, there is still concern for the future conservation of native mussels, as survey efforts have shown little recruitment (Neves and Widlak 1987, Brenderman 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 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 et al 1999, Weinstein 2001).

Jacobson et al. (1997) conducted a comprehensive study that examined the effects of copper exposure on 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 fish host). Released glochidia were impacted at lower copper concentrations (36-80 µg Cu/L) than encysted glochidia (>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-h 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-h LC50 for adults was >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, 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 longer exposure duration. The 96-LC50 values for juveniles of pheasantshell (*Actinonaias pectorosa*) and paper pondshell (*Utterbackia imbecillis*) were 14.05 and 10.60 mg total ammonia as N/L, respectively, while the

corresponding 48-h value for glochidia were 3.76 and 5.85 mg total ammonia/L. Similarly, the mean 96-h LC50 for the rainbow mussel (*V. iris*) was 6.75 mg total ammonia/L, and the 24-h value for glochidia was 3.79 mg total ammonia /L. Keller and Ruessler (1997) examined the toxicity of malathion to early life stages of U. imbecillis, little spectaclecase (V. lienosa), and downy rainbow mussel (V. 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 (US EPA) 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 6th (88µg Cu/L), and 14th (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*, *D. magna*, and *Oncorhynchus mykiss* (rainbow trout) (Mummert et al. 2003, Goudreau et al. 1993). 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 24-h acute glochidia LC50s with 48-h acute LC50s for *D. magna* and 7-day fecundity EC50s 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 for the effectiveness of glochidia as test organisms in the laboratory.

Guidelines for conducting acute toxicity tests with early life stages of freshwater mussels were drafted in 1990 (US EPA 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 et al. 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, but efforts will go unwarranted unless better techniques for assessing the health of glochidia are developed.

1.2.1 Study goals

The primary objective of this study was to compare the sensitivities of glochidia of different species of freshwater mussels to Hg by conducting laboratory tests with organic and inorganic Hg salts. Many freshwater systems are contaminanted by Hg pollution, as anthropogenic sources, such as the incineration of medical wastes, disposal of Hg-laden

material, industrial processing, pesticide use, and the burning of fossil fuels, have made it more available in ecosystems. Although most Hg 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 methyl-mercury (MeHg) in water (Barkay et al. 1997, Wiener and Shields 2000, Mauro et al. 2002). This organic form of Hg is highly toxic to aquatic life, and has been documented to bio-accumulate in food webs (Barkay et al. 1997, French et al. 1999, Mason et al. 2000, Wiener and Shields 2000, Mauro et al. 2002). The US EPA is currently reassessing the WQC for Hg, as researchers have become more aware of the threat it poses to humans and wildlife (Moore et al. 2003). Fish Consumption Advisories (FCA) for Hg have been issued in nearly every U.S. state (French et al. 1999, Mason et al. 2000, Webber and Haines 2003). However, recent studies examining the sensitivities of freshwater organisms are sparse, and results from earlier studies may be flawed because technology for measuring low concentration of Hg did not exist. Furthermore, there is little known about the sensitivity of freshwater mussels to Hg, despite documented declines in polluted (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 Hg 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). I wanted to determine whether standard, freshwater regulatory test organisms are suitable surrogate test organisms for assessing Hg 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 objective of this study was to expose glochidia to sodium chloride (NaCl) to determine whether it is an appropriate reference toxicant. Tests were conducted based on methods described in protocol for standard freshwater test organisms (US EPA 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.

1.3 Methods

1.3.1 Test organisms

Gravid specimens of *Lampsilis fasciola* (wavyrayed lampmussel), *V. iris* (rainbow mussel), *Epioblasma capsaeformis* (oyster mussel), and *E. 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 Aquatic Wildlife Conservation Center in Marion, VA. Adult mussels were acclimated to laboratory conditions for at least 48 h before the glochidia were extracted. Glochidia were removed 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 the glochidia. They were loaded into test chambers < 2 h after extraction.

Daphnids, *C. dubia* and *D. magna* (< 24 h-old), were cultured at the VPI&SU Aquatic Toxicology Laboratory according to standard procedure (APHA 1998). Organisms were cultured in a 80:20 mixture of moderately hard, synthetic water (EPA^{100}) (US EPA 1993) and filtered reference water at 25±1° C under 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).

1.3.2 Preparation of mercury test solutions

Mercuric chloride (MC) and methyl-mercuric 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.

1.3.3 Toxicity tests

1.3.3.1 Glochidia

Because a protocol has yet to be established for glochidia bioassays, we attempted to adhere to the test design described in US EPA 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, and therefore, I 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 h, and the remaining four replicates were assessed after 48 h. Tests were conducted at 20±1° C, under a 12:12 light:dark photoperiod.

Glochidia viability was assessed through a NaCl response test, similar to that described by Goudreau et al. (1993) and Huebner and Pynnonen (1992). 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, after which a concentrated NaCl solution was added. Any glochidia closed prior to, or remaining open after the addition of the salt solution were recorded as functionally dead.

1.3.3.2 EPA test organisms

Acute 48-h toxicity tests were conducted with *C. dubia*, *D. magna*, and *P. promelas* according to US EPA standard protocol (1993). Cladoceran bioassays were conducted in 50-ml glass beakers with approximately 35 ml of test solution. There were 4 replicates of 5 individuals for each treatment. *Pimephales* bioassays were conducted in 300-ml glass beakers filled with ~250 ml of test solution. There were 2 replicates of 10 individuals for each concentration. Mortality was assessed after 24 and 48 h. All tests were conducted at 20±1° C under 12:12 light:dark photoperiod, and organisms were not fed during the tests.

1.3.4 Reference toxicant tests

Reference toxicity tests were conducted with glochidia of *L. fasciola*, *E. capsaeformis* and *E. brevidens*. Sodium chloride (NaCl) was used as the toxicant because it is the suggested contaminant for reference bioassays with standard freshwater regulatory

test organisms (US EPA 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 NaCl was used as the toxicant, and EPA^{100} water was used as the diluent, and control treatment. Viability of glochidia was assessed after 24 and 48 h of exposure. Bioassays were conducted at 20±1° C under a 12:12 light:dark photoperiod.

Results of monthly acute NaCl 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 between January 2001 and August 2003 according to standard protocol (US EPA 1993).

1.3.5 Water chemistry and Hg 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 < ± 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 et al. (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 US EPA (1991) standard methods. Trace metal-grade pure hydrochloric acid was used to

reduce the sample pH to ≤ 2. The prepared samples were refrigerated until analysis at the VA Tech Soil Laboratory (Blacksburg, VA).

1.3.6 Data analysis

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

1.4 Results

1.4.1 Control survivorship

The combined mean glochidia viability in control treatments for all of the bioassays was ≥89% for the species tested after 24 h (Fig. 1.1 [28]). Mean control survivorship remained >80% after 48 h for all species, except *L. fasciola*, which declined to 78%. Overall, viability substantial decreased with increased exposure time for all species except *V. iris*.

1.4.2 Hg salt results

1.4.2.1 Mercuric chloride

Glochidia from the different species of freshwater mussels had similar tolerances to MC, as 24-h and 48-h LC values for *L. fasciola*, *E. capsaeformis*, and *E. brevidens* ranged between 25 – 54 and 27 - 40 µg Hg/L, respectively (Table 1.1 [23]). Although not evident by the LC50 values, viability decreased with increased exposure time in nearly every treatment. Survivorship remained high in the control (24 h =>89% and 48 h =>81%), but was substantially reduced in treatments containing elevated concentrations of Hg. After 48 h, 100% mortality was observed in treatments ≥120 µg Hg/L.

Ceriodaphnia was far more sensitive to MC than *D. magna*, as the respective 48-h LC50 values were 7 and 19 µg Hg/L (Table 1.2 [24]). Sensitivity increased with exposure time in both tests, and the largest contrast in 24- and 48-h LC50 values (90 and 15 µg Hg/L) was observed with *D. magna*. Survivorship in the control remained 100%, but was substantially reduced in treatments with measurable concentration of Hg for both species.

1.4.2.2 Methyl mercuric 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 h ranged between 21 – 26 µg Hg/L for the two species (Table 1.3 [25]). However, 48-h LC50 values could not be calculated because mortality was >50% in the lowest test treatment, 8 µg Hg/L. Therefore, these values were reported conservatively as < 8 µg Hg/L. *Villosa iris* glochidia were far more tolerant than the two other species. A 24-h LC50 could not be calculated because only 38% of the individuals exposed to 120 µg Hg/L died; however, the value was reported as > 120 µg Hg/L for comparative purposes. After 48 h, the LC50 for *V. iris* declined substantially to 43 µg Hg/L, but was still 5 times higher compared to the values found for glochidia of the other species.

Ceriodaphnia was the most sensitive organism tested to MMC, as 100% mortality occurred in treatments ≥8 µg Hg/L, despite 100% survivorship in the control (Table 1.4 [26]). The 48-h LC50 could not be calculated in either *C. dubia* test because of high mortality in low concentrations. Subsequently, these values were reported conservatively as < 4 and < 8 µg Hg/L. *Daphnia* were also quite sensitive to MMC, as 48-h LC50 values for the two trials were 18 and 15 µg Hg/L. *Pimephales promelas* was extremely tolerant to MMC exposure, as a 24-h LC50 values could not be calculated due to only 15% mortality in the highest treatment; this value was expressed as > 120 µg Hg/L. A 48-h 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.

1.4.3 Reference toxicant results

1.4.3.1 Glochidia

Glochidia of *L. fasciola*, *E. capsaeformis*, and *E. brevidens* had similar tolerances to NaCl, as the upper and lower 95% confidence limits for the 24- and 48-h LC50 values nearly overlapped (Table 1.5 [27]). After 48 h, control survivorship was extremely low in the *L. fasciola* bioassay (68%) and 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 Hg tests, viability decreased with increased exposure times in most treatments. The average 48-h LC50 for glochidia from all three species combined was 2.46 g NaCl/L (Fig 1.2 [29]).

1.4.3.2 Standard regulatory test organisms

The three different standard regulatory organisms, *C. dubia*, *D. magna*, and *P. promelas*, had very distinct NaCl tolerances. The most sensitive species was *C. dubia*, as the average 48-h LC50 was 2.33 g NaCl/L. Similar values for *D. ma*gna and *P. promelas* were 4.96 and 9.84 g NaCl/L (Fig. 1.2 [29])

1.4.4. Water chemistry and Hg concentrations

Water chemistry parameters and Hg concentration analysis results for the different test treatments are summarized in Table 1.6 [30]. Dissolved oxygen remained > 5.0 mg/L in all bioassays. Other water parameters for in- and out-water did not differ substantially, except for total Hg concentration, which was substantially lower in out-water. Treatments

≤ 15 µg Hg/L were below detection limit (BDL). The in-water values for treatments ≥ 30 µg Hg/L were very close to nominal concentrations.

1.5 Discussion

1.5.1 Mercury tests

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 NaCl, that is known to elicit this avoidance behavior (Huebner and Pynnonen 1992, Goudreau et al. 1993, Jacobson et al. 1997, and 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, Goudrea et al. 1993, Jacobson et al. 1997, Keller and Ruessler 1997, Cherry et al. 2002). These observations have encouraged 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 reproductive potential of impacted individuals. In my study, very low concentrations of Hg drastically affected the viability of glochidia. However, additional research is needed to more accurately determine species tolerances because impairment was observed in treatments with Hg concentrations below detection limit (BDL) in this study. Regardless, this study provided substantial evidence that released glochidia from some species of freshwater mussels are sensitive to Hg at measurable concentrations in the environment.

Interestingly, I also noted substantial inter-specific variability in Hg 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. Few studies have conducted experiments with glochidia from numerous species of freshwater mussels, and those 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-h LC50 value for the most sensitive species, *Lampsilis siliquoidea* was 7 mg/L, compared to 324 mg/L for the most tolerant species tested, *Utterbackia imbecillis*.

There was also a distinct difference in the toxicity of the different Hg 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 Hg concentrations were tested for both salt treatments, the 48-h LC50 values were approximately three times lower for glochidia exposed to MMC. Several other studies have documented similar differences in the toxicity of Hg salts with test organisms other than glochidia. Baby and Menon (1987) observed that juvenile marine bivalves were more sensitive to Hg, 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 et al. (1982) cited that *D. magna* in a chronic study excreted Hg slower when exposed to MMC than MC.

1.5.2 Glochidia tolerance compared to standard test organisms

Both *C. dubia* and *D. magna* were more sensitive to MC than glochidia of all species tested, as 48-h 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 (US EPA 1985), and of them, only 4 genera had mean LC50 values lower than the GMAV of 30 µg Hg/L for *Epioblasma* in my 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. Methyl-mercury is more toxic than other forms because it is more available to biota and accumulates in aquatic food webs. Inorganic mercury is converted into methyl-mercury through microbial respiration in aquatic systems, and we assume that this conversion occurred in our test chambers. Though we did not measure MeHg concentrations, MMC salt may have been more toxic than MC salt because a greater portion of the total measured Hg in solution already existed in the organic form. It will be important to have lower detection limits, and analyze both total Hg and MeHg concentrations in future bioassays. Overall, this study suggests that aquatic organisms are highly sensitive to Hg, regardless of the salt form being tested.

1.5.3 NaCl 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 NaCl concentrations. Furthermore, glochidia were quite sensitive to NaCl, 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 NaCl as a reference toxicant for glochidia, but additional studies are needed to verify these results, and improve the precision of acceptable tolerance ranges for species.

Standard methods (US EPA 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 data bases based on the premise that 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 thesholds are typically $\geq$ 90 % for acute, and $\geq$ 80% for chronic bioassays (US EPA 1994, 1994). Additional requirements have also been established for chronic *C. dubia* bioassays, and include a minimum control survivorship of $\geq$ 80%, $\geq$ 60% of organisms in control treatments have three broods within eight days, and surviving organisms in controls average $\geq$15 neonates. Chronic *P. promelas* bioassay test results are only acceptable if control organisms survivorship is $\geq$ 80%, and average growth is $\geq$ 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 ≥90% after extraction, or ≥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. I advocate conducting reference bioassays concurrently with other glochidia toxicant tests as a means for inferring the relative health of organisms used. Currently, additional research to determine the acceptable range in NaCl tolerances of glochidia for a species of mussel is needed before reference test endpoints can be used effectively as QA/QC measures. Since seasonal variation is anticipated, it will also be important to conduct tests at different times throughout the year to evlauted temporal effects.

1.6 Acknowledgment

The authors thank John Schmerfeld, Cindy Kane, and Jess Jones for their active roles in the project. The research was supported by the U.S. Fish and Wildlife Service.

1.7 References

1) American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environmental Federation (WEF). 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th Edition. American Public Health Association, Washington, DC.

2) Augspurger T, AE Keller, MC Black, WG Cope, FJ Dwyer. 2003. Water quality guidance for protection of freshwater mussels (*Unionidae*) from ammonia exposure. *Environ Tox Chem* 22: 2569-2575.

3) Baby KV and NR Menon. 1987. Salt forms of metals & their toxicity in the brown mussel, *Perna indica* (Kuriakose & Nair). *Indian J Mar Sci* 16: 107-109.

4) Barkay T, M Gillman, RR Turner. 1997. Effects of dissolved organic carbon and salinity on bioavailability of mercury. *Applied Environ Micro*: 4267-4271.

5) Beckvar N, S Salazar, M Salazar, K Finkelstein. 2000. An *in situ* assessment of mercury contamination in the Sudbury River, Massachusetts, using transplanted freshwater mussels (*Elliptio complanata*). *Can J Fish Aquat Sci* 57: 1103-1112.

6) Biesinger KE, LE Anderson, JG Eaton. 1982. Chronic effects of inorganic and organic mercury on *Daphnia magna*: Toxicity, accumulation, and loss. *Arch. Environ Contam Toxicol* 11: 769-774.

7) Brenderman SA, Neves RJ. 1993. Life history of the endangered fine-rayed pigtoe, *Fusconaia cuneolus* (Bivalvia: Unionidae), in the Clinch River, Virginia. *Am Malacol Bull* 10:83-91.

8) Cherry DS, Van Hassel JH, Farris JL, Soucek DJ, Neves RJ. 2002. Site-specific derivation of the acute copper criteria for the Clinch River, Virginia. *Hum Ecol Risk Assess* 8:591-601.

9) Diamond JM, DW Bressler, VB Serveiss. 2002. Assessing relationships between human land use and the decline of native mussels, fish, and macroinvertebrates in the Clinch and Powell River Watershed, USA. *Environ Toxicol Chem* 21: 1147-1155.

10) French KJ, DA Scruton, MR Anderson, DC Schneider. 1999. Influence of physical and chemical characteristics on mercury in aquatic sediment. *Water Air Soil Pollut* 110: 347-362.

11) Goudrea SE, RJ Neves, RJ Sheehan. 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. *Hydrobiologia* 252: 211-230.

12) Gulley DD. 1993. Toxstat Version 3.3. University of Wyoming, Department of Zoology and Physiology, Laramie, WY.

13) Henley WF, Neves RJ. 1999. Recovery status of freshwater mussels (Bivalvia: Unionidae) in the North Fork Holston River, Virginia. *Am Malacol Bull* 15:65-73.

14) Huebner JD, KS Pynnonen. 1992. Viability of glochidia of two species of *Anodonta* exposed to low pH and selected metals. *Can J Zool*. 70: 2348-2355.

15) Jacobson PJ, Neves RJ, Cherry DS, Farris JL. 1997. Sensitivity of glochidial stages of freshwater mussels (Bivalvia: Unionidae) to copper. *Environ Toxicol Chem* 16:2384-2392.

16) Keller, AE. 1993. Acute toxicity of several pesticides, organic compounds and wastewater effluent to the freshwater mussel, *Anodonta imbecillis*, *Ceriodaphnia dubia* and *Pimephales promelas*. *Bull Environ Contam Toxicol* 51:696-702.

17) Keller AE, Ruessler DS. 1997. The toxicity of malathion to unionid mussels: Relationship to expected environmental concentrations. *Environ Toxicol Chem* 16:1028-1033.

18) Keller AE, Zam SG. 1991. The acute toxicity of selected metals to the freshwater mussel, *Anodonta imbecillis*. *Environ Toxicol Chem* 10:539-546.

19) Masnado RG, SW Geis, WC Sonzogni. 1995. Comparative acute toxicity of a synthetic mine effluent to *Ceriodaphnia dubia*, larval fathead minnow and the freshwater mussel, *Anodonta imbecillis*. *Environ Toxicol Chem* 14: 1913-1920.

20) Mason RP, JM Laporte, S Andres. 2000. Factors controlling the bioaccumulation of mercury, methylmercury, arsenic, selenium, and cadmium by freshwater invertebrates and fish. *Arch Environ Contam Toxicol* 38: 283-297.

21) Mauro JBN, JRD Guimaraes, H. Hintelmann. 2002. Mercury methylation in macrophytes, periphyton, and water- comparative studies with stable and radio-mercury additions. *Applied Bio Chem* 374: 983-989.

22) Milam CD, Farris JF. 1998. Risk identification associated with iron-dominated mine discharges and their effect upon freshwater bivalves. *Environ Toxicol Chem* 17:1611-1619.

23) Moore DRJ, RS Teed, GM Richardson. 2003. Derivation of an ambient water quality criterion for mercury: Taking account of site-specific conditions. *Environ Tox Chem* 22: 3069-3080.

24) Mummert AK, RJ Neves, TJ Newcomb, DS Cherry. 2003. Sensitivity of juvenile freshwater mussels (*Lampsilis fasciola*, *Villosa iris*) to total and un-ionized ammonia. *Environ Tox Chem* 22: 2545-2553.

25) Naimo TJ. 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4:341-362.

26) Neves RJ, Widlak JC. 1987. Habitat ecology of juvenile freshwater mussels (Bivalvia: Unionidae) in a headwater stream in Virginia. *Am Malacol Bull* 5:1-7.

27) Olson GF, DI Mount, VM Snarski, TW Thorslund. 1975. Mercury residues in fathead minnows, *Pimephales promelas* Rafinesque, chronically exposed to methylmercury in water. *Bull Environ Contam Tox*. 14: 129-134.

28) US Environmental Protection Agency. 1985. Ambient water quality criteria for mercury-1984. EPA 440/5-84-026. Washington, DC.

29) US Environmental Protection Agency. 1990. Proposed guide for conducting acute toxicity tests with the early life stages of freshwater mussels. EPA/68/02/4278. Washington, DC.

