Proceedings Summary Report

Mussel Toxicity Testing Procedures Workshop

August 23-24, 2005
Crowne Plaza-Chicago Metro Hotel, Chicago, IL

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DISCLAIMER

This report provides a basic overview of the United States Environmental Protection Agency (EPA)-sponsored workshop entitled “Mussel Toxicity Testing Procedures” held on August 23-24, 2005 at the Crowne Plaza-Chicago Metro Hotel in Chicago, Illinois. The information presented reflects summary notes taken during the meeting by the contractor, Great Lakes Environmental Center (GLEC), and is not intended to quote any speaker nor participant at the workshop. Additionally, EPA does not necessarily endorse the statements made by non-EPA participants.

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In July 2004, the United States Environmental Protection Agency (EPA) notified the public in the Federal Register (69 *FR* 41262) of its intent to re-evaluate the current aquatic life criteria for ammonia in response to recent studies suggesting that some freshwater mussel species may be more sensitive to ammonia exposure than the aquatic organisms considered in deriving the current ammonia criteria. As part of the Federal Register notice, EPA requested additional pertinent data on the acute or chronic toxicity of ammonia to aquatic life, particularly freshwater mussels, and scientific views on the use of mussel data that may help EPA in re-evaluating the ammonia criteria.

EPA convened a workshop on August 23-24, 2005 in Chicago, Illinois to provide a forum for information exchange on science issues related to freshwater mussel toxicity testing, including those raised in response to the Federal Register notice. At the workshop, approximately 60 interested citizens, scientific experts and government officials shared and discussed information, data, studies and the latest research findings relevant to mussel toxicity testing. Attendees had the opportunity to comment on this information, ask questions and otherwise participate in the information exchange. This was neither an EPA decision-making meeting nor a public hearing.

The workshop was divided into a total of seven sessions. During each session, scientific experts presented information regarding their specific area of expertise, which was followed by an audience question/comment period. Two additional sessions were included: a background information session at the beginning of the workshop and a summary session to conclude the workshop.

This report summarizes the presentations and discussions at the workshop.

AGENDA

Session I: Introduction
- Welcome
- Purpose of workshop, U.S. EPA
- Summary of issues raised in notice of intent to re-evaluate the aquatic life ambient water quality criteria for ammonia, U.S. EPA

Session II: Background
"Overview of North American freshwater mussels," Chris Barnhart, Southwest Missouri State University

Session III: Propagation and Culturing
"Methods for culturing freshwater mussels," Richard Neves, Virginia Polytechnic Institute and State University

"Propagation and Culture of Freshwater Mussels: *In Vitro*," Author: Cristi Bishop, EA Engineering; Presented by Richard Neves, Virginia Polytechnic Institute and State University

Session IV: Methods for Conducting Laboratory Toxicity Tests
"ASTM International standard guide for conducting laboratory toxicity tests with freshwater mussels," Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

"Control survival of mussels in toxicity tests," Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

"Precision of acute copper and ammonia toxicity tests conducted with glochidia or juvenile mussels," Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

Session V: Relative Sensitivity of Laboratory Toxicity Tests
"Acute toxicity of ammonia to freshwater mussels: How new data and emerging data quality guidelines affect data synthesis," Tom Augspurger, Ecological Services Field Office, U.S. Fish and Wildlife Service

"Acute and chronic effects of ammonia and copper on 2-month old juvenile mussels," Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

"Comparison of the acute toxicity of ammonia and copper of freshwater mussels and surrogate species ," Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

"Comparison of the sensitivity of glochidia and juvenile mussels to ammonia, metals, and chlorine," Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

"Comparison of the sensitivity of early life stages of native freshwater mussels to current use pesticides," Robert Bringolf, North Carolina State University

"Effects of hypoxia on juvenile mussels," Brianna Kaiser, Southwest Missouri State University

"The relative sensitivity of mussels to sulfates," David Soucek, Illinois Natural History Survey

Session VI: Lab-to-Field, Water Chemistry-to-Sediments
"Sensitivity of juvenile unionids to ammonia in sediment and water-only toxicity tests," Teresa Newton, Upper Midwest Environmental Sciences Center, U.S. Geological Survey

"Cultural importance of freshwater mussels to Native American tribes: a field study of caged mussels," Meredith Garvin, Tribal Environmental Management Services

"Potential sources of variability in unionid mussel metal burdens in the great rivers confluence area," David Soucek, Illinois Natural History Survey

"Residual effects of lead and zinc mining on freshwater mussels in the Tri-State Mining District," Robert Angelo, Kansas Department of Health and Environment

Session VII: Discussion of Salient Issues and Closing Remarks

<u>TUESDAY, AUGUST 23, 2005</u>

Session 1: Introduction

The mussel toxicity testing procedures workshop was initiated by Mr. Brian Thompson, from the water quality standards program at EPA Region 5, in Chicago, IL. Mr. Thompson welcomed all participants, and provided the basic format of the workshop. He noted that all correspondence at the workshop would be captured with an audio recorder to facilitate accurate representation of the discussions when the proceedings report was developed. He indicated that any objections to this activity could be voiced either at the present time or to him between sessions. Mr. Thompson then introduced Mr. Bill Swietlik, from the Health and Ecological Criteria Division at EPA Headquarters, in Washington, DC.

