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U.S. FISH AND WILDLIFE SERVICE
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FINAL REPORT FOR
ENVIRONMENTAL CONTAMINANTS PROGRAM
OFF-REFUGE INVESTIGATIONS SUB-ACTIVITY FUNDING

Developing Water Quality Standards for Recovery of Imperiled Freshwater Mussels (Family Unionidae)

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Abstract

Many factors are cited in the decline of freshwater mussel (Family Unionidae) populations in North America and for the listing of greater than 70% of native unionid species as endangered, threatened, or of special concern. For the nearly 300 species of mussels, habitat alteration, introduction of exotic species, over-utilization, disease, predation and pollution are considered causal or contributing factors to declines in many areas of the United States. Almost all of the nearly 70 species of federally-listed endangered or threatened freshwater mussels in the United States occur in U.S. Fish and Wildlife Service (Service) regions 3, 4, and 5.

There were four objectives for this investigation: 1) Assess the acute toxicity of chlorine, ammonia and copper – three common pollutants - to early lifestages (glochidia and juvenile) of freshwater mussels and assess the chronic toxicity of chlorine, copper and, if possible, ammonia to one of the species used in the acute toxicity assessments. Test organisms included endangered and non-endangered mussel species from the central and eastern United States; 2) Document instream water quality conditions (particularly for copper, ammonia and chlorine) in habitats for these species; 3) Employ captive propagation to supply test organisms and elucidate various life-history and culture attributes (fish hosts, culture methods, rearing methods, etc.); and 4) Evaluate the scientific validity of acute toxicity assessments using mussel glochidia and refine methodologies for conducting acute and chronic toxicity tests with juvenile mussels.

Six manuscripts for publication documenting the research and findings of this investigation are in either co-author review or final development. These papers are to published in a proposed series in the journal *Environmental Toxicology and Chemistry*. These manuscripts will more completely describe the research and findings of this investigation. This Final Report provides a brief review of the results found during this investigation and emphasizes their importance relative to mussel recovery management actions.

A great amount of effort went to developing guidance for conducting tests with freshwater mussels. ASTM Committee E47 on Biological Effects and Environmental Fate approved a new standard entitled a S*tandard guide for conducting laboratory toxicity tests with freshwater mussels* (E2455-06) through ASTM International. The standard describes methods for conducting laboratory toxicity tests with early life stages of freshwater mussels including glochidia and juvenile mussels in water-only exposures.

Results of this investigation indicate that mussels are generally sensitive to ammonia and copper. There has been little change in the overall interpretation of ammonia data since the publication by Augspurger et al. (2003) and mussels routinely rank among the more sensitive organisms to ammonia. Augspurger et al (2003) and the additional data generated by this investigation lead EPA to hold a Mussel Toxicity Testing Workshop in Chicago, Illinois (August 23-24, 2005). This workshop provided EPA with information regarding the standardization of mussel toxicity testing methods as well as the sensitivity of mussels to ammonia.

As was the case with ammonia, results from tests conducted with copper during this investigation as well as those results found in the literature, indicate that mussels rank among the more sensitive organisms to copper.

The field assessment demonstrated a direct application of the laboratory toxicity data generated for site-specific risk evaluation of pollutants in important mussel habitats. Monitoring in North Carolina demonstrated that water quality in important unionid streams exceed criteria

and standards developed to be protective of aquatic life as well as site-specific guidelines for mussel protection. Further it demonstrates that since mussels are among the more sensitive taxa for pollutants such as ammonia and copper, that exceedences of these criteria or standards, or lack of mussel toxicity data when generating the criteria or standard, may be a contributing factor to the decline of these species.

In summary, this investigation has documented 1) the relative sensitivity of a number of mussel species, including listed species, to chlorine, copper and ammonia 2) that mussel toxicity tests can be routinely and reliably conducted, and 3) that field monitoring can document instream concentrations of copper and ammonia that in laboratory studies have been shown to cause adverse effects to early lifestages of mussels. In addition, the project has generated a wealth of data which can be used to remedy the identified concerns, and the study participants are already working with state and federal agencies to integrate the investigations' data into environmental protection programs.

Key words: (ID: 200230003.1; FFS: 3F27; freshwater mussels, unionid, glochidia, juvenile, acute, chronic, ammonia, copper, chlorine, ASTM, streams, monitoring)

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INTRODUCTION

Many factors are cited in the decline of freshwater mussel (Family Unionidae) populations in North America and for the listing of greater than 70% of native unionid species as endangered, threatened, or of special concern (Williams et al. 1993; Neves et al. 1997). For the nearly 300 species of mussels, habitat alteration, introduction of exotic species, over-utilization, disease, predation and pollution are considered causal or contributing factors to mussel decline in many areas of the United States (Fuller 1974; Havlik and Marking 1987; Lydeard et al. 2004). Almost all of the nearly 70 species of federally-listed endangered or threatened freshwater mussels in the United States occur in U.S. Fish and Wildlife Service (Service) Regions 3, 4, and 5 (Neves 1997).

Freshwater mussel life history is complex because their life cycle includes a brief parasitic stage on a host fish. Mussels produce larvae called glochidia, which are released by the adult female. Method of host attachment depends on how glochidia are released from the female. Some species expel glochidia from their exhalant siphon into the water. Expelled glochidia can either attach to the gills of fish through normal fish gill activity or can attach to the fins or skin. Other unionids bind glochidia into conglutinates, which are released from female mussels' gills. These conglutinates often resemble fish prey items. Fish gills become infected when fish are lured to, and consume the conglutinate. Between release from the gills and attachment to the fish host, glochidia may be directly exposed to contaminants in the water. Fish hosts for many mussels are unknown. However, studies have shown some freshwater mussels require a single fish species while others may transform on multiple species. Once on the fish, the glochidia encyst for several days or weeks, transform into juvenile mussels and excyst. Juveniles then begin an independent existence on the benthos as filter-feeding mussels where they are continually exposed to contaminants that occur in the water column.

Impaired water quality may be a limiting factor in the recovery of listed mussels. Toxic substances were one of the top five stressors cited as limiting freshwater mussels in a recent survey of experts for this taxa (Richter et al. 1997). A separate review identified industrial, municipal, and agricultural pollution as significant threats to listed mussels (Wegner et al. 2001). Keller (1993, 1996), Masnado et al. (1995), and Keller and Ruessler (1997) found the cladocerans *Ceriodaphnia dubia* and *Daphnia magna* to be more sensitive than the freshwater mussel *Utterbackia (*formerly *Anodonta) imbecillis* to the acute effects of organic chemicals (herbicides, organochlorine insecticides, an organophosphate, and solvents) and a simulated mine effluent. However, information from limited toxicity testing indicates a potential for early lifestages of mussels to be among the most sensitive aquatic organisms yet tested for impacts of short term exposure to ammonia (Horne and McIntosh 1979; Wade 1992; Goudreau et al. 1993; Scheller 1997; Myers-Kinzie 1998; Augspurger et al. 2003), chlorine (Goudreau et al. 1993), metals (Keller and Zam 1991; Jacobson et al. 1993), and paper mill effluent (Wade et al. 1993; McKinney and Wade 1996).

In a comprehensive review of the toxicological literature available for freshwater mussels as part of the derivation of water quality criteria for the Great Lakes ecosystem, it was concluded that proposed criteria for copper, zinc, nickel, cadmium, and chromium (VI) may not be protective of listed freshwater mussels (Blankenship 1995). Additionally, Augspurger et al. (2003) summarized acute ($\leq$96 hours) ammonia exposure assays, covering nine species and eight genera of freshwater mussels and found mussels were uniformly at the sensitive end of the

range of ammonia toxicity relative to other tested invertebrates and fishes.

While natural resource management agencies and academic institutions have invested considerable effort in determining the pollutant sensitivity of early lifestages of freshwater mussels over the last 20 years, many findings have not been embraced by water quality regulators due to a lack of consistent or standardized test methods. This Environmental Contaminants Program Investigation was initiated to fill this void as well as provide location-specific pollutant information for recovery planning in important habitats. These data needs have been identified as high priorities in the National Strategy for the Conservation of Freshwater Mussels (NNMCC 1998).

There were four study objectives for this investigation:

1) Assess the acute toxicity of chlorine, ammonia and copper – three common pollutants - to glochidia and juvenile lifestages of freshwater mussel including endangered and non-endangered mussels from the central and eastern United States. In addition, assess the chronic toxicity of chlorine, copper and, if possible, ammonia to one of the species used in the acute toxicity assessments.

2) Document water quality conditions (particularly for copper, ammonia and chlorine) in habitats for these species. Habitats selected included areas where mussels historically occurred and water quality was identified as a potential cause for the loss, or areas that currently contained the best remaining populations.

3) Employ captive propagation to supply test organisms and reap a number of ancillary benefits. Data to foster direct management actions expected from this part of the project include those related to elucidation of various life-history and culture attributes (fish hosts, culture methods, rearing methods, etc.).

4) Evaluate the scientific validity of acute toxicity assessments using mussel glochidia and refine methodologies for conducting acute and chronic toxicity tests with juvenile mussels.

Many individual studies were conducted with freshwater mussels to evaluate these factors and provide supporting data necessary to ensure that the toxicity data produced by the project were embraced by the scientific and regulatory communities. Following is a brief review of work conducted and results for the four objectives. Currently, there are six manuscripts (proposed as a series in the journal *Environmental Toxicology and Chemistry*) either in co-author review or final development (Appendix 2). These manuscripts will more completely describe the research and findings of this investigation. Other products have already been generated and published as a result of this investigation and those will be noted throughout this final report and are listed in Appendix 1. For purposes of providing information to the public and other government agencies, it will be appropriate - because of specificity and completeness - to provide reprints of the final publications expected to be available within the next year.

METHODS AND MATERIALS

Initiation of investigation

Upon notification of funding (February 2001), the Service entered into cooperative agreements with the U.S. Geological Survey, Biological Resources Division's (USGS-BRD) Virginia Cooperative Fish and Wildlife Research Unit at Virginia Tech (Blacksburg, VA) and Missouri State University (Springfield, MO) for captive propagation to produce glochidia and juvenile freshwater mussels for use in toxicological evaluations. The research to support captive propagation for supply of test organisms produced other benefits, such as culture methods and rearing methods. The Service's Endangered Species program funding in Regions 3, 4, and 5 supported this and other aspects of the project.

Cooperative agreements were also developed between the Service USGS-BRD's Columbia Environmental Research Center (CERC), Columbia, MO for freshwater mussel toxicity testing method refinement, conductance of ammonia, copper and chlorine toxicity tests with mussels, and analytical chemistry. The Service (Jim Dwyer, Tom Augspurger, Andy Roberts, Cindy Kane) and CERC (Chris Ingersoll, Ning Wang, Eugene Greer) met with Chris Barnhart (Missouri State University), Jerry Farris and Cristi Milam (Arkansas State University), Dick Neves (Virginia Cooperative Fish and Wildlife Research Unit at Virginia Tech), and Don Cherry (Virginia Tech) to review and discuss methods for conducting toxicity tests with glochidia and juvenile mussels. Information gathered during these visits was used to develop a draft response to a list of questions raised by the U.S. Environmental Protection Agency (EPA) regarding freshwater mussel toxicity tests with glochidia as well as to establish the preferred test procedures for this study. Further, this method review provided direction for the development of standard guidance for conducting toxicity tests mussels as well as to identify research needs that, if filled, would facilitate use of data generated by the project.

Toxicity testing

Glochidia ammonia, chlorine and copper acute toxicity tests were conducted with 11 species (Table 1). In addition, ammonia, chlorine and copper toxicity tests were conducted with juvenile mussels for 7 of the 11 species. Acute toxicity tests were conducted in basic accordance with ASTM - Standard guide for conducting acute toxicity tests on the test materials with fishes, macroinvertebrates, and amphibians (E729-96). Test durations of 6, 24 and 48-h were conducted

While the ASTM E729-96 guidance was generally followed, the described procedures were not completely appropriate for use with freshwater mussels. For example, endpoint identification – shell closure upon addition of a saline solution - was not described. To document the best practices developed during these tests and to address EPA concerns regarding test methods with mussel glochidia, efforts were made to develop mussel-appropriate ASTM guidance. Conditions to be considered included, but were not limited to, test duration, test temperature, feeding, end-point identification, control survival, and test repeatability including round-robin testing. Generating supporting data for guidance ensured that the toxicity data produced during the investigation was utilized by the scientific and regulatory communities.

In addition to acute toxicity tests, longer term 10 day lethality (copper, ammonia and chlorine) and 28 day chronic (growth – shell length - and survival; copper and ammonia) tests were also conducted. The 10 day tests were generally conducted with the same species as used in the shorter acute exposures (6, 24 and 48 h). Chronic copper studies were conducted with

rainbow (*Villosa iris*), fatmucket (*Lampsilis siliquoidea*) and oyster mussels (*Epioblasma capsaeformis*) while chronic ammonia studies were conducted with rainbow, fatmucket and wavy-rayed lampmussel (*Lampsilis fasciola*).

Field assessment

In addition to the toxicity testing, a field assessment of total copper, total residual chlorine (TRC) and total ammonia was performed in three North Carolina streams which support the endangered Carolina heelsplitter (*Lasmigona decorata*) and dwarf wedgemussel (*Alasmidonta heterodon*). Samples for TRC analyses were collected in amber glass bottles and were analyzed immediately onsite. Continuous power for the spectrophotometer was provided by a 12-volt vehicle battery using a cigarette-lighter adaptor and power inverter. Grab samples for total ammonia were collected in 125 mL polyethylene chemically cleaned bottles and acidified with concentrated H_2SO_4 to pH < 2. Grab samples for copper analyses were collected unfiltered in 125 mL polyethylene chemically-cleaned bottles. Samples were stored on ice (~4^{o}C) in the field and shipped overnight to CERC for analysis. Copper samples were acidified at CERC with Ultrex HNO_3 (1%, v/v) upon receipt. Total ammonia was measured by an ion electrode method following protocols described in Method 4500-D (APHA, AWWA, AWRF 1998). The MDL was 0.04 mg/L and the method quantitation limit (MQL) was 0.13 mg/L unionized ammonia (computed as 3.3 times the MDL). All ammonia results presented are for total ammonia as nitrogen (TAN) unless otherwise noted. Samples for total recoverable copper were digested with nitric acid. Digestates were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The MDL ranged from 0.38 to 1.70 µg/L and the MQL limit ranged from 1.25 to 5.61 µg/L. All copper results reported are for the total recoverable fraction unless otherwise noted.

Results from this investigation, in addition to data obtained from NC Division of Water Quality ambient monitoring stations in the study area (USEPA 2006) were compared to estimates of chemical concentrations likely to be protective of mussels as well as state standards and USEPA criteria to evaluate the risk of these water quality parameters to the Carolina heelsplitter and the dwarf wedgemussel.

To date, only Region 4 (North Carolina Field Office) has completed its field data collection and analysis. Region 5 has an on-going effort. Region 3 has yet to initiate the field component.

RESULTS

Currently, there are six manuscripts either in co-author review or final development. These manuscripts will more completely describe the research and findings of this investigation. In addition, ASTM guidance for conducting toxicity tests with mussels has been developed, balloted and published (ASTM - S*tandard guide for conducting laboratory toxicity tests with freshwater mussels* (E2455-06)). A brief review of the results to be published will be described.

Toxicity testing

The toxicity of ammonia, chlorine, and copper on glochidia and juvenile mussels was measured in acute toxicity tests. The toxicity endpoint measured in 6-h, 24-h, or 48-h glochidia toxicity tests was survival based on valve closure in response to the addition of NaCl. The

toxicity endpoint measured in 2-, 4-, or 10-d juvenile toxicity tests was survival based on foot movement. Copper EC50s for glochidia ranged from 7 ug/L (48-h wavy-rayed lampmussel) to >100 ug/L (6-h rainbow, mucket, wavy-rayed lampmussel, dwarf wedge mussel, pink mucket; 24-h wavy-rayed mussel). Ammonia EC50s for glochidia ranged from 3 mg total ammonia as N/L (6-h oyster mussel; 48-h mucket) to >16 mg total ammonia as N/L (6- h ellipse, wavy-rayed lampmussel, fatmucket, pink papershell, mucket, dwarf wedge mussel; 24-h pink papershell, dwarf wedgemussel, fatmucket) and chlorine ranged from 16 ug/L (48-h mucket) to >100 (most species tested at 6-h exposure). For copper, ammonia, and chlorine, the strongest correspondence in EC50s was observed between the 2-d glochidia test and the 4-d juvenile test for several species.

The sensitivity of freshwater mussels to ammonia and copper was compared with several commonly-tested surrogate species. Glochidia (2-d exposures) and juvenile mussels (4-d exposures) of up to 15 species of mussels were tested. Surrogate species evaluated included *Daphnia magna* and *Ceriodaphnia dubia* (cladocerans, 2-d exposures), *Hyalella azteca* (amphipod, 2-d exposures), *Pimephales promelas* (fathead minnows, 4-d exposures), and *Oncorhynchus mykiss* (rainbow trout, 4-d exposures). The EC50s for ammonia ranged from 13 to >16 mg total ammonia-N/L for the surrogate species and ranged from 3.0 to 13 mg total ammonia-N/L for glochidia and from 2.3 to 11 mg total ammonia-N/L for juvenile mussels (EPA acute water quality criterion is 5.6 mg total ammonia-N/L at pH 8 [including salmonids]). The EC50s for copper ranged from 15 to >100 ug/L for the surrogate species and from 7 to 86 ug /L for glochidia and from 6.8 to 60 ug/L for juvenile mussels (EPA acute water quality criterion is 23 ug/L at a hardness of 170 mg/L). Results of this study indicate that ammonia and copper are frequently toxic to mussels at concentrations that are not toxic to the surrogate species.

Chronic toxicity tests were conducted for 28 d. Endpoints measured were 28-d survival (based on foot movement) and growth (based on shell length). Control survival for the copper test with rainbow was 88%, all other tests with copper and ammonia had control survivals of 98 to 100%. In the 28-d copper studies, chronic values for growth (ChV; geometric mean of the no-observed-effect concentration and the lowest-observed-effect concentration; Stephan et al, 1985) ranged from 4.6 ug/L (oyster mussel) to 8.5 ug/L (fatmucket), while growth ChVs in toxicity tests with ammonia ranged from <0.4 mg total ammonia -N/L (rainbow) to 0.48 mg N/L (wavy-rayed lampmussel). In the copper and ammonia exposures ChVs for survival were always equal to or greater than the growth ChVs.

Intra- and inter-laboratory acute toxicity tests with copper were conducted with glochidia and juvenile mussels. The CERC also evaluated variability in intra-laboratory acute toxicity tests with ammonia and chlorine with glochidia and juvenile mussels. Copper intra-laboratory toxicity tests were conducted with glochidia of mucket and fatmucket. Intra-laboratory variability of EC50s for toxicity tests conducted with copper, ammonia, or chlorine over exposure periods of 24 and 48 h, expressed as the coefficient of variation (CV), ranged between 13 and 36% for toxicity tests conducted with glochidia of mucket and between 14 and 34% for toxicity tests conducted with glochidia of fatmucket. Intra-laboratory variability of EC50s for toxicity tests conducted with juvenile fatmucket and copper over exposure periods of 48 and 96 h expressed as the CV ranged from 13 to 26%. Copper inter-laboratory toxicity tests were conducted with glochidia and juvenile fatmucket. The testing laboratories included 2 federal facilities and 3 university facilities. Inter-laboratory variability of EC50s for glochidia, expressed as the CV, was 13% for the 24-h EC50s and was 24% for the 48-h EC50s. Inter-laboratory variability of

EC50s for juvenile mussels, expressed as the CV, was 22% for the 48-h EC50s and was 42% for the 96-h EC50s. These measures of intra- and inter-laboratory precision in glochidia or juvenile mussel toxicity tests were at or below the variation reported for previous inter-laboratory studies with commonly-tested organisms in water-only exposures or in sediment exposures (e.g., CVs ranging from 14 to >100%).

Field Assessment

Three streams were monitored for ammonia, copper and chlorine. Selected streams were Goose Creek, Tar River and Swift Creek. The three streams were selected because 1) they support the endangered Carolina heelsplitter and dwarf wedgemussel, and 2) discharge monitoring reports indicate that point source discharges to these sites may limit water quality. Ammonia, copper and chlorine results were screened against previously recommended guidance for the protection of mussels (based on the results of the toxicity testing conducted by CERC) as well as water quality criteria and State standards. EPA criteria for copper were exceeded periodically in all three streams with a percent range of samples exceeding the criteria of 35% to 94%. No samples collected from Swift creek exceeded the State copper action level, the federal criteria/state standard for chlorine or the acute or chronic proposed freshwater ammonia concentration for the protection of mussels. Tar River had 10% of the samples exceeding the State copper action level and 35% of the samples exceeding the federal criteria/State standard for chlorine. Site-specific mussel protection guidelines were rarely exceeded in the Tar River. For Goose Creek, 26% of the samples exceeded the state copper action level, 15% of the samples exceeded the federal criteria/state standard for chlorine. Additionally for Goose Creek, 6% of the samples exceeded the acute proposed freshwater ammonia concentration for the protection of mussels while 18% of the samples exceeded the chronic proposed freshwater ammonia concentration for the protection of mussels.

Site-specific hardness-adjusted copper screening values for acute and chronic mussel protection are frequently exceeded in all streams, and State monitoring datasets confirm our finding that copper concentrations in these streams are frequently above thresholds of concern. The frequency of exceedance of site-specific guidelines reported is concerning and suggests risks to mussels may be excessive; however, it is possible that a significant portion of the exceedences are associated with suspended copper (i.e., that attached to suspended sediment). No data for dissolved copper, the most toxic form to aquatic life, are available; they were not collected as part of this effort because the State regulatory framework for copper is based on unfiltered (total) copper. In the absence of dissolved copper data, estimating the dissolved fraction of copper allows a more realistic interpretation of total copper data presented here, albeit with some uncertainty.

Using EPA protocols (USEPA 1993, 1996), the geometric mean TSS value for each drainage was applied to a default partitioning relationship to develop a site-specific translator for converting total copper to dissolved copper was determined for each drainage. Estimated dissolved copper concentrations (determined using these translators) also exceeded site-specific protective concentrations for freshwater mussels for acute (47 to 57 percent of results) and chronic (53 to 80 percent of results) exposures as well as the State action limit for copper (9 to 14 percent of results) of 7 mg/L. The relationship between total copper and total suspended solids (TSS) indicated that total copper concentrations could not be fully be explained by sediment load in these areas as might be anticipated if copper sorption to suspended solids was

driving total copper loads. Therefore, the estimated frequency with which dissolved copper concentrations exceed protective thresholds is uncertain, but based on instream TSS loads it is likely that dissolved copper concentrations present a risk to freshwater mussels in these streams.

DISCUSSION

Short-term tests with glochidia may be useful for screening the sensitivity of mussels to some chemicals (i.e., copper, ammonia, and chlorine), but response of juvenile mussels may be more ecologically relevant. Use of glochidia to screen the relative sensitivity of a particular mussel species to select chemicals would be particularly useful when evaluating species where only a limited number of adult mussels are available for methods development or for generating juvenile mussels for toxicity testing. Moreover, the host fish for some species of mussels or techniques for transforming juvenile mussels in the laboratory may be unknown for some species. Tests with juvenile mussels provide an opportunity to conduct longer exposures which is important for organisms which may live 20+ years.

Results of this investigation indicate that mussels are not generally more sensitive to chlorine (Figure 1A and 1B) but are sensitive to copper (Figure 2A and 2B) ammonia (Figure 3A and 3B). Results from tests conducted during this investigation with copper as well as those found in the literature which meet ASTM test acceptability criteria, indicate that mussels rank among the more sensitive organisms to copper. Augspurger et al (2003) compiled acute ammonia toxicity data for freshwater mussels, screened the data against data quality objectives, and summarized the information to derive estimates of concentrations that would be safe for mussels. Since then, additional tests have been performed and a standard method for conducting mussel toxicity tests has been balloted. We compared the number of toxicity tests, the number of species tested, and the magnitude of the results among (1) the 2003 dataset, (2) that same dataset as modified by applying test acceptability recommendations from ASTM, and (3) a 2005 dataset which includes new data generated from this investigation that also meet the test acceptability recommendations in the draft ASTM guide. There was little change in the overall interpretation of ammonia data since the publication by Augspurger et al (2003) and mussels routinely rank among the more sensitive organisms to ammonia.

A great amount of effort went to developing guidance for conducting tests with freshwater mussels. ASTM Committee E47 on Biological Effects and Environmental Fate approved a new standard entitled a S*tandard guide for conducting laboratory toxicity tests with freshwater mussels* (E2455-06) through the ASTM International. The standard describes methods for conducting laboratory toxicity tests with early life stages of freshwater mussels including glochidia and juvenile mussels in water-only exposures. Recommended test conditions for conducting these toxicity tests and test acceptability criteria outlined in the standard are based on various methods published in the literature and on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels. The standard states that toxicity tests with glochidia and juvenile mussels should be conducted at 20°C with a 16L:8D photoperiod at a luminance of about 100 to 1000 lux. Toxicity tests with glochidia are typically started within 2 h of isolation from the gills of the female mussels; however, some toxicity tests have been conducted with glochidia isolated from female mussels about 24 h before the start of a test. The endpoint measured in toxicity tests with glochidia is survival (viability) as determined by the response of organisms to the addition of a solution of NaCl. Glochidia that close their

valves with the addition of a salt solution are classified as alive (viable) in a toxicity test. For most species, the duration of a toxicity test conducted with glochidia should be up to 24 h with survival measured at 6 and 24 h. Control survival is typically >90% at the end of 24-h toxicity tests conducted with glochidia. Longer duration toxicity tests with glochidia (e.g., 48 h) can be conducted as long as control survival >90% is achieved. Effect concentrations are typically calculated based on the percentage of viable glochidia in the control at a particular sampling time. Survival can be determined throughout the toxicity test by subsampling each replicate. Toxicity tests with juvenile mussels are typically started with organisms <5 d after release from the host; however, some toxicity tests have been started with 2- to 4-month-old juvenile mussels. Acute toxicity tests with juvenile mussels are typically conducted for 96 h with survival measured at 48 and 96 h. Chronic toxicity tests started with 2- to 4-month-old juvenile mussels have been conducted for 21 to 28 d with measures of survival (based on movement of the foot) and growth (based on shell length). Control survival is typically >90% at the end of 96-h toxicity tests conducted with juvenile mussels and is typically >80% at the end of 10- to 28-d toxicity tests conducted with juvenile mussels. Juvenile mussels are not typically fed during toxicity tests conducted for up to 10 d. Algae have been used as a food source in toxicity tests conducted for 10 to 28 d.

The field assessment was a critical evaluation of the laboratory toxicity data generated in this investigation. In the North Carolina monitoring, it was demonstrated that important unionid streams exceed criteria and standards developed to be protective of aquatic life. Water quality data collection in three North Carolina streams indicates that risks associated with exposure to ammonia, copper, and chlorine are most pronounced in the Goose Creek drainage. Copper concentrations were above levels of concern for mussels in all drainages; however, collection of dissolved copper samples is recommended to further refine a risk estimate for these areas. Chlorine concentrations were infrequently above levels of concern, and further study is needed to determine the extent of impacts to instream habitats when significant exceedences occur. Combined, the data demonstrate that since mussels are among the more sensitive taxa for pollutants such as ammonia and copper, exceedances of aquatic life criteria or standards for ammonia and copper are likely contributing factors to the decline of these species and mussel toxicity data must be included when generating criteria or standards in order to protect this faunal group.

In summary, this investigation has documented 1) the relative sensitivity of a number of mussel species, including listed species, to chlorine, copper and ammonia 2) that mussel toxicity tests can be routinely and reliably conducted, and 3) that field monitoring can document instream concentrations of copper and ammonia that in laboratory studies have been shown to cause adverse effects to early lifestages of mussels. In addition, the project has generated a wealth of data which can be used to remedy the identified concerns, and the study participants are already working with state and federal agencies to integrate the investigations' data into environmental protection programs.

Accomplishments and Management Recommendations:

1. The study helped provide the data for the new the S*tandard guide for conducting laboratory toxicity tests with freshwater mussels* (E2455-06) through ASTM International. This promotes sound science by providing a consensus-based list of test acceptability conditions for evaluating the quality of data in previously conducted tests as well as future tests. The publication of ASTM E2455-06 and the use of its protocols helps ensure data generated by this project will be embraced by the regulatory community.

2. Service risk assessments should carefully evaluate existing data against the protocols for data quality in the ASTM E2455-06. Any future Service investigations that include toxicity tests with freshwater mussels should follow ASTM guidance so that results are comparable to other studies and acceptable to regulators.

3. The project provided reliable and robust toxicity data with which to derive water quality recommendations. Analysis of the protectiveness of existing criteria, standards, and action levels being conducted by the Service, EPA, and others will need to take into advisement the results from this investigation. Mussels appear to be under protected by existing copper and ammonia criteria, standards or action levels, and protective water quality standards for important mussel habitats should be developed. Re-examination of copper and ammonia criteria is warranted. Augspurger et al (2003) and the additional data generated by this investigation lead EPA to hold a Mussel Toxicity Testing Workshop in Chicago, Illinois (August 23-24, 2005). This workshop provided EPA with information regarding the standardization of mussel toxicity testing methods as well as the sensitivity of mussels to ammonia.

4. This investigation demonstrates that recovery teams should conduct a threat analysis based on comparison of ambient water quality data and discharge monitoring reports to derived water quality standards for mussels. The identification of threats to a species' survival constitutes much of the initial endangered species management and recovery effort. Where threats are indicated, a site-specific risk assessment approach may be a valuable tool to guide regulatory and non-regulatory mechanisms (e.g., modified discharge permit conditions, local water quality management plans, or adoption of site-specific water quality standards) too improve water quality conditions for mussel recovery. Further, recovery teams working on threatened and endangered mussel habitat characterizations can use these data to better describe water quality needs or methods to determine water quality needs to recover mussel species. Service Contaminants Specialists can use this approach to assist recovery teams with assessing the extent to which water quality is an important limiting factor for mussels and provide data needed to remedy identified problems.

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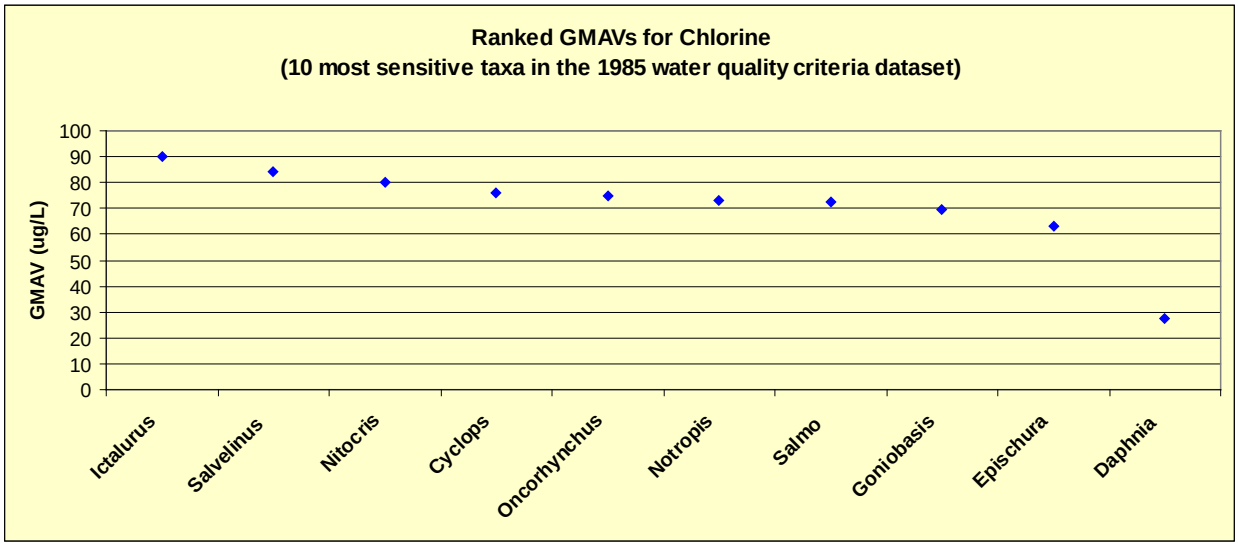
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Table 1. List of freshwater unionid mussel species used in glochidia and juvenile toxicity testing with ammonia, chlorine and copper.

Species	**Common Name**	**Glochidia**	**Juvenile**
Actinonaias ligamentina	Mucket	X	
Alasmidonta heterdon	Dwarf wedgemussel	X	
Epioblasma capsaeformis	Oyster mussel	X	X
Lampsilis abrupta	Pink mucket	X	X
Lampsilis fasciola	Wavy-rayed lampmussel	X	X
Lampsilis rafinesqueana	Neosho mucket	X	X
Lampsilis siliquoidea	Fatmucket	X	X
Leptodea leptodon	Scaleshell	X	X
Potamilus ohiensis	Pink papershell	X	
Venustaconcha ellipsiformis	Ellipse	X	
Villosa iris	Rainbow	X	X

Figure 1A and 1B.



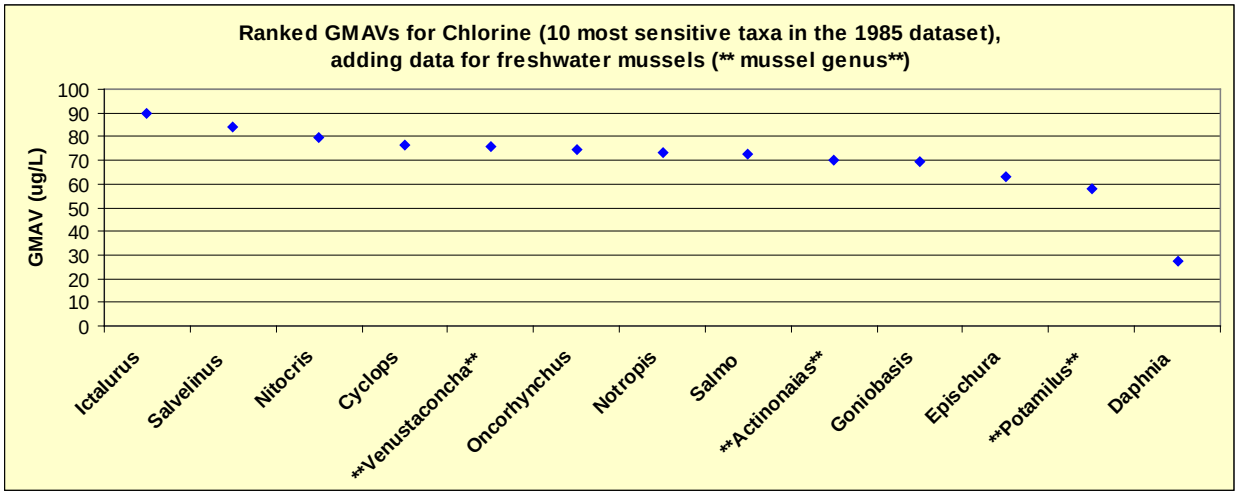
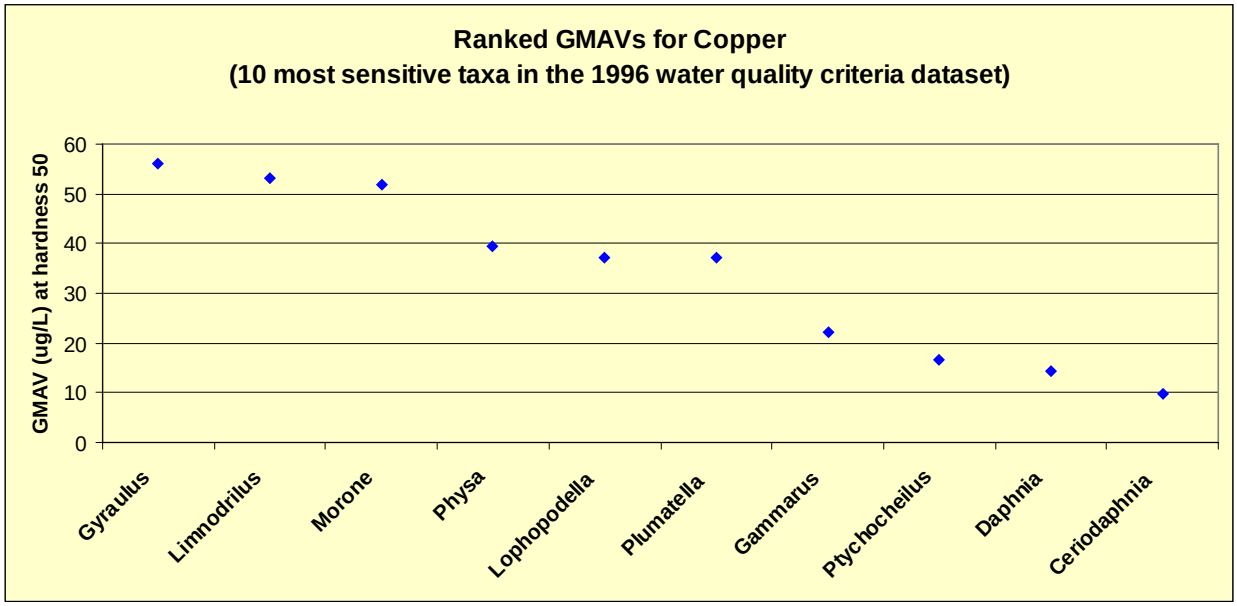


Figure 2A and 2B.



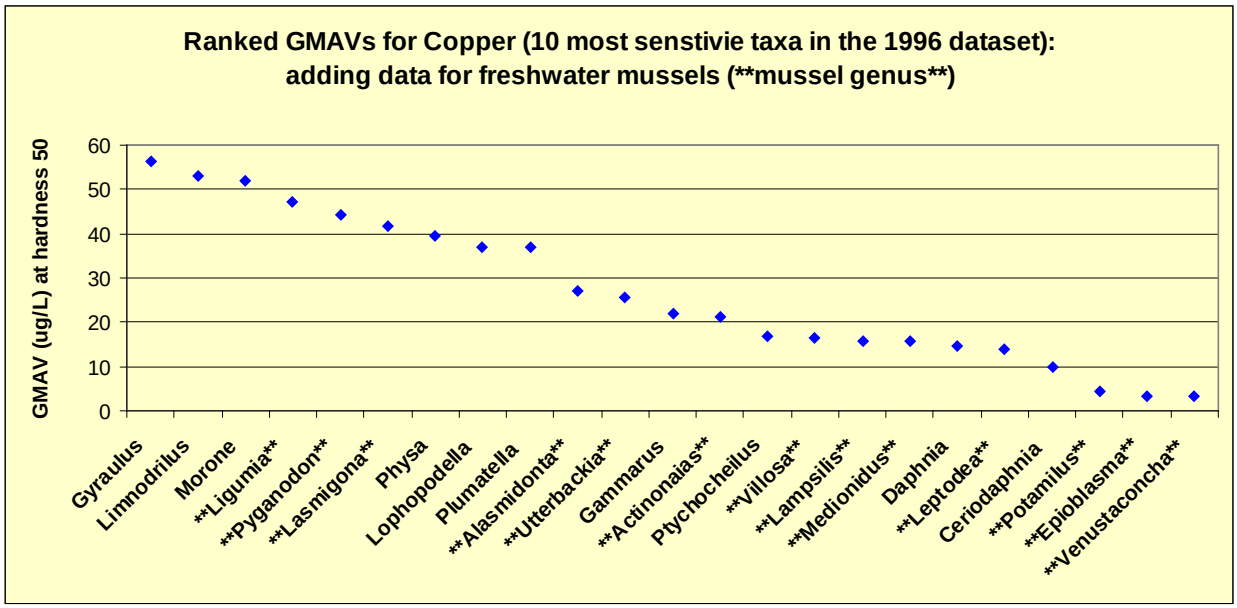
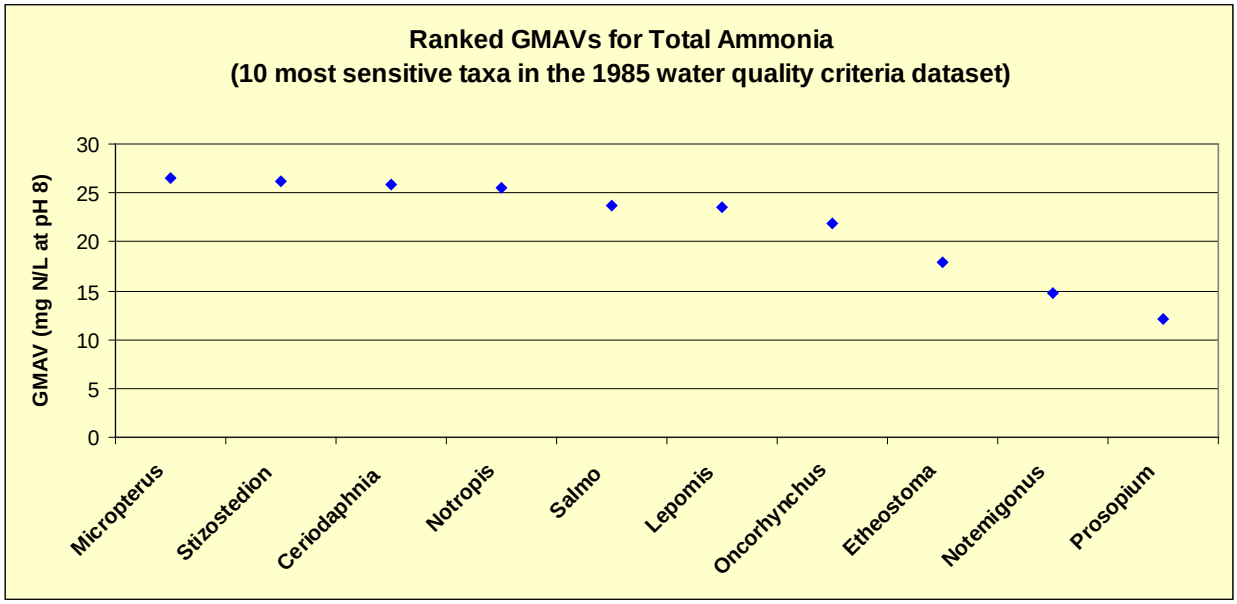
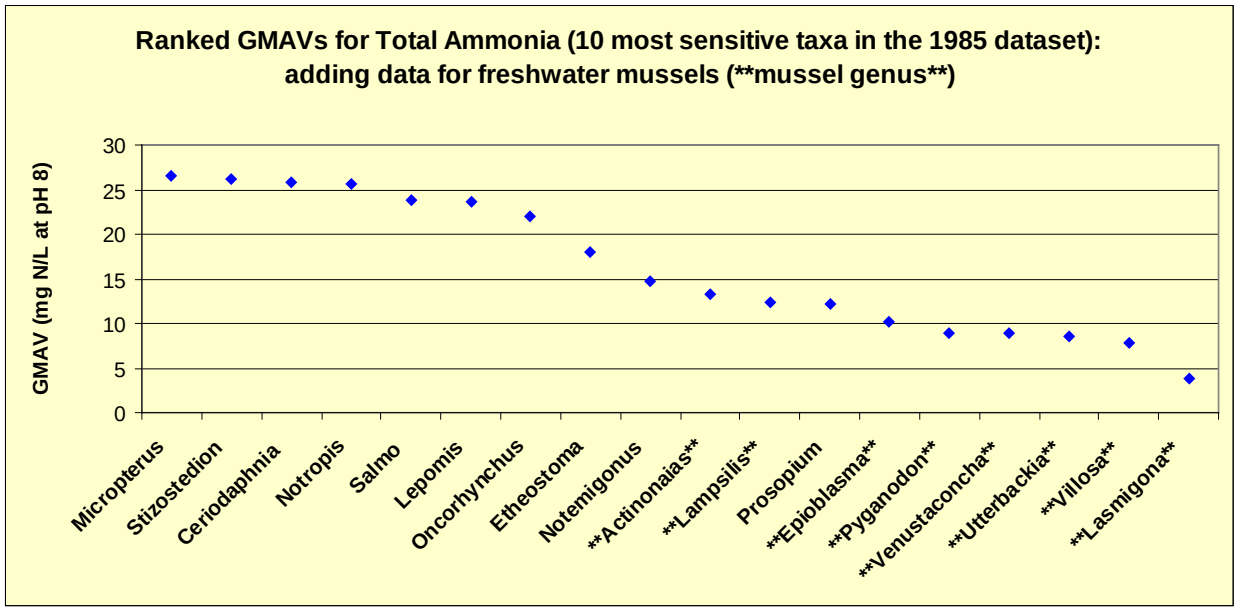


Figure 3A and 3B.





APPENDICES

Appendix 1 - Presentation of Findings

Augspurger TP, Ward SE. Site-specific water quality standards for an impaired stream with endangered freshwater mussels. Presented at the 24th meeting of SETAC, Austin, TX, November 9-13, 2003.

Ingersoll CG, Wang N, Greer IE, Whites DW, Dwyer FJ, Roberts AD, Augspurger T, Kane C, Tibbott C. Acute toxicity of ammonia, chlorine, and copper to glochidia and juveniles of freshwater mussels. Presented at the 24th meeting of SETAC, Austin, TX, November 9-13, 2003.

Wang N, Ingersoll CG, Greer IE, Whites DW, Dwyer FJ, Roberts AD, Augspurger T, Kane C, Tibbott C, Neves RJ, Barnhart MC. Developing standardized guidance for conducting toxicity tests with glochidia of freshwater mussels. Presented at the 24th meeting of SETAC, Austin, TX, November 9-13, 2003.

Ward SE, Augspurger TP. Comparison of low level chlorine measurement instrumentation for stream monitoring. Presented at the 24th meeting of SETAC, Austin, TX, November 9-13, 2003.

Ingersoll CG, Besser JM, Wang N. Using water quality criteria and surrogate species to protect sensitive life stages of freshwater mussels and other aquatic organisms. Seminar presented at University of North Texas, Denton, TX, January 30, 2004.

Ingersoll CG, Besser JM, Wang N. Using water quality criteria and surrogate species to protect sensitive life stages of freshwater mussels and other aquatic organisms. Presented at the USEPA Surface Water Monitoring and Standards Meeting, Chicago, IL, February 4, 2004.

Wang N, Ingersoll CG, Greer IE, Whites DW, Dwyer FJ, Roberts AD, Augspurger T, Kane C, Tibbott C, Neves RJ, Barnhart MC. Developing standardized guidance for conducting toxicity tests with glochidia of freshwater mussels. Presented at the joint meeting of the Midwestern chapter and Ozark-Prairie Region chapter of SETAC, La Crosse, WI, March 5, 2004.

Ingersoll CG, Wang N, Greer IE, Whites DW, Dwyer FJ, Roberts AD, Augspurger T, Kane C, Tibbott C. Acute toxicity of ammonia, chlorine, and copper to glochidia and juveniles of freshwater mussels. Presented at the joint meeting of the Midwestern chapter and Ozark-Prairie Region chapter of SETAC, La Crosse, WI, March 5, 2004

Wang N, Ingersoll CG, Greer IE, Whites DW, Roberts A, Dwyer FJ, Augspurger T, Kane C, Tibbott T, Neves RJ, Barnhart MC. Developing standard guidance for conducting toxicity tests with glochidia of freshwater mussels. Seminar presented at Peking University in Beijing China (June 16, 2004), at the Research Center for Eco-environmental Sciences in Beijing China (June 18, 2004) and at the Institute of Hydrobiology in Wuhan China (June 21, 2004).

Ingersoll C, Augspurger T, Barnhart C, Bidwell J, Bishop C, Black M, Cope G, Bringolf R, Dwyer J, Greer E, Keller A, Linder G, Neves D, Newton T, Pepin R, Roberts A, Roberts C, Salazar M, Samel A, Stephan C, Van Hassel J, Wang N, Watters T. ASTM International standard guide for conducting laboratory toxicity tests with freshwater mussels. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Ingersoll C, Wang N, Barnhart C, Neves D. Control survival of mussels in toxicity tests. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Wang N, Barnhart C, Bidwell J, Cope G, Geis S, Ingersoll C, Newton T. Precision of acute copper and ammonia toxicity tests conducted with glochidia or juvenile mussel. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Augspurger T, Dwyer J, Ingersoll C, Wang N. Acute toxicity of ammonia to freshwater mussels: How new data and emerging data quality guidelines affect data synthesis. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Wang N, Ingersoll C, Barnhart C, Neves D, Augspurger T, Dwyer J, Kane C. Acute and chronic effects of ammonia and copper on 2-month old juvenile mussels. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Wang N, Ingersoll C. Comparison of the acute toxicity of ammonia and copper of freshwater mussels and surrogate species. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Ingersoll C, Wang N, Augspurger T, Dwyer J, Kane C. Comparison of the sensitivity of glochidia and juvenile mussels to ammonia, metals, and chlorine. Mussel Toxicity Testing Workshop, U.S. Environmental Protection Agency. Chicago, Illinois. August 23-24, 2005.

Ingersoll CG, Augspurger T, Barnhart C, Cope G, Dwyer FJ, Bishop C, Neves RJ, Newton TJ, Roberts A, Wang N. Development of an ASTM standard guide for conducting laboratory toxicity tests with freshwater mussels. Presented at the 26th meeting of SETAC, Baltimore, MD, November 13-17, 2005.

Ivey CD, Ingersoll CG, Soucek DJ, Wang N. Influence of water hardness and chloride on the acute toxicity of sulfate to fatmucket (*Lampsilis siliquoidea*) and fingernail clams (*Sphaerium simile*). Presented at the 26th meeting of SETAC, Baltimore, MD, November 13-17, 2005.

March FA, Dwyer FJ, Ingersoll CG, Wang N, Augspurger, T. Evaluation of copper water quality standards and criteria for freshwater mussels. Presented at the 26th meeting of SETAC,

Baltimore, MD, November 13-17, 2005.

Wang N, Ingersoll CG, Greer IE, Whites DW, Dwyer FJ, Roberts AD, Augspurger T, Kane C, Neves RJ, Barnhart MC. Acute and chronic toxicity of copper, ammonia, and chlorine to early life stages of freshwater mussels. Presented at the 26th meeting of SETAC, Baltimore, MD, November 13-17, 2005.

Ward S, Augspurger T. 2005. Risk assessment of water quality in streams supporting federally-endangered freshwater mussels in North Carolina. Presented at the 26th meeting of SETAC, Baltimore, MD, November 13-17, 2005.

Appendix 2 - Publications

Published:

ASTM International. S*tandard guide for conducting laboratory toxicity tests with freshwater mussels* (E2455-05). In Annual Book of ASTM Standards. Vol 11.05. ASTM, West Conshohocken, PA.

Ingersoll C.G., N.J. Kernaghan, T.S. Gross, C.D. Bishop, N Wang, and A. Roberts. Laboratory Toxicity Testing with Freshwater Mussels. *In* J.L. Farris J.H. VanHassel eds. Laboratory Toxicity Testing of Freshwater Mussels. In Press. SETAC Press.

Proposed Publications (in author review or near completion)

Assessing contaminant sensitivity of early life stages of freshwater mussels (Unionidae) Editorial. Augsupurger, Dwyer, Kane et al

Assessing contaminant sensitivity of early life stages of freshwater mussels: Intra- and Interlaboratory variability in acute toxicity tests with glochidia and juvenile freshwater mussels. Wang et al.

Assessing contaminant sensitivity of early life stages of freshwater mussels: Acute toxicity of copper, ammonia, and chlorine. Wang et al

Assessing contaminant sensitivity of early life stages of freshwater mussels: Chronic toxicity of copper and ammonia. Wang et al.

Assessing contaminant sensitivity of early life stages of freshwater mussels: An evaluation of dervivation of water quality guidance for copper. March et al.

Assessing contaminant sensitivity of early life stages of freshwater mussels: Ecological risk assessment of copper, ammonia and chlorine. Ward et al.