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Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels[1]

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1. Scope

1.1 This standard guide describes methods for conducting laboratory toxicity tests with early life stages of freshwater mussels including glochidia and juvenile mussels in water-only exposures (Annex A1). Future revisions to this standard may describe methods for conducting toxicity tests with *(1)* adult freshwater mussels and *(2)* contaminated sediments using various life stages of freshwater mussels.

1.2 Many factors are cited as potentially contributing to the decline of freshwater mussel populations in North America. Of the nearly 300 taxa of freshwater mussels in North America, 70 species (23 %) are listed as endangered or threatened and another 40 species (14 %) are candidates for possible listing (Williams et al 1993 (**1**); Neves 1997, 2004 (**2, 3**)).[2] Habitat alteration, introduction of exotic species, over-utilization, disease, predation and pollution are considered causal or contributing factors in many areas of the United States (Neves et al 1997) (**4**). Over the past decade, there have been over 75 published studies conducted that have evaluated the role of contaminants in the decline of populations of freshwater mussels (Kernaghan et al 2005) (**5**). In these studies, early life stages of mussels of several species are highly sensitive to some metals and ammonia in water exposures when compared to many of the most sensitive species of other invertebrates, fish, or amphibians that are commonly used to establish U.S. Environmental Protection Agency Water Quality Criteria (WQC; Augspurger et al 2003 (**6**), Keller et al 2005 (**7**), Kernaghan et al 2005 (**5**); USGS (2005a,b) (**8, 9**) section 1.5). Importantly, results of these previous studies indicate WQC for individual chemicals established for the protection of aquatic organisms may not be adequately protective of sensitive stages of freshwater mussels.

1.3 *Summary of Life History of Freshwater Mussels:*

1.3.1 Freshwater mussels are bivalve mollusks belonging to the family Unionidae or Margaritiferidae (section 10.1). Adults are sedentary animals, spending their entire lives partially or completely burrowed in the bottoms of streams, rivers, or lakes. Adult mussels are filter feeders, using their gills to remove suspended particles from the water column. The microscopic, juvenile stage uses foot (pedal) feeding to some degree for the first several months of their lives, feeding on depositional materials in pore water of sediment, including bacteria, algae, and detritus. Freshwater mussels have an unusual and complex mode of reproduction, which includes a brief, obligatory parasitic stage on fish or other host organisms called glochidia (Fig. 1).

1.3.2 The successful transfer of mature glochidia to a suitable host constitutes a critical event in the life cycle of most freshwater mussels. Once the glochidia are released from the female, the glochidia need to attach to the gills or the fins of an appropriate fish host and encyst to complete development. Although glochidia may survive for months during brooding in the female mussel, glochidia typically survive for only a few days after release unless the glochidia reach a compatible host. Encystment on the host occurs by overgrowth of host tissue. Metamorphosis of juvenile mussels on the fish host occurs within days or weeks, depending on species and temperature. Host fish specificity varies among mussels. While some mussel species appear to require a single host organism, other species can transform their glochidia into juvenile mussels on several species of host fish. Following proper host infestation, glochidia transform into microscopic juveniles and excyst (drop off) and settle into suitable habitat to survive. The transformation of glochidia to juveniles results in the development of internal organs necessary for self-sustained existence as a benthic organism.

1.3.3 Newly-transformed juvenile mussels have a life style different from adult mussels. Transformed juvenile mussels may be at the sediment-water interface or may burrow several centimeters into sediment and rely on water percolating between substrate particles of sediment for food and oxygen. Newly-transformed juvenile mussels feed using ciliary currents

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

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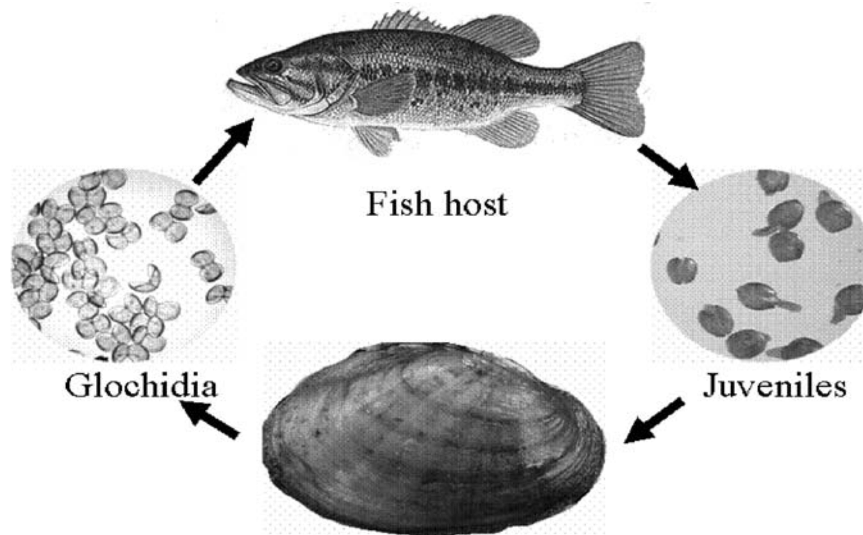

FIG. 1 Life Cycle of a Freshwater Mussel (Chris Barnhart, Missouri State University, Springfield, MO)

on the foot and mantle. Older juvenile and adult mussels likely use different food types when living in different microenvironments. Given that glochidia and juvenile mussels are ecologically and physiologically different from adult mussels, protection of habitat quality of adult life stages may not be protective of glochidia or juvenile life stages of freshwater mussels. Distributions of adult mussels are dependent both on the presence of host fish and on microhabitat conditions. Efforts to assess effects of contaminants on mussels need to evaluate potential exposure to host fish in addition to exposure to each unique life stage of freshwater mussels.

1.4 *Summary of Toxicity Testing Conditions:*

1.4.1 Section 4 provides a summary of conditions for conducting toxicity tests with glochidia and juvenile mussels. Annex A1 provides guidance for conducting water-only toxicity tests with glochidia and juvenile mussels. Recommended test conditions for conducting these toxicity tests are based on various published methods outlined in Table A1.1 and Table A1.4 in Annex A1 and are based on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels (section 16.5). Glochidia and juvenile mussels are only available on a seasonal basis. Section 10 describes procedures for collecting adult female mussels from the field to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host organism.

1.4.2 In the field, mussels may be exposed to contaminants in water, sediment, or food. This standard only addresses effects associated with exposure of mussels to contaminants in water.

1.4.3 Guide E724 describes procedures for conducting acute 48-h toxicity tests with embryos or larvae of saltwater bivalve mollusks. Endpoints measured in Guide E724 include survival or shell deposition. Procedures outlined in Guide E724 may be useful in helping to design studies for conducting toxicity tests with freshwater mussels as outlined in Annex A1.

1.4.4 Results of tests, even those with the same species, using procedures different from those described in Annex A1 may not be comparable. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting toxicity tests with aquatic organisms. If tests are conducted with procedures different from those described in this standard, additional tests are required to determine comparability of results. General procedures described in this standard might be useful for conducting tests with other aquatic organisms; however, modifications may be necessary.

1.5 *Summary of Results of Toxicity Tests Conducted with Freshwater Mussels:*

1.5.1 Keller et al (2005) **(7)** summarized results of acute laboratory toxicity tests conducted with glochidia and juvenile mussels described in 16 published studies. Freshwater mussels tended to be less sensitive in exposures to some pesticides and other organic compounds compared to other commonly-tested aquatic organisms. In contrast, Keller et al (2005) **(7)** concluded that U.S. Environmental Protection Agency (USEPA) water quality criteria (WQC) for some metals and ammonia may not be protective of freshwater mussels.

1.5.2 Augspurger et al (2003) **(6)** evaluated ammonia toxicity data generated for glochidia and juvenile of freshwater mussels in laboratory toxicity tests. Specifically, these toxicity data were used to estimate concentrations that would not likely be harmful to mussels in acute and chronic exposures and were used to evaluate the protectiveness of the WQC for ammonia. Results of acute toxicity tests (24 to 96 h) for 10 species in 8 genera were used to calculate genus mean acute values (GMAVs) ranging from 2.56 to 8.97 mg/L (total ammonia as N at pH 8 at 25°C). The freshwater mussels are at the sensitive end of the range when added to the GMAVs from the database used to derive the acute WQC for ammonia. Recalculation of the criteria maximum concentration (CMC) including these mussel data resulted in a CMC 75 % lower than the CMC of 5.62 mg/L total ammonia as N at pH 8 at 25°C (for application when salmonids absent). No chronic ammonia toxicity data (for example, 21 to 28-d exposures) were available for freshwater mussels; however, when a range of acute to chronic ratios were used to estimate a criteria continuous concentration

(CCC), the estimated CCC for mussels was 20 to 75 % less than the CCC of 1.24 mg/L total ammonia as N at pH 8 and 25°C. Hence, Augspurger et al (2003) (**6**) concluded that the acute and chronic WQC for ammonia may not be protective of freshwater mussels.

1.5.3 Milam et al (2005) (**10**) conducted a series of 24-h acute toxicity tests with glochidia of six freshwater mussel species, *Leptodea fragilis*, *Utterbackia imbecillis*, *Lampsilis cardium*, *Lampsilis siliquoidea*, *Megalonaias nervosa*, and *Ligumia subrostrata*, and with two commonly-tested organisms, *Ceriodaphnia dubia* and *Daphnia magna*. Chemicals selected for testing (carbaryl, copper, 4-nonylphenol, pentachlorophenol, permethrin, and 2,4-dichlorophenoxyacetic acid [2,4-D]) represented different chemical classes and different toxic modes of action (Dwyer et al 2005a,b) (**11, 12**). No single chemical elicited consistently high or low toxicity; however, carbaryl and 2,4-D were generally the least toxic to the species tested. Milam et al (2005) (**10**) concluded that the toxicity data generated with *C. dubia* and *D. magna* were relatively protective of the range of sensitivities exhibited by glochidia of the mussels species tested. However, toxicity data generated with the commonly-tested *U. imbecillis* were not always protective of the range of sensitivities exhibited by the other mussel species tested.