30) US Environmental Protection Agency. 1991. Methods for determining metals in environmental samples. EPA/600/4-91/010. Washington, DC.

31) US Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms- 4th ed. EPA/600/4-90/027F, Washington, DC.

32) US Environmental Protection Agency. 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms-3rd ed. EPA/600/4/91/002. Washington, DC.

33) Webber HM, TA Haines. Mercury effects on predator avoidance behavior of a forage fish, golden shiner (Notemigonus crysoleucas). *Environ Tox Chem* 22: 1556-1561.

34) Weinstein JE. 2001. Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel, *Utterbackia imbecillis. Environ Tox Chem* 20: 412-419.

35) Weinstein JE. 2002. Photoperiod effects of the UV-induced toxicity of fluoranthene to freshwater mussel glochidia: Absence of repair during dark periods. *Aquat Toxicol* 59: 153-161.

36) Weinstein JE, Polk KD. 2001. Phototoxicity of anthracene and pyrene to glochidia of the freshwater mussel *Utterbackia imbecillis*. *Environ Toxicol Chem* 20:412-419

37) Wiener JG, PJ Shields. 2000. Mercury in the Sudbury River (Massachusetts USA): pollution history and synthesis of recent research. *Can J Fish Aquat Sci* 57: 1053-106120:2021-2028.

38) Williams JD, Warren ML, Cummings KS, Harris JL, Neves RJ. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18:6-22.

39) Wobeser G. 1975. Acute toxicity of methyl mercury chloride and mercuric chloride for rainbow trout (*Salmo gairdneri*) fry and fingerlings. *J Fish Res Board Can* 32:2005-2013.

40) Yeager MM, RJ Neves, DS Cherry. 1999. Competitive interactions between early life stages of *Villosa iris* (Bivalvia: Unionidae) and adult Asian clams (*Corbicula fluminea*). Proceeding of the First Freshwater Mollusk Conservation Society Symposium.

Table 1.1. The 24- and 48-h LC50 values, respective 95% confidence intervals, and mean survivorship of glochidia of *Lampsilis fasciola*, *Epioblasma capsaeformis*, and *Epioblasma brevidens* exposed to different concentrations of mercuric chloride ($HgCl_2$).

Species	Concentration (µg Hg/L)	n	24-h % Mortality	24-h LC_{50} (95% CI)	n	48-h % mortality	48-h LC_{50} (95% CI)
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	
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				
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	
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	
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	

Table 1.2. The 24- and 48-h LC50 values, respective 95% confidence intervals, and mean survivorship of *Ceriodaphnia dubia* and *Daphnia magna* exposed to different concentrations of mercuric chloride ($HgCl_2$).

Species	Concentration (µg Hg/L)	n	24-h % Mortality	24-h LC_{50} (95% CI)	n	48-h % mortality	48-h LC_{50} (95% CI)
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	
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 1.3. The 24- and 48-h LC50 values, respective 95% confidence intervals, and mean survivorship of glochidia of *Epioblasma capsaeformis, Epioblasma brevidens,* and *Villosa iris* exposed to different concentrations of methyl-mercuric chloride (CH_3HgCl_2).

Species	Concentration (µg Hg/L)	n	24-h % Mortality	24-h LC_{50} (95% CI)	n	48-h % mortality	48-h LC_{50} (95% CI)
	Control	50	4		50	18	
	8	50	10		50	70	
E. capsaeformis	15	50	36	**21 µg Hg/L**	50	80	**8 µg Hg/L**
(individual A)	30	50	68	**(17- 24)**	50	100	**(4 - 9)**
	60	50	100		50	100	
	120	50	100		50	100	
	Control	100	3		100	10	
	8	100	4		100	49	
E. capsaeformis	15	100	13	**26 µg Hg/L**	100	100	**< 8 µg Hg/L**
(individual B)	30	100	60	**(23 - 28)**	100	100	**(n/a)**
	60	100	100		100	100	
	120	100	100		100	100	
	Control	100	11		100	17	
	8	100	10		100	56	
E. brevidens	15	100	26	**25 µg Hg/L**	100	100	**< 8 µg Hg/L**
	30	100	51	**(22 - 28)**	100	100	**(n/a)**
	60	100	100		100	100	
	120	100	100		100	100	
	Control	326	6		305	5	
	8	246	4		316	5	
V. iris	15	257	6	**> 120 µg Hg/L**	309	8	**43 µg Hg/L**
	30	316	6		325	15	**(41 - 45)**
	60	276	8		314	90	
	120	255	38		336	100	

Table 1.4. The 24- and 48-h LC50 values, respective 95% confidence intervals, and mean survivorship of *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* exposed to different concentrations of methyl-mercuric chloride (CH_3HgCl_2).

Species	Concentration (µg Hg/L)	n	24-h % Mortality	24-h LC_{50} (95% CI)	n	48-h % mortality	48-h LC_{50} (95% CI)
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	
	15	20	15		20	100	
	30	20	60		20	100	
	60	20	90		20	100	
	120	20	100		20	100	
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	
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	
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	
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	

Table 1.5. The 24- and 48-h LC50 values, respective 95% confidence intervals, and percent mortality of glochidia of *Lampsilis fasciola*, *Epioblasma capsaeformis*, and *Epioblasma brevidens* exposed to the reference toxicant NaCl.

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

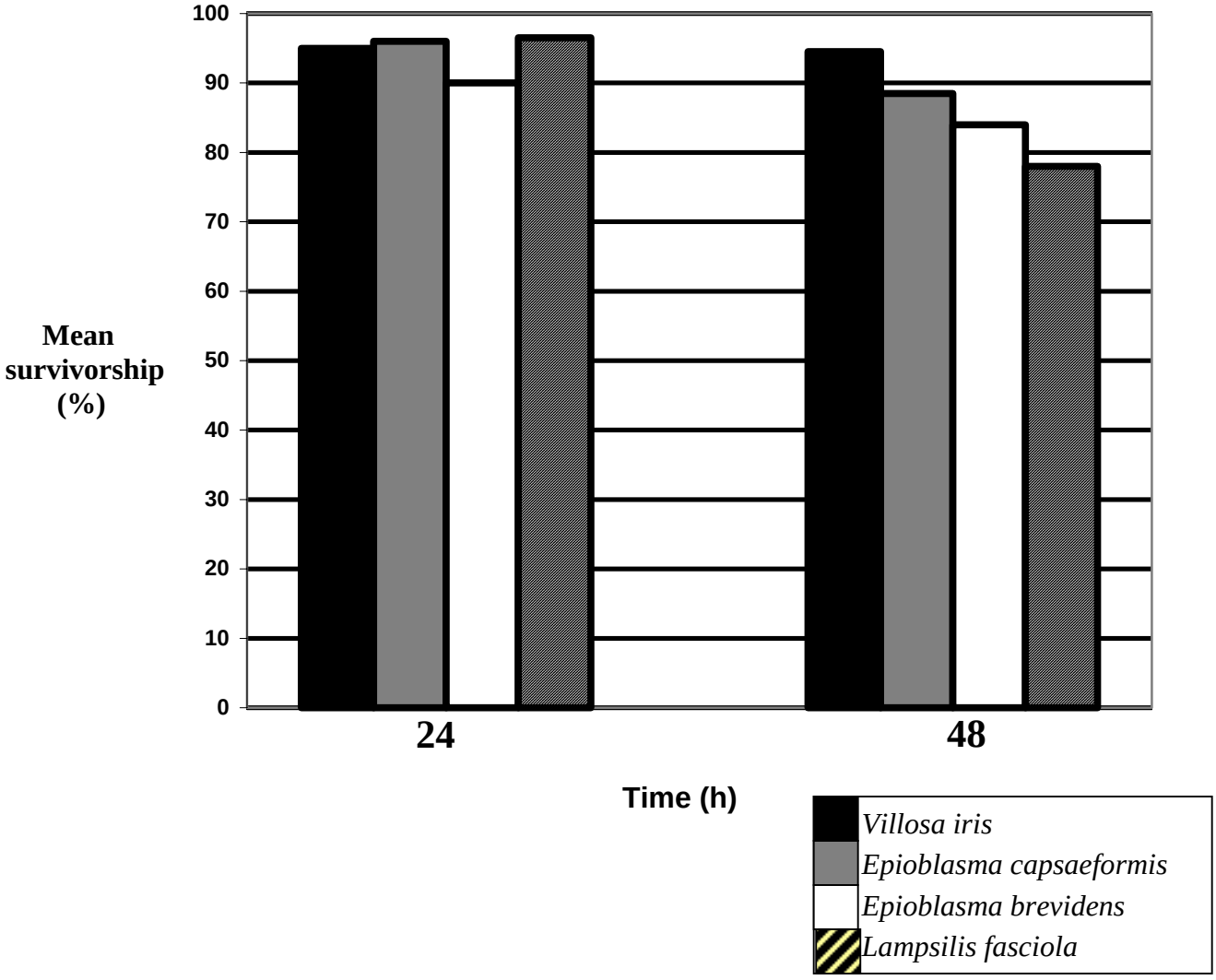


Figure 1.1. Mean viability of mussel glochidia from *Villosa iris, Epioblasma capsaeformis, Epioblasma brevidens,* and *Lampsilis fasciola* in control treatments after 24 and 48 h.

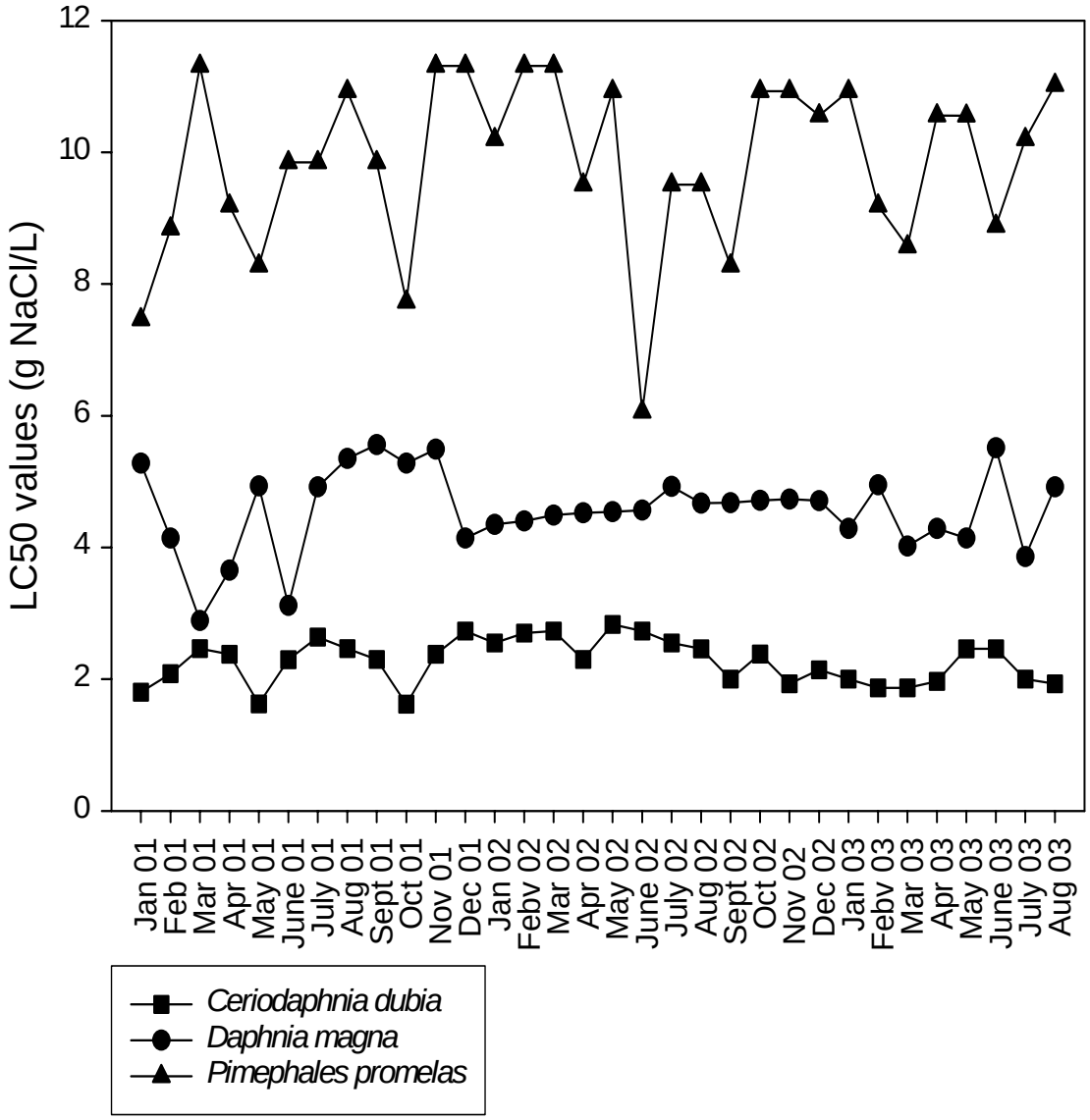


Figure 1.2. The48-h LC50 values of reference toxicant tests conducted with standard regulatory test organisms *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimphales promelas*. Bioassays conducted according to US EPA Protocol (1999)

Table 1.6. Mean conductivity, pH, Alkalinity, Hardness, and in- and out-water mercury concentrations for the acute mercury and reference toxicant tests.

Test	Treatment	Conductivity (µmhos)	pH (su)	Alkalinity (mg/L as CaCO3)	Hardness (mg/L as CaCO3)	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
	120	297 ± 11	7.81 ± 0. 08	63.1 ± 5.7	86.2 ± 7.8	117.7 ± 21.6	98.4 ± 27
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
	60	299 ± 11	7.81± 0.14	n/a	n/a	62.0 ± 18.4	46.7 ± 22
	120	294 ± 9	7.83 ± 0. 12	61.9 ± 7.2	84.8 ± 5.6	133.6 ± 38.8	86.6 ± 41
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	14570 ± 342	7.81 ± 0.13	62.6 ± 3.9	82.9 ± 5.8	n/a	n/a

Chapter 2: The use of sodium chloride as a reference toxicant for evaluating the quality of freshwater mussel glochidia.

2.1 Abstract

Glochidia have been proposed as a surrogate life stage for juvenile mussels in bioassays because of their greater availability and demonstrably lower tolerances to some contaminants. However, the validity of glochidia test results are under scrutiny by regulatory agencies for lack of quality assurance / quality control (QA/QC) measures, and concerns about assessing the health of test organisms. Sodium chloride was used as a reference toxicant to assess the health of freshwater mussel glochidia. Glochidia from four mussel species were tested, and LC50 values were significantly different among species; respective means were 2.5 g NaCl/L for *Alasmidonta heterodon*, 1.7 g NaCl/L for *Epioblasma capsaeformis*, 2.1 g NaCl/L for *Lampsilis fasciola,* and 0.98 g NaCl/L for *Villosa iris* after 24 or 48 h of exposure. Mean control survivorship remained > 90% after 24 h, and > 80% after 48 h for all species. More importantly, I observed a high degree of variability among LC50s for glochidia collected from different gravid adults of the same species, despite minimal differences in control survivorship. The study also provides tentative methods for conducting glochidia reference toxicant tests that may serve as QA/QC measures in future tests.

Keywords: glochidia freshwater mussel sodium chloride quality viability

2.2 Introduction

The imperiled status of many freshwater mussel species has intensified interest in identifying potential factors leading to their demise. For several decades, scientists have recognized the value of using adult mussels as bio-indicators because of their sedentary nature, close association with benthic and pelagic zones, and ability to tolerate the accumulation of high levels of heavy metals in their tissue (Salanki and Balogh 1989, (Metcalfe-Smith 1994, Warren et al. 1995, Malley et al. 1999, Beckvar et al. 2000). However, not until recent advances in culturing techniques were sufficient numbers of juvenile mussels available to conduct toxicity tests. It is well documented that early life stages of most aquatic organisms are more sensitive to toxicants than adults, and this trend is also apparent in studies focused on juvenile and adult mussels (Naimo 1995, Jacobson et al. 1997, Keller Ruessler 1997). The discrepancy in sensitivities between the two life stages may be due to differences in physiology (i.e., thinners shells that are more permeable to toxicants), metabolism (i.e., faster accumulation and slower excretion rates of toxicants relative to body mass), or behavior (i.e., adults rely primarily on filter-feeding whereas juveniles rely on both filter- and pedal-feeding) (Yeager et al. 1994, Naimo 1995).

Observations in rivers also indicate that juveniles have greater sensitivities than adults, as recent mussel surveys have shown disproportionately low percentages of young mussels in populations and lack of recruitment at sites of adequate habitat (Yeager et al. 1994, Henley and Neves 1999). This has led researchers to question whether current United States Environmental Protection Agency (EPA) Water Quality Criteria (WQC) are suitable for protecting the early life stages of mussels (Augspurger et al. 2003). Additional studies are needed, but it has proven difficult and costly to obtain sufficient

numbers of juveniles for conducting tests, for lack of mussel culturing facilities in the United States (Newton et al. 2003). These difficulties are exacerbated when threatened or endangered species are involved. Therefore, researchers are considering glochidia, the parasitic larval stage, as a surrogate for the juvenile life stage in toxicity tests because greater numbers are more readily available (Valenti et al. 2003)

While in the water column, glochidia may be exposed to toxicants that impair their ability to attach to host fish (Keller and Ruessler 1997), thus reducing reproductive potential. Laboratory tests have shown that exposure to stressors may provoke a positive response by glochidia, which includes sporadic snapping or complete closure of their valves (Goudreau et al. 1993, Jacobson et al. 1997); however, this behavior is undocumented in rivers. Researchers speculate that closed glochidia are unable to attach to host fish, and therefore are functionally unavailable (Goudreau et al. 1993, Jacobson et al. 1993, Weinstein and Polk 2001). Closed glochidia may not re-open, and the response is likely influenced by the toxicant, concentration, and exposure time. The valve closure response can be easily quantified, and has proven to be an effective means for assessing glochidia viability in toxicological studies. Several researchers have used concentrated NaCl solutions to elicit this behavior (Goudreau et al. 1993, Jacobson et al. 1997, Keller and Ruessler 1997). Open glochidia that respond to NaCl by closing their valves are considered viable, whereas those with closed valves prior to, or whose valves remain gaped after the salt solution exposure, are classified as functionally dead.

Few studies have compared the relative sensitivities of glochidia and juveniles of the same species, although Keller and Ruessler (1997) compared acute toxicity of the insecticide malathion for both life stages of *Utterbackia imbecillis*, *Villosa lienosa*, *and V. villosa*. The glochidia of the three species had lower 48-h LC50 values than newly

transformed juveniles, which is indicative of greater sensitivity. Valenti et al. (2003) tested the sensitivities of *V. iris* glochidia and 2-month old juveniles to mercuric chloride ($HgCl_2$) and found 48-h LC50 values of 52 µg Hg/L and 135 µg Hg/L, respectively. Furthermore, 24- and 48- h LC50 values in similar tests with 2-mo old *Epioblasma capsaeformis* juveniles were 0.16 and 0.14 mg Hg/L, respectively, (DS Cherry, unpublished data), which are substantially higher than those reported for glochidia (0.04 and 0.03 mg Hg/L, respectively) (Valenti et al. 2003). Augspurger et al. (2003) reported *V. iris* and *U. imbecillis* glochidia to be equally or more sensitive to total ammonia in acute laboratory studies. The greater sensitivities of glochidia noted in previous acute studies further support their use as surrogate test organisms for juvenile mussels in short-term exposure toxicity tests.

Cherry et al. (2002) compared the acute sensitivities of glochidia and standard test organisms in a study to develop an acute site-specific Criterion Maximum Concentration (CMC) for copper. Acute bioassays were conducted with 17 species to assess the potential impact of a coal-burning power plant's effluent on mussel populations in the Clinch River, VA. They reported that glochidia from three mussel species were more sensitive than *Ceriodaphnia dubia* and *Pimephales promelas*. The Genus Mean Acute Values (GMAV) for glochidia of the four most sensitive mussel species ranged from 37-60 mg/L, compared to 88 and 310 mg/L for *C. dubia* and *P. pimephales*, respectively. In a study examining the toxicity of different mercury salt forms, Valenti et al. (2003) reported sensitivities of glochidia of *Epioblasma capsaeformis* and *E. brevidens* to methyl-mercuric chloride comparable to that of *C. dubia*, and greater than *Daphnia magna* and *P. promelas* after 48-h exposures. Milam and Farris (1998) examined the toxicity of partially treated mine water and reported glochidia of *Leptodea fragilis* to be

more sensitive than *P. promelas*, but less sensitive than *D. magna* and *C. dubia*. However, their study contrasted 24-h acute glochidia LC50s with 48-h acute LC50s for *D. magna* and 7-day fecundity EC50s for *C. dubia*. Additional studies have suggested that glochidia are extremely sensitive when compared to other aquatic organisms (Goudreau et al. 1993, Weinstein 2001, Weinstein and Polk 2001).

The suitability of using glochidia as test organisms for assessing environmental impairment is currently under evaluation, and regulatory agencies are hesitant to accept the results of previous studies for a lack of standardized test protocols and Quality Assurance/ Quality Control (QA/QC) measures. Many of these concerns were expressed after guidelines for conducting laboratory tests with early life stages of freshwater mussels were proposed (US EPA 1990). The primary criticism of using glochidia as test organisms was difficulty in accurately verifying organism health at test initiation. Test validity endpoints previously suggested include criteria such as viability $\geq$90% immediately after extraction, or control survivorship $\geq$80% over the test duration (Jacobson et al. 1997, Keller and Ruessler 1997). Although these endpoints are useful and may eventually be incorporated as principle components of acceptable glochidia bioassay criteria, there is still concern for the accuracy and precision of these approaches.

Current U.S. EPA protocol for standard test organisms require not only a minimum control survivorship (typically >90%), but also the completion of reference toxicant tests to ensure adequate organism health. Reference toxicity tests are required 1) monthly at test organism culturing facilities, and 2) concurrently with permit tests conducted with organisms collected from the field or obtained from commercial suppliers (US EPA 1993, 1994). Reference toxicant tests allow researchers to better infer the relative health of test organisms, ensure suitability of test conditions, and verify that test

procedures were appropriate by contrasting reference test endpoints with those of an established database. Overall, the current reference toxicant system for standard test organisms aids in validating test results, and helps maintain uniformity among test facilities.

2.2.1 Study Objectives

The study goal was to propose a potential reference toxicant test protocol for glochidia supported by tests results, and to begin to develop tentative species data bases. I hope to encourage future reference tests trials with glochida so that a quality control assurance criterion can be established.

2.3 Materials and Methods

2.3.1 Test Organisms

Gravid adults of Villosa iris (rainbow mussel), Epioblasma capsaeformis (oyster mussel), and Lampsilis fasciola (wavyrayed lampmussel) from the Clinch River, VA, and Alasmidonta heterodon (dwarf wedgemussel) from the Ashuilot River, NH were collected during the 2003 summer field season. The oyster mussel and dwarf wedgemussel are federally endangered species. Adult mussels were held in a recirculating trough at 21 $\pm$ 1° C to acclimate them to laboratory conditions before the glochidia were extracted. Glochidia were removed by gently prying open a gravid female, puncturing the marsupium with a water-filled sterile syringe, and then injecting the water to flush the glochidia.

2.3.2 NaCl Test Design

The glochidia reference test design was based on established US EPA protocols (1993) for other freshwater organisms (Table 2.1 [54]). In the initial reference test with *V. iris*, test concentrations were a 0, 0.5, 1.0, 2.0, 4.0, and 8.0 g NaCl/L. However, due to 100%

mortality at the two highest concentrations, the 8.0 g NaCl/L concentration was eliminated in all subsequent tests and replaced with a 0.25 g NaCl/L concentration. Moderately hard, synthetic water (US EPA 1993, 1994) served as diluent and control. The reference toxicant was Certified A.C.S. grade NaCl salt (Fisher Scientific S271-3). A 0.5 serial dilution was used to prepare the test concentrations. Bioassays were conducted for 48 h, and viability was assessed after each 24-h interval. However, *A. heterodon* bioassays were conducted for only 24 h because it was essential that most glochidia of this endangered species be used to infest host fish for production of juveniles. Similarly, one female *E. capsaeformis* partially released glochidia while in captivity, leaving only sufficient glochidia for a 48 h test.

Bioassays were conducted at 20$\pm$1° C under a 16:8 light:dark photoperiod, with eight replicates for each concentration. Four replicates were randomly sampled after 24 h and glochidia viability was assessed, and the remaining four replicates were checked after 48 h. The test chambers consisted of 50-ml glass beakers filled with ~ 40 ml of test solution. Glochidia (25-75 total) were loaded into each test chamber with a fine tip glass pipette. Precautions were taken to evenly distribute glochidia in test chambers, to minimize potential interactions among test organisms in the same replicate. To assess viability, a sample of glochidia was drawn out of the test chamber with a fine-tip glass pipette and transferred to a glass petri dish for observation with a dissecting microscope. After the numbers of open and closed glochidia were recorded, they were then exposed to a concentrated NaCl solution. Glochidia were recorded as functionally dead if they were closed before or did not show a response after addition of the salt solution. A response was defined as either the complete, partial, or sporadic closure of the valves.

2.3.3 Water chemistry

Temperature, dissolved oxygen, conductivity, and pH were measured at the initiation and completion of each bioassay. Alkalinity and hardness were measured for the control and highest concentration. An Accumet® (Fisher Scientific, Pittsburgh, PA, USA) pH meter with an Accumet gel-filled combination electrode (accuracy < ± 0.05 pH at 25° C) was used to measure pH. Dissolved oxygen was measured with a Yellow Springs model 54A meter® (Yellow Springs, Yellow Springs, OH, USA), and a Yellow Springs model 30 conductivity meter® (Yellow Springs, Yellow Springs, OH, USA) was used to measure specific conductivity (accuracy ± 0.5%). Total hardness and alkalinity (as mg/L $CaCO_3$) were measured in accordance with APHA et al. (1998) through colorimetric titrations. Incubator temperature was monitored twice daily.

2.3.4 Data analysis

Data from the two separate trials of testing with *V. iris* and *E. capsaeformis* were pooled for statistical analysis, as there were no significant differences in control survivorship or LC50 values between testing periods for either species ($p<0.05$). Spearman-Karber LC50 values were calculated using the Toxstat® program. One-way ANOVAs and Student's t test pair-wise comparisons were calculated with the computer program Jump In 4.0® ($\alpha = 0.05$ level). Statistics were not computed for concentrations ≥ 4.0 g NaCl/L because mortality was 100% for all species.

2.4 Results

2.4.1 Intraspecific variation

2.4.1.1 *Villosa iris*

The 24-h control survivorship means for *V. iris* glochidia from the various adult mussels ranged from 89.2 - 98.0% and were not significantly different (p=0.12) (Table 2.2 [55]). Glochidia from mussel # 8 had the lowest control survivorship (89.2%), which was slightly lower than that of the other mussels (93.1-98.0%). However, the ANOVA for all other concentrations < 4.0 g NaCl/L had significant variation in survivorship ($p<0.001$).

The Student's t pair-wise comparisons at the 0.5, 1.0, 2.0 g NaCl/L concentrations had significant variation in mean survivorship among glochidia of different individuals after 24 h of exposure. At the 0.5 g NaCl/L concentration, mean survivorship of glochidia from mussel # 8 was approximately three-times lower than that of the other seven individuals tested; 28.5% compared to 72.6 – 90.5%. Glochidia collected from mussel #8 also had the lowest survivorship (20.5%) at the 1.0 g NaCl/L concentration. Survival also was greatly reduced for glochidia from mussel #7 at this concentration, with mean survivorship declining from 74.5% at 0.5 g NaCl/L to 41.0% at 1.0 g NaCl/L. There was also significant variation in survivorship of glochidia from different individuals at 2.0 g NaCl/L; however, mean viability was drastically reduced for all glochidia (1.6 – 16.5%).

The 24-h LC50s also varied markedly for glochidia collected from different *V. iris* females, as values ranged from 0.31-1.51 g NaCl/L. The LC50 of 0.31 g NaCl/L for mussel #8 was 3-5 times lower than for the other seven individuals. Six of the eight

individuals had LC50s $\geq$1.0 g NaCl/L, and of these, five had confidence intervals that nearly overlapped. The LC50 of 1.51 g NaCl/L for glochidia from mussel #1 did not overlap, and was substantially higher than the values for glochidia from the other females.

There was a highly significant difference in 48-h control survivorship among glochidia from different individuals of *V. iris* (p=0.0016). The lowest mean survivorship of 82.9% occurred with glochidia obtained from mussel #8, and was significantly lower than the control survivorship means (89.6 – 97.0%) for the glochidia from the other mussels. Overall, there was a significant difference between 24- and 48-h control survivorship for glochidia of *V. iris* (p=0.045), as the means were 95.2 $\pm$ 4.9% and 92.5 $\pm$ 5.7%, respectively.

The 0.5, 1.0, and 2.0 g NaCl/L concentrations also had significant variations in survivorship among glochidia collected from the various *V. iris* females after 48 h of exposure. Similar trends to the 24-h data were apparent. Again, individual #8 had the lowest survivorship at the 0.5 and 1.0 g NaCl concentrations, 29.0 and 6.0%, respectively. Glochidia from individual #7 also had significantly impaired survivorship, when compared to the other individuals at these concentrations (50.0 and 37.5%, respectively), despite 95.0% control survivorship.

The 48-h LC50 values for glochidia from the eight female *V. iris* varied substantially. An LC50 value could not be calculated for mussel #8 because mortality was >50% at the lowest test concentration, so the value was expressed conservatively as <0.25 g NaCl/L. Individual #7 had an LC50 of 0.57 g NaCl/L, which was 2-4 fold lower than those recorded for the other six individuals. Though glochidia from individuals #5 and #6 had LC50 values $\leq$ 1.0 g NaCl (0.99 and 0.97 g NaCl/L, respectively), the upper

95% confidence intervals exceeded this limit. The LC50s for glochidia from the remaining four *V. iris* ranged from 1.15-1.60 g NaCl/L, and mussel #1 again had the highest value. The 24- and 48-h LC50s were not significantly different for glochidia of *V. iris* (p=0.66), as mean values were 1.07 $\pm$ 0.36 and 0.98 $\pm$ 0.41 g NaCl/L, respectively.

2.4.1.2 *Epioblasma capsaeformis*

The 24-h control mean survivorships for *E. capsaeformis* glochidia from the various adults were not significant different (p=0.11), and ranged between 87.3 – 93.7% (Table 2.3 [56]). Survivorship also was not significant different at the 0.25 and 0.5 g NaCl/L concentrations (p= 0.063 and p=0.21, respectively); however, there were highly significant differences at the 1.0 and 2.0 g NaCl/L concentrations (p= <0.0001 and p= 0.0003).

At the 1.0 g NaCl/L concentration, the mean survivorship for glochidia from mussel #5 (46.3%) was approximately half the value recorded for glochidia from the other individuals, which ranged from 86.9 – 92.2%. A similar trend was also apparent at the 2.0 g NaCl/L concentration for glochidia from mussel #5, as mean survivorship was only 11.5%; approximately 3-5 times lower than the values (35.9 – 50.9%) recorded for glochidia from the other four mussels.

There was also variation in the 24-h LC50 values for glochidia of *E. capsaeformis*. Glochidia from mussel #5 had a substantially lower LC50, 1.10 g NaCl/L, compared to the other four mussels, 1.81 – 1.98 g NaCl/L. Notably, glochidia from adults that maintained mean control survivorship $\geq$90% also had markedly higher LC50 values.

There were highly significant differences among control survivorship values for glochidia of *E. capsaeformis* after 48 h of exposure to NaCl (p= 0.0069). Control survivorship for glochidia from mussels # 3 (80.4%) and #4 (76.9%) were significantly lower than the values recorded for mussels # 1 (91.5%) and #2 (89.1%). No comparison could be made with mussel #5, which had the lowest control survivorship after 24 h, because there were inadequate glochidia to run a 48-h test. Overall, control survivorship was significantly reduced after 48 h compared to 24 h (p=0.0023), and the 24- and 48-h means were 90.8 $\pm$ 3.4 and 84.4 $\pm$ 7.8%, respectively.

Statistically significant variations in survivorship of glochidia from different *E. capsaeformis* females were noted at the 0.5, 1.0, and 2.0 g NaCl/L concentrations after 48 h. Mean survivorship for glochidia from mussel #4 was only 66.5%, compared to 86.6 - 88.9% for glochidia from the other three mussels. A similar trend was apparent at the 1.0 g NaCl/L concentration. The most striking variation in sensitivity among *E. capsaeformis* glochidia was noted at the 2.0 g NaCl/L concentration as the survivorship for glochidia from mussel #4 was significantly lower (4.2%), compared to the other four mussels (24.8 – 74.4%).

The 48-h LC50 values for glochidia of *E. capsaeformis* also varied markedly. The lowest LC50 recorded was for glochidia from mussel #4 (1.38 g NaCl/L), but the values were higher for glochidia from the other mussels (1.65-1.90 g NaCl/L). Glochidia from mussels #1-3 had LC50s with overlapping confidence limits and control survivorship >80%. Overall, the 24- and 48-h LC50 values were not significantly different for glochidia of *E. capsaeformis*, as means were 1.73 $\pm$ 0.36 and 1.70 $\pm$ 0.24 g NaCl/L, respectively.

2.4.1.3 *Alasmidonta heterodon*

There was no significant difference in control survivorship of glochidia between the two *A. heterodon* females (p=0.996), as means were 95.5 and 95.4%, respectively (Table 2.4 [57]). Furthermore, survivorship for glochidia from the different adults did not vary significantly at any concentration. The 24-h LC50s were 2.62 and 2.41 g NaCl/L, and their 95% confidence limits overlapped.

2.4.1.4 *Lampsilis fasciola*

A significant difference was evident among 24-h glochidia control survivorship from the three *L. fasciola* females (p=0.02) (Table 2.5 [58]). Glochidia from mussel #3 had a significantly lower mean control survivorship of 84.9% compared to 94.2 and 94.0% for the other two mussels. Significant variation in 24-h survivorship also occurred among the glochidia at all concentrations < 2.0 g NaCl/L. Glochidia from mussel #3 consistently had the lowest survivorship at most concentrations, although the minimum mean viability remained >80%. Conversely, mussel #3 had the highest mean survivorship of 64.8% at the 2.0 g NaCl/L concentration; however, it was not significantly different from the means (57.8 and 62.3%) for the glochidia from the other mussels (p=0.86).

The 24-h LC50s ranged from 2.09 – 2.41 g NaCl/L. Glochidia from mussel #3 had the highest LC50 despite having the lowest control survivorship, which was atypical of the pattern observed in our other experiments. However, this discrepancy was minor, as 95% confidence limits nearly overlapped for glochidia from all three individuals.

After 48 h of exposure, mean control survivorship for glochidia from mussel #3 was 80.0%, and was significantly lower than values from the other mussels (87.5 and

91.3%). *Lampsilis fasciola* was the only species tested in which glochidia did not have a significant difference in control survivorship between 24 and 48 h (p=0.06); however, a larger sample size is needed to confirm this observation. The respective means were 91.0 ± 6.0 and 86.2 ± 6.0%.

There were significant differences among the survivorships of glochidia from different adult mussels at concentrations ≤ 2.0 g NaCl/L, though no decisive trend was apparent. The 48-h LC50s did not vary greatly for glochidia from different adults, and values ranged from 2.00 – 2.30 g NaCl/L. The 24- and 48-h LC50s were not significantly different (p=0.40), and means were 2.24 ± 0.16 and 2.12 ± 0.16 g NaCl/L, respectively.

2.4.2 Interspecific variation

2.4.2.1 Control Survivorship

The 24-h control survivorships for glochidia from the various species were significantly different (p=0.0041). Student's t pair-wise comparison revealed that *V. iris* and *A. heterodon* had significantly higher control survivorships than *E. capsaeformis* and *L. fasciola* glochidia (p=0.05). However, glochidia from all species averaged >90% control survivorship after 24 h of exposure (Fig. 2.1 [59]). The results of the one-way ANOVA contrasting 48-h control survivorship among species showed a highly significant difference (p= 0.0002). *Villosa iris* was the only species that sustained a mean control survivorship >90%. No 48-h data were available for *A. heterodon*.

2.3.2.2 LC50 values

The 24-h LC50s of glochidia from the four mussel species tested were significantly different (p<0.0001) (Fig 2.1 [59]). The most NaCl-tolerant species was *A. heterodon* (2.52 ± 0.15 g NaCl/L), while the most sensitive species was *V. iris* (1.07 ±

0.36 g NaCl/L). The average LC50 values for *L. fasciola* and *E. capsaeformis* were 2.24 ± 0.16 and 1.73 ± 0.36 g NaCl/L, respectively. Forty-eight hour LC50s also varied significantly among species (p=0.008), as glochidia of *V. iris* again had a significantly lower value of 0.98 ± 0.41 compared to 2.12 ± 0.16 and 1.70 ± 0.24 g NaCl/L for *L. fasciola* and *E. capsaeformis*, respectively ($p<0.05$).

2.4.2.3 Water Chemistry

Temperature was 20±1° C, dissolved oxygen was >6.5 mg/L, and pH ranged from 7.84 – 8.19 in all treatments. Alkalinity and hardness ranged from 62-68 mg $CaC0_3$ and 82-86 mg $CaC0_3$, respectively. Conductivity (µS/cm) increased with concentration: control = 264-357, 0.25 = 724-774, 0.5 = 1100- 1290, 1.0 = 2047-2340, 2.0 = 3740-4086, 4.0 = 7050 – 7430, and 8.0 g NaCl/ L = 13910-15240.

2.5 Discussion

2.5.1 Intra-specific variation

The NaCl tolerances and control survivorships of glochidia collected from different gravid females varied substantially within some species. Intra-specific variability was most pronounced for glochidia of *V. iris,* as 48-h LC50 values ranged from <0.25 – 1.60 g NaCl/L, and control survivorship varied from 82.9 – 97.0%. The greater variability reported for V. iris may be attributed to the larger sample size (n=8) of experiments. More tests were run with *V. iris* because they are a relatively common mussel, making glochidia easier to obtain and expandable. Intra-specific variability will likely become evident for other species if larger sample sizes are used in future tests.

The substantial intra-specific variability in survival observed in our study confirms previous toxicological efforts that report a broad range in LC50 values for

glochidia from females of the same species. In experiments assessing the toxicity of copper, Cherry et al. (2002) reported large ranges in LC50 values (μg Cu/L) for glochidia of *L. fasciola* (26 – 51), *V. iris* (36 – 98), *Medionidus conradicus* (16 – 81), *Ptychobranchus fasciolaris* (17 – 212), and *Actinonaias ligamentina* (30 –172). Similarly, Jacobson et al. (1997) reported 24-h LC50 values (μg Cu/L) of 26 and 46 for *L. fasciola* and 36, 37, 39, 46, 46, 65, 73, 75, and 80 for *V. iris*.

There are several factors that may affect the tolerances of glochidia and result in intra-specific variation; of these, many are associated with obtaining gravid females from different field sites. Habitat disturbances such as pollution, sedimentation, reduction in food quality/availability, and flood events may affect the health and development of glochidia in gravid females. Furthermore, glochidia may also be directly exposed to toxicants while *in situ* through contact with marsupial fluids or the water column. Additional factors that likely influence glochidia tolerances include variation in developmental stage and maturity, genetics, and acclimation of the gravid female or glochidia to laboratory conditions.

The individual or cumulative effects that these disturbances have on glochidia health are unknown, as is their potential influence on test results. Lower LC50 values are anticipated in bioassays that use glochidia collected from gravid females in a weakened state, which may lead to false-positive test results that overestimate environmental risk. The range of NaCl tolerances observed in our study shows that intra-specific variability is common among glochidia obtained from different gravid females, and we expect a similar trend in studies with other toxicants. This observation is pertinent in the development of a standardized test protocol, and emphasizes the need for an improved approach for assessing the health of glochidia used in bioassays.

2.5.2 Inter-specific variation

Glochidia from different freshwater mussel species exhibited varying NaCl tolerances, corroborating previous studies that reported substantial differences in species tolerances for other toxicants. Keller and Ruessler (1997) examined the toxicity of the organophosphate insecticide malathion to glochidia from several species, and found a wide range in tolerances. The lowest 24-h LC50 was recorded for glochidia of *V. lienosa* (22 mg/L), while the highest was for *U. imbecillis* (366 mg/L). Similarly, Huebner and Pynnonen (1992) reported the tolerance of glochidia from *Anodonta cygnea* and *A. anatina* to be different in experiments testing pH and aluminum. Studies have also shown substantial differences in species tolerances to copper, as there was a three-fold difference in the LC50 values for the most and least sensitive species (Cherry et al. 2002, Jacobson et al. 1997)

Few published studies have tested glochidia from multiple species, but the relative sensitivities of species would be expected to fluctuate depending on the toxicant. For example, a high sensitivity to NaCl is not always indicative of greater sensitivity to other toxicants. In this study, *V. iris* glochidia were most sensitive to NaCl, but we found the glochidia of this species to be far more tolerant to mercury than those of *E. capsaeformis*, *E. brevidens*, and *L. fasciola* (Valenti et al. 2003). Therefore, researchers should be cautious when attempting to use glochidia of common mussel species as surrogate test organisms for other species in environmental risk assessments.

2.5.3 Comparison of LC50 and Control Survivorship

Glochidia of several mussel species had greater variability in LC50 values than control survivorships. This difference was most evident in experiments with *V. iris,* as 48-h LC50 values varied 7-fold, while control survivorship remained >80% for glochidia

from the various adults. A similar trend has also been noted for *C. dubia, D. magna*, and *P. promelas* in monthly acute NaCl reference tests, conducted according to US EPA protocol (1993), as LC50 values among months were more variable than the control survivorship, which was > 90% in all tests (D.S. Cherry, unpublished data). These results suggest that the comparative evaluation of NaCl tolerances provides researchers with a more accurate measurement of test organism quality compared to monitoring control survivorship, because subtle differences are more readily quantifiable.

2.6 Conclusion

My study showed significant differences among the tolerances of glochidia from different gravid adults to NaCl within (intra-specific variability) and between (inter-specific variability) species. These findings are important because despite reported differences in tolerances among species (Huebner and Pynnonen 1992, Jacobson et al. 1997, Keller and Ruessler 1997, Augspurger et al. 2003), no publications have examined the variability in sensitivities of glochidia from different gravid females of the same species. Although my study lacks sufficient results to establish acceptable ranges of NaCl tolerances for glochidia of the species tested, it does confirm that variability in tolerances is common within a species. The study also provides a tentative protocol for conducting reference toxicant tests that appears acceptable for most species, common and endangered. The incorporation of reference toxicant tests with glochidia will provide an additional QA/QC measure that may be critical in the future development of a standardized test protocol.

Furthermore, my study supports implementing a QA/QC criterion of $\geq$ 80.0% viability in control treatments as a validation of the health of glochidia used in bioassays, as proposed by Keller and Ruessler (1997). The results of toxicity tests in which >20%

mortality occurred in control glochidia are questionable. As a result, glochidia from some mussel species may be less effective test organisms because maintaining acceptable levels of control survivorship may only be feasible when test durations are substantially reduced. High levels of control mortality jeopardize test results by making it more difficult for researchers to verify that observed mortality is related to the toxicant(s), and not to other extraneous factors. However, as with standard test organisms, monitoring control survivorship may not be sufficient in itself to confidently verify the health of glochidia used in toxicity tests.

Regulatory agencies have implemented reference toxicant tests into current protocols for standard test organisms as additional QA/QC measures. The reference toxicant system is based on two premises: 1) healthy individuals of the same species should have similar sensitivities to a toxicant, and 2) unhealthy organisms are more stressed by additional burdens of strain than healthy organisms. Organisms with lower fitness will be more sensitive in toxicity tests, and yield lower LC50 values because their ability to tolerate a toxicant is reduced. When reference test results are compared to those of a long-term data base, reduced organism health should become evident if reference LC50 values are not within an established species range.

Despite $\geq$ 80.0% control survivorship, markedly lower NaCl tolerances for glochidia from some gravid females reinforces the need to improve QA/QC methods for evaluating the health of glochidia. However, additional studies are needed to establish adequate species data bases before QA/QC reference toxicant systems, similar to those used for standard regulatory test organisms, can be effective. Larger sample sizes of adults of the same species are important for establishing acceptable ranges of glochidia tolerances. Future studies should also verify that NaCl is an acceptable reference toxicant

for glochidia, despite the fact that the US EPA currently uses it as a standard reference toxicant for their freshwater organisms. Therefore, reference bioassays and other toxicant tests need to be conducted concurrently with glochidia collected from individual females to evaluate the relationships in tolerances If NaCl is an acceptable reference toxicant, then glochidia obtained from females who exhibit a low tolerance to it relative to other individuals of that species, should also have lower tolerances to other toxicants.

Reference toxicant tests may also serve as monitors for assessing the health of the broods of gravid females collected from a population over time. As alluded to earlier, a mussel's ability to produce and release viable glochidia is likely associated with environmental conditions, and may be limited by anthropogenic, biotic, and abiotic stress factors. Monitoring the viability of glochidia from females using a reference toxicant may become an easy, cost-effective approach for the long-term monitoring of mussel assemblages. However, base-line reference toxicant tolerance criteria must first be established for a given population before the system can be implemented as a biological monitoring tool.

2.7 Acknowledgements

A federal Endangered Species Permit enabled researchers to collect specimens for this study. Special thanks goes to Dr. Rebecca Currie, Branden Locke, and Jennie Uerz for their assistance.

2.8 References

1) Augspurger T, AE Keller, MC Blacks, WG Cope, FJ Dwyer. 2003. Water quality guidance for protection of freshwater mussels (Unionidae)from ammonia exposure. *Environ Toxicol Chem* 22: 2569-2575.

2) Beckvar N, S Salazar, M Salazar, K Finkelstein. 2000. An *in situ* assessment of mercury contamination in the Sudbury River, Massachusetts, using transplanted freshwater mussels (*Elliptio complanata*). *Can J Fish Aquat Sci* 57: 1103-1112.

3) Cherry DS, Van Hassel JH, Farris JL, Soucek DJ, Neves RJ. 2002. Site-specific derivation of the acute copper criteria for the Clinch River, Virginia. *Hum Ecol Risk Assess* 8:591-601.

4) Goudrea SE, RJ Neves, RJ Sheehan. 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. *Hydrobiologia* 252: 211-230.

5) Henley WF, Neves RJ. 1999. Recovery status of freshwater mussels (Bivalvia: Unionidae) in the North Fork Holston River, Virginia. *Am Malacol Bull* 15:65-73.

6) Huebner JD, KS Pynnonen. 1992. Viability of glochidia of two species of *Anodonta* exposed to low pH and selected metals. *Can J Zool*. 70: 2348-2355.

7) Jacobson PJ, Neves RJ, Cherry DS, Farris JL. 1997. Sensitivity of glochidial stages of freshwater mussels to copper. *Environ Toxicol Chem* 16:2384-2392.

8) Keller AE, Ruessler DS. 1997. The toxicity of malathion to unionid mussels: Relationship to expected environmental concentrations. *Environ Toxicol Chem* 16:1028-1033.

9) Malley F, A Stewart, B Hall. 1996. Uptake of methyl mercury by the floater mussel, *Pyganodon grandis* (Bivalvia, Unionidae), caged in a flooded wetland. *Environ Toxicol Chem* 15: 928-936.

10) Metcalfe-Smith J. 1994. Influence of species and sex on metal residues in freshwater mussels (Family: Unionidae) from the St. Lawrence River, with implications for biommonitoring programs. *Environ Toxicol Chem* 13: 1433-1443.

11) Milam CD, Farris JF. 1998. Risk identification associated with iron-dominated mine discharges and effect upon freshwater bivalves. *Environ Toxicol Chm* 17:1611-1619.

12) Naimo TJ. 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4:341-362.

13) Newton TJ, JW Allran, JA O'Donnell, MR Bartsch, WB Richardson. 2003. Effects of ammonia on juvenile unionid mussels (*Lampsilis cardium*) in laboratory sediment toxicity tests. *Environ Toxicol Chem* 22: 2554-2560.

14) Salanki J, K Balogh. 1989. Physiological background for using freshwater mussels in monitoring copper and lead pollution. *Hydrobiologia* 188/189: 445-454.

15) Unpublished data. Donald Cherry. Virginia Tech. Biology Department. Blacksburg, VA. 24060.

16) US Environmental Protection Agency. 1990. Proposed guide for conducting acute toxicity tests with the early life stages of freshwater mussels. EPA/68/02/4278. Washington, DC.

17) US Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms- 4th ed. EPA/600/4-90/027F, Washington, DC.

18) US Environmental Protection Agency. 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms-3rd ed. EPA/600/4/91/002. Washington, DC.

19) Valenti TW, DS Cherry, RJ Neves, BA Locke. November 8-13th, 2003. Sensitivities of freshwater mussel glochidia and regulatory test organisms to mercury and a reference toxicant. SETAC 24th Annual Meeting in North America. Austin, TX. Platform Presentation

20) Warren L, S Klaine, M Finley. 1995. Development of a field bioassay with juvenile mussels. *J N Am Benthol Soc* 14: 341-346.

21) Weinstein JE, Polk KD. 2001. Phototoxicity of anthracene and pyrene to glochidia of the freshwater mussel *Utterbackia imbecillis. Environ Toxicol Chem* 20:2021-2028.

22) Weinstein JE. 2001. Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel, *Utterbackia imbecillis. Environ Toxicol Chem* 20:412-419.

23) Yeager M, D Cherry, and R Neves. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, Villosa iris (Bivalvia: Unionidae). *J N AM Benthol Soc* 13: 217-222.

Table 2.1. Summarized test parameters for a 48-h acute reference toxicity test protocol with freshwater mussel glochidia. Test conditions are based on United States Environmental Protection Agency standard protocol USEPA/600/4-90/027F.

Parameter	Conditions
Test condition	Static, non-renewal.
Temperature	20 $\pm$ 1° C.
Test duration	48 h.
Toxcant	Chemical grade NaCl.
Dilution factor	0.5.
Test concentration	Five test concentrations and a control. [0, 0.25, 0.5, 1.0, 2.0, 4.0 g NaCl/L].
Light quality	Fluorescent lighting.
Photoperiod	16:8 light:dark.
Test chamber	50-ml beaker filled with ~ 40 ml of test solution.
Test water	Moderately hard, synthetic water (EPA^{100}).
Test organisms	Glochidia obtained from gravid mussels that are extracted < 4 h prior to test initiation.
Organisms/replicate	25-75 organisms per replicate.
Replicates/concentration	Four replicates per monitoring interval.
Water quality	Dissolved oxygen, pH, conductivity, hardness and alkalinity measured.
Feeding	None.
Aeration	None.
Assessing viability	Exposure glochidia to a concentrated NaCl solution. Any glochidia closed prior to or remaining open after the addition of the NaCl solution are to be recorded as dead.
Endpoints	Lethal concentration 50 (LC50) is the calculated concentration that results in mortality for 50% of the exposed test organisms.
Test acceptability	Control survivorship > 80%.
Equipment	Incubator, dissecting scope, and appropriate meters.

Table 2.2. The LC50 values and mean survivorship for *Villosa iris* glochidia from eight female mussels in the control, 0.5, 1.0, and 2.0 g NaCl/L treatments.

		Survivorship				
Time	**Individual**	**Control**	**0.5**	**1.0**	**2.0**	**LC50 [95% CI]**
24	1	97.5 ^{A}	90.5 ^{A}	92.0 ^{A}	16.5 ^{A}	1.51 [1.45-1.58]
	2	98.0 ^{A}	85.5 ^{AB}	72.0 ^{B}	13.5 ^{AB}	1.25 [1.19-1.31]
	3	97.0 ^{A}	90.4 ^{A}	74.5 ^{AB}	8.1 ^{BC}	1.22 [1.15-1.29]
	4	95.7 ^{AB}	72.6 ^{CD}	59.8 ^{BC}	7.5 ^{BC}	1.06 [0.95-1.18]
	5	93.5 ^{AB}	87.0 ^{AB}	74.5 ^{A}	11.0 ^{B}	1.31 [1.23-1.38]
	6	93.1 ^{AB}	76.7 ^{CD}	58.5 ^{BC}	4.9 ^{CD}	1.06 [0.97-1.16]
	7	97.5 ^{A}	74.5 ^{CD}	41.0 ^{C}	5.0 ^{CD}	0.85 [0.78-0.93]
	8	89.2 ^{B}	28.5 ^{E}	20.5 ^{D}	1.6 ^{D}	0.31 [0.25-0.36]
48	1	96.0 ^{AB}	94.5 ^{A}	91.0 ^{A}	22.5 ^{A}	1.60 [1.53-1.68]
	2	97.0 ^{A}	85.5 ^{AB}	53.0 ^{CD}	14.0 ^{B}	1.06 [0.98-1.14]
	3	90.8 ^{BC}	91.8 ^{A}	74.6 ^{AB}	3.3 ^{C}	1.27 [1.21-1.33]
	4	89.6 ^{C}	75.8 ^{BC}	61.0 ^{BC}	3.4 ^{C}	1.15 [1.07-1.23]
	5	93.5 ^{ABC}	87.5 ^{A}	48.5 ^{CD}	0 ^{C}	0.99 [0.94-1.05]
	6	95.0 ^{ABC}	66.2 ^{C}	57.5 ^{C}	0 ^{C}	0.97 [0.86-1.08]
	7	95.0 ^{ABC}	50.0 ^{D}	37.5 ^{D}	0.5 ^{C}	0.57 [0.42-0.78]
	8	82.9 ^{D}	29.0 ^{E}	6.0 ^{E}	0 ^{C}	< 0.25 [n/a]

Different letters represent significant differences ($p<0.05$)

Table 2.3. The LC50 values and mean survivorship for *Epioblasma capsaeformis* glochidia from five female mussels in the control, 0.5, 1.0, and 2.0 g NaCl/L treatments.

		Survivorship				
Time	Individual	**Control**	**0.5**	**1.0**	**2.0**	**LC50 [95% CI]**
24	1	90.7 ^{A}	91.0 ^{A}	86.9 ^{A}	42.0 ^{AB}	1.91 [1.78-2.06]
	2	93.7 ^{AB}	96.3 ^{A}	91.6 ^{A}	50.9 ^{A}	1.98 [1.87-2.10]
	3	91.7 ^{AB}	93.7 ^{A}	90.5 ^{A}	36.7 ^{B}	1.81 [1.69-1.93]
	4	91.0 ^{AB}	90.3 ^{A}	92.2 ^{A}	35.9 ^{B}	1.84 [1.75-1.93]
	5	87.3 ^{B}	90.5 ^{A}	46.3 ^{B}	11.5 ^{C}	1.10 [1.02-1.19]
48	1	91.5 ^{A}	86.6 ^{A}	88.2 ^{A}	50.4 ^{A}	1.90 [1.74-2.08
	2	89.1 ^{A}	87.4 ^{A}	85.5 ^{A}	24.8 ^{B}	1.65 [1.56-1.76]
	3	80.4 ^{B}	88.9 ^{A}	84.8 ^{A}	74.4 ^{C}	1.86 [1.75-1.98]
	4	76.9 ^{B}	66.5 ^{B}	70.2 ^{B}	4.2 ^{D}	1.38 [1.34-1.42]
	5	n/a	n/a	n/a	n/a	n/a

Different letters represent significant differences ($p<0.05$)

Table 2. 4 The LC50 values and mean survivorship for *Alasmidonta heterodon* glochidia from two female mussels in the control, 0.5, 1.0, and 2.0 g NaCl/L treatments.

		Survivorship				
Time	Individual	**Control**	**0.5**	**1.0**	**2.0**	**LC50 [95% CI]**
24	1	95.5 [A]	98.8 [A]	92.0 [A]	80.6 [A]	2.62 [2.51-2.74]
	2	95.4 [A]	93.4 [A]	92.7 [A]	87.7 [A]	2.41 [2.25-2.58]
48	n/a	n/a	n/a	n/a	n/a	n/a

Table 2.5. The LC50 values and mean survivorship for *Lampsilis fasciola* glochidia from three female mussels in the control, 0.5, 1.0, and 2.0 g NaCl/L treatments.

		Survivorship				
Time	**Individual**	**Control**	**0.5**	**1.0**	**2.0**	**LC50 [95% CI]**
24	1	94.3 ^{A}	91.6 ^{A}	87.0 ^{B}	57.9 ^{A}	2.09 [1.96-2.22]
	2	94.1 ^{A}	94.8 ^{A}	92.4 ^{A}	62.3 ^{A}	2.22 [2.12-2.32]
	3	84.9 ^{B}	82.0 ^{B}	81.9 ^{C}	64.8 ^{A}	2.41 [2.29-2.53]
48	1	87.5 ^{A}	84.6 ^{AB}	80.1 ^{B}	71.8 ^{A}	2.30 [2.16-2.44]
	2	91.3 ^{A}	90.3 ^{A}	87.6 ^{A}	50.4 ^{B}	2.00 [1.87-2.13]
	3	80.0 ^{B}	82.4 ^{B}	81.2 ^{B}	48.5 ^{B}	2.06 [1.94-2.19]

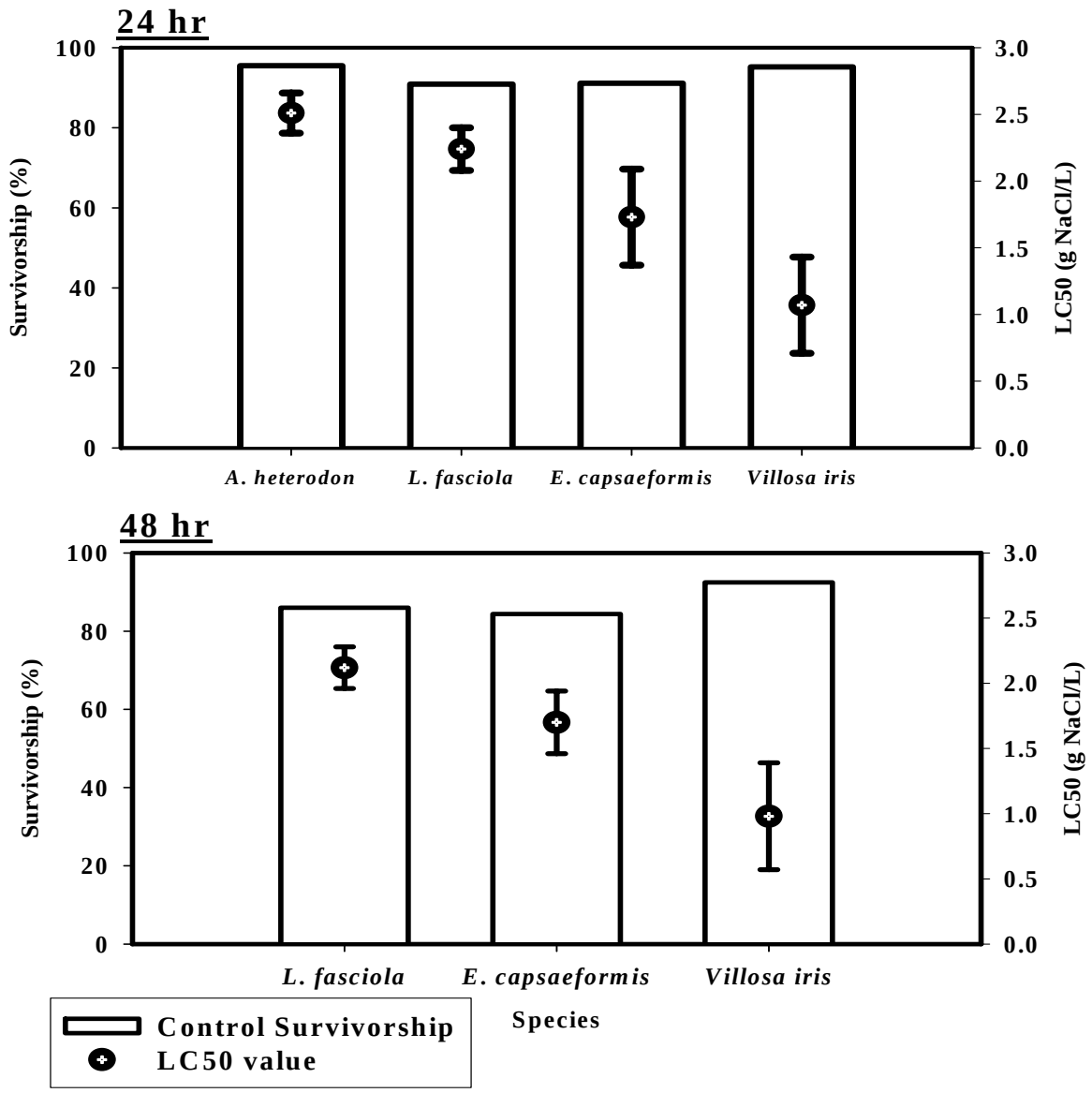


Figure 2.1. The mean percent control survivorship and LC50 with corresponding standard deviation for glochidia from the mussel species *Alasmidonta hererodon*, *Lampsilis fasciola*, *Epioblasma capsaeformis*, and *Villosa iris* for a) 24-h test results, and b) 48-h test results.

Chapter 3: Acute and chronic sensitivity of early life stages of freshwater mussels (Bivalvia: Unionidae) to total residual chlorine relative to water quality criteria of 1984

3.1 Abstract

Chlorine is a highly toxic, widely used halogen disinfectant that is present in point source pollution discharges from wastewater treatment plants and industrial facilities. Although it dissipates quickly in natural waters, Cl pollution poses a substantial threat to biota located downstream from these sources due to its continuous discharge. The current United State Environmental Protection Agency freshwater criteria for Cl is 19 μg/L total residual Cl (TRC) as a maximum 1-h average concentration, and 11 μg/L as a maximum 3-day average. Researchers are growing increasing concerned about declines not only in the number of species and densities of individuals, but also in recruitment. Earlier life stages of freshwater mussels are far more sensitive than adults to contaminants other than Cl. Acute tests were conducted with glochidia from several species, while chronic bioassays were conducted with 2-mo old *Epioblasma capsaeformis* and 3-, 6-, and 12-mo old *Villosa iris* juveniles. The 24-h LC50 values for glochidia from *V. iris*, *Lampsilis fasciola*, *Alasmidonta heterodon*, *E. capsaeformis*, and *E. brevidens* were 22, 15, 12,10, and 7 μg TRC/L, respectively. LOAEC(s) for *E. capsaeformis* was 20 μg TRC/L, while similar values for *V. iris* by increasing age were 30, 30, and 60 μg TRC/L. Younger age classes were more sensitive, which was far more evident for survivorship endpoints, as mortality in concentrations of 30-250 μg TRC/L was between 20-50% for 3-mo old, 60 – 100% for 6-mo old, and 90 – 100% for 12-mo old juveniles. Acute endpoints for glochidia tests were 2.5 - 37 times higher than values for cladoceran species, *Ceriodaphnia dubia* and *Daphnia magna*. Comparing chronic sensitivities for the same species results in endpoints 3.7 – 16.2-fold lower for juveniles. Overall, the greater sensitivity of cladocerans suggest that they may be used effectively as surrogates.

Key words: chlorine, freshwater mussels, acute, chronic, glochidia, juveniles.

3.2 Introduction

Chlorine is a halogen disinfectant often used by wastewater treatment facilities to eliminate pathogenic organisms in discharges before their release into aquatic systems (Pratt et al. 1988). It has also been used effectively as an agent to control biofouling of exotic bivalves in waterlines of industrial and electrical plants (Cherry et al. 1986, Doherty et al. 1986, Ramsay et al. 1988, Rajagopal et al. 1995, 1997). The high toxicity and relative quick dissipation rate of chlorine from the water column make it an appealing chemical alternative (Stewart et al. 1996). Because human activities cause high concentrations of chlorine to be released into the environment, considerable research has been conducted examining its effect on freshwater systems. Numerous studies conducted in the late 1970s and early 1980s assessed environmental impairment at either species or community levels (US EPA 1985). Extensive toxicity testing in the laboratory with several early life stages of aquatic organisms, along with survey and in situ experiments, allowed researchers to infer what concentrations would pose a threat to biota in river systems. Based upon data from these experiments, the United States Environmental Protection Agency (EPA) drafted the water quality criteria (WQC) for chlorine in 1984. This document provided guidance to state regulatory agencies by suggesting safe chlorine concentrations in freshwater and marine systems, and its overall goal was to ensure the protection of aquatic life in waterways receiving permitted outfalls. The maximum average one-hour concentration (acute) was not to exceed 19 μg/L more than once every three years on average, and the 4-d average (chronic) concentrations was not to exceed 11 μg/L more than once every 3 yrs. The values were based on 3-yr running averages because researchers assumed that it would take perturbed systems that amount of time to recover fully if exposed to TRC above recommended

criteria. For comparison, chlorine concentrations in tap water are typically 1500-2000 µg/L.

These criteria were based on Total Residual Chlorine (TRC) to minimize the complexity of monitoring chlorine since it may exist as one of several interim forms depending upon the pH and temperature, along with the presence of organic matter and nitrogenous compounds (Stewart et al. 1996). When placed in water, chlorine hydrolyzes into one of two free chlorine forms (FRC), hypochlorous (HOCl) or hypochlorite ion (OCl^-). If ammonia is present, combined forms of chlorine will be formed (CRC), mono- and dichloramine (Cairns et al. 1990, Wan et al. 2000 a, b). All four of these are highly toxic to aquatic biota, and their total sum equals TRC. The Cl^- ion by itself is relatively benign to most freshwater organisms, and poses little toxicological threat (US EPA 1994). The predominant form of chlorine found in freshwater is monochloramine because most streams and rivers have circum-neutral pH, along with high levels of organic matter and ammonia, especially below wastewater treatment plants (Goudreau et al, 1993).

Toxicity data for thirty-three freshwater species were considered in drafting the WQC for chlorine. Acute values ranged from 28 µg TRC/L for the cladoceran *Daphnia magna* to 710 µg TRC/L for the threespine stickleback (*Gasterosteus aculeatus*). Chronic bioassays were conducted with only three species; however, endpoints were far lower (3.4 – 26 µg TRC/L) with acute/chronic ratios ranging from 3.7 - 78. The most sensitive organism was again *D. magna*, and confidence limits for chronic tests with this species ranged from 2-14 µg/L (Arthur et al. 1975). However, at the time during which experiments used to create WQC for chlorine were conducted (1958-1982), accurate detection limits for TRC were approximately only 10 µg/L. Adverse effects have been

observed at chlorine concentration well below this threshold. Several studies have revealed that algae and periphyton communities are impaired at chlorine concentrations as low as 2 µg/L (Pratt et al. 1988, Cairns et al. 1990). Arthur et al. (1975) reported chronic endpoints for *D. magna* ranging from 3.7 - 7.5 µg/L.

Additional information concerning the behavior of TRC in freshwater, coupled with advancement of analytical methods, have allowed researchers to more accurately assess toxicity since the drafting of the 1984 WQC. The high reactivity of compounds that comprise TRC makes it difficult to maintain desired treatment concentrations during bioassay, as dissipation from the water column is rapid, and varies distinctively for each (Fisher et al. 1999, Taylor 1993). Many TRC constituents undergo photolysis, and therefore concentrations in the water column decline rapidly when light is present. Since bioassays are typically conducted with photoperiods that have 12-16 h of light, substantial TRC loss may occur from the water column within hours. Deviation from desired concentration will grow precipitously as test length increases, and to account for the loss, test solutions must be intermittently renewed or continuously spiked with chlorine to maintain stable treatment levels. Similar complexities of rapid loss are also perpetuated by the use of natural waters during tests, since it often has substantial chlorine buffering capacity due to the presence of fine particulate matter (referring to both organic matter (FPOM) and geomorphic sediments), microscopic biota, and plant material.

With progressive improvements in laboratory testing approaches with chlorine, researchers have generated test endpoints that are likely more accurate estimates of susceptibility. Many of these values are far lower than those reported in WQC, which is attributed to exposures that were continuous rather than static or intermittent. Because

most permitted outfalls containing chlorinated effluents are released continuously, these values may reflect toxicity more accurately. A study by Taylor (1993) emphasized the importance of this in a series of experiments examining the acute tolerance of *Ceriodaphnia dubia* to different forms of FRC and CRC. The researcher observed that both forms of FRC (HOCl and OCl^-) dissipated in less than 1 min in static treatments; however, CRC concentrations were far more stable. Experiments comparing test designs revealed the substantial affect that this may have on toxicological endpoints, as LC50 values for tests with FRC were 7-8 times lower for organisms in continuous versus static, non-renewal exposures, whereas a difference was not evident in tests with CRC. Twenty-four h LC50 values for *C. dubia* were 6 μg OCl^-/L and 5 μg HOCl/L in continuous unfed exposure tests, both well below the WQC, while static unfed values were 48 and 35 μg/L, respectively. Feeding also substantially affected the acute toxicity, as endpoints for these bioassays were well above those of the other experiments, as values were 80 μg OCl^-/L and 140 μg HOCl/L, which may be due to FRC binding to available food and being lost from the water column. Additional studies have report substantial differences in the endpoints of intermittent and continuous exposure tests with chlorine. Fisher et al. (1999) documented far lower LC50 values for several species if exposed during tests to treatments that were continuously spiked, rather than those intermittently exposed, as respective values were 32 and 55 μg/L for *D. magna*, 78 and 301 μg/L for *Hyalella azteca* (amphipod), 59 and 374 μg/L for *Oncorhychus mykiss* (rainbow trout), and 304 and 572 μg/L for *Notemigonus crysoleucas* (golden shiner).

The Clean Water Act of 1977 (Section 304 a:1) states that the EPA is to publish WQC based on data that reflect the latest scientific knowledge; however, as criteria for chlorine are based on data which are more than 20 yrs old, it does not appear to be the

case. Recently, laboratory toxicity testing with freshwater mussels has received increased attention, as several researchers have documented that early life stages are more susceptible to contaminants than organisms used to derive safe water concentrations and/or assess environmental risk (Cherry et al. 2002, Augspurger et al. 2003). Researchers are growing increasingly concerned of current population trends because successful recruitment seems to be sparse, exemplified by the lack of young mussels, even at sites where diverse assemblages of adults are found (Jacobson et al 1997, Henley and Neves 1999). However, since unionids have multiple immature life stages during reproduction, including a larval parasitic stage that requires a specific fish host, it has proven challenging for researchers to evaluate the severity to which potential factors are impacting recruitment. Although in situ studies and surveys are useful for identifying impairment at specific sites, it is extremely difficult for researchers to isolate variables and distinguish direct cause-effect relationships because of the complexity of river systems. This is exemplified by interaction between multiple non-point and point source stressors, competitive interaction among organisms, host-fish demographics, and varying abiotic and biotic components. Laboratory testing allows researchers to assess the relative tolerances of different life stages of unionids (i.e., glochidia, juvenile, adult) far easier than could be achieved in the field.

The intent of this research was to determine whether current WQC for chlorine are stringent enough to protect early life stages of native freshwater mussels. To achieve this goal, a series of experiments were to be conducted with glochidia from various species of freshwater mussels to determine their tolerance to TRC. These tests shall be renewed intermittently so that accurate TRC concentrations can be maintained. However, glochidia are only suitable as test organisms for acute tests, as researchers

report substantial declines in viability during laboratory studies after only short periods, ranging from hours to days depending on the species (Telda and Fernando 1969, Huebner and Pynnonen 1992, Jacobson et al. 1997). Therefore, several 21-d chronic toxicity tests were conducted with juvenile mussels to assess their sensitivity to TRC. Bioassays conducted with 3-, 6-, and 12-mo old *Villosa iris* juveniles will allow researchers to determine which age class is most susceptible to chlorine. An additional chronic test will also be conducted with juveniles from a species that is currently listed as federally endangered, *Epioblasma capsaeformis,* to determine its tolerance relative to a species with more stable populations.

3.3 Material and Methods

3.3.1 Test organisms

3.3.1.1 Glochidia

Gravid females of *V. iris* (rainbow mussel), *E. capsaeformis* (oyster mussel), *E. brevidens* (Cumberlandian combshell), and *Lampsilis fasciol*a (wavyrayed lampmussel) were collected from the Clinch River, VA, whereas females of *Alasmidonta heterodon* (dwarf wedgemussel) were collected from the Ashuilot River, NH. *Epioblasma capsaeformis*, *E. brevidens*, and *A. heterodon* are federally endangered species. Specimens obtained from the Clinch River were transported back to the laboratory immediately after collection, while those from the Ashuilot River were mailed over night in chilled coolers. Adults were acclimated to laboratory conditions in recirculating troughs maintained at 20 ± 2°C and fed a tri-algal diet for at least 24 h prior to extraction of glochidia. Glochidia were extracted by gently prying open a gravid female's shell and puncturing the marsupial tissue with a 100 cc water-filled syringe. Injecting the water

caused glochidia to be flushed out. The process was repeated for each gill. Glochidia were then rinsed with clean water to remove unwanted material, and four samples of 25-50 individuals were assessed for viability with a NaCl solution, as described by Jacobson et al. (1997) and Goudreau et al. (1993). Viable glochidia were those that were open, and responded to the addition of NaCl by closing, or repeatedly closing and opening their valves. Glochidia that were closed prior to addition of the brine solution, or open but showed no movement to the noxious substance, were recorded as ecologically dead since it was assumed that they would be unable to attach to host fish. Only glochidia from adults that had average viabilities of at least 90% prior to the initiation of tests were used in experiments

3.3.1.2 Juvenile mussel

Juvenile mussels were obtained from the Virginia Polytechnic Institute and State University Aquaculture Center, where they were produced *in vivo* on appropriate host fish. The host species used for *V.iris* was *Ambloplites rupestris* (rockbass), while for *E. capsaeformis* it was *Cottus carolinae* (banded sculpin). Infestation of host fish followed the protocol of Zale and Neves (1982). Once fish hosts were infested with glochidia, they were transferred to 20-l glass aquaria. Aquaria were monitored every few days to determine when juvenile mussels began releasing from host fish. Once sufficient numbers had dropped, juveniles were siphoned from the tank bottom and transferred to cylindrical PVC chambers (diameter x height = 30 x 8 cm) that were sealed on one side with a fine plastic mesh (55 microns). These chambers were held in large recirculating troughs, and each contained a small amount of previously aerated and autoclaved sediment. Troughs were filled with 50/50 mix of dechlorinated tap water and well water,

and treated daily with 30,000 cells/mL unicellular algae (*Neochloris oleoabundans*). Juveniles were monitored periodically to assess health and renew sediment until they reached their appropriate age for the bioassays. Only individuals in good health, as evident by full guts, greenish hues, and pedal feeding, were used in bioassays.

I decided to use juveniles that were no younger than 2-mo old as test organisms during chronic bioassays rather than newly transformed juveniles for several reasons. Researchers have documented extremely low juvenile survivorship (<50%) 2 wk after excystment during culturing experiments, despite high production yields immediately following transformation (Jones and Neves 2002, Jones et al. 2004). Some researchers speculate that this is attributable to some populations exhibiting normal bottlenecks, which may be inevitable due to genetics. Zimmerman et al. (2003) also noted the substantial impact that predators may have on survivorship in culturing experiments. To account for these concerns, we used older mussels that we anticipated were beyond the age of high mortality, and less susceptible to predators, such as flatworms. Substrate is needed in chronic tests to meet the living requirements of juveniles, which also makes the use of older mussels advantageous because they are far easier to relocate. However, growth rates is also reduced with age. This makes it harder for researchers to distinguish differences between treatments means, thereby reducing statistical power and making predictions of NOAECs less accurate. Furthermore, Ingersoll et al. (2003) observed that 2-mo old individuals of *V. iris* were far more susceptible to 2- and 4-d exposures to ammonia and chlorine than newly transformed juveniles.

3.3.2 Acute toxicity tests with glochidia

Treatments were 5, 10, 30, 60, 120, 250, and 500 µg TRC/L, plus a control. Calcium hypochlorite (HTH-High Test Hypochlorite) was used as the toxicant and

moderately hard synthetic water (EPA^{100}) described in EPA standard methods (2002), was used as the diluent and control. Solutions for each treatment were prepared in 3-L plastic nalgene beakers and then measured. If needed, concentrations were readjusted using stock solutions until accurate. Concentrations were measured intermittently throughout the test, and re-adjusted accordingly with stock solutions (approximately 2 times concentration of the treatment) to account for loss of chlorine. Concentrations were more difficult to maintain target levels during light periods, and in higher treatments (i.e., those > 120 µg/L).

Glochidia (approximately 25-50) were transferred with a fine-tip glass pipette to test chambers (Fig. 3.1 [87]) that were randomly appropriated to the different treatments. For each treatment, there were four replicates of 25-50 glochidia for each time interval that a test was monitored. So if a test was conducted for 48 h, eight test chambers would be loaded, whereas a test for 72 h would have twelve. As the availability of *E. capsaeformis* and *E. brevidens* gravid females were limited, and glochidia were needed for culturing, tests with these species were only conducted for 24 h. Three rounds of bioassays were conducted with *A. heterodon* and *E. capsaeformis*, two rounds with *L. fasciola*, and one round with *V. iris* and *E. brevidens*.

Viability was assessed after each 24 h interval, and was achieved by transferring glochidia with fine-tip glass pipettes from test chambers to glass petri dishes for observation with a dissecting microscope. The numbers of open and closed glochidia were tallied, after which a concentrated NaCl solution was used to assess viability as previous described. The computer Toxstat® Version 3.5 (West, Inc., Laramie, Wyoming) was used to calculate LC50 values.

3.3.3 Chronic toxicity tests with juveniles

Chronic toxicity tests were conducted with six chlorine treatments that approximately doubled in concentration and ranged between 5 and 500 µg TRC/L. For the different tests, several treatment concentrations overlapped to allow for easy comparisons. Bioassays with 2-mo old *E. capsaeformis* and 3-mo old *V. iris* had lower concentration ranges beginning at 5 µg TRC/L, whereas tests with 6- and 12-mo *V. iris* instead had a higher concentration of 500 µg/L. Treatments were prepared in 140-L re-circulating systems (n=7) powered by 1.5 amp pumps. Each trough was filled with 120-L of a 50/50 mix of dechlorinated tap water and reference water from a river site (Sinking Creek, Newport, Virginia). One system served as the control, while the remaining six were spiked with HTH to desired chlorine concentrations. Concentrations in the systems were maintained by adding chlorine stock at a rate of ~10 L per day to compensate for loss; un-spiked water was delivered to the control treatment. Stocks of different concentrations were used on the treatments, and were prepared with HTH and the 50/50 water mixture. New stocks were prepared every 48 h and 20 L of water was removed with a siphon from each trough. Systems were covered with Plexiglas to impede chlorine loss. Treatments were maintained for a week prior to loading mussels to ensure that concentrations were stable, and chlorine levels were monitored twice daily thereafter

In each trough were 20 individual test chambers (Fig. 3.1 [87]), each containing 2 ml of <150 micron, autoclaved, and aerated sediment from a reference site on the New River, VA. Chambers were held in position with plastic test tube holders and oriented to maximize uniform flow. The shell length of a juvenile was measured with the ocular lens of a dissecting microscope, after which the individual was randomly allocated to one of the individual test chambers (n=140). Troughs were dosed daily with 30,000 cells/ml

Neochloris oleoabundans. Temperature was maintained at 23 ± 1°C, and a photoperiod of 16 light: 8 dark was established using an automatic timer.

After 21 d, juveniles were retrieved from the test chambers. The same magnification was used to measure initial and final shell length for a given test, although it varied among tests depending on test organism size. Survivorship was evaluated by observing individuals at the highest magnifications (40 X). Individuals that did not move for 2 min were recorded as dead. Movement was defined as pedal feeding, active filtering, shell movement, or visceral mass movement observed through the shell. To make statistical analysis more meaningful and universally conceptualized, ocular shell lengths were converted to metric units (μm). Total growth was calculated by subtracting initial length from final length. Proportional growth was calculated by dividing total growth by initial length. No observable adverse effect concentrations (NOAEC) and lowest observable adverse effect concentrations (LOAEC) were determined for growth and survivorship based on the statistical approach described for *Pimephales promelas* in standard protocol (EPA 2002) using Toxstat Version 3.5 (West, Inc., Laramie, Wyoming) (α=0.95).

3.3.4 Water chemistry

The temperature, dissolved oxygen, pH, alkalinity, and hardness of each sample were measured at the initiation and completion of acute tests, and biweekly for chronic bioassays. An Accumet® (Fisher Scientific, Pittsburgh, PA, USA) pH meter with an Accumet gel-filled combination electrode (accuracy <± 0.05 pH at 25°C) was used to measure pH. Dissolved oxygen and conductivity were measured with Yellow Springs model 54A meter®, and a model 30 conductivity meter®, respectively (Yellow Springs,

OH, USA). Total hardness and alkalinity (as mg/L $CaCO_3$) were measured in accordance with APHA et al (1998) through colorimetic titration.

3.5 Results

3.5.1 Acute toxicity of TRC to glochidia

Average viability was greater than 90% for glochidia of all species after 24 h in control treatments, but was 0% at concentrations of 500 µg TRC/L (Table 3.1 [84]). *Epioblasma brevidens* (LC50 = 70 µg/L), *E. capsaeformis* (LC50 = 107 µg/L), and *A. heterodon* (LC50 = 107 µg/L) were slightly more sensitive to chlorine than *L. fasciola* (LC50 = 145 µg/L), and far more sensitive than *V. iris* (LC50 = 220 µg/L) after 24 h of exposure. At 250 µg TRC/L, average viability for sensitive species (<20%) was nearly half that of the respective value for *L. fasciola* (35%), and less than a third of that for *V. iris* (66%). In concentrations of 30 µg TRC/L and higher, viability remained greater than 90% for all species after 24 h, except *E. brevidens* (79-87%).

After 48 h of exposure, viability in treatments containing measurable chlorine, in general, differed only slightly for *V. iris* and *A. heterodon*, although it decreased substantially for *L. fasciola*. However, average viability of *L. fasciola* declined to less than 80% in the control after 48 h, so results are suspect and may be inaccurate. Forty-eight hour LC50 values for *V. iris* and *A. heterodon* were 260 and 95 µg/L, respectively. Control viability remained greater than 90% for *V. iris* after 72 h, yet the LC50 value (180 µg/L) was still higher than the 24 h value for the other species tested.

3.5.2 Test results for chronic bioassays with *V. iris*

Early life stages of *V. iris* juveniles were more susceptible to TRC exposure than older stages. After 21 d of exposure, growth was significantly reduced at the same concentration for all three age groups (Table 3.3 [86]). Juveniles of all classes in

treatments of 30 µg TRC/Land lower had no significant growth impairment compared to individuals in controls ($p<0.05$). Average growth for individuals was reduced by 37-80% in treatments with TRC concentration of 30-120 µg/L, and by 90% in treatments above 250 µg/L relative to the growth of juveniles in controls.

Survivorship was significantly reduced at the same concentration as growth for 3-mo old juveniles (LOAEC = 30 µg/L), but at higher relative concentrations for 6- and 12-mo old juveniles (LOAECs = 250 and >500 µg/L, respectively). Survivorship for 3-mo old juveniles was less than 50% in treatments with TRC concentrations of 30 µg and above. No 3-mo old individuals survived in the 120 µg TRC/L treatment. Six-month old juveniles had > 85% survivorship in treatments with TRC concentrations less than 250 µg/L, 60% at 250 µg TRC/L, and 50% at 500 µg TRC/L. Few deaths were observed in treatments with 12-mo old juveniles, as survivorship remained 80% or above in TRC treatments up to 500 µg/L (Fig. 3.2 [88]).

3.5.3 Test results for chronic bioassay with *E. capsaeformis*

Two month-old *E. capsaeformis* juveniles were more sensitive than any age class of *V. iris*. Growth was significantly reduced at concentrations of 20 µg TRC/L and higher, as exposed individuals grew less than 20% relative to those in the control. Growth in the control, and NOAEC treatment (10 µg/L) were 400 and 375 µm, respectively, which differed by only ca. 6%. The numbers of observed mortalities were also considerably high in the test, as 50% or more of the individuals perished at concentrations of 30 µg/L and higher. All individuals in the 120 µg/L treatment died after 21-d of exposure, whereas those in the control and 5 µg/L had average survivorship of 80 and 100%, respectively (Table 3.3 [86]).

3.6 Discussion

3.6.1 Toxicity of TRC to glochidia

Glochidia from freshwater mussels that are currently listed as endangered were more sensitive to chlorine exposure than those of species with relatively stable populations, as 24-h LC50 values for *E. capsaeformis*, *E. brevidens*, and *A. heterodon* were 50-70 % lower than those for *V. iris* (220 µg TRC/L). Ingersoll et al. (2004) reported an LC50 value similar to the one reported in this study. Goudreau et al. (1993) reported lower endpoints for *V. iris* (84 µg/L), which is attributable to tests being conducted with CRC rather than TRC. Regardless, glochidia from all species tested were far more tolerant to chlorine than most other aquatic organisms (Table 3.2 [85]). Acute endpoints for glochidia in our study were 2.5-37 times higher than respective values for cladoceran species (Taylor 1993, Fisher et al. 1999, EPA 1984). For comparison, Taylor (1993) reported LC50 values of 5 µg/L hypochlorus ion (OCl-) and 6 µg/L hypochlorus acid (HOCl-) in continuous exposure experiments. Researchers have also documented endpoints for other genera, including several fish species and a freshwater copepod, that are approximately half that of the values calculated for glochidia in this study.

The water quality criteria for chlorine drafted by the U.S. EPA in 1984 stated that freshwater aquatic organisms, and their uses, would not be adversely affected by chlorine if: 1) the maximum average 1 h concentration does not exceed 19 µg/L more than once every three years on average, and 2) the 4-d average TRC concentration does not exceed 11 µg/L more than once every 3 yrs on average. Average viability of glochidia in treatments containing 30 µg TRC/L was above 90% for all species except *E. brevidens,* which had a respective value of 83%. Based upon these results, the risk that glochidia will be adversely effected by concentrations in the environment seems minimal

Moreover, during experiments longer in duration, average viability of *V. iris* glochidia after 48 and 72 h in the control and 10 µg/L treatments were nearly identical. After 48 h, the mean viabilities in these treatments were 95 and 90%, respectively, and after 72 h, 90% and 88 µg/L, respectively. Similar trends are also apparent for data generated with *L. fasciola*; however, since average viability of individuals in the control treatment was below 80% after 48 h, test results may be unreliable. Based upon these observations, and the fact that 24-h LC50 values are 6-20- fold higher, 11 µg TRC/L appears to be sufficient to protect glochidia of most species. Furthermore, as the overall mean acute values for freshwater mussels was 38 µg/L, it seems likely that surrogate organisms, such as *C. dubia* and *D. magna*, will be effective for assessing environmental risk since they are far more susceptible to chlorine.

When relating the results of laboratory bioassays to environmental scenarios, there are additional factors that suggest glochidia will not be adversely affected by permitted release of chlorine into aquatic systems. First of all, measured concentrations of chlorine in the water column of river systems are highest just below the source, and dissipate rapidly with increased distance downstream (Stewart et al. 1996). Therefore, chlorine concentrations are often well below WQC in the water column of most river reaches, whereas areas of concern are confined to short reaches below the outfall. As glochidia were tolerant of chlorine concentrations far above the WQC, it seems unlikely that actual concentrations in rivers will have toxic effects on glochidia. Furthermore, glochidia are most susceptible to contaminants after being released by the gravid female, but prior to encysting on a fish host. Consequently, the most susceptible phase is exposed to contaminants for only brief periods. Although numerous species of glochidia have been observed in stream drift, it is highly probable that those coming in contact

immediately with appropriate fish host species make up an overwhelming percentage of individuals that successfully transform into juveniles.

3.6.2 Chronic juvenile test

The 21-d exposure tests with juveniles yielded more sensitive toxicological endpoints than acute glochidia bioassays, as demonstrated by LOAECs of 20 – 60 µg/L TRC compared to LC50 values of 70 – 220 µg TRC/L. However, the lowest measured chlorine concentration in treatments that caused adverse effects was still above safe concentrations established in the 1984 WQC for chlorine. The most sensitive test endpoint for chlorine toxicity was derived from data for the chronic bioassays conducted with *E. capsaeformis* juveniles (20 µg TRC/L). At this concentration, growth was reduced by 85% relative to the control, whereas at the next lower concentration (NOAEC) of 10 µg TRC/L, which was very close to the maximum allowable 4-day TRC average (11 µg TRC/L), the difference was negligible (6%). Data generated in my study suggest that *E. capsaeformis* is more sensitive than *V. iris,* as adverse effects were observed for the former at lower concentrations in less time. The smaller body size of *E. capsaeformis* relative to *V. iris* may be attribute to a difference in susceptible to contaminants, and also accounts for the substantial difference in growth of individuals in controls for the respective species, 400 compared to 700 µm.

Respective LOAEC values for the three age classes of *V. iris* show were the same (30 µg/L). However, a difference in the sensitivity of the three age classes is far more distinguishable if survivorship alone is considered, especially in higher treatment concentrations (pg 88). After 21 d, only 20% of 3-mo old juveniles survived in the 120 µg TRC/L treatment, whereas all 6- and 12-mo old juveniles survived. At 250 µg TRC/L, survival of 3-mo old juveniles was again 20%, but dropped substantially for 6-

mo olds at this concentration to 60%. No 12-mo old individuals died at this concentration, and even at the highest chlorine treatment of 500 μg TRC/L, 80% of exposed individuals survived. Similar size class effects have been observed in experiments evaluating varying approaches for controlling biofouling by non-native bivalves with chlorination. An extensive study by Rajagopal et al. (2003) showed that size classes (10, 25, and 35 mm) of the marine brown mussel, *Perna perna,* had different tolerances. Like my study, difference in sensitivities of the three age classes are most striking when comparing survivorship at high concentrations. The number of hours to reach 100% mortality for individuals exposed to 500 μg/L varied substantially among the different classes as values for species in ascending order of size class were 84, 102, and 120 h, respectively.

During my study, I observed significant reductions in growth of individuals exposed to measurable concentrations of TRC. Although studies examining effects of chlorine on native freshwater mussels are rare, considerable research has been conducted with introduced bivalves while developing biofouling treatment strategies (Cherry et al. 1986, Doherty et al. 1986, Ramsay et al. 1988, Rajagopal et al. 1995, 1997). These experiments provide useful information pertaining to behavioral and sublethal effects caused by chlorine exposure to bivalves, although applying toxicological endpoints to infer relative tolerance of unionids has only limited merit because of substantial differences between genera. Bivalves reduce periods of activity and filtration when exposed to a noxious substance, such as Cl, and in some cases, completely avoid exposure by sealing their valves and changing metabolism type (Naimo et al. 1995, Chen et al. 2001). Irrevocably, the ability of bivalves to avoid exposure often makes mortality a poor endpoint for juvenile experiments, especially for tests that are short in duration.

Therefore, sub-lethal effects are often far better indicators of impairment than survivorship, and are especially important to consider for freshwater mussels since reductions in fitness will become more evident over their long life spans. Reduced growth is a definitive illustration of lower fitness, as juvenile mussels can neither propagate nor survive if they do not grow. Individuals unable to obtain sufficient energy reserves may perish due to latent mortality, especially younger juveniles that are unable to develop sufficiently during summer-fall months to endure winter.

3.7 Conclusion

Overall, data obtained in my study strongly suggest that early life stages of freshwater mussels are less sensitive to TRC than many other aquatic organisms. The mean acute test endpoint for freshwater mussel glochidia in my study was 108 μg/L TRC, which is more than twice the average value reported in the 1984 WQC for the five most sensitive species (51 μg/L), and 4-16 times higher than more recent values reported for cladoceran species. Chronic endpoints for juveniles were substantially lower than those for glochidia, as values were 5 – 7 times lower for youngest age class of juvenile for a given species. Test endpoints for all acute (70 – 220 μg TRC/L) and chronic tests (20 – 60 μg TRC/L) were above WQC for chlorine, which indicates that the toxicity threat is only minimal. Furthermore, as adverse effects have been observed at far lower concentrations for other species during chronic tests with test organisms commonly used to assess environmental risk, such as *C. dubia*, *D. magna*, and *P. promelas*, it seems that they may be more suitable as test organisms to assess chlorine toxicity. Furthermore, as the sensitivities of the more typically-used test organisms to TRC were greater than early life stages of mussels during my 39999study, it appears that they may be suitable as surrogate test organisms to assess chlorine toxicity.

3.8 Acknowledgements

Research was conducted thanks to support from the United States Fish and Wildlife Service. We thank Patrick Berry for his contributions during the initial phases of this study.

3.9 Work Cited

1) Augspurger T, Keller AE, Black MC, Cope WG, Dwyer FJ. 2003. Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. *Environ Toxicol Chem* 22: 2569-2575.

2) Cairns J, BR Niederlehner, JR Pratt. 1990. Evaluation of joint toxicity of chlorine and ammonia to aquatic communities. *Aquatic Toxicol* 16:87-100.

3) Chen LY, AG Heath, RJ Neves. 2001. Comparison of oxygen consumption in freshwater mussels (Unionidae) from different habitats during declining dissolved oxygen concentrations. Hydrobiologia 450: 209-214.

4) Cherry DS, Van Hassel JH, Farris JL, Soucek DJ, Neves RJ. 2002. Site-specific derivation of the acute copper criteria for the Clinch River, Virginia. *Hum Eco Risk Assess* 8:591-601.

5) Cherry DS, RL Roy, RA Lechleitner, RA Dunhardt, GT Peters, J Cairns. 1986. *Corbicula* fouling and control measures at the Celco plant, Virginia. *Am Malacol Bull* Special Edtion 2: 69-81.

6) Doherty FG, JL Farris, DS Cherry, J Cairns. 1986. Control of the freshwater fouling bivalve *Corbicula fluminea* by halogenation. *Arch Environ Contam Toxicol* 15: 535-542.

7) Fisher DJ, DT Burton, LT Yonkos, SD Turley, GP Ziegler, BS Turley. 2003. Derivation of acute ecological risk criteria for chlorite in freshwater ecosystems. *Water Res* 37: 4359-4368.

8) Gulley DD, West, Inc. 1996. TOXSTAT version 3.5. Fish Physiology and Toxicology Laboratory, Department of Zoology and Physiology. University of Wyoming. Laramie, Wyoming.

9) Henley WF, RJ Neves. 1999. Recovery status of freshwater mussel (Bivalvia: Unionidae) in the North Fork Holston River, Virginia. *Am Malacol Bull* 15: 65-73.

10) Huebner JD, KS Pynnonen. 1992. Viability of two species of Anodonta exposed to low pH and selected metals. *Can J Zool* 70: 2348-2355.

11) Ingersoll C, N Wang, E Greer, D Whites, J Dwyer, A Roberts, T Augspurger, C Kane, C Tibbott. 2003. Acute toxicity of copper, ammonia, and chlorine to glochidia and juveniles of freshwater mussels. Published abstract in Proceeding of Society of Environmental Toxicology and Chemistry 24th annual meeting, Austin ,TX, USA.

12) Jacobson PL, JL Farris, DS Cherry, RJ Neves. 1993. Juvenile freshwater mussel (Bivalvia: Unionidae) response to acute toxicity testing with copper. *Environ Toxicol Chem* 12:879-883.

13) Jones J, RJ Neves. 2002. Life history and propagation of the endangered fanshell pearlymussel, *Cyprogenia stegaria* Rafinewque (Bivalvia: Unionidae). *J N Am Benthol* Soc 21: 76-88

14) Jones J, Neves RJ, Ahlstedt SA, Mair RA. 2004. Life history and propagation of the endangered dromedary peralymussel (*Dromus dromas*) (Bivalvia: Unionidae). *J N Am Benthol Soc* 23: 515-525.

15) Pratt JR, NJ Bowers, BR Neiderlehner, J Cairns. 1988. Effects of chlorine on microbial communities in naturally derived microcosms. *Environ Toxicol Chem* 7:679-687.

16) Rajagopal SV, KVK Nair, G van der Velde, HA Jenner. 1997. Response of the mussel, *Brachidontes striatulus,* to chlorination: an experimental study. *Aquatic Toxicol* 3:135-149.

17) Rajagopal SV, P Venugopalan, KVK Nair, J. Azariah. 1995. Response of green mussel, Perna viridis (L), to chlorine in the context of power plant biofouling control. *Mar Behav Physiol* 25:261-274.

18) Ramsay GG, J H Tackett, DW Morris. 1988. Effect of low-level continuous chlorination on *Corbicula fluminea* - short communication. *Environ Toxicol Chem* 7: 855-856.

19) Stewart AJ, WR Hill, KD Ham, SW Christensen, JJ Beauchamp. 1996. Chlorine dynamics and ambient toxicity in receiving streams. *Ecol. Appl*. 6:458-471.

20) Taylor, P. A. 1992. An evaluation of the toxicity of various forms of chlorine *Ceriodaphnia dubia*. *Environ Toxicol Chem* 12:925-930.

21) Telda S, CH Fernando. 1969. Observations of *Lampsilis radiata* (Gmelin) infesting yellow perch, *Perca flavescens* (Mitchell), in the Bay of Quinte, Lake Ontario. *Can J Zool* 47: 705-712.

22) U.S. Environmental Protection Agency. 1985. Ambient Water Quality Criteria for Chlorine - 1984. Office of Water Regulations and Standards Criteria and Standards Division, Washington, DC. EPA 440/5-84-030.

23) U. S. Environmental Protection Agency. 1994. Chemical Summary for Chlorine. EPA 749-F-94-010a.

24) U.S. Environmental Protection Agency. 2002. Short-term methods for estimation the chronic toxicity of effluents receiving water to freshwater organisms- 4th Ed. EPA/821-R-02-013. Washington, DC.

25) Wan MT, V Aggelen, W Cheng, C Buday. 2000a. Validation of the acute toxicity of inorganic chloramines to the freshwater invertebrate *Daphnia magna*. *Bull Environ Toxicol Chem* 64: 213-220.

26) Wan MT, RG Watts, W Cheng. 2000b. Acute toxicity of inorganic chloramines to *Daphnia magna* in two types of dilution water. *Bull Environ Toxicol Chem* 65: 147-152.

27) Zale AV, RJ Neves. 1992. Fish hosts of four species of lampsiline mussels (Mollusca: Unionidae) in Big Moccasin Creek, Virginia. *Can J Zool* 60: 2335-2542.

28) Zimmerman LL, Neves RJ, Smith DG. 2003. Control of predacious flatworms *Macrostomum* sp. in culturing juvenile freshwater mussels. *N Amer J Aquatic* 65: 28-32.

Table 3.1. The average viability of glochidia from five species of freshwater mussels exposed to total residual chlorine (TRC).

		Average Viability (% ± SD)					
Species	Concentation (µg TRC/L)	n[b]	24- h	n[b]	48-h	nb	72-h)
Villosa iris	Control[a]	4	95 ± 1	4	95 ± 2	4	90 ± 4
	5	4	96 ± 3	4	93 ± 3	4	87 ± 4
	10	4	94 ± 2	4	90 ± 6	4	88 ± 5
	30	4	90 ± 7	4	95 ± 2	4	80 ± 6
	60	4	91 ± 4	4	89 ± 6	4	75 ± 7
	120	4	71 ± 10	4	80 ± 7	4	67 ± 17
	250	4	66 ± 7	4	69 ± 7	4	49 ± 10
	500	4	0 ± 0	4	0 ± 0	4	0 ± 0
Lampsilis fasciola	Control[a]	8	93 ± 3	8	79 ± 9	4	55 ± 4
	5	8	94 ± 4	8	78 ± 10	4	54 ± 8
	10	8	93 ± 4	8	78 ± 6	4	48 ± 9
	30	8	91 ± 4	8	72 ± 9	4	48 ± 11
	60	8	86 ± 4	8	52 ± 19	4	42 ± 13
	120	8	49 ± 20	8	27 ± 13	4	24 ± 10
	250	8	35 ± 13	8	4 ± 4	4	5 ± 4
	500	8	0 ± 0	8	0 ± 0	4	0 ± 0
Alasmidonta heterodon	Control[a]	12	93 ± 6	8	93 ± 4		
	5	4	93 ± 4	na	na		
	10	12	91 ± 4	8	90 ± 6		
	30	12	90 ± 6	8	87 ± 7		na
	60	12	67 ± 10	8	58 ± 12		
	120	12	44 ± 11	8	35 ± 11		
	250	12	19 ± 13	8	11 ± 9		
	500	12	0 ± 0	8	0 ± 0		
Epioblasma capsaeformis	Control[a]	12	94 ± 4				
	5	12	93 ± 3				
	10	12	95 ± 3				
	30	12	90 ± 3		na		na
	60	12	73 ± 7				
	120	12	42 ± 9				
	250	12	12 ± 8				
	500	12	0 ± 0				
Epioblasma brevidens	Control[a]	4	91 ± 7				
	5	4	87 ± 11				
	10	4	79 ± 11				
	30	4	83 ± 14		na		na
	60	4	61 ± 11				
	120	4	22 ± 10				
	250	4	4 ± 5				
	500	4	0 ± 0				

a = Concentration below detection limit.

b = Number of replicates, each containing 25 or 50 individual glochidia.

Table 3.2. Comparison of acute toxicological endpoints for common freshwater test organisms, and those for freshwater mussel glochidia generated in our study.

Typical regulatory test organisms		Freshwater mussel glochidia		
Species	Mean Acute Value (µg/L)	Species	Time (h)	Mean EC50 value (µg/L)
Cladoceran	6 – 27[a]	Rainbow mussel	24	220
Ceriodaphnia dubia	< 80[b]	*Villosa iris*	48	260
	120[E]			
Cladoceran	28[c]		72	180
Daphnia magna	32[d]	Wavyrayed lampmussel	24	145
Pugnose shiner	45[c]	*Lampsilis fasciola*	48	80*
Notropis arogenus			72	90*
Common shiner	51[c]	Oyster mussel	24	107
Notropis cornutus		*Epioblasma capsaeformis*		
Lake trout	60[c]	Cumberland combshell	24	70
Salvelinus namaycush		*Epioblasma brevidens*		
Rainbow trout	62[c]	Dwarf wedgemussel	24	107
Salmo gairdneri	59[d]	*Alasmidonta heterodon*	48	95
Copepod	63[c]			
Epischura lacustris				
Amphipod	78[d]			
Hyalella azteca				

* Control survivorship below 80%.
a = Taylor PA (1993). Values are for concentrations of free residual or combined forms of chlorine rather than TRC.
b = Stewart et al. (1996)
c = Fisher et al. (1999)
d = 1984 Chlorine water quality criteria.
e = Manning et al. 1996.

Table 3.3. Initial length and growth of mussel juveniles exposed to different concentrations of Total Residual Chlorine (TRC) for 21 days.

Species	Age	Concentration (µg TRC/L)	Initial length (µm)	Final length (µm)	Total growth (µm)
Epioblasma capsaeformis	2-mo	Control	500	900	400
		5	530	930	400
		10[a]	490	860	370
		20[b]	510	580	70*
		30	510	550	40*
		60	520	590	70*
		120	500	500	0*
Villosa iris	3-mo.	Control	1290	1970	680
		5	1230	1900	670
		15	1300	1850	550
		30[a]	1330	1750	420
		60[b]	1340	1360	20*
		120	1390	1490	100*
		250	1190	1260	70*
	6-mo.	Control	1900	2580	680
		15	1660	2040	380
		30[a]	1840	1980	140
		60[b]	1840	1950	110*
		120	1730	1880	150*
		250	1690	1730	40*
		500	1820	1830	10*
	12-mo.	Control	6630	7980	1350
		15	6550	7790	1240
		30[a]	6920	7380	460
		60[b]	6580	6690	110*
		120	6870	7110	240*
		250	6330	6340	10*
		500	7230	7280	50*

a = No observable adverse effects concentration.
b = Lowest observable adverse effects concentration.
* Significantly different that the control treatment ($p<0.005$).

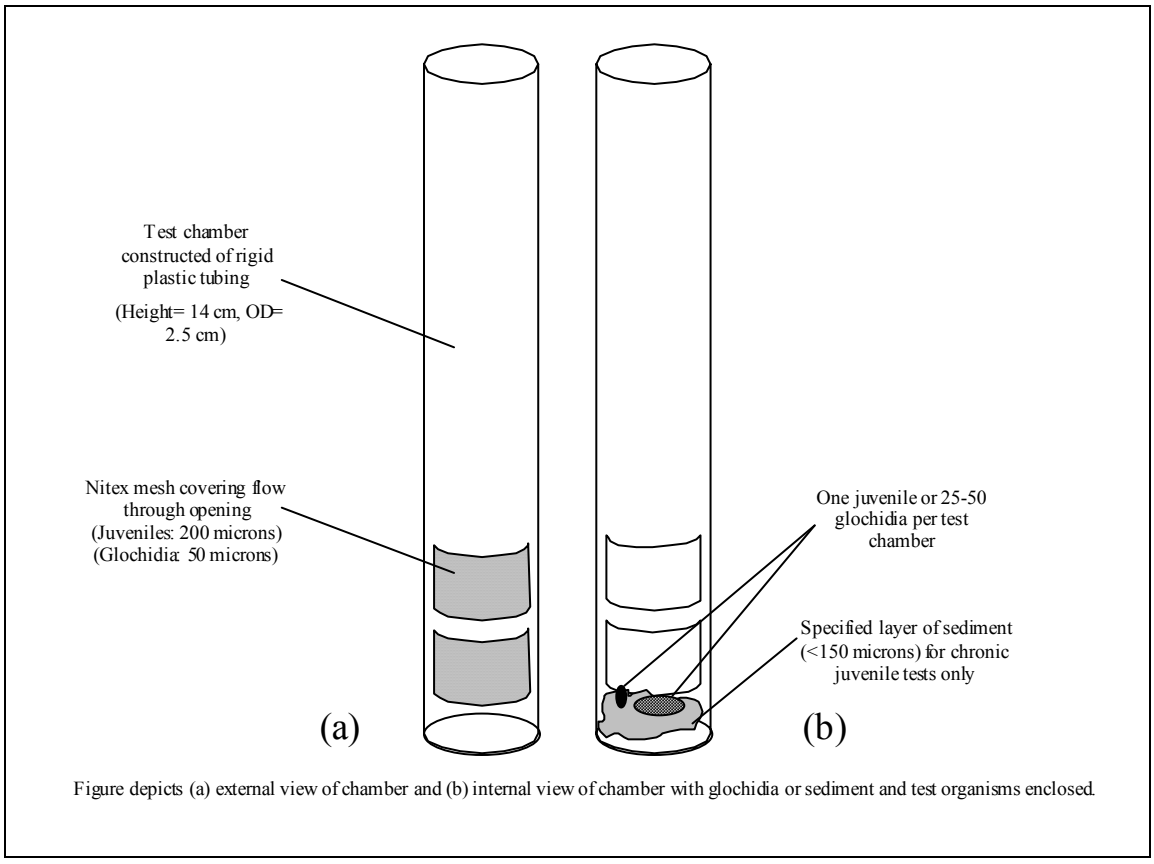
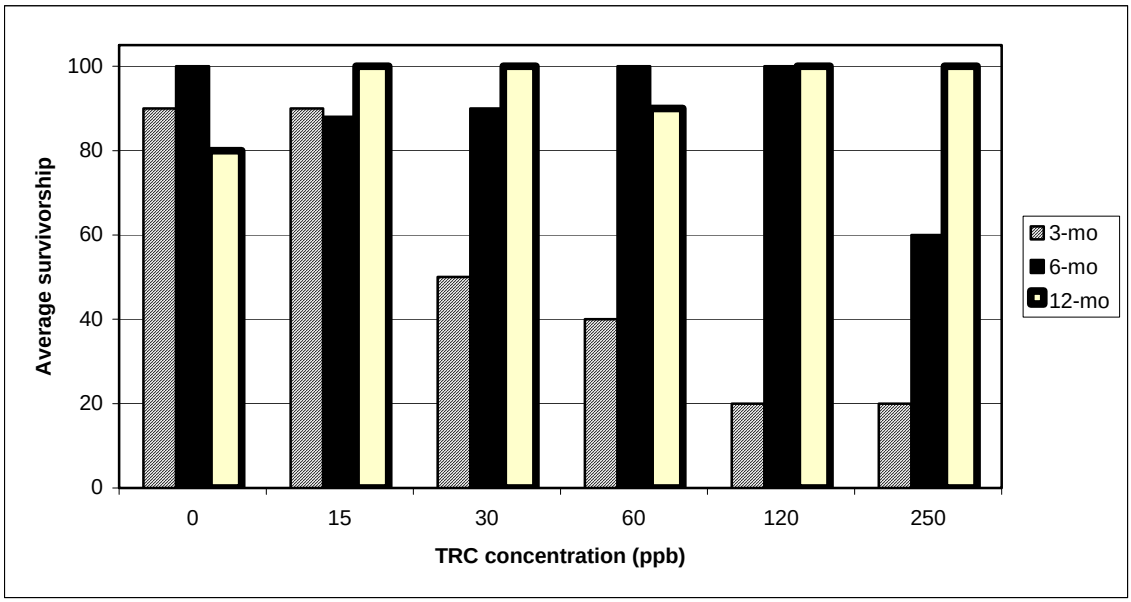


Figure 3.1. Diagram of test chamber used for acute glochidia and chronic juvenile toxicity tests. Not drawn to scale.



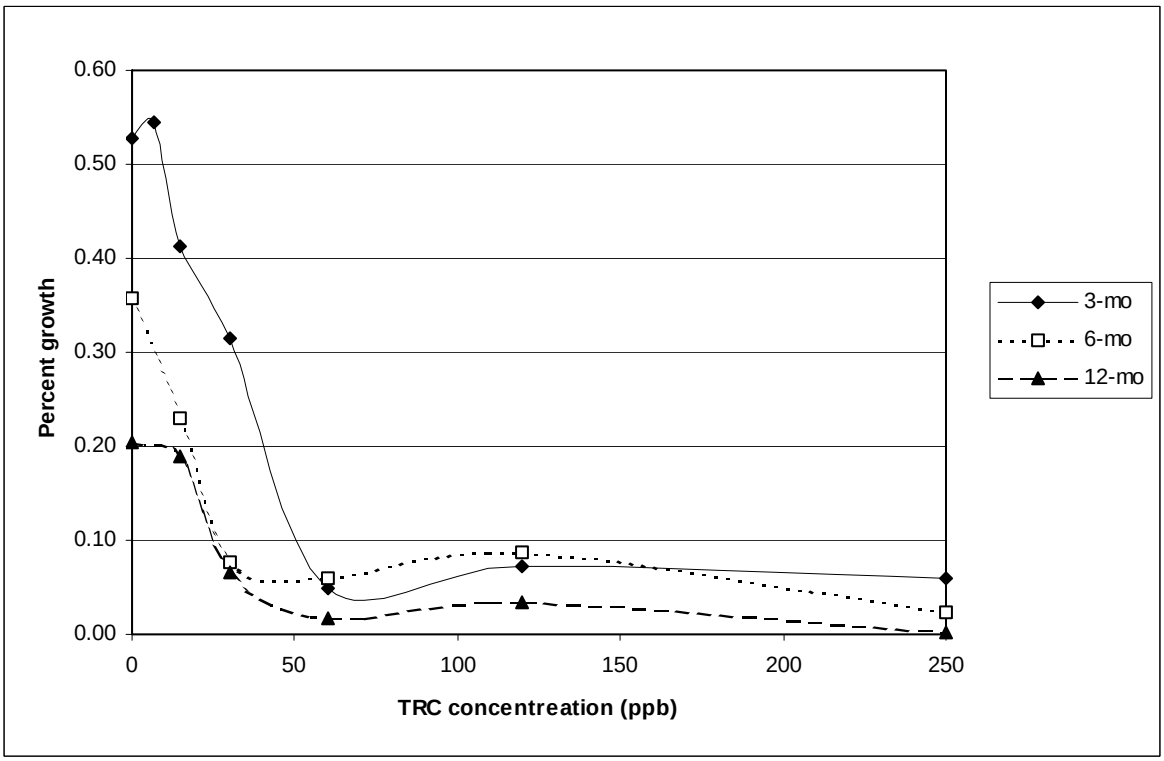


Figure 3.2. Survivorship and proportional growth for 2-, 6-, and 12-mo old *Villosa iris* exposed to different concentration of TRC.

Chapter 4: Acute and chronic toxicity of mercury to early life stages of the rainbow mussel, *Villosa iris* (Bivalvia: Unionidae) -Short Communication.

4.1 Abstract

Mercury (Hg) contamination is receiving increased attention globally due to 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-day chronic juvenile toxicity tests) were conducted by exposing *Villosa iris* to mercuric chloride salt ($HgCl_2$). Glochidia were more sensitive to acute exposure than juvenile mussels, as 24-, 48, and 72-h LC50 values 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 control organisms. The substantial difference in juvenile test endpoints emphasizes the importance of assessing chronic exposure and sub-lethal effects. Overall, my study supports the use of glochidia as an alternative life stage to juveniles in acute toxicity tests. However, as glochidia may only be used only in short-term tests, it is imperative that a 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

4.2 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 (Yeager et al. 1994), and these areas likely have higher Hg concentrations due to its affinity to bind with fine particulate matter (Cattani et al. 1999, French et al. 1999, Wiener and Shields 2000). Several in situ studies have shown that bivalves have a propensity to bioaccumulate Hg (Cattani et al. 1999, Wiener and Shields 2000), which may be due to their close association with the water column- sediment boundary, and their feeding behavior(Salanki and Balogh 1989, Naimo 1995). The United States Environmental Protection Agency (US EPA) is currently reviewing the Water Quality Criteria (WQC) for Hg, but only limited toxicological data are available for most 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 mussels are more sensitive to contaminants than adults (Yeager et al. 1994, Naimo 1995, Jacobson et al. 1997). This finding is also supported by field observations, as alarmingly few young mussels have been found in assemblages with diverse adult populations (Jacobson et al. 1997, Weinstein 2001). Furthermore, immature stages of unionids have been documented to be more sensitive than other aquatic species (Goudreau et al. 1993, Weinstein and Polk 2001), including commonly used regulatory

test organisms (Cherry et al. 2002, Valenti et al. In review). However, with standard test protocols not yet established for freshwater 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 since they are obtained from gravid females 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 (Tedla and Fernando 1969, Huebner and Pynnonen 1992, Jacobson et al. 1997). This may be attributable to their limited energy reserves, and therefore glochidia are only effective as test organisms for assessing acute. 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 meets the unique living requirements of juvenile mussels, and as a result, there is little published literature documenting their chronic sensitivities (Keller et al. 1999).

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 2-mo old juveniles, and a 21-day, 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 2 wk after

successful transformation (<50%) (Jones and Neves 2002, Jones et al. 2004), which may be attributable to high predation (Zimmerman et al. 2003). 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 since concerns about control survivorship have brought the validity of previous unionid tests into question.

4.3 Methods

4.3.1 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 re-circulating 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. (1997) and Goudreau et al. (1993). Glochidia from three adult females 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 ~ 2 mo before being used as test organisms. Over this period, mussels were housed in sediment (150 micron) within recirculating troughs, and fed a daily diet of $3x10^7$ cells *Neochloris oleoabundans* algae/ L.

4.3.2 Acute toxicity tests

4.3.2.1 Glochidia

Test conditions are summarized in Table 4.1 [105]. Viability of glochidia was determined by transferring a sub-sample (~50) of glochidia from a replicate to a glass petri dish for observation under magnification (40x). 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 (Goudreau et al. 1993, Jacobson et al. 1997).

4.3.2.2 Juveniles

Test conditions are summarized in Table 4.1 [105]. 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 (40x) for movement (defined as pedal feeding, active filtering, valve contraction(s), or visceral mass movement observed through the shell). Individuals showing no movement for 3 min were recorded as dead.

4.3.3 Chronic toxicity test with juveniles

Test conditions are summarized in Table 4.1[105]. The test apparatus was a modified version of the self-contained simulated lotic microcosm (SLM) described by Kennedy et al. (2003). that provides flow for lotic organisms. Each SLM consisted of five small, glass vials (outside diameter x height = 28 mm x 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 approximately 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.

A juvenile was randomly selected and measured under magnification (40x) using an ocular micrometer lens before being transferred to test chambers. Shell length was converted to mm. Treatment water was renewed every third day by siphoning and replacing 50% with fresh test solution. The test chambers were supplied daily with 30,000 *Neochloris oleoabundans* cells /ml 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.

4.3.4 Mercury analysis

Samples were prepared for Hg analysis through Inductively Coupled Plasma (ICP) spectrometry at Virginia Tech's soil testing laboratory, according to US EPA standard methods (1991). For the glochidia bioassay, initial treatment Hg concentrations were measured at time 0 h 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 due to complete mortality. During the chronic juvenile test, treatment concentrations were measured for in-water (days 1, 4, 7, 10, 13, 17, 20) and out-water (days 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.

4.3.5 Statistical analysis

The Toxstat® Version 3.5 (West, Inc., Laramie, Wyoming) (Gulley 1996) computer program was used to calculate trimmed Spearman-Karber LC50 values for the acute bioassays, and No Observable Adverse Effects Concentration (NOAEC) and Lowest Observable Adverse Effects Concentration (LOAEC) for the chronic test (p=0.05). Data analysis for survivorship and growth followed US EPA protocol (2002) for chronic bioassays with *Pimephales promelas*. Growth (mm) was calculated by subtracting initial length from final length. Measured Hg concentrations were used when values were not below detection limit.

4.4 Results and Discussion

4.4.1 Acute glochidia and juvenile toxicity tests

Villosa iris glochidia were more sensitive than 2 mo-old juveniles to acute Hg exposure, as there was nearly a 10-fold difference in 72-h LC50 values. In both tests, there was a dose-dependant response, and toxicity increased with exposure time. After 24 h, glochidia viability remained high, and a LC50 value could not be calculated because survivorship was >50% in the highest test treatment (107 µg Hg/L, Table 4.2 [106]). The viability of glochidia decreased substantial after 72 h, and was ≤31% for treatments ≥12 µg Hg/L. Viability in the control and lowest test treatment remained high throughout both tests, >90% and ≥85%, respectively. During the acute test with juveniles, all individuals died in the highest test concentration after 24 h (234 µg Hg/L< Table 4.3 [107]), but survivorship remained high for the other treatments (≥90%, pg 107). Survivorship decreased slightly at each time interval the test was monitored, but remained >95% after 96 h in treatments >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. (in review) reported 48-h LC50 values spanning < 8 to 43 μg/L Hg/L for glochidia of several species, with *V. iris* being the most tolerant species. Keller and Zam (1991) conducted bioassays with newly transformed *Anodonta imbecilis* juveniles (1-2 day 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 3-mo old endangered juvenile oyster mussels (*Epioblasma capsaeformis*), I recorded 24-h and 48-h LC50 values of 160 and 140 μg/L total Hg, respectively. Toxicological endpoints similar to those calculated in my study have been generated in studies with juvenile marine bivalves, as LC50 values for different species were between 125-161 μg Hg/L (Nelson et al. 1988, Krishnakumar et al. 1989).

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 a contaminant(s) to glochidia and juveniles of the same species. Jacobson et al. (1997) 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, while those for *Pyganodon grandis* were 46-347 μg/L and 33-44 μg/L. Augspurger et al. (2003) 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 (1997) 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 easily obtained, and more cost effective 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 has been observed in control treatments (≥90%) after 48 h repeatedly in experiments with glochidia from *V. iris* and other species (Valenti et al. 2003). In addition, glochidia, unlike juveniles, are less able to avoid toxicants because they have thinner, more permeable shells (Hoggarth and Gaunt 1988). 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.

4.4.2 Chronic 21-day juvenile toxicity test

The mean growth of individuals in the control and 4 µg Hg/L treatments were >0.5 mm, and did not vary significantly (Table 4.4 [108]). Growth was reduced by 25% at 8 µg Hg/L, and by ≥50% in the remaining treatments. Individuals exposed to ≥32 µg Hg/L grew only ca. 10% as much as the control. No dead juveniles were found, and the only apparent mortalities recorded were for individuals not located. NOAEC and LOAEC for growth were 4 and 8 µg Hg/L, respectively.

Several studies have shown that bivalves decrease oxygen consumption, growth, and byssal thread production when exposed to Hg (Nelson et al. 1988, Naimo et al. 1995,

Jacobson et al. 1997). Salanki and Balogh (1989) reported that Hg affects filtration rates of bivalves, as exposed individuals had shorter periods of activity, and extended periods of rest. The lower growth observed in treatments containing higher Hg concentrations may be due to 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 sub-lethal 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. (1999) 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 only be intermittently, rather than chronically exposed to Hg. Furthermore, not feeding mussels in the acute test also may have expedited their uptake rate, as Naimo (1995) 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.

4.5 Conclusions

My study supports the use of glochidia as an alternative life stage for juveniles in acute bioassays since their ability to avoid exposure is limited, and 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, since individuals may be able to avoid toxicants by closing their valves for sustained periods. However, there are limitations with using glochidia as test organisms, since 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. I 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 be cautioous 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 female and before encysting on host fish (Jacobson et al. 1997), which is often only a short time interval. Conversely, juvenile mussels are more likely threatened by chronic exposure to toxicants in sediment or interstitial water, as Yeager et al. (1994) noted that juvenile *V. iris* burrow into the substrate and rely 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.

4.6 Acknowledgements

I thank the United States Fish and Wildlife Service for their support of this project. I also thank Jess Jones and Rachel Mair of the Virginia Tech Aquaculture Center for their assistance in obtaining and culturing test organisms.

4.7 References

1) Yeager MM, Cherry DS, Neves RJ. 1994. Feeding and burrowing behavior of juvenile rainbow mussels, *Villosa iris* (Bivalvia: Unionidae). *J North Am Benthol Soc* 13:217-222.

2) Cattani O, Fabbri D, Salvati M, Trombini C, Vassura I. 1999. Biomonitoring of mercury pollution in wetland near Ravenna, Italy by translocated bivalves (*Mytilus galloprovincialis*). *Environ Toxicol Chem* 18: 1801-1805.

3) French KJ, Scruton DA, Anderson MR, Schneider DC. 1999. Influence of physical and chemical characteristics on mercury in aquatic sediment. *Water Air Soil Poll* 110: 347-362.

4) Wiener JG, Sheilds PJ. 2000. Mercury in the Sudbury River (Massachusetts, USA): pollution history and synthesis of recent research. *Can J Fish Aquat Sci*. 57: 1053-1061.

5) Salanki J, Balogh KV. 1989. Physiological background for using freshwater mussels in monitoring copper and lead pollution. *Hydrobiologia* 188/189: 445-454.

6) Naimo TJ. 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4:341-362.

7) Jacobson PJ, Neves RJ, Cherry DS, Farris JL. 1997. Sensitivity of glochidial stages of freshwater mussels (Bivalvia: Unionidae) to copper. *Environ Toxicol Chem* 16:2384-2392.

8) Weinstein JE. 2001. Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel *Utterbackia imbecillis*. *Environ Toxicol Chem* 20:412-419.

9) Goudrea SE, Neves RJ, Sheehan RJ. 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch River, Virginia, USA. *Hydrobiologia* 252: 211-230.

10) Weinstein JE, Polk KD. 2001. Phototoxicity of anthracene and pyrene to glochidia of the freshwater mussel *Utterbackia imbecillis*. *Environ Toxicol Chem* 20:2021-2028.

11) Cherry DS, Van Hassel JH, Farris JL, Soucek DJ, Neves RJ. 2002. Site-specific derivation of the acute copper criteria for the Clinch River, Virginia. *Human Ecol Risk Assess* 8:591-601.

12) Valenti TW, Cherry DS, Neves RJ, Locke BA. (In review). Bivalve Ecotoxicology. eds. Van Hassel and Farris. Sensitivity of mussel glochidia and regulatory test organisms to mercury and a reference toxicant. Presented at SETAC.

13) Tedla S, Fernando CH. 1969. Observations of *Lampsilis radiata* (Gmelin) infesting yellow perch, *Perca flavescens* (Mitchell), in the Bay of Quinte, Lake Ontario. *Can J Zool* 47: 705-712.

14) Huebner JD, Pynnonen KS. 1992. Viability of glochidia of two species of *Anondonta* exposed to low pH and selected metals. *Can J Zool* 70: 2348-2355.

15) Keller AE, Ruessler DS, Kernaghan NJ. 1999. Effect of test conditions on the toxicity of copper to juvenile unionid mussels. In *Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption*, Vol 8. STP 1364. American Society for testing and Materials, Philadelphia, PA, pp 329-340.

16) Jones J, Neves RJ. 2002. Life history and propagation of the endangered fanshell pearlymussel, *Cyprogenia stegaria* Rafinesque (Bivalvia: Unionidae) *J North Am Benthol Soc* 21: 76-88.

17) Jones J, Neves RJ, Ahlstedt SA, Mair RA. 2004. Life history and propagation of the endangered dromedary peralymussel (*Dromus dromas*) (Bivalvia: Unionidae). *J N Am Benthol Soc* 23: 515-525.

18) Zimmerman LL, Neves RJ, Smith DG. 2003. Control of predacious flatworms *Macrostomum* sp. in culturing juvenile freshwater mussels. *N Am J Aquatic* 65: 28-32.

19) Zale AV, Neves RJ. 1982. Fish host of four species of *Lampsiline* mussels (Mollusca: Unionidae) in Big Moccasin Creek, VA. *Can J Zool* 60: 2535-2542.

20) Kennedy AJ, Cherry DS, Currie RJ. 2003. Evaluation of ecologically relevant bioassays for a lotic system impacted by a coal-mining effluent using *Isonychia biocolor*. *Environ Monit Assess* 44: 324-331.

21) U.S. Environmental Protection Agency. 1991. Methods for determining metals in environmental samples. EPA/600/4-91/010. Washington, DC.

22) Gulley DD, West, Inc. 1996. TOXSTAT version 3.5. Fish Physiology and Toxicology Laboratory, Department of Zoology and Physiology. University of Wyoming. Laramie, Wyoming.

23) U.S. Environmental Protection Agency. 2002. Short-term methods for estimation the chronic toxicity of effluents receiving water to freshwater organisms- 4th Ed. EPA/821-R-02-013. Washington, DC.

24) Keller AE, Zam SG. 1991. The acute toxicity of selected metals to the freshwater mussel, *Anodonta imbecillis*. *Environ Toxicol Chem* 10:539-546.

25) Nelson DA, Miller JE, Calabrese A. 1988. Effect of heavy metals on bay scallops, surf clams, and blue mussels in acute and long-term exposures. *Arch Environ Contam Tox* 17: 595–600.

26) Krishnakumar PK, Damondaran R, Nambisan PNK. Acute toxicity of selected heavy metals to the green mussel *Perna viridis*. *Indian J Marine Sci* 16:263-264.

27) Augspurger T, Keller AE, Black MC, Cope WG, Dwyer FJ. 2003. Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. *Environ Toxicol Chem* 22: 2569-2575.

28) Keller AE, Ruessler DS. 1997. The toxicity of malathion to unionid mussels: Relationship to expected environmental concentration. *Environ Toxicol Chem* 16: 1028-1033.

29) Hoggarth MA, Gaunt AS. 1988. Mechanics of glochidial attachment (Mollusca: Bivalvia: Unionidae). *J Morphol* 198: 71-81.

Table 4.1. Summary of test parameters for acute tests with glochidia and juveniles, and the chronic test with juvenile freshwater mussels.

	TEST TYPE AND LIFE STAGE		
PARAMETERS	Acute-Glochidia	Acute-Juvenile	Chronic-Juvenile
Test type	Static / non-renewal	Static / non-renewal	Dynamic / renewal
Temperature	20 + 1 º C	20 + 1 º C	20 + 1 º C
Test duration	72 h	96 h	21 days
Toxicant	Mercuric chloride (MC)	MC	MC
Test concentrations[A]	0, 8, 15, 30, 60 120 µg/L	0, 15, 30, 60 120, 250 µg/L	0, 4, 8, 15, 30, 60 120 µg/L
Test chamber	50-ml glass beaker	50-ml glass beaker	1-L glass beaker
Test solution	40 ml	40 ml	950 ml
Diluent water[B]	EPA^{100}	EPA^{100}	EPA^{100}
Sediment	none	none	2 ml sieved to 200 microns
Test organisms	< 2 h after extraction	2 months old	2 months old
Organisms/replicate	50 – 75	5	5
# of Replicates	4 per time interval	4 total	4 total
Feeding	none	none	30,000 cells daily
Aeration	none	none	yes
Endpoints	LC50	LC50	Survivorship and Growth NOAEC + LOAEC

A= Nominal concentrations.
B= Moderate hard synthetic water, prepared according to standard US EPA protocol EPA-821-R-02-012.

Table 4.2. Mean survivorship and LC50 values after 24, 48, and 72 h for *Villosa iris* glochidia exposed to different concentrations of mercuric chloride ($HgCl_2$).

Total [Hg] (µg/L)	24 h Survivorship (%)	48 h Survivorship (%)	72 h Survivorship (%)
Control[A]	94	95	91
8[A]	96	95	85
12[B]	94	92	31
25.5	94	86	0
62	92	10	0
107	62	0	0
LC50 Value	> 107[C]	39	14
95% Confidence Limits	na	37-41	12-15

A= Below detection limit (<8.4 µg/L) and expressed as nominal value.
B= To calculated mean concentration, 8.4 µg/L was used as the 72-h value since actual concentration was below detection limit.
C= Insufficient mortality to generate an LC50 value.

Table 4.3. Mean survivorship and LC50 values after 24, 48, 72, and 96 h for *Villosa iris* juveniles exposed to different concentrations of mercuric chloride ($HgCl_2$).

Total [Hg] (µg/L)	24 h **Survivorship** (%)	48 h **Survivorship** (%)	72 h Survivorship (%)	96 h Survivorship (%)
0[A]	100	100	100	95
13[B]	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

A= Below detection limit (<8.4 µg/L) and expressed as nominal value.

B= To calculated mean concentration, 8.4 µg/L was used as the 72-h value since actual concentration was below detection limit.

Table 4.4. Mean survivorship and growth of 2-mo old juvenile *Villosa iris* exposed to different concentrations of mercuric chloride for 21-d.

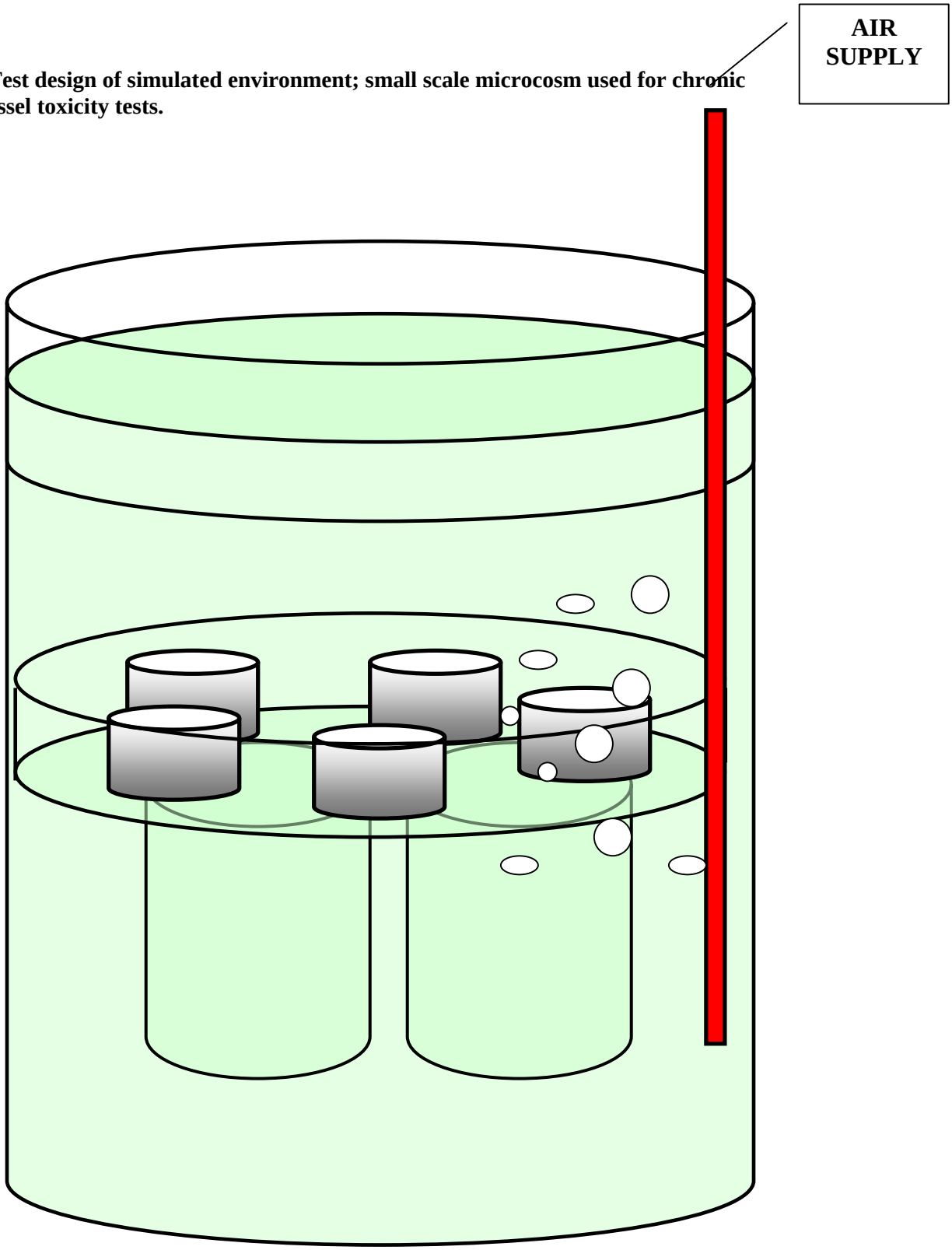
Total [Hg][A] (µg/L)	Survivorship (%)	Growth ± SD (mm)
0[B]	90	0.51 ± 0.17
4[B]	95	0.53 ± 0.11
8[B]	100	0.37 ± 0.13*
15	95	0.26 ± 0.13*
32	100	0.06 ± 0.05*
62	100	0.01 ± 0.02*
114	100	0 ± 0*

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

A= Mean in-water concentrations; out-water for all concentrations was below detection limit (<8.4 µg/L).

B= Below detection limit (<8.4 µg/L) and expressed as nominal value.

Figure 4.1 Test design of simulated environment; small scale microcosm used for chronic juvenile mussel toxicity tests.



Curriculum Vitae

Theodore Walter Valenti
Home address:
PO Box 373
Constableville, NY 13325
tvalenti@vt.edu

EDUCATION

Graduate

Master's in Biology with emphasis in Environmental Toxicology 2004.
Virginia Polytechnic Institute and State University, Blacksburg VA.
Primary Advisor: Dr. Donald S. Cherry.
GPA: 3.6.

Undergraduate

Bachelor of Arts Degree 2001. Biology Major / Environmental Studies Minor.
Hamilton College, Clinton NY.
Dean's List Fall 2000, Spring 2001.
Final GPA: 3.0.

PROFESSIONAL INTERESTS/AFFILIATIONS

Ecotoxicology
- Environmental risk assessment of freshwater systems through the examination of laboratory and field toxicological parameters.
- Integration of science and public affairs to create sound policy.
- Development of standard test protocols.

Sigma Xi National Honor Society
Society of Environmental Toxicology and Chemistry (SETAC)
Save Our Streams Conservation Society (SOS)

TEACHING EXPERIENCE

Graduate Teaching Assistant. *Virginia Tech, Department of Biology, Blacksburg VA.*
Devised teaching schedules, lectured classes, administered examinations, and graded students' work for introductory biology class (Spring 2002).

Substitute Teacher. *South Lewis Central School District, Turin NY.*
Taught a variety of classes ranging in grade from K – 12. Also served as an assistant coach for varsity football and wrestling (Fall 2001).

Teaching Assistant. *Hamilton College, Biology Department, Clinton NY*
Prepared laboratories, assisted students, and conducted review sessions for introductory biology classes. (Fall 2000 – Spring 2001).

Teacher and Laboratory Assistant. *Johns Hopkins' Center for Talented Youth, Clinton NY.*
Taught classes in Paleo-biology and Primate Evolution to gifted students ranging in age from 9 – 14 years (Summer 1999).

RESEARCH EXPERIENCE

Graduate Research Assistant. *Virginia Tech, Department of Biology, Blacksburg VA.* Conducted laboratory bioassays, carried out experiments in the field, and completely tasks pertinent to the daily operations of a toxicology laboratory (Fall 2002 – Fall 2004).

Research Assistant. *New York State Department of Environmental Conservation, Rome NY.* Conducted laboratory bioassays on non-target aquatic organisms to assess the impact of pyrethroid insecticides currently applied to control the spread of the West Nile Virus (Fall 2000 - Spring 2001).

Research Assistant. *Ernest Williams, Hamilton College, Biology Department, Clinton NY.* Observed organisms in the field, conducted behavioral studies, and examined host plant relationships (Summer 2000).

PUBLICATIONS

Valenti TW, DS Cherry, RJ Neves, BA Locke, J Schmerfeld. 2004. Sensitivities of freshwater mussel glochidia and regulatory test organisms to mercury and a reference toxicant. Invited chapter in SETAC book entitled "Bivalve Toxicology." (In review).

Valenti TW, DS Cherry, RJ Neves, J Schmerfeld. 2004. Acute and chronic toxicity of mercury to early life stages of the rainbow mussel, *Villosa iris* (Bivalvia: Unionidae)- Short Communication. *Environ Toxic Contam* 24 (in press).

Valenti TW, JL Chaffin, DS Cherry, ME. Schreiber, HM Valett, M Charles. 2004. Bioassessment of an Appalachian headwater stream influenced by an abandoned arsenic mine. *Arch Environ Toxicol Chem* (In review).

Valenti TW, DS Cherry, RJ Neves, JW Jones, & RA Mair. Inter- and intra-specific variability in the tolerance of glochidia exposed to sodium choride. (In prep).

Valenti TW, DS Cherry, RJ Neves. Acute and chronic exposure of early life stage of freshwater mussels to chlorine. (In prep).

GRANTS

Cherry DS, RJ Currie, TW Valenti. An ecotoxicological evaluation of mercury in the North Fork Holston River at Saltville, Virginia. United States Fish & Wildlife Service ($58,000).

Cherry DS, TW Valenti, RJ Currie, RJ Neves. First preliminary report on preassessment screen data gaps Saltville national pollution list site natural resource damage assessment. United States Fish & Wildlife Service ($82,800).

Cherry DS, RJ Currie, TW Valenti, ML Simon, RJ Neves. Coal fines / particulate impact upon mussel assemblages. United States Geological Survey ($32,000).

PRESENTATIONS / PUBLISHED ABSTRACTS

Valenti TW, DS Cherry, RJ Neves, BA Locke. November 8-13th, 2003. Sensitivities of freshwater mussel glochidia and regulatory test organisms to mercury and a reference toxicant. SETAC 24th Annual Meeting in North America. Austin, TX. Platform Presentation.

Valenti TW, DS Cherry, RJ Neves, BA Locke, JM Uerz, RJ Currie. November 8-13th, 2003. Acute and chronic mercury exposures to different life stages of the rainbow mussel, *Villosa iris*. SETAC 24thAnnual Meeting in North America. Austin, TX. Poster Presentation.

Paul EA, HA Simonin, TW Valenti. November 16-20th, 2002. Toxicity of several mosquito insecticides to crayfish. SETAC 23rd Annual Meeting in North America. Salt Lake City, UT. Poster Presentation.

Valenti TW, DH Catlin, EW Williams. July 28-30th, 2000. Some behavior of frosted elfins in lupine patches. The Annual Meeting of Lepidopterist's Society, Wake Forest, Winston Salem, NC. Poster Presentation.

Catlin DH, TW Valenti, EW Williams. November 3-4th, 2000. The effect of plant chemistry on oviposition of *Phyciodes tharos* butterflies. The 25th Annual Biological Sciences Research Symposium, Binghamton University, Binghamton, NY. Poster Presentation.

TECHNICAL REPORTS

Cherry DS, TW Valenti, RJ Currie, RJ Neves. First preliminary report on preassessment screen data gaps Saltville national pollution list site natural resource damage assessment. (2003).

Cherry DS, TW Valenti, RJ Currie, RJ Neves, JW Jones. Chlorine toxicity to early life stages of freshwater mussels (2002-2004, quarterly).

Cherry DS, RJ Currie, TW Valenti, BA Locke, JM Uerz, ML Simon. Toxicity test laboratory performance evaluation (DMR-QA 23 WET) for acute and/or chronic tests. (2003).

Cherry DS, RJ Currie, TW Valenti, BA Locke, JM Uerz. Quarterly biomonitoring results of water flea and fathead minnow to Pine Bluff Mill Effluent, International Paper Co., Pine Bluff AR (2002-2003).

Cherry DS, RJ Currie, TW Valenti, BA Locke, & JM Uerz. Acute / chronic toxicity evaluation of Effluent 001 / 002 / 003 Celanese Acetate, Celco Plant, VA (2002-2004, quarterly).