Mr. Swietlik welcomed all attendees and thanked the speakers and participants for attending. He then stated the purposes of the workshop: to discuss the science of mussel toxicity and to openly share scientific information regarding mussel toxicity. Mr. Swietlik then shared background information, including information on EPA's effort to re-evaluate the ammonia criteria. He noted that EPA requested additional data on issues of science in July 2004, and the public responded with both policy/programmatic comments and scientific comments. Mr. Swietlik reminded participants that this workshop was initiated to address the science issues raised in the data call and not policy/programmatic issues. He indicated that the primary science areas he hoped would be addressed in the workshop include: (1) mussel toxicity testing as a scientifically acceptable procedure; (2) the relationship between field and laboratory studies and (3) the reliability of mussel toxicity data for criteria development. He also specified the outcomes of the workshop, which included: (1) a summary of the workshop available to the public; (2) the information from the workshop will be considered in the possible derivation of the new ammonia criteria; (3) the results may be pertinent to other criteria and (4) the information may be applied to EPA's criteria methodology revisions.

Session 2: Background

2.1 "Overview of North American Freshwater Mussels," Mr. Chris Barnhart, Southwest Missouri State University

Unionoidae are a diverse and widespread group of animals. There are approximately 300 species found in North America. Although the greatest number of species are found in the Southeast, freshwater mussels are distributed throughout the United States. Mussels are often the dominant component of the invertebrate benthos in streams and are ecologically significant as suspension feeders. They continue to be harvested for the cultivated pearl industry. Many mussels are threatened by numerous human activities, and they are the most endangered group of animals in North America. Approximately 70% of North American freshwater mussel species are extinct, imperilled or vulnerable, and almost every genera has at least one species that is presumed extinct.

Historically, mussels were very common, and they were most often harvested for button making. In 1916, the commercial shell harvest in North America reached approximately 140 million pounds. Today pearls are often harvested from mussels raised in aquaculture facilities.

Mussels have a vulnerable life history because: (1) the long lived adults require stable benthic habitats in rivers; (2) they are filter feeders and therefore are exposed to water-borne pollutants; (3) they have a high-risk parasitic larval stage that are dependent on native fish hosts and (4) the tiny juvenile mussels are susceptible to drift, siltation, micropredators and sediment toxicity.

Mussels feed primarily through filtration of the water column or the sediment pore water. Food sources include detritus, bacteria, phytoplankton and zooplankton. Freshwater mussels may deposit and/or suspension feed; data suggesting both feeding methods exist. Filtration rates may approach 6 L/hour.

Reproduction in freshwater mussels occurs when the male-released spermatozeugmata are captured by the females, and the eggs are fertilized. Developing eggs are brooded in the gills of the female, and eventually develop into glochidia, the larval stage of the mussel. The glochidia, which have "teeth" that facilitate attachment to the host fish, are then released by the female mussel and attach to the gills of a host fish species. Glochidia survival time after being released from the female mussel ranges from less than two days to nearly 10 days. Once on the fish host, the glochidia are encysted by host tissue. Host fish suitability depends on the mussel species. Some mussel species have only one suitable host fish while others may have many.

Many different techniques are used by the female mussel to attract the fish host. Examples include broadcasting the glochidia, packaging glochidia as "bait", attracting hosts with anatomical "lures" and grabbing the fish by the head. The attached glochidia develop into juveniles, which eventually excyst and fall into the substrate and develop into adult mussels, which are often long-lived. Generally, growth rates are most dramatic in the first years of existence and then decline over time.

Every life stage of the mussel may be sensitive to pollutants, particularly the long-lived adult stage and the vulnerable larval stage. Mussels may be exposed to dissolved, suspended and deposited pollutants.

In summary, mussels are a highly diverse, historically abundant and widespread group. They are ecologically important and, as a group, are at a critical stage for conservation because of their large number of imperiled species.

2.1.1 Audience questions/comments

– Participant: What are mussel beds?
– Barnhart: Mussel beds are a concentration of mussels in a given stable area, usually 5 mussels/m^2. Mussel beds are often linked to reproductive success. In these areas, a loss of one species may affect other mussel species.

– Participant: Do mussels ever migrate?
– Barnhart: Mussels do migrate vertically and/or horizontally depending on the season or other environmental factors.

– Participant: Can juveniles tolerate low DO?
– Barnhart: Juveniles are sensitive to lowered DO concentrations. A subsequent presentation will address this in some detail.

– Participant: Do juveniles bury in the substrate?
– Participant 2: Yes, but not very deeply because it is difficult to filter in the substrate.

– Participant: Is there variation in life history across the country?
– Participant 2: Generally there is little variation.
– Barnhart: There are hermaphroditic mussels, but the vast majority reproduce in the manner described in the presentation. Additionally, most species require one specific fish host, while others are less selective.

– Participant: Why is there a low diversity (or fewer numbers) of mussels in the western United States relative to the Mississippi basin?
– Barnhart: There are large numbers of organisms, with less diversity because of unsuitable habitat.

– Participant 2: Dispersion is also an issue because of the geographic boundaries present in the western United States.
– Participant 3: Some other factors that can affect diversity include water temperature and urbanization.

Session 3: Propagation and Culturing

3.1 "Methods for Culturing Freshwater Mussels," Mr. Richard Neves, Virginia Polytechnic Institute and State University

Mussels are a diverse group of organisms and are most abundantly found in the southern and central United States, especially in Southeastern drainages. Mussels are beneficial in that they have the ability to improve water quality by filtering suspended materials and producing pseudo-feces, essentially capturing particles from the water and depositing them on the substrate.

The propagation facility at Virginia Polytechnic Institute and State University, which was funded by state and federal money, was developed to propagate federally endangered species. The facility has a pond that can simulate riverine conditions to raise the mussels, a fish holding component to infest fish hosts and an algae-culturing component to raise food for the mussels. The culturing occurs in a closed, re-circulating system so that the culture water can be reused. Not all propagation facilities are closed systems.

The steps of culturing the mussels are: (1) fish hosts and gravid females are collected; (2) the glochidia are removed from the gills of the gravid female mussel; (3) the fish are infested with the glochidia; (4) the fish are placed in rearing tanks and (5) the tanks are siphoned to remove the juveniles, which are then transferred to culturing systems with a natural substrate to grow to larger size over several months. Appropriate feeding rates and water chemistry parameters are maintained throughout the culturing process to promote optimum growth and development.

Other mussel culturing facilities exist (currently 13 total facilities) throughout the United States. Not all of these facilities use exactly the same culturing techniques, but most follow the same general methods. For example, one facility uses water extracted directly from a river rather than using well water to rear the mussels.

3.1.1 Audience questions/comments

- Participant: How specific is the algae food for the mussels?

- Neves: The algae are not highly specific, and most are green algae of suitable size for consumption by juveniles. Commercial preparations of algae are also available for mussel culturing needs.

3.2 "Propagation and Culture of Freshwater Mussels: *In Vitro*," Author: Ms. Cristi Bishop, EA Engineering; Presented by Mr. Richard Neves, Virginia Polytechnic Institute and State University.

In Vitro propagation of mussels began in the early 1900's primarily for the restoration of populations from exploitation for mother-of-pearl buttons. In 1926, the first transformation medium for mussel culture was developed. Since then, media have been developed that successfully transform glochidia. The main components of the artificial media are: (1) an ionic balance; (2) a whole protein; (3) serum replacements; (4) antibiotics/antimycotics and (5) other medium components like glucose or cod liver oil.

There are advantages and disadvantages to using *in vitro* culturing techniques. Advantages include: (1) more juveniles per unit effort and space; (2) juveniles can be produced when the host fish is unknown and (3) ability to observe growth and development directly. Disadvantages include: (1) the costs are generally greater; (2) microbial and fungal infestation can be a problem and (3) *in vitro* raised juveniles may be more sensitive to stressors than *in vivo* raised mussels for bioassays.

Today, *in vitro* propagation is somewhat successful; 20 species have been successfully propagated. Growth and survival of *in vitro* raised mussels are also relatively good. However, additional research is needed to answer the primary concern of physiological health of *in vitro* raised mussels.

3.2.1 Audience questions/comments

- Participant: Are the energy reserves between *in vitro* and *in vivo* raised mussels significantly different?
- Neves: There is a greater energy reserve in the *in vivo* raised mussels, as judged by available research results.

Session 4: Overview of Criteria Development

Chuck Stephan from U.S. EPA's Office of Research and Development, in Duluth, MN, gave a brief overview of the review of toxicity test results that were considered for use in the derivation of aquatic life criteria in the 1980s and early 1990s. The 1985 Guidelines gave three examples of reasons for rejecting test results and described acceptable acute and chronic toxicity tests for a variety of aquatic species. In addition, criteria documents that were based on the 1985 Guidelines identified test results that were not used and explained why. The current goal should be to ensure that all possibly useful results of aquatic toxicity tests are reviewed by adequately trained scientists using a written comprehensive procedure to evaluate quality.

Session 5: Methods for Conducting Laboratory Toxicity Tests

5.1 "ASTM International Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels," Mr. Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

Freshwater mussels are the most vulnerable aquatic animal group in the United States. There are many causes for their present decline including: (1) habitat alteration; (2) introduced species; (3) over-utilization; (4) disease; (5) predation and (6) pollution.

ASTM (American Society for Testing and Materials) is a global forum for developing consensus based standards, with nearly 30,000 world-wide members. These standards are developed as guides, specifications and test methods. The technical standards, developed by members, are then approved by consensus in a committee through a relatively rigid review process. The mussel toxicity testing ASTM method was developed by the committee E47 on biological effects and environmental fate, sediment assessment and toxicology subcommittee. The standard was recently approved and a revision to this document is currently being considered by the Task Group that developed the original standard. Additionally, the standard provides guidance on general methods and does not require that a single method be used to conduct toxicity tests with mussels.

Based on the recommendations in the ASTM manual, juvenile mussels cultured *in vitro* should not be used for numerous reasons unless it can be proven that *in vitro* and *in vivo* raised mussels react similarly. The specific test conditions for the 6 and 24-hour glochidia test and the acute and chronic juvenile mussel tests were described in this presentation (these conditions are included in the ASTM manual). The test acceptability criteria were also described in this presentation (and the ASTM manual). In the future, the following issues will be addressed in the ASTM manual's mussel toxicity testing method: (1) conditions for conducting sediment toxicity tests with glochidia or juvenile mussels and (2) conditions for conducting tests with adult mussels.

5.1.1 Audience questions/comments

- Participant: What is the acceptable variability in endpoints for reference toxicant testing?
- Ingersoll: Usually plus or minus two standard deviations is the standard for a reference toxicant test.

- Participant: What is the definition of a chronic versus an acute test?
- Ingersoll: Acute tests are typically conducted for up to 4 days with lethality measured as an endpoint. Chronic tests with mussels are conducted for 21 to 28 days with survival and growth measured as endpoints.
- Participant: Does handling the mussels affect test mortality?
- Ingersoll: No, mussels are relatively tough organisms.

- Participant: How long should one examine for mortality in a given toxicity test?
- Ingersoll: Technicians examine the mussels for approximately five minutes.

5.2 "Control Survival of Mussels in Toxicity Tests," Mr. Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

Based on the ASTM manual, the test acceptability requirements for mussel toxicity testing are as follows: (1) a 24-hour glochidia test must have 90% control survival; (2) a 96-hour juvenile test must have 90% control survival and (3) a 28-day juvenile test should have 80% control survival. These acceptability requirements were based on historic data, USGS laboratory data (ammonia and copper tests) and cultured juvenile mussel survival in the laboratory. Most of these data for most species of mussels showed greater than 90% survival in glochidia and juvenile control samples, however some species did not fare as well. USGS discovered that different water types were not significantly affecting control survival. USGS also discovered that cultured mussels have increased survivorship, and that mussels raised in buckets ("muckets in buckets") were able to live for a relatively long duration, provided they were fed properly.

5.2.1 Audience questions/comments

- Participant: ASTM and EPA should better define the health of the test organisms used in these toxicity tests.

- Participant: To test fitness, could one collect juveniles from the field and compare them to the lab raised animals?
- Ingersoll: This could be completed, but it would be difficult.

- Participant: Generally, lowered survival in control organisms is likely due to the "batch" of the mussels rather than species of the mussel.

- Participant: Glochidia tests should be at least 24 hours long, but some should have a longer duration because the glochidia of some species of mussels may naturally survive longer than 24 hours in the environment.
- Ingersoll: It is important to consider how long the organism is exposed to pollution "in the real world".

5.3 "Precision of Acute Copper and Ammonia Toxicity Tests Conducted with Glochidia or Juvenile Mussels," Mr. Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

The goal of this study was to examine the intra- and inter-laboratory precision of acute toxicity tests with glochidia and juvenile mussels using a round-robin study with five separate laboratories, each with varying degrees of toxicity testing experience with mussels. ASTM testing methods were used for glochidia and juvenile tests. For the 24- and 48-hour glochidia tests, fatmucket and mucket mussels were used, and copper, ammonia and chlorine were tested. For the 48- and 96-hour juvenile mussel tests, fatmucket mussels were used, and copper was tested.

The intra-laboratory EC50 coefficient of variation ranged from 13% to 36% for ammonia, copper and chlorine glochidia tests using muckets, and from 14% to 34% for glochidia tests using fat muckets. The inter-laboratory EC50 coefficient of variation for glochidia tests was 13% for the 24-hour test and 48% for the 48-hour test. Inter-laboratory variability of the EC50 for juvenile mussels, expressed as the coefficient of variation, was 22% for the 48-hour EC50s and was 42% for the 96-hour EC50s. Based on these coefficients of variation, which are very similar to other published studies using other common test organisms (e.g. EPA Methods for Measuring the Acute Toxicity of Effluents and Receiving Water to Freshwater and Marine Organisms, 1993), mussel glochidia and juvenile toxicity tests can be repeated reliably between and within laboratories.

5.3.1 Audience questions/comments

- Participant: For copper, juveniles and glochidia acted similarly (they had similar EC50s), but this was not true for other chemicals.
- Wang: Sometimes glochidia are more sensitive (compared to juveniles) when other chemicals are considered.

- Participant: Why were ammonia, copper and chlorine used in these tests?
- Wang: Copper is a common reference toxicant. Ammonia and chlorine are two chemicals of interest to the US Fish and Wildlife Service.
- Participant 2: Sodium chloride is also used as a reference toxicant during each test.
- Participant 3: It is difficult to determine organism health by reference toxicant tests.

- Participant: The presenter stated that the inter- and intra-laboratory coefficients of variation derived from this study were similar to those derived with other organisms in other studies. This is misleading; it's similar to comparing apples to oranges.
- Participant 2: These data were given for comparison to show the similarity between USGS and EPA data.

Session 6: Relative Sensitivity of Laboratory Toxicity Tests

6.1 "Acute Toxicity of Ammonia to Freshwater Mussels: How New Data and Emerging Data Quality Guidelines Affect Data Synthesis," Mr. Tom Augspurger, Ecological Services Field Office, U.S. Fish and Wildlife Service

In 2003, acute ammonia toxicity data were compiled by the USFWS because of concerns with ammonia toxicity to mussels, and because the state of North Carolina has 7 threatened or endangered mussel species and no ammonia water quality standard. These data were collected from a variety of sources using data quality objectives outlined in Augspurger et al. 2003. Acute toxicity data were normalized for pH and genus mean acute values (GMAVs) were then calculated. Based on these data, it was determined that mussels were very sensitive to ammonia.

Since that study, a new ASTM standard on mussel toxicity testing has been approved, and new ammonia data for mussels are available. Therefore, USFWS compared the 2003 dataset to datasets developed using: (1) the 2003 dataset as modified with the data quality objectives of the ASTM standard and (2) a 2005 dataset with all new ammonia toxicity data that also met the data quality objectives of the ASTM standard. Using the new test acceptability criteria from the ASTM standard, certain data were removed from the analysis, including 48-hour glochidia exposures for two mussel species, and tests with control survival between 80 and 90%.

All three datasets showed that mussels were a sensitive group based on GMAVs. Therefore, it can be concluded that mussel toxicity data can be useful for water quality management purposes, among other things. It was also noted that agencies can apply different data quality objectives based on their need. Additional similar work is being completed for copper, and will be available at SETAC in November 2005.

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

6.1.1 Audience questions/comments

- Participant: When were the copper studies that you referred to completed?
- Augspurger: They were completed in the late 1980s through 2005.

6.2 "Acute and Chronic Effects of Ammonia and Copper on 2-Month Old Juvenile Mussels," Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

The goal of this study was to evaluate acute and chronic toxicity of ammonia and copper to 2-month old juvenile mussels of rainbow mussel, fatmucket or pink mucket. The 2-month old juveniles were used because this is a critical life stage and they generally show acceptable control survival in 28-day toxicity tests. Three test species were examined using both static renewal and flow through ASTM methods, with the endpoints of survival at day 4 and survival and growth at day 28.

For both test chemicals (copper and ammonia), control survival was greater than 90% for the 4-day tests and greater than 88% for the 28-day tests. The 4-day ammonia EC50 was highest for all species under both renewal and flow-through procedures over 10- or 28-day exposures. For the rainbow mussel, survival and growth were affected negatively by increased ammonia concentrations. The chronic value (geometric mean of the no-observed-effect concentration and the lowest-observed-effect concentration) for the rainbow mussel based on survival and growth was <0.5 total ammonia mg N/L, which is less than EPA's water quality criteria.

The 4-day copper EC50 was highest for all species under the renewal and flow-through procedures. Both survival and growth were negatively affected with increased copper concentrations, however growth was more sensitive than survival. The chronic value was 4.4 µg Cu/L for rainbow mussel or 8.8 µg Cu/L for fatmucket, which was also lower than EPA's chronic ambient water quality criteria.

Based on these data, it can be concluded that: (1) growth was generally more sensitive than survival in chronic tests; (2) effect concentrations decreased with exposure period for both chemicals; (3) the 96-hour copper EC50s for 2-month old juvenile mussels were above the EPA's acute water quality criteria, whereas the 96-hour ammonia EC50s for 2-month old juvenile mussels were at or below the water quality criteria and (4) chronic effect concentrations were below the EPA chronic ambient water quality criteria for copper and ammonia.

6.2.1 Audience questions/comments

- Participant: Growth rates of mussels will always show natural variability, so we must pick the peak exist date for juveniles in toxicity testing to reduce variability in control growth throughout the toxicity tests.

- Participant: Why not measure weight rather than length?
- Participant 2: It's difficult to measure a weight with these small organisms.

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6.3 "Comparison of the Acute Toxicity of Ammonia and Copper on Freshwater Mussels and Surrogate Species," Mr. Ning Wang, Columbia Environmental Research Center, U.S. Geological Survey

The goal of this study was to evaluate the acute sensitivity of glochidia and newly-released juvenile mussels to copper and ammonia, and compare these data to commonly tested surrogate species, including *Daphnia magna*, *Ceriodaphnia dubia*, *Hyalella azteca*, *Pimephales promelas* and *Oncorhynchus mykiss*. ASTM mussel toxicity testing methods were followed for 24- or 48-hour glochidia and 48- and 96-hour juvenile mussel tests.

Based on these studies, it was concluded that: (1) newly-released juvenile mussels and glochidia tend to be more sensitive to ammonia than surrogate species; (2) *C. dubia*, newly-released juvenile mussels and glochidia were the most sensitive to copper compared to other test species and (3) the EC50 of copper and ammonia for glochidia and newly-released juvenile mussels is at or below EPA acute ambient water quality criteria.

6.3.1 Audience questions/comments

None.

6.4 "Comparison of the Sensitivity of Glochidia and Juvenile Mussels to Ammonia, Metals and Chlorine," Mr. Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey

The goal of this study was to determine which life stage of mussel is the best for toxicity testing, and which may be more sensitive to certain chemicals, including ammonia, chlorine, copper, zinc, lead and cadmium. Typical ASTM endpoints were measured. However, it is important to consider the ecological relevance of such endpoints (i.e. glochidia tests greater than 24 hours may not be relevant for all species, but glochidia tests greater than 24 hours may be relevant for certain species of mussels).

The 2-day glochidia and 4-day juvenile EC50s observed during the copper, ammonia and chlorine tests showed the strongest relationship, while 2-day glochidia tests with zinc, lead and cadmium showed less sensitivity then juvenile fat mucket 4-day tests. Based on these data, it can be concluded that short term glochidia tests may be useful for some chemicals, however the response of juveniles may be more significant from an ecological standpoint.

There are additional research needs in this area, including the evaluation of relative endpoint sensitivity in acute and chronic toxicity tests for glochidia, brooding females, various stages of juvenile mussels and adult mussels. The endpoints measured in these tests should include: (1) glochidia survival and ability to transform on a host fish; (2) survival, growth and behavior of juvenile mussels and (3) survival, growth, behavior, bioaccumulation and reproduction of adult mussels.

6.4.1 Audience questions/comments

- Participant: Have you ever examined the difference in health between lab and field raised mussels?
- Ingersoll: Organism health is difficult to define, but the growth rates between the lab and field raised mussels appear to be similar.

- Participant: Are any west coast species ever included in these toxicity studies?
- Ingersoll: Most mussels that have been tested are from southeastern or central North America.

- Participant: Do mussels exposed to toxins recover after the toxicity tests have been performed?
- Ingersoll: Yes. Mussels can be transferred to clean water to observe recovery. However, juveniles may be less likely to recover. Additionally, mussels can (and do) die after being placed in the clean water.

- Participant: It seems as though there is a general concern among participants with the health of lab verses field raised organisms.

- Participant: Mussels probably can’t be maintained in a lab indefinitely, and this is a concern. Additionally, there may not be enough resources to determine how long aquacultured mussels have lived in the laboratory.
- Participant 2: It is possible to raise mussels long term in a hatchery environment (it has been done in Virginia).

- Ingersoll: Does EPA use lab raised organisms that have not completed all life cycles in a lab setting?

- Participant: Yes, but there are inherent concerns in using these organisms. Additionally, mussels are a unique group relative to these organisms because they have a parasitic life stage.

- Participant: Mussels are a sensitive group. We are at this workshop because of this fact.

- Participant: Has work been completed comparing toxicity and/or health in different culture waters or natural waters?
- Participant 2: In Virginia, where there has been a long success with lab cultured mussels, no substantive work has been completed to examine the toxicity in culture water versus reconstituted water.

6.5 "Comparison of the Sensitivity of Early Life Stages of Native Freshwater Mussels to Current Use Pesticides," Mr. Robert Bringolf, North Carolina State University

Mussels comprise one of the most imperiled faunal groups in the world, and there has been a sharp decline in the last 50 years. Pesticides are one of the factors that may be leading to the recent decline of mussels because pesticide application often coincides with mussel reproduction, and therefore may affect early life stages. The goal of the study, therefore, was to generate toxicology data for a suite of technical grade and formulation pesticides relevant to early life stages of freshwater mussels.

For technical grade pesticides (atrazine, fipronil, pendimethalin and permethrin), ASTM methods were utilized for toxicity tests with 6 species of glochidia and 5 species of juveniles. Chlorpyrifos was the only technical grade pesticide that caused acute toxicity with glochidia or juveniles, and the toxicity was similar for the two lifestages. For acute tests with all other technical grade pesticides completed with glochidia and juveniles, there was low or no toxicity. A chronic test was also completed with *L. siliquoida* using technical grade atrazine. Growth and survival were negatively affected at environmentally relevant concentrations.

For formulation pesticides, using ATSM approved methods, Roundup® was substantially more acutely toxic to both glochidia and juvenile *L. siliquoida* than was the technical grade glyphosate. LC50s for other formulation grade pesticides were similar to those reported for the technical grade pesticides.

The results of this study indicate that: (1) some pesticides and pesticide formulations are acutely toxic to glochidia; (2) some pesticide formulations may be more toxic than technical grade pesticides; (3) glochidia and juveniles have a similar sensitivity to pesticides; and (4) chronic effects of atrazine on survival and growth warrant further investigation. Additional research includes the exploration of survival and growth in chronic juvenile tests and the effects of pesticides on adult mussels.

6.5.1 Audience questions/comments

- Participant: Atrazine kills algae, so were the chronic effects observed a result of reduced food availability?
- Bringolf: It is doubtful because algae were added daily; however, the current ASTM protocol recommends use of prepared, non-living algal cells as the diet for chronic tests. This type of algae would not be affected by an herbicide and would not confound the results.

6.6 "Effects of Hypoxia on Juvenile Mussels," Ms. Brianna Kaiser, Southwest Missouri State University

Dissolved oxygen (DO) is affected by many environmental factors, including temperature, aquatic photosynthesis, reaeration, BOD, groundwater/surface water mixing and hypolimnetic releases. Mussels are affected by both water column and interstitial water DO, the latter of which tends to be lower and more variable. DO concentration in the interstitial water is altered by flow, sediment size and sediment oxygen demand. Mussels are affected by altered DO concentrations; lowered DO may limit aerobic aeration, reduce growth, cause behavioral responses or mortality.

In this study, 4 species of juvenile mussels were chronically exposed to seven concentrations of DO for up to 49 days at a constant water temperature. Mussels were not fed, and survival was assessed at 2-day intervals throughout the duration of the study. The results of the study indicate that survival was different depending on the species of mussel, and the critical DO (that point where the predicted days to death of the mussels was equal to 90% of that at 7 mg/L) for the four species was 0.65 mg/L at 0 months. This critical DO increased with juvenile age.

Future studies will examine the following issues: (1) hypoxia affects on growth of juveniles; (2) influence of temperature on hypoxia tolerance and (3) hypoxia tolerance of brooding females and brooded embryos.

6.6.1 Audience questions/comments

- Participant: Did not feeding the mussels affect the results?
- Kaiser: Yes, not feeding may have affected the results.

- Participant: What is the mussel's syphon?

- Participant 2: It's a part of the mussel that may be extended above the surface, but it is not very pronounced.

6.7 "The Relative Sensitivity of Mussels to Sulfates," Mr. David Soucek, Illinois Natural History Survey

There are few data describing the toxicity of sulfates on invertebrates or the effects of hardness and chloride on sulfate toxicity. Therefore, *C. dubia*, *H. azteca*, *C. tentans*, *S. simile* and *L. siliquoidea* were exposed to various concentrations of sulfates.
Juvenile mussels were the least sensitive organism to sulfates when chloride concentrations in moderately hard reconstituted water ranged between 25 and 33 mg/L and hardness was near 100 mg/L, and alterations to hardness and chloride concentrations on sulfate toxicity to fatmuckets was not apparent. Variation in water chloride concentration and hardness did affect sulfate toxicity to *C. dubia* and *H. azteca*. Increased hardness reduced the toxicity of sulfate. When chloride concentration was less than 25 mg/L, increased chloride increased sulfate toxicity to both species. However, at concentrations between 5 and 25 mg/L, increased chloride concentrations reduced toxicity of sulfate to *H. azteca*.

To tease apart these relationships for the mussel, additional chemistry characteristics of the water were examined, specifically the Ca:Mg ratio. When examining this ratio, it was determined that sulfate was more toxic to juvenile fatmuckets in water with a lower Ca:Mg ratio at the same water hardness than in water with an elevated Ca:Mg ratio.

6.7.1 Audience questions/comments

- Participant: For *H. azteca*, osmoregulation was likely the problem rather than the direct toxicity of sulfates.

- Participant: This study suggests that mussels may be exposed to increased concentrations of toxicants and still survive, provided that their health is adequate and that the proper mineral water chemistry is present.

Session 7: Lab-to-Field, Water Chemistry-to-Sediments

7.1 "Sensitivity of Juvenile Unionids to Ammonia in Sediment and Water-Only Toxicity Tests," Ms. Teresa Newton, Upper Midwest Environmental Sciences Center, U.S. Geological Survey

Much of the toxicity testing now completed in the laboratory is due to concerns with field studies. Mussels can be exposed to contaminants through many routes, including overlying water, sediments, pore water, fish hosts and food. Adult mussels likely suspension and deposit feed, while juveniles may pedal feed.

We continued this work by conducting a series of 96-hour sediment and 96-hour water-only laboratory tests with two species of juvenile mussels. These tests were similar in design to those mentioned above, except that LC50s and EC50s were generated based on TAN (Total Ammonia as Nitrogen) concentrations in (1) pore water; (2) water overlying the sediments in the sediment exposures and (3) overlying water in the water-only exposures. Differences in toxicity between species were minimal. Survival exceeded 95% in the controls across all tests. LC50s were similar when based on concentrations in the overlying water in the water-only tests and the pore water in the sediment tests. However, LC50s were about 4-fold greater when based on concentrations in the water overlying the sediments in the sediment exposures, even though TAN concentrations in the water overlying the sediments were only about 20% greater than in the pore water. It is unclear why the LC50s were greater in the water overlying sediments. The pH in the exposures varied among the overlying water (8.0), the sediment-water interface (7.8) and at a sediment depth of 2.5 cm (7.2) - the depth the juveniles were placed. However, if differences in LC50s were driven by differences in pH, the LC50s should be lower in the higher pH exposures. Because pH can affect toxicity, understanding the location of the mussels in the sediment exposures is important in understanding the toxicity of ammonia to juveniles. Growth of mussels in the controls was consistently suppressed in the water-only exposures, relative to the sediment exposures. In the sediment exposures, growth was consistently reduced with increasing TAN concentrations; whereas in the water-only exposures, there was no consistent relation between growth and TAN concentrations.

Additionally, a series of *in situ* sediment tests were completed in the St. Croix River with juvenile *L. cardium*. In these tests, survival was negatively correlated and growth was positively correlated with TAN concentrations in pore water. These results likely differ from the laboratory studies for the following reasons: (1) in the laboratory, mussels received a constant supply of ammonia, whereas in the field, ammonia may be pulsed; (2) there was considerable spatial and temporal variability in ammonia in the field and (3) presumably due to a recent flood three months prior to this study, ammonia concentrations in the St. Croix River were quite low and thus, there was not a large enough range in ammonia concentrations to see an effect on the test endpoints.

7.1.1 Audience questions/comments

- Participant: Was a DO profile ever completed in the sediment during the laboratory tests?

- Newton: No, we completed DO profiles in the water, but not in the sediments.

7.2 "Cultural Importance of Freshwater Mussels to Native American Tribes: a Field Study of Caged Mussels," Ms. Meredith Garvin, Tribal Environmental Management Services

Historically, mussels were very important to native American tribes; they were often used in tribal religious ceremonies and even for food. Recently Tribal elders and other concerned tribal members noted a decline in mussels in the Spring River, located in the Tri-State Superfund site in northeastern Oklahoma. Because of this, a caged mussel study was initiated in the river to determine the effects of the drainage, containing elevated levels of metals, on mussels. Adult mussels, measured and weighed, were placed in each of five cages and placed in the river at varying distances downstream of the Superfund site. At designated intervals, survival and growth were recorded for each cage, and tissues were collected and sent for metal burden analyses. At the time of the presentation, no data were available, however all mussels in the Spring River were dead after 9 months.

7.2.1 Audience questions/comments

- Garvin: A sediment and water quality analysis study was also completed as part of this study. These results are also not available.

- Participant: Were the sediment types consistent at all sites?
- Garvin: To some degree they were similar, but the mussel cages were placed at the same depth to attempt to address this issue.

- Participant: What was the size distribution of the adult mussels?
- Garvin: All were relatively the same size, with some degree of variability.

- Garvin: The data will be made available at a later date.

7.3 "Potential Sources of Variability in Unionid Mussel Metal Burdens in the Great Rivers Confluence Area," Mr. David Soucek, Illinois Natural History Survey

Mussels can absorb contaminants through different routes, including the water column, sediment pore water and food. The objectives of this study were: (1) to determine the metal levels in mussels collected from the Mississippi River and Illinois River confluence area; (2) to address spatial and interspecific variability in tissue metal burdens and (3) assess the risk of metals to mussels and their consumers. Three sites were examined; two in each river and one below the confluence. Mussels at these sites were weighed, measured and the tissues were removed to examine tissue metal burdens.

The data suggest that there was no difference in mussel length, height or age between the three sites. ^{15}N enrichment, an indication of consumer trophic level, was elevated at the three sites relative to previously published data. Mussels collected from the most downstream site generally had the most elevated tissue metal burden, and certain species of mussels had elevated tissue metals relative to other species. Differences in mussel tissue burdens between species may be explained by differences in mussel age (size) and differences in feeding. For example, older mussels had a greater metal tissue burden, and mussels with tissues having a higher ^{15}N enrichment had greater tissue metal burdens. Tissue metals in some species of mussels were near published thresholds.
Future studies will examine: (1) contaminant sources and bioavailability issues; (2) more sensitive measures of mussel stress to determine effects of Cu, Se and Zn and (3) species specific filtration and assimilation rates and their effect on ^{15}N enrichment and contaminant uptake.

7.3.1 Audience questions/comments

- Participant: Are mussels able to reject Zn contaminated particles through pseudo-feces?
- Soucek: There are no data to refute nor accept this question.

7.4 "Residual Effects of Lead and Zinc Mining on Freshwater Mussels in the Tri-State Mining District," Mr. Robert Angelo, Kansas Department of Health and Environment

Historically, the Tri-State Mining district Superfund site, including portions of southeastern Kansas, southwestern Missouri and northeastern Oklahoma, has been polluted with various metals. This widespread heavy metal contamination continues today. The goals of this study were to: (1) examine the occurrence and distribution of native mussels in the Spring River basin; (2) evaluate densities, dominant taxa and age classes of mussels in selected reaches; (3) determine mussel metal tissue burdens and (4) compare community and tissue burdens to environmental concentrations.

Mussel communities and prevailing metal contaminant levels in fluvial sediment and surface water were surveyed at 23 sites within the basin, and a total of 29 species were found in the Spring River (13 of which are listed as a special concern species). The number of mussels at each site generally decreased from upstream to downstream. In the tributaries of the Spring River, a total of 23 species were observed (8 of which are listed as a special concern species and 2 of which were not found in the Spring River). The relative abundance of mussels varied by site, and mussels were seemingly absent from sites exhibiting the highest concentrations of cadmium, lead and zinc in sediment and surface water.

Lead, cadmium and zinc concentrations in mussel tissues increased dramatically downstream of the confluence with Center Creek. Tissue metal burden in mussels was correlated with tissue metal burdens in the Asian Clam, which were found in abundance in the surveys. Within the basin, increases in sediment lead, cadmium and zinc concentrations were strongly correlated with mussel species richness and metal contaminant burdens in mussel tissue.

Based on this study, it can be concluded that: (1) The Spring River basin supports a rich fauna of mussels, but drainage from mined areas negatively impacts mussel richness; (2) mussel densities and species richness in the Spring River declined abruptly below the confluence of Center and Turkey creeks, two heavily impacted tributaries no longer supporting mussel populations in their lower reaches; (3) mussels downstream of mining areas have elevated levels of metals in their tissues and (4) tissue metal burdens correlate well with environmental concentrations.

7.4.1 Audience questions/comments

- Participant: Are these data published?
- Angelo: No, not yet.

- Participant: It was stated that the Asian clam could be used as a surrogate for native mussel tissue burden analyses. Why?
- Angelo: Asian clams are much more abundant, and there is a strong relationship between Asian clam and native mussel tissue metal burdens. The difficulty is in trying to convince people of this relationship.

- Participant: Why aren’t biomarkers used in these type of studies?
- Angelo: Using biomarkers is expensive. Two or more agencies working together may be able to alleviate this financial burden.

- Participant: Have you worked with Gastropods in this system?
- Angelo: To some degree we have, but not a great deal. The focus has been on mussels.

- Participant: Are there any thoughts of developing an IBI for mussels?
- Angelo: Yes, to some degree.

Session 8: Discussion of Salient Issues and Closing Remarks

Mr. Bill Swietlik closed the workshop by thanking all attendees and speakers. He then reviewed the purpose and focus of the mussel toxicity testing workshop. Overall, Mr. Swietlik was pleased with the progress and outcomes of the workshop. He believed that most of the goals of the workshop were addressed, yet indicated the potential for additional work to better understand the relationship between mussel toxicity testing and field response.

Mr. Brian Thompson then thanked all individuals for attending, and opened the floor to comments or questions by the attendees.

8.1 Audience questions/comments

- Participant: It was unfortunate that many state representatives did not attend the workshop in light of the implications of the possible ammonia criteria change.
- Participant 2: The states need to be involved with any policy issue changes.

- Participant: Will the workshop materials be made available to the public?
- Thompson: Yes, the proceedings, slide presentations, and abstracts will be posted on EPA's website.

- Participant: If the ammonia criteria is changed, it must be done quickly because many species of mussels are currently being lost.

- Participant: There needs to be a mechanism for EPA's defined research needs to further the mussel toxicity testing process.
- Swietlik: Issues needing more research include the health of mussels used in toxicity testing and lab versus field studies.
- Swietlik: Potential additional research needs discussed at the workshop include: (1) effects of ammonia on mussels at different pH values; (2) ammonia sensitivity in mussels of different ages; (3) effects of temperature on ammonia toxicity to mussels; (4) data showing relationship between degraded sites and absence of mussels needs to be published; (5) vertical placement of mussels in sediment needs to be investigated, including exposure to sediment versus water column ammonia; (6) there is a need for more information on different categories of toxicants with alternative modes of action, and their effects on mussels; (7) more data on west coast species of mussels and (8) best feeding methods for chronic tests.

Mr. Thompson then gave a final thanks to all attendees, the presenters and GLEC.