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1.7 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D4447 Guide for Disposal of Laboratory Chemicals and Samples
E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods
E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method
E724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes
E1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates
E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates
E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates
E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines
E1850 Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests
IEEE/ASTM SI 10 Standard for Use of the International System of Units (SI) (the Modernized Metric System)

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that a test ought to be designed to satisfy the specified conditions, unless the purpose of the test requires a different design. "Must" is used only in connection with the factors that relate directly to the acceptability of a test. "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although the violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable,"

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions*—For definitions of other terms used in this standard, refer to Guides E729 and E1241 and Terminology E943 and D1129. For an explanation of units and symbols, refer to Practice E380. A listing of the common and scientific names of freshwater mussels in North America can be found in AFS (1998) (**13**).

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *acute test*—a comparative study in which organisms, that are subjected to different treatments, are observed for a short period usually not constituting a substantial portion of their life span (for example, 24- to 96-h exposures).

3.3.2 *chronic test*—a comparative study in which organism, that are subjected to different treatments, are observed for a long period or a substantial portion of their life span (for example, 21- to 28-d exposures). There is no test duration that represents a distinct boundary between acute and chronic test durations for any species. Although acute or chronic test procedures may specify standard duration(s), these durations have not been intended to define an acute:chronic boundary. Acute tests often utilize mortality as the only measure of effect; chronic tests usually include additional measures of effect such as growth or reproduction.

3.3.3 *EC50*—a statistically or graphically estimated concentration that is expected to cause one or more specified effects in 50 % of a group of organisms under specified conditions.

3.3.4 *IC50*—a point estimate of the toxicant concentration that would cause a 50 % reduction in a non-quantal measurement such as fecundity or growth.

3.3.5 *LC50*—a statistically or graphically estimated concentration that is expected to be lethal to 50 % of a group of organisms under specified conditions.

3.3.6 *lowest-observed-effect concentration (LOEC)—in a toxicity test*, the tested concentration of one or more chemicals immediately above the highest tested concentration that did not result in a statistically significant change in the particular toxicological variable compared to that value in the control.

3.3.7 *no-observed-effect concentration (NOEC)— in a toxicity test*, the test concentration of one or more chemicals immediately below the lowest tested concentration that resulted in a statistically significant change in a particular toxicological variable compared to the control.

3.3.8 *reconstituted water*—a dilution water that is prepared by adding appropriate amounts of selected chemicals to water, which is usually prepared using deionization or reverse osmosis, so that the concentrations and ratios of the major ions in the dilution water are similar to those in comparable natural surface waters.

3.3.9 *surrogate species*—a species that is tested to estimate responses of another species, for which direct testing is impractical.

3.3.10 *toxicity test*—an experiment used to study the adverse effect(s) of one or more chemicals on whole organisms, tissues, or cells.

3.3.11 *Unionoidea*—the super family of freshwater bivalves that includes the North American families Unionidae and Margaritiferidae. The family Unionidae includes three subfamilies (Unioninae, Anodontinae, and Lampsliniae).

3.3.12 *unionoid*—any mussel species in the super family Unionoidea.

3.3.13 *unionid*—any mussel species in the family Unionidae.

3.3.14 *margaritiferid*—any mussel species in the family Margaritiferidae.

3.3.15 *bradytictic*—a mussel species spawning its gametes in late summer and the female broods the glochidia over winter for release the following spring (also called long-term brooders).

3.3.16 *tachytictic*—a mussel species spawning its gametes in spring and the female releases the glochidia in late spring or summer of that year (also called short-term brooders).

3.3.17 *glochidia*—bivalve larvae of unionid mussels which are generally parasitic on the gills of fish.

3.3.18 *marsupium*—a brood pouch for developing eggs and glochidia in unionid mussels, formed by a restricted portion of the outer gill, the complete outer gill, or all gills.

4. Summary of Guide

4.1 Annex A1 provides guidance for conducting water-only toxicity tests with glochidia and juvenile mussels. Recommended test conditions for conducting these toxicity tests are based on various published methods outlined in Table A1.1 and Table A1.4 in Annex A1 and are based on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels (section 16.5). Glochidia and juvenile mussels are only available for a limited time on a seasonal basis. Section 10 describes procedures for collecting adult female mussels from the field to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host organisim.

4.1.1 Toxicity tests with glochidia and juvenile mussels should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux. Toxicity tests with glochidia are typically started within 2 h after glochidia are isolated from the gills of the female mussels; however, some toxicity tests have been started with glochidia isolated from female mussels for 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 (for example, 48 h) can be conducted as long as control survival

>90 % is achieved. For example, toxicity tests conducted for 48 h with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates).Effect concentrations are typically calculated based on the percentage of viable glochidia in the control at a particular sampling time. Glochidia are not fed during the toxicity test. Survival can be determined throughout the toxicity test by subsampling each replicate.

4.1.2 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 toxicity tests conducted for 10 to 28 d 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.

5. Significance and Use

5.1 Protection of a species requires prevention of unacceptable effects on the number, weight, health, and uses of the individuals of that species. Toxicity tests can be used provide information about the toxicity of a test material to a specific life stage of a particular species of mussel. The primary adverse effects studied are reduced survival or growth.

5.2 Results of toxicity tests might be used to predict effects likely to occur on mussels in field situations as a result of an exposure under comparable conditions.

5.3 Results of toxicity tests might be used to compare the sensitivities of different mussel species and the toxicity of different test materials, and to study the effects of various environmental factors on results of such tests.

5.4 Results of toxicity tests conducted with mussels might be an important consideration when assessing the risks of test materials to aquatic organisms or when deriving USEPA Water Quality Criteria for aquatic organisms (Guide E1241).

5.5 Results of acute toxicity tests (for example, 24- to 96-h tests) might be useful for predicting the results of chronic tests on the same test material with the same species in another water or with another species in the same or a different water. Most predictions take into account the results of acute toxicity tests, and so the usefulness of the results of a chronic toxicity test is greatly increased by reporting also the results of an acute toxicity test conducted with a similar life stage of the same species under the same conditions (Guide E729).

5.6 Results of toxicity tests might be useful for studying the biological availability of, and structure-activity relationships between, test materials.

5.7 Results of toxicity tests will depend on temperature, composition of the dilution water, condition of the test organisms, and other factors.

5.8 *Interferences*—A number of factors can impede or prevent selection and use of freshwater mussels for toxicity testing (Guide E1850). The following should be considered when selecting a test species and measuring the sensitivity of the test species during toxicity tests.

5.8.1 Handling of field-collected adult mussels resulting from collection or transport to the laboratory might cause excessive mortality or sublethal effects.

5.8.2 The age, health, and physical condition of adult mussels (for example, the presence of parasites, bacteria, and disease) collected from a resident population might not be adequately known.

5.8.3 The physical characteristics of the testing environment (such as water quality, temperature, water flow, light) and food requirements might affect the ability of the test organisms to acclimate, recover from handling, or adapt to the laboratory environment conditions.

5.8.4 The degree of contamination and the history of contamination at the collection of the adult mussels might not be adequately known.

5.8.5 In the field, mussels may be exposed to contaminants in water, sediment, or food. This standard only addresses effects associated with exposure of mussels to contaminants in water. Future revisions to this standard may describe methods for conducting toxicity tests with *(1)* adult freshwater mussels and *(2)* contaminated sediments using various life stages of freshwater mussels.

5.8.6 There are insufficient data available to determine if juvenile mussels are able to avoid exposure to chemicals by valve closure. If it is suspected that juvenile mussels are avoiding exposure to a chemical in a toxicity test, it may be desirable to place the suspected live test organisms into dilution water that does not contain any added test material for 1 to 2 d after the end of the toxicity test to determine whether these test organisms are alive or dead (section A1.4.7; Guide E729).

6. Apparatus

6.1 *Facilities*—Although some small organisms can be held and acclimated in static or renewal (for example, static renewal) systems, most organisms are held, acclimated, and cultured in flow-through systems. Test chambers should be in a constant-temperature room, incubator, or recirculating water bath. For static and renewal tests a dilution-water tank, which may be used to prepare reconstituted water, is often elevated so that dilution water can be delivered by gravity into holding and acclimation tanks and test chambers. For flow-through tests an elevated head box is often desirable so that dilution water can be delivered by gravity into holding and acclimation tanks and into the metering system (6.4), which prepares the test solutions and delivers them to the test chambers. Strainers and air traps should be included in the water-supply system. Head boxes and holding, acclimation, culture, and dilution-water tanks should be equipped for temperature control and aeration. Air used for aeration should be free of fumes, oil, and water;

filters to remove oil and water are desirable. Filtration of air through a 0.22-µm bacterial filter might be desirable (Guide E729). The facility should be well-ventilated and free of fumes. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. A timing device should be used to provide a controlled photoperiod. A15 to 30-min transition period when the lights go on might be desirable to reduce the possibility of organisms being stressed by large, sudden increases in light intensity. A transition period when the lights go off might also be desirable (Guide E729).

6.2 *Special Requirements*—Some organisms may require special conditions during holding, acclimation, and testing. For example, adult mussels should be provided a substrate suitable for burrowing.

6.3 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect test organisms. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize dissolution or leaching. Concrete and rigid plastics may be used for holding, acclimation, and culture tanks in the water-supply system, but these materials should be soaked, preferably in flowing dilution water, for a week or more before use (Guide E729). Cast iron pipe should not be used for water-supply systems because colloidal iron may be added to the dilution water, and strainers will be needed to remove rust particles. Brass, copper, lead, galvanized metal, and natural rubber should not contact dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber or other materials not previously mentioned should not be used unless it has been shown that either *(1)* unfed individuals of a sensitive aquatic species (for example, *Daphnia magna*) do not show more signs of stress, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water that does not contain the item or *(2)* their use will not adversely affect survival, growth, or reproduction of a sensitive species (Section 8 and Guide E729).

6.4 *Metering System:*

6.4.1 For flow-through tests, the metering system should be designed to accommodate the type and concentration(s) of the test material and the necessary flow rates of test solutions. The system should permit the mixing of test material with dilution water immediately before entrance to the test chambers and permit the supply of the selected concentration(s) of test material (section 9.3) in a reproducible fashion. Various metering systems, using different combinations of such as syringes, siphons, pumps, saturators, solenoids, valves have been used successfully to control the concentrations of test material in, and the flow rates of, test solutions. Proportional diluters use an intermittent flow design and various devices for metering the test material. Continuous-flow metering systems are also available, as are systems that prepare the different test solutions independently of each other. See Guide E729, E1241 and Test Method E1706 for additional detail on metering systems.

6.4.2 The metering system should be calibrated before and after the test by determining the flow rate through each test chamber and by measuring either the concentration of test material in each test chamber or the volume of solution used in each portion of the metering system. The general operation of the metering system should be visually checked daily in the morning and afternoon throughout the test. The metering system should be adjusted during the test if necessary. It is usually desirable to construct the metering system so that it can provide at least ten-volume additions per 24 h, if desired, in case *(1)* the loading is high or *(2)* there is rapid loss of test material due to microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization. At any particular time during the test, the flow rates through any two test chambers should not differ by more than 10 %.

6.4.3 The frequency of water addition to the each test chamber should be based on the duration of the exposure and on the stability of the exposure concentrations (for example, based on degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization). Ideally, preliminary tests should be conducted to determine how frequently water should be added to maintain water quality and exposure concentrations of the test material. For example, in 96-h exposures with ammonia and juvenile mussels, water was renewed every two days to maintain relatively consistent exposure concentrations (USGS 2005a(**8**)). In 28-d exposures starting with 2-month-old juvenile mussels, about 4 volume additions/d were delivered to each test chamber in copper and ammonia toxicity tests (USGS 2005b (**9**)).

6.4.4 Speciation of some metals (for example, lead or copper) and perhaps other test materials is not instantaneous and may change over a period of time (perhaps hours or days), even in test solutions that do not contain test organisms. Water-renewal systems have been designed with "equilibration chambers" that provide a residence time for test solution before the test solution is delivered to the exposure chambers (Kim et al. 1999, Besser et al. 2005(**14, 15**)).

6.5 *Test Chambers:*

6.5.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which no water connections exist. However, screens, cups may be used to create two or more compartments within each chamber. Therefore, the test solution can flow from one compartment to another within a test chamber, but, by definition, cannot flow from one chamber to another. Because the solution can flow from one compartment to another in the same test chamber, the temperature, concentration of test material, and levels of pathogens and extraneous contaminants are likely to be more similar between compartments in the same test chamber than between compartments in different test chambers in the same treatment. All chambers (and compartments) in a test must be identical.

6.5.2 Test chambers may be constructed by welding, but not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicone adhesive. Stoppers and silicone adhesive sorb some organic chemicals, which are then difficult to remove. Therefore, as few stoppers and as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed for strength, the extra adhesive should be on the outside of chambers rather than on the inside. Especially in static and renewal tests, the size and shape of the test chamber might affect the results of tests on materials that volatilize or sorb onto the chambers in substantial quantities.

6.5.3 The dimensions of test chambers and volume of water to test depends on the age and number of the organisms being tested (Annex A1).

6.6 *Cleaning*—The metering system, test chambers, and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid (HCl)), and rinsed at least twice with deionized or dilution water. Reagent grade solvents are recommended. If lesser grades are used, possible contaminants should be considered with respect to the purpose of the test (some lots of some organic solvents might leave a film that is insoluble in water). A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and the acid, but it might attack silicone adhesive. At the end of the test, all items that are to be used again should be immediately *(1)* emptied, *(2)* rinsed with water, *(3)* cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases, detergent, organic solvent, or activated carbon to remove organic chemicals), and *(4)* rinsed at least twice with deionized or dilution water. Acid can be used to remove mineral deposits, and 200 mg of hypochlorite (ClO^-)/L can be used to remove organic matter and for disinfection. A solution containing about 200 mg of ClO^-/L may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water. However, ClO^- is quite toxic to many aquatic animals and is difficult to remove from some construction materials. It can be removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, by autoclaving in deionized water for 20 min, or by drying the item and letting it sit for at least 24 h before use. An item cleaned or disinfected with hypochlorite should not be used unless it has been demonstrated at least once that unfed individuals of a sensitive aquatic species do not show more signs of stress, such as discoloration, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water containing a similar item that was not treated with ClO^- (Guide E729). The metering system and test chambers should be rinsed with dilution water just before use.

6.7 *Acceptability*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain dilution water without added test material. Determine before the first test: *(a)* whether test organisms will meet test acceptability requirements outlined in Annex A1, *(b)* whether the food, water, or handling procedures are acceptable, *(c)* whether there are any location effects on either survival or growth of organisms, and *(d)* the magnitudes of the within-chamber and between-chamber variances.

7. Hazards

7.1 *General Precautions:*

7.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management and includes: *(1)* the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, *(2)* the preparation of a formal, written health and safety plan, which is provided to each laboratory staff member, *(3)* an ongoing training program on laboratory safety, and *(4)* regular safety inspections.

7.1.2 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses, and by using dip nets, forceps, or tubes to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans, recommended handling procedures, and biological, chemical, and physical properties of the test material should be studied before a test is begun (section Appendixes X2, X3, and X4 in Guide E1023). **Warning—**Special procedures might be necessary with radiolabeled test materials and with test materials that are, or are suspected of being, carcinogenic (Guide E729).

7.1.3 Collection and use of environmental samples (for example, sediments, effluents) may involve substantial risks to personal safety and health. Chemicals in field-collected samples may include carcinogens, mutagens, and other potentially toxic compounds. Inasmuch as testing is often started before chemical analyses can be completed, worker contact with field-collected samples needs to be minimized by *(1)* using personal safety gear, *(2)* manipulating samples under a ventilated hood or in an enclosed glove box, and *(3)* enclosing and ventilating the exposure system. Personnel collecting samples and conducting tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation because of lack of oxygen or presence of noxious gases.

7.2 *Safety Equipment:*

7.2.1 Before beginning sample collection or laboratory work, personnel should determine that all required safety equipment and materials have been obtained and are in good condition.

7.2.2 *Personal Safety Gear*—Personnel should use safety equipment, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, face shields, hard hats, and safety shoes.

7.2.3 *Laboratory Safety Equipment*—Laboratories should be provided with safety equipment such as first-aid kits, fire extinguishers, fire blankets, emergency showers, and eye wash stations. Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

7.3 *General Laboratory and Field Operations:*

7.3.1 Special handling and precautionary guidance in Material Safety Data Sheets (MSDS) should be followed for reagents and other chemicals purchased from supply houses.

7.3.2 It is advisable to wash exposed parts of the body with bactericidal soap and water immediately after collecting or manipulating field-collected samples.

7.3.3 Strong acids and volatile organic solvents should be used in a fume hood or under an exhaust canopy over the work area.

7.3.4 **Warning—**An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.3.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only under a fume hood.

7.3.6 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

7.3.7 Use of ground-fault systems and leak detectors is strongly recommended to help prevent electrical shocks. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories should not be used. Ground-fault interrupters should be installed in all "wet" laboratories where electrical equipment is used.

7.3.8 All containers should be adequately labeled to indicate their contents.

7.3.9 A clean and well-organized work place contributes to safety and reliable results.

7.4 *Disease Prevention*—Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio. Thorough washing of exposed skin with bactericidal soap should follow handling of samples collected from the field.

7.5 *Safety Manuals*—For further guidance on safe practices when handling field-collected samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals (Test Method E1706).

7.6 *Pollution Prevention, Waste Management, and Sample Disposal*—Work with some field-collected samples may require compliance with rules pertaining to the handling of hazardous materials. Guidelines for the handling and disposal of hazardous materials should be strictly followed (Guide D4447). The Federal Government has published regulations for the management of hazardous waste and has given the States the option of either adopting those regulations or developing their own. If States develop their own regulations, these regulations are required to be at least as stringent as the Federal regulations. As a handler of hazardous materials, it is your responsibility to know and comply with the pertinent regulations applicable in the State in which you are operating (Test Method E1706).

8. Dilution Water

8.1 *Requirements*—The dilution water should *(a)* be available in adequate supply, *(b)* be acceptable to the test organisms, *(c)* be of uniform quality, and *(d)* except as stated in 8.1.4, not unnecessarily affect results of the test. Additional details on dilution water for use in culture or toxicity testing can be found in Guide E729.

8.1.1 The minimal requirement for an acceptable dilution water for toxicity tests is that healthy test organisms survive in it through acclimation and testing without showing signs of stress, such as discoloration, unusual behavior, or death. A better criterion for an acceptable dilution water is that at least one species of aquatic animal (preferably of the one being tested or one taxonomically similar) will survive, grow, or reproduce satisfactorily in the water. Because daphnids are more sensitive to some test materials than many other aquatic animal species, water in which daphnids (less than 24-h old) will survive for 48 h without showing signs of stress is probably acceptable for toxicity tests with most freshwater animal species. Water in which daphnids will survive, grow, and reproduce satisfactorily in a life-cycle test is probably an acceptable dilution water for tests with most freshwater animal species.

8.1.2 The quality of the dilution water should be uniform so that the test organisms are cultured or acclimated and the test conducted in water of the same quality. The range of hardness should be within 10 % of the average.

8.1.3 The dilution water should not unnecessarily affect the results of a toxicity test because of such things as sorption or complexation of test material. Except as in accordance with section 8.1.4, it is desirable for the purpose of reducing inter-laboratory variability that the concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L.

8.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of a toxicity test, it will be necessary to use a water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such a water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or be conducted in a more usual dilution water to facilitate interpretation of the results in the special water.

8.2 *Source:*

8.2.1 *Reconstituted Water:*

8.2.1.1 Tables 1 and 2 in Guide E729 provide recipes for preparing a variety of reconstituted waters that have been used successfully to conduct toxicity tests. Reconstituted water is prepared by adding specified amounts of reagent grade chemicals to high-quality water with *(a)* resistivity greater than 1 MΩ water and *(b)* either total organic carbon (TOC) less than 2

mg/L or chemical oxygen demand (COD) less than 5 mg/L. Acceptable water can usually be prepared using properly operated deionization or reverse osmosis units. Conductivity should be measured on each batch and TOC or COD should be measured at least twice a year and whenever substantial changes might be expected. If the water is prepared from surface water, TOC or COD should be measured on each batch. The reconstituted water should be aerated before use. Problems have been encountered with some species in reconstituted waters, but sometimes these problems have been overcome by aging the reconstituted water for one or more weeks.

8.2.2 *Natural Dilution Water:*

8.2.2.1 If natural dilution water is used, it should be obtained from an uncontaminated, uniform quality source. The quality of water from a well or spring is usually more uniform than that of water from a surface water. If a surface water is used as a source of water, the intake should be positioned (for example, about one meter below the surface) to minimize fluctuations in quality and the possibility of contamination, and to maximize the concentration of dissolved oxygen to help ensure that the concentrations of sulfide and iron are not high.

8.2.2.2 Water quality characteristics (such as hardness, conductivity, pH) may be adjusted, if desired, by addition of appropriate reagent grade chemicals, acid, base, or deionized water if desired (Guide E729). Chlorinated water should not be used as, or in the preparation of, dilution water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals (Guide E729). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines. Some organic chloramines, however, react slowly with sodium bisulfite. In addition to residual chlorine, municipal drinking water often contains high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. The concentrations of most metals can usually be reduced with a chelating resin, but use of different dilution water might be preferable. If dechlorinated water is used as dilution water or in its preparation, during the test it should be demonstrated that a sensitive aquatic species (for example, daphnids less than 24-h old) do not show more signs of stress, such as discoloration, unusual behavior, or death, when held in the water for at least 48 h without food than when similarly held in a water that was not chlorinated and dechlorinated).

8.3 *Treatment:*

8.3.1 Dilution water should be aerated intensively by such means as air stones, surface aerators, or column aerators before adding test material. Adequate aeration will bring the pH and the concentrations of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % of saturation to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Super-saturation by dissolved gases, which might be caused by heating the dilution water, should be avoided (Guide E729).

8.3.2 Filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low and as a pretreatment before ultraviolet sterilization or filtration through a finer filter.

8.3.3 Dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm or less (Guide E729).

8.4 *Characterization*—The following items should be measured at least twice each year, or more often *(a)* if such measurements have not been made semiannually for at least two years, or *(b)* if a surface water is used: pH, particulate matter, TOC, organo-phosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc, hardness, alkalinity, conductivity, sodium, and chloride. For each analytical method used the detection limit should be below either *(a)* the concentration in the dilution water or *(b)* the lowest concentration that has been shown to unacceptably affect the test species (Guide E729).

9. Test Material

9.1 *General*—The test material should be reagent grade or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed (Guide E729). Before a test is begun, the following should be known about the test material: *(1)* Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material, *(2)* Solubility and stability in the dilution water, *(3)* Measured or estimated acute or chronic toxicity to the test species, *(4)* Precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentrations are to be measured, *(5)* Estimate of toxicity to humans, and *(6)* Recommended handling procedures (Section 7).

9.2 *Stock Solution:*

9.2.1 In some cases the test material can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is dilution water, although filtration or sterilization, or both, of the water might be necessary. If the hardness of the dilution water will not be affected, deionized water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials (Guide E729). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more

soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than use of the minimum necessary amount of a strong acid or base.

9.2.3 If a solvent other than dilution water is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect the test species. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals, low volatility, and high ability to dissolve many organic chemicals (Guide E729). Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but these materials might stimulate undesirable growths of microorganisms (Guide E729; **Warning—**Acetone is also quite volatile). If an organic solvent is used, it should be reagent grade or better and its concentration in any test solution must not exceed 0.5 mL/L in 96-h tests (Guide E729) or 0.1 mL/L in longer-term tests (Guide E1241).A surfactant must not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions (these limitations do not apply to any ingredient in a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution or if the test is on a solvent or surfactant).

9.2.4 If a solvent other than dilution water is used, at least one solvent control using solvent from the same batch used to make the stock solution must be included in the test. If no solvent other than water is used, a dilution-water control must be included in the test and the survival and growth of test organisms in the dilution-water control must meet test acceptability requirements in order for the test to be considered acceptable (Annex A1). Using no solvent other than dilution water is the most desirable option because using any other solvent means that antagonism, synergism, and confounding are possible (Guide E1241). Using different concentrations of a solvent at the different concentrations of the test material should be avoided because both the concentration of the solvent and the concentration of the test material vary across the treatments, potentially resulting in confounding. Therefore, it is desirable to test the same concentration of solvent in all of the test solutions.

9.2.4.1 If the concentration of solvent is the same in all test solutions that contain test material, the solvent control must contain the same concentration of solvent.

9.2.4.2 If the concentration of solvent is not the same in all test solutions that contain test material, either *(a)* a toxicity test must be conducted to determine whether survival or growth of the test organisms is related to the concentration of the solvent over the range used in the toxicity test, or *(b)* such a toxicity test must have been conducted on the solvent using the same dilution water and test species. If survival or growth are related to the concentration of solvent, a toxicity test with that species in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival nor growth are related to the concentration of solvent, a toxicity test with that same species in that same water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments (Guide E1241).

9.2.4.3 There may be instances when a toxicity test is to be conducted with a species that is not routinely available for testing (for example, such as with an endangered species.) In these instances, the toxicity test used to evaluate potential effects of a solvent outlined in 9.2.4.2 may be conducted with species in the same family (preferably the same genus) as long as the concentrations of solvent are at least double the concentration of solvent used in the toxicity test on the test material. Testing at least double the concentration of solvent used in the toxicity test would provide some margin of safety in extrapolating results of toxicity tests between species in the same family. For example, Dwyer et al (2005a,b) **(11, 12)** and Besser et al (2005) **(16)** reported the sensitivity of endangered species of fish was within a factor of about 2 of commonly-tested surrogate fish species for a variety of organic and inorganic chemicals in acute or chronic toxicity tests. Similarly, USEPA (2003) **(17)** reported similar sensitivity of aquatic species to a variety of organic or inorganic chemicals in toxicity tests conducted within a family.

9.2.4.4 If the test contains both a dilution-water control and a solvent control, the survival and growth of the organisms in the two controls should be compared. If a statistically significant difference in survival or growth is detected between the two controls, only the solvent control may be used for meeting the requirements of outlined in Table A1.3 or Table A1.5 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be pooled for meeting the requirements outlined in Table A1.3 or Table A1.5 and as the basis for calculation of results.

9.2.5 If a solvent other than water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvent on the toxicity of the test material or the sensitivity of the test species.

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to allow calculation of an LC50, EC50, or IC50, the test concentrations should bracket the predicted concentration. The prediction might be based on the results of a test on the same or a similar test material with the same or a similar species. In acute toxicity tests, if a useful prediction is not available, it is usually desirable to conduct a range-finding toxicity test in which groups of five or more organisms are exposed for 24 to 96 h to a control and three to five concentrations of the test material that differ by a factor of ten. Replicate chambers are not typically evaluated in range-finding toxicity tests. The greater the similarity between the range-finding test and the definitive test, the more useful the range-finding test will be. If necessary, concentrations above solubility should be used because organisms in the real world are sometimes exposed to concentrations above solubility and because solubility in dilution water is often not well known. The use of concentrations that are more than ten times greater than solubility are probably not worthwhile. With some test materials it might be found that concentrations above solubility do not kill or affect a greater percentage of test organisms than

does the concentration that is the solubility limit; such information is certainly worth knowing.

9.3.2 In chronic toxicity tests, the test concentrations should bracket the best prediction of that concentration. Such a prediction can be based on the results of an acute toxicity test using the same dilution water, test material, and species (Guide E729). If an acute-chronic ratio has been determined for the test material with a species of comparable sensitivity, the result of the acute test can be divided by the acute-chronic ratio. Except for a few materials, acute-chronic ratios with sensitive species are often less than five. Thus, if no other useful information is available, the highest concentration of test material in an early life-stage test is often selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test (Guide E1241).

9.3.3 In some (usually regulatory) situations, it is necessary only to determine *(a)* whether a specific concentration of test material is acutely toxic to the test species, or *(b)* whether the LC50, EC50, or IC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When there is interest only in a specific concentration, it is often necessary only to test that concentration, and it is not necessary to actually determine the LC50, EC50, or IC50.

10. Test Organisms

10.1 *Life History of Freshwater Mussels:*

10.1.1 Freshwater mussels are bivalve mollusks belonging to the family Unionidae or Margaritiferidae. Adults are sedentary animals, spending their entire lives partially or completely burrowed in the bottoms of streams, rivers, or lakes. Adult mussels are filter feeders, using their gills to remove suspended particles from the water column (Murray and Leonard 1962) (**18**), such as detritus, phytoplankton, zooplankton, diatoms, bacteria, and other microorganisms (Fuller 1974 (**19**), Strayer et al 2004 (**20**)). The extent of selectivity exhibited by mussels feeding on each of these food groups is poorly understood and is likely to vary by species (Beck and Neves 2003) (**21**). Recent evidence suggests that detritus, bacteria, and zooplankton may be important food sources (Silverman et al 1997 (**22**), Nichols and Garling 2000 (**23**)). The early juvenile stage use foot (pedal) feeding to some degree for the first several months of their lives, feeding on depositional materials in pore water of sediment, including bacteria, algae, and detritus (Yeager et al 1994 (**24**), Silverman et al 1997 (**22**)) in addition to unicellular algae (Gatenby et al 1997 (**25**), O'Beirn et al 1998 (**26**), Parker et al 1998 (**27**), Beck and Neves 2003 (**21**)). Pedal feeding in juvenile mussels is accomplished by movements of microscopic cilia lining the foot that carry food particles into the mantle cavity and into the mouth. Juvenile mussels also use the foot in a sweeping motion to draw particles toward the mantle cavity (Reid et al 1992) (**28**).

10.1.2 Unionid mussels have an unusual and complex mode of reproduction, which for most species includes a brief, obligatory parasitic stage on fish (Fig. 1). Freshwater mussels are typically dioecious, but some species may be hermaphroditic (for example, *Toxolasma parvus*, *Lasmigona compressa*, *Utterbackia imbecillis*; Watters 2005). During the breeding season, males release sperm into the water column and females draw the sperm in through the incurrent aperture. The eggs are fertilized in the suprabranchial chambers in the gills and are moved to the marsupial region of the gill until released as mature glochidia by the thousands to millions (Fig. 2)

10.1.3 Spawning takes place in the spring for most amblemines and in the summer for most anodontines and lampsilines (Watters 2005) (**30**). Depending on the species, mature glochidia may be brooded for several months or may be released shortly after maturation. Winter-brooding mussels produce glochidia in the late summer or fall, but do not release the glochidia until the following spring or summer (bradytictic or long-term brooders). Summer-brooding mussels produce glochidia in the late spring or early summer and release them in the summer (tachytictic or short-term brooders). Some mussels release glochidia in the fall or winter and after attaching to a host, the glochidia remain dormant over winter until a threshold temperature is reached in the spring, at which time the glochidia metamorphose and excyst as juvenile mussels (for example, *Pyganodon grandis* and *Leptodea fragilis*; Watters 2005) (**30**).

10.1.4 The successful transfer of mature glochidia to a suitable host constitutes a critical event in the life cycle of most freshwater mussels. Various adaptations have evolved to facilitate this process. High levels of mortality occur during the passage of glochidia from the female mussel to the host fish due to low incidence of fish host contact. Once encysted in the gill, glochidia may be relatively protected from *in situ* exposure contaminants in water (Jacobson et al 1997) (**31**). The method of host infestation greatly varies among species. While some species simply broadcast glochidia into the surrounding water to haphazardly come into contact with the appropriate host, the process is more intricate and direct for other species. For example, females in the genus *Lampsilis* have an extension of the mantle tissue that resembles a small fish or invertebrate complete with eye spots and appendages. This lure is displayed outside the shell between the valves and is twitched repetitively to attract a predaceous fish host. The host is infested while attempting to eat the lure when the marsupial gills of the female are ruptured (Kraemer 1970 (**32**), Barnhart and Roberts 1997 (**33**)). Some species release conglutinates (small structures containing glochidia) freely into the water. In many conglutinate-producing species (for example, *Elliptio*, *Fusconaia*, *Pleurobema*, *Plethobasus*, *Cyprogenia*, and *Quadrula*), conglutinates are released as cohesive masses made up of unfertilized eggs that hold together mature glochidia. Conglutinates of some species (for example, *Ptychobranchus*) are made up of gelatinous material that enclose large numbers of glochidia (Hartfield and Hartfield 1996) (**34**). Conglutinates may resemble prey items of the host fish; the host fish are infested with glochidia when fish attempt to eat conglutinates (Chamberlain 1934 (**35**), Barnhart and Roberts 1997 (**33**), Jones et al 2004 (**36**)).

10.1.5 Glochidia range in size from about 50 to 400 µm (Hoggarth 1999 (**37**), McMahon and Bogan 2001 (**29**), Wachtler et al 2001 (**38**)). The only visible behavior of which

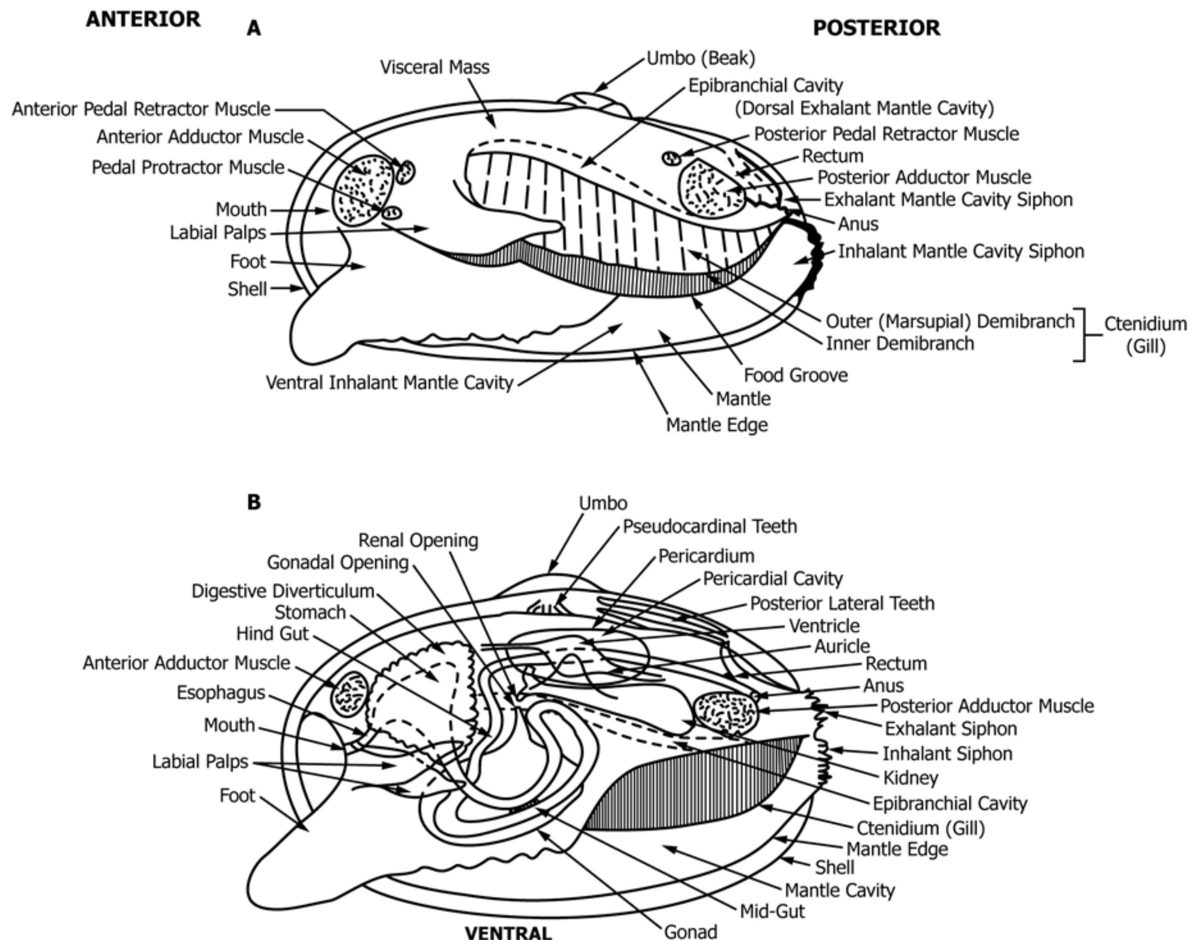


FIG. 2 General External Anatomy of the Soft Tissues (A), and Internal Anatomy, Organs, and Organ Systems of Soft Tissues of a Unionid Mussel (B); adapted from McMahon and Bogan, Academic Press, 2001, (29) Copyright Academic Press

glochidia are capable is closure of the valves, which is accomplished by a single adductor muscle. The valves close in response to a variety of artificial tactile and chemical stimuli such as insertion of objects placed between valves, hypo-osmotic solutions, saturated NaCl or KCl solutions, or the blood of vertebrates (LeFevre and Curtis 1912 (39), Arey 1921 (40)). In nature, glochidia will attach to the gills or the fins of a host fish upon contact. The sharp valves cut into the epithelium of the host, enclosing and compressing the tissue (LeFevre and Curtis 1912 (39), Arey 1932 (41)). After glochidia are released from the female, glochidia need to attach to the gills or the fins of an appropriate fish host and encyst to complete development. Although glochidia may survive for months during brooding in the female mussel, glochidia typically survive for only a few days after release unless the glochidia reach a compatible host. Encystment on the host occurs from encapsulation by host tissue (Zimmerman and Neves 2002) (42).

10.1.6 Metamorphosis of juvenile mussels on the fish host occurs within days or weeks, depending on species and temperature. Host fish specificity varies among mussels. While some mussel species appear to require a single host organism, other species can transform their glochidia into juvenile mussels on many species of host fish. Following proper host infestation, glochidia transform into microscopic juveniles and excyst (drop off) and settle into suitable habitat to survive. The transformation of glochidia to juveniles results in the development of internal organs necessary for self-sustained existence as a benthic organism. Newly-transformed juvenile mussels have a life style different from adult mussels. Transformed juvenile mussels may be at the sediment-water interface or may burrow several centimeters into sediment and rely on water percolating between substrate particles of sediment for food and oxygen (Neves and Widlak 1987) (43).

10.1.7 Newly-transformed juvenile mussels feed using ciliary currents on the foot and mantle. Older juvenile and adult mussels likely use different food types when living in different micro-environments. Given that glochidia and juvenile mussels are ecologically and physiologically different from adult mussels, protection of habitat quality of adult life stages may not be protective of glochidia or juvenile life stages of freshwater mussels (Watters 2005) (30). Distributions of adult mussels are dependent both on the presence of host fish and on microhabitat conditions. Efforts to assess effects of contaminants on mussels need to evaluate potential exposure to host fish in addition to exposure to each unique life stage of freshwater mussels (Watters 2005) (30).

10.1.8 Photographs of lures and conglutinates that mimic prey items of the host fish can be found at the following websites: *(1)* http://unionid.smsu.edu/default.htm and *(2)* http://courses.smsu.edu/mcb095f/gallery/. Additional information on the life history or propagation techniques for freshwater mussels can be found in Gordon and Layzer (1989) **(44)**, Parmalee and Bogan (1998) **(45)**, Bishop et al (2005) **(46)**, and Watters (1995, 2005) **(47, 30)**.

10.1.9 *Anatomy of Adult Mussels*—Fig. 2 illustrates the *(a)* general external anatomy of the soft tissues and *(b)* internal anatomy, organs, and organ systems of soft tissues of a unionid mussel. McMahon and Bogan (2001) **(29)** provide an overview of the basis anatomy and physiology of freshwater mussels. Information is also provided in McMahon and Bogan (2001) **(29)** on the ecology and evolution and on the collection, identification and rearing freshwater mussels. Unlike most epibenthic marine bivalves, North American freshwater mussels lack true siphons or tubes for water intake and release. Because of this, freshwater mussels frequently burrow only to the posterior edge of the shell (Watters 2005) **(30)**. However, anecdotal observations suggest that certain freshwater species are routinely found near the sediment-water interface (that is, *Amblema plicata*), while other species maybe be found well below the sediment-water interface (for example, *Obliquaria reflexa*). In temperate locations, mussels may burrow deeper into the substrate during the winter.

10.1.10 *Tolerance Limits of Mussels:*

10.1.10.1 Dimock and Wright (1993) **(48)** reported oxygen, pH and temperature requirements for juvenile *Utterbackia imbecillis* and *Pyganodon cataracta* and found that 7- to 10-d old juvenile mussels could not survive 24 h in an anoxic condition. Temperatures above 30°C were lethal (for example, 96-h median lethal effect at 31.5°C for *Utterbackia imbecillis* and 33°C for *Pyganodon cataracta*). Slight acidity was tolerated with >70 % survival in all groups above a pH value of 5.0 with LC50s of pH 4.5 for both species. Chen et al (2001a) **(49)** summarizes oxygen consumption by 9 species of freshwater mussels. Sparks and Strayer (1998) **(50)** reported that juvenile *Elliptio complanata* were sensitive to low concentrations of dissolved oxygen with survival significantly reduced at 1.3 mg/L and behavior affected at 2 to 4 mg/L.

10.2 *Test Species and Life Stage:*

10.2.1 Table A1.1 and Table A1.4 lists examples of species that have been used to conduct toxicity tests with glochidia or juvenile mussels. These species were selected for testing based on availability, past successful testing, and ease of handling in the laboratory. Selection of the test species or the life stage to be tested depends on the purpose and scope of the study and should be appropriate to the overall objective of the study (Guide E1850). For example, early life stages of a species might be sensitive to a certain toxicant and readily acclimate to the laboratory environment. These organisms may be used in an acute toxicity test or sublethal test designed to assess toxicity using a growth endpoint (Annex A1), but would not provide information on reproduction.

10.2.2 Before mussels are collected from the field, appropriate federal or state permits for collection of mussels are mandatory. In addition, permission is needed to collect mussels from private landowners. Specific guidance on collection of adult mussels in the field can be obtained from Strayer and Smith (2003) **(51)**.

10.2.3 When selecting the appropriate test species, the following selection criteria should be considered in order of importance (Guide E1850):

10.2.3.1 *Ease of Organism Procurement and Laboratory Culture and Handling*—Species should be screened for ease of handling, ease of collection, and resistance to shock and handling. Preference might be given to those species that can be successfully cultured in the laboratory and are amenable to laboratory testing (Table A1.1 and Table A1.4). Organisms for use in testing should not have had prior exposure to contaminants or other known sources of stress. Potential criteria to determine whether a given batch of field-collected organisms is suitable for laboratory testing should include the following:

(1) Adult mussels collected from the field should not have signs of obvious physical abnormalities such as broken shells or lesions. High survival of adult mussels several days after placement in the laboratory environment should indicate that the organisms have adapted to the new environment.

(2) Organisms should exhibit normal behavior (for example, feeding or locomotory, if appropriate).

(3) Reference-toxicant tests should be performed with subsamples of each batch of glochidia or juvenile mussels used in toxicity tests (following the recommended conditions for conducting toxicity tests in Table A1.1 and Table A1.4). Results of these reference-toxicant tests can be used to compare test organism sensitivity over time either with previously reported results of toxicity tests or with laboratory data being developed for that species and life stage (section 16.3).

10.2.3.2 *Ease of Method Development*—Test procedures might exist for the species of interest or an ecologically similar species (Table A1.1 and Table A1.4). Alternatively, preliminary tests should be conducted with the species and life stage of interest to determine how well the selected species will respond in laboratory conditions.

10.2.3.3 *Potential Sensitivity to Contaminants*—A variety of references are available that categorize species in terms of general sensitivity to organic enrichment and other contaminants (Guide E1850). It is desirable to use species for which data are available, indicating their relative sensitivity to a given test material or class of test materials (for example, Keller et al 2005) **(7)**.

10.2.3.4 *Test Performance Characterization*—To document the quality of the data produced from a given test organism (and surrogate species as well) and to determine the comparability of the selected test organism with other species data for the same test material, method performance characteristics should be determined, preferably before definitive toxicity testing of the test material of interest (Guide E1850). The degree to which a toxicity test with selected test organisms yields meaningful data will depend on how well the test performance characteristics meet the data quality objectives of the study (for example, Table A1.3 and Table A1.5). Test performance characterization should include the following steps:

(1) Different batches of the same species and the same life stage should be collected and tested over time in order to obtain a measure of the variability associated with testing the particular species. The relative sensitivity and quality of test organisms can then be determined through an assessment of test organism response to a known toxicant or, preferably, different classes of toxicants (for example, NaCl, metals, chlorinated organic compounds, or polycyclic aromatic hydrocarbons) in which the toxicity effect is theoretically constant across tests. Repeated tests using standard or reference materials could be used to compare the sensitivity of the selected test organism with existing data for surrogate test species, through the development of a reference-toxicant control chart for the species and the test material being used (Section 16.3).

(2) The appropriate exposure time required for testing should be determined. Different life stages of the same species (for example, glochidia versus juvenile mussels) might require different exposure durations in order to obtain meaningful test endpoints (section 10.3, Annex A1). As a general rule, acute toxicity tests should conducted for at least 24 h with glochidia and for 96 h with juvenile mussels. However, shorter time periods for glochidia toxicity tests might be needed for a particular species depending on the survival time of the glochidia (Table A1.2, section A1.5.2). A 48-h toxicity test with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates). Longer exposure periods may be required for older life stages of mussels that are capable of avoiding exposure for short periods of time (older juvenile mussels and adult mussels; Guide E729 and 5.8.6).

(3) If a hypothesis test is used, the statistical power of a particular toxicity testing method (Guide E1850, Section 14). This information will provide a measure of test reliability, given the method and test species used. For regression, probit, or logit-based endpoints such as LC50 or IC25, test reliability and data quality of objectives are best stated in terms of the range of the 95 % confidence limit around the endpoint; the tighter the confidence intervals of the endpoint, the more reliable the test.

(4) The method precision (degree to which independent tests using the same concentration of test material elicits a similar response or test endpoint) should be determined and compared in relation to the decision criteria or data quality objectives to the study (for example, Section 16). For certain applications, it might be desirable or necessary to determine test precision before conducting the definitive testing of a particular test material.

(5) Appendix X3 in Guide E1850 provides a flow chart that summarizes the factors described above that should be considered when selecting a test species.

10.3 *Age:*

10.3.1 Annex A1, Table A1.1, and Table A1.4 describe the age of test organisms to be used and recommended to start a toxicity test.

10.4 *Source:*

10.4.1 Adult mussels collected from the field should be representative of the organisms that could occur at the study site based on habitat features available and historic species records for the region and should not have been previously exposed to contaminants or pathogens (Guide E1850). Therefore, adult mussels should be obtained from reference areas (Test Method E1706), outside of the direct influence of point- or non-point sources of contamination. Adult mussels collected to produce either glochidia or juvenile mussels should be obtained from the same location. Priority pollutant analyses of the site water, sediment, or organism tissues might be used to determine whether organisms have had exposure to source-related contaminants at the collection site. The taxonomic identity of test species should be determined by appropriate keys and verified by an appropriate expert (section 11.5).

10.4.2 Table 1 provides a summary of facilities that have cultured juvenile mussels as of May 2005. Table 2 and section 10.5 provide a summary of techniques that have been used to transform juvenile mussels. Transformation of juvenile mussels has been reported for many species using either fish hosts (*in vivo*) or artificial media (*in vitro*; Bishop et al 2005 **(46)**; section 10.5). Additionally, Watters (1994) **(52)** reported over 150 species of fish hosts for 95 species of freshwater mussels. While the main focus of the culture facilities listed in Table 1 is propagation of juvenile mussels for release into the environment, these facilities may also be a source of either glochidia or juveniles for use in toxicity tests. Individuals at these facilities will be able to provide additional guidance on handling and culturing of freshwater mussels. The following sections briefly summarize activities at each of the facilities listed in Table 1.

10.4.2.1 *Mammoth Spring National Fish Hatchery, AR—* Over 2500 individuals comprising 28 species of native mussels from the White and Ouachita Rivers in Arkansas have been held in refugia at the Mammoth Spring National Fish Hatchery since 1995. This facility was designed to hold adult mussels in response to a zebra mussel infestation predicted by personnel at the state game and fish commission. Species were held and monitored for survival and physiological condition (cellulolytic enzyme activity), using surrogate species, for four years (some species are still surviving in the hatchery raceways nearly seven years after initial collection). Survival from year one (90 %) to year four (60 %) was measured and indicated that the hatchery provided suitable conditions (high water quality, adequate food source, and continuous water temperatures throughout the year) for short- and long-term holding of native mussels. Since 1994, this hatchery has supported freshwater mussel propagation for recovery and restoration projects in Arkansas and Ohio. Six species (including two federally-endangered species), have been propagated using a combination of host fish and artificial media for the production of juvenile mussels (*L. streckeri*, *Arkansia wheeleri*, *P. grandis*, *L. siliquoidea*, *L. ventricosa*, *Fusconaia flava*, and *U. imbecillis*). About 10 000 juvenile mussels of these species were maintained in recirculating streams for several weeks and reintroduced into watersheds to support restoration goals of the U.S. Fish and Wildlife Service.

TABLE 1 Facilities Currently Conducting Freshwater Mussel (Unionidae) Propagation and Refugium Efforts

Facility	State or Province	Species	Contact
Mammoth Spring National Fish Hatchery	AR	*Lampsilis streckeri* FE[A] *Arkansia wheeleri* FE *Pyganodon grandis* *L. siliquoidea* *L. ventricosa* *Fusconaia flava* *Utterbackia imbecillis*	Richard Shelton (Hatchery Manager) 870/625-3912 mammothspring@fws.gov http://mammothspring.fws.gov
Lost Valley State Fish Hatchery	MO	*Epioblasma triquetra* SR[B] *L. teres*	Ken Neubrand (Hatchery Manager) 660/438-4465 Ken.neubrand@mdc.mo.gov http:// www.conservation.state.mo.us/areas/hatchery/lostvalley/
Warm Springs National Fish Hatchery	GA	*Villosa vibex* *V. lienosa* *L. subanbulata* FE	Curtis Echevarria (Hatchery Manager) 706/655-3382 warmsprings@fws.gov http://warmspringshatchery.fws.gov
White Sulphur Springs National Fish Hatchery	WV	*L. fasciola* *V. iris* *E. rangiana* FE *P. clava* FE *V. fabalis* CD[C] *Amblema plicata* *Cyclonalas turberculata* *A. ligamentina* *E. dilatata* *Epioblasma* spp.	Catherine Gatenby (Project Leader) 304/536-1361 catherine_gatenby@fws.gov hltp://northeast.fws.gov/wv/wssnfh.html
Genoa National Fish Hatchery	WI	*L. higginsi* FE *O. Fragosa* FE *L recta* SR *O. olivaria* SR *L. cardium* *L. siloquoidea* SR *L. teres* SR	Tony Brady (Mussel Biologist) Doug Aloisi (Hatchery Manager) Roger Gordon (Mussel Program Supervisor) 608/689-2605 Doug_Aloisi@fws.gov http://midwest.fws.gov/Genoa
Aquatic Wildlife Conservation Center at Buller Fish Culture Station	VA	*Actinonaias ligamentina* *A. pectorosa* *Epioblasma capsaeformis* FE *E. brevidens* FE *E. f. walkeri* FE *Epioblasma triquetra* SR *Lampsilis fasciola* *L. ovata* *Polamilus alatus* *Villosa iris* *V. perpurpurea* FE	Nathan Eckert (SW VA Mussel Recovery Coordinator) Joe Ferraro (Mussel Propagation Specialist) 276/783-2138 Nathan.Ekert@DGIF.virginia.gov http:// www.dgif.virginia.gov/wildlife/freshwater_mussels.html
Tennessee Aquarium Research Institute	GA	*Lampsilis altilis* *L. virescens* *Lasmigona holstonia (etowahensis)* *Medionidus acutissimus* *Pleurobema decisum* *P. georgianum* *Ptychobranchus greenii* *Villosa nebulosa* *V. umbrans*	Paul Johnson (Director) 706/694-4419 pdj@tnari.org http://www.tennis.org/get_involved/research_tnari.asp
Kentucky Department of Fish and Wildlife Resources	KY	59 species including 7 federally-listed species	Monte McGregor (Aquatic Scientist) 502/564-7109 monte.mcgregor@ky.gov www.kdfwr.state.kv.us
Missouri State University	MO	Various species	Chris Barnhart 417/836-5166 chrisbarnhart@smsu.edu http://biology.smsu.edu/aquatic/smsuwebs.htm
Cooperative Fish and Wildlife Research Unit, Virginia Polytech and State University	VA	Various species	Richard Neves 540/231-5927 mussel@vt.edu http://www.fw.vt.edu/fisheries/neves.htm
Arkansas State University	AR	Various species	Jerry Farris 501/972-3082 jlfarris@astate.edu
Department of Fisheries and Wildlife, University of Minnesota	MN	Various species	Mark Hove 612/624-3019 mark_hove@umn.edu http:// www.fw.umn.edu/Personnel/staff/Hove/Personal.Page
Cooperative Fisheries Research Unit, Tennessee Tech University	TN	Various species	Jim Layzer 931/372-3032 Jim-layzer@tntech.edu

TABLE 1 *Continued*

Facility	State or Province	Species	Contact
Department of Zoology, University of Guelph	ONT	Various species	Gerald Mackie 519/767-6684 http://www.uoguelph.ca/cbs/

[A]FE: Federally endangered
[B]SR: State rare
[C]CD: Candidate for listing

TABLE 2 Summary of Techniques Used to Transform Juvenile Mussels (adapted from Bishop et al, 2005) (46) (reprinted with permission)

Species	Technique	Purpose	Reference
Alasmidonta raveneliana	Fish host	Toxicity testing	Keller and Augspurger 2005 (**53**)
Amblema plicata	Fish host	Reintroduction	Hubbs 2000 (**54**)
	Media	Culture development	B Hudson and M Barfield (personal communication)
Anodonta suborbiculata	Fish host	Host suitability	Barnhart and Roberts 1997 (**33**)
Anodontoides ferussacianus	Fish host	Host suitability	Hove et al 1997 (**55**)
Toxolasma cylindrellus	Fish host	Unknown	Hudson and Isom 1984 (**56**)
Cyclonaias tuberculata	Fish host	Host suitability	Hove et al 1997 (**55**)
Elliptio angustata	Media	Toxicity testing	Hudson et al 1996 (**57**)
E. complanata	Media	Culture development	B Hudson and M Barfield (personal communication)
E. crassidens	Media	Unknown	D Simbeck (personal communication)
E. icenterina	Fish host	Toxicity testing	Keller and Ruessler 1997 (**58**)
Fusconaia ebena	Media	Culture development	Isom and Hudson 1982 (**59**)
Fusconaia flava	Media	Reintroduction	Milam et al 2000 (**60**)
Lampsilis cardium	Fish host	Toxicity testing	Keller and Ruessler 1997 (**58**)
	Media	Reintroduction	Milam et al 2000 (**60**)
	Fish host	Toxicity testing	Newton et al 2003 (**61**)
L. fasciola	Fish host	Reintroduction	Morgan et al 1997 (**62**)
	Media		D Simbeck (personal communication)
L. ovata	Fish host	Culture development	Isom and Hudson 1982 (**59**)
L. rafinesqueana	Fish host	Host suitability	Barnhart and Roberts 1997 (**33**) Shiver 2002 (**63**)
L. reeveiana	Fish host	Host suitability	Barnhart and Roberts 1997 (**33**)
L. siliquoidea	Media	Reintroduction	Milam et al 2000 (**60**)
	Media	Survival and growth	Myers-Kinzie 2000 (**64**)
L. streckeri	Fish host	Host suitability and reintroduction	Winterringer 2003 (**65**)
L. subangulata	Fish host	Host suitability	C Echevarria (personal communication)
L. teres	Media	Unknown	Keller and Zam 1990 (**66**)
Ligumia recta	Media	Culture development	Isom and Hudson 1982 (**59**) Milam et al 2000 (**60**)
Medionidus conradicus	Fish host	Reintroduction	Morgan et al 1997 (**62**)
Megalonaias gigantia	Media	Unknown	B Isom, D Simbeck (personal communication)
M. nervosa	Fish host	Reintroduction	Hubbs 2000 (**54**)
Pleurobema coccineum	Fish host	Host suitability	Hove et al 1997 (**55**)
P. cordatum	Media	Culture development	Hudson and Isom 1984 (**56**)
Ptychobranchus occidentalis	Fish host	Host suitability	Barnhart and Roberts 1997 (**33**)
Pyganodon cataracta	Media	Unknown	Dimock and Wright 1993 (**48**)
P. grandis	Fish host	Toxicity testing	Keller and Ruessler 1997 (**58**)
	Fish host	Reintroduction	Milam et al 2000 (**60**)
	Media		B Isom (personal communication)
Strophitus undulatus	Fish host	Host suitability	Hove et al 1997 (**55**)
Utterbackia imbecillis	Fish host	Toxicity testing	Keller and Zam 1991 (**67**)
	Media	Culture development	Warren 1996 (**68**)
	Fish host	Toxicity testing	Clem 1998 (**69**)
	Fish host	Physiological effects	Isom and Hudson 1982 (**59**)
	Media	Viability	Barfield et al 1997 (**70**)
		Unknown	Hudson and Shelbourne 1990 (**71**) Wade et al 1989 (**72**) Dimock and Wright 1993 (**48**) Fisher and Dimock 2000 (**73**) Keller and Zam 1990 (**66**)
Venustaconcha ellipsiformis	Fish host	Host suitability	Riusech and Barnhart 2000 (**74**)
V. pleasii	Fish host	Host suitability	Riusech and Barnhart 2000 (**74**)
Villosa iris	Fish host	Toxicity testing	Jacobson et al 1993 (**75**)
	Fish host	Behavior	Yeager et al 1994 (**24**)
	Media	Unknown	D Simbeck (personal communication)
V. liensosa	Fish host	Toxicity testing	Keller and Ruessler 1997 (**58**)
	Fish host	Host suitability	C Echevarria (personal communication)
	Media	Unknown	Keller and Zam 1990 (**66**)
V. taeniata	Fish host	Reintroduction	Morgan et al 1997 (**62**)
V. vibex	Fish host	Host suitability	C Echevarria (personal communication)

10.4.2.2 *Lost Valley State Fish Hatchery, MO*—Since 2002, personnel at the Lost Valley State Fish Hatchery have propagated, via host fish, about 5000 *Epioblasma triquetra* and 40 000 *Lampsilis teres* juvenile mussels. *Epioblasma triquetra* is considered rare by the state of Missouri and is currently listed as a candidate species by U.S. Fish and Wildlife Service.

10.4.2.3 *Warm Springs National Fish Hatchery, GA*—Due to drought conditions that were occurring in a small tributary of the Flint River, Georgia, 1500 individual mussels were transported to the Warms Springs National Fish Hatchery in the late 1990s. Two species federally listed as endangered (*Lampsilis subangulata* and *Pleurobema pyriform*) have been propagated at the hatchery. Most of the mussels recovered from the dry tributary were maintained at the hatchery in recirculating tanks for about one year. Propagation efforts at the hatchery began in 2000 using a variety of host fish. Hatchery managers reported the successful transformation juvenile *Villosa vibex*, *V. lienosa*, and *L. subangulata*. *Lampsilis subangulata* is listed as endangered by the federal government and consideration of this listing has prompted hatchery personnel to focus efforts on propagating this and other species in the region. From these three species, nearly 8000 juvenile mussels were released into Spring Creek, GA. An additional 20 000 juvenile mussels have been maintained in laboratory conditions and are being monitored for growth and survival of viable juvenile mussels in these hatchery conditions.

10.4.2.4 *White Sulphur Springs National Fish Hatchery, WV*—In response to an emergency salvage order, White Sulphur Springs was involved in the collection and holding of various mussels species from the Ohio River in 1995. While high mortality occurred in mussels held in <5 cm of substrate during winter months, the following years yielded a much high survival of mussels held in containers with at least 20 cm of substrate. The propagation of two common mussels, *Lampsilis fasciola* and *V. iris* indicated that conditions at the hatchery may be limiting for the successful transformation of other species. While juvenile mussels were successfully propagated using fish host techniques, mean survival of *V. iris* and *L. fasciola* juvenile mussels following three months was 50 % and 6 %, respectively.

10.4.2.5 *Genoa National Fish Hatchery, WI*—The Genoa hatchery is focusing its recovery efforts on the propagation and reintroduction of federally endangered juvenile *Lampsilis higginsi*, and *Quadrula fragosa*. Various propagation techniques are being implemented including hatchery propagation (using host fish) and holding of juvenile mussels for survival and growth. Over 4 years, about 1 500 000 juvenile mussels were released into watersheds known to maintain existing or historic populations of *L. higginsi*. The majority of juvenile mussels produced are by cage propagation in river systems using host fish. Other propagation techniques include the free release of infested host fish. Nearly 20 500 host fish were released in 2003 and 2004 and results indicate that for cage releases, over 7000 sub-adults are living and growing from these 2 year classes. Other mussel work includes host fish studies and propagation for the native mussel species. In 2004, channel catfish were infested and held with *Q. fragosa* glochidia and held until releases are favorable in the spring.

10.4.2.6 *Aquatic Wildlife Conservation (AWCC), Buller Fish Cultural Station, VA*—AWCC was established in 1998 to recover mussels within the Upper Tennessee River Drainage of Virginia. The facility has held over 30 species of adult mussels with a survival rate of 95 %. Additionally, at least 16 species have spawned at the AWCC including both state and federally listed species. These mussels are held in 1 meter round diameter tanks fed with natural river water. Propagation and release has been successful for *Actinonaias ligamentina*, *A. pectorosa*, *Epioblasma brevidens*, *E. capsaeformis*, *E. florentina walkeri*, *Lampsilis fasciola*, *L. ovata*, *Villosa iris* and *V. perpurpurea*. Over 70 000 individuals, ranging from 1 week to 6 years of age, have been released into the Powell and Clinch Rivers. Grow-out of propagated juvenile mussels past one year has been attempted and successful for 4 species (*E. brevidens*, *E. capsaeformis*, *L. fasciola* and *V. iris*). Due to concerns over impacts in Indian Creek, Tazewell County, VA, an Ark population of 2 federally endangered species, *E. florentina walkeri* and *V. perpurpurea*, was established at AWCC. Both species have spawned providing a number of females on hand for propagation during the upcoming season.

10.4.2.7 *Tennessee Aquarium Research Institute (TNARI), GA*—To stem the tide of extinction in southeastern rivers and streams, TNARI surveys and monitors mollusks within the region and to propagate mussels and snails in captivity for reintroduction into the wild. TNARI scientists have successfully bred in captivity the Georgia rocksnail, the plicate rocksnail and the spiny riversnail—snails selected for propagation because habitat destruction has resulted in the loss of these species from over 85 percent of their historical range. In 2002, TNARI researchers produced about 12 000 snails in captivity. More than 2700 spiny riversnails were released into the Tennessee River in 2002. The TNARI has propagated the following species since 2000: *Io fluvialis*, *Lampsilis altilis*, *L. virescens*, *Lasmigona holstonia*, *Leptoxis foremani*, *Leptoxis plicata*, *Medionidus acutissimus*, *Pleurobema decisum*, *P. georgianum*, *Ptychobranchus greenii*, *Villosa nebulosa* and *V. umbrans*.

10.4.3 Bishop et al (2005) (**46**) reported both successful and unsuccessful shipment of gravid mussels of various species based on numerous personal communications with facilities involved in mussel transport. Shipping gravid mussels is often necessary because mussels are not in the area where the propagation laboratory is located.

10.4.3.1 Long-term brooders (Lampsilinae and Anodontinae) tend to hold their embryos or glochidia during shipping and handling. Adult mussels can be transported to the laboratory at about 4 to 10°C using ice bags or ice packs placed in a cooler. The ice bags or ice packs should not be in direct contact with the mussels or with the water containing the mussels (if mussels are shipped with water). Specifically, there should be some insulation around the ice bags or ice packs. Cope et al (2004) (**76**) recommends shipping adult mussels in moist burlap in coolers with ice in plastic bags for transport duration <12 h at a temperature within 2°C of the collection water (if possible). Alternatively, Chen et al (2001b) (**77**) and Gordon (2001) (**78**) recommend shipping adult mussels in well-aerated water. The approach used may be dependent on the species of

mussel being shipped. For species that are relatively tolerant of low of oxygen, it may not matter which approach is used for short intervals of time (Chen et al 2001b) (**77**). Lampsilinae and Anodontinae mussels will not likely abort glochidia during transport, but can abort glochidia after been warmed and placed into culture systems. Once received in the laboratory, the temperature of the water containing the mussels should be gradually adjusted to the test temperature (for example, increase by no more than about 3°C/h). Some culture facilities have had better success when adult mussels are held for a day or two before the glochidia are extracted for propagation of juvenile mussels (Bishop et al 2005) (**46**).

10.4.3.2 Short-term brooders (Unioninae) tend to abort embryos or glochidia during shipping or following shipping (although less than 5 % may abort, resulting in partial demibranch release during transportation). Adult amblemine mussels transported in wet towels in an ice chest often abort when returned to water. *Quadrula* species seem to be especially prone to aborting glochidia when disturbed (Bishop et al 2005b) (**46**).

10.4.4 Glochidia have been shipped free from the marsupia in cool, well oxygenated natural or reconstituted water (Gordon 2001 (**78**); section 10.4.4). Excised gravid marsupia have also been shipped for use in propagation efforts. However, the most appropriate way to ship glochidia is free from the marsupia because the female mussel is not killed (section 10.5.3). Alternatively, cold storage at about 4°C of inflated marsupia for up to 4 d has been shown to be effective in maintaining the condition of encapsulated glochidia for toxicity testing (Bishop et al 2005) (**46**). Glochidia of *Lampsilis higginsi* were held at 8 to 12°C for 24 h without a substantial reduction in viability (Gordon 2001) (**78**). Zimmerman and Neves (2002) (**42**) compared glochidia from two species over time in different temperature regimes and found that glochidia in the cooler temperatures (0 and 10°C) remained viable longer than those at 25°C (75 % survival at 7.5 days for *Villosa iris* and at 14.4 days for *Actinonaias pectorosa*) and were able to be transformed on fish following this time period (Table A1.2).

10.4.5 *Shipping Glochidia or Juvenile Mussels:*

10.4.5.1 Section 10.5.3 describes procedures for isolation of glochidia from female mussels and section 10.5.4 describes procedures for culturing juvenile mussels. It may be desirable to ship adult mussels containing glochidia rather than ship glochidia isolated from female mussels. Once glochidia have been isolated, the female mussel can be returned to the collection site (Keller and Augspurger 2005) (**53**).

10.4.5.2 Young juvenile mussels or glochidia isolated from female mussels are fragile and should be shipped with care. The glochidia or juvenile mussels should be shipped from the source to the laboratory in as short of a period of time as possible using an over night delivery service. Check to determine that the vendor accepts live organisms for shipment. Before shipping, empty shells or detritus should be separated from the glochidia or juvenile mussels. The mussels should then be placed into clean culture water or acclimated to the dilution water before shipment (section A1.4.2.2). It is not necessary to feed the juvenile mussels during shipping. In fact, food may adversely affect the water quality during transit.

10.4.5.3 Either plastic bags or square, wide mouth polyethylene bottles (for example, 250 to 1000 mL) work well for contain mussels when placed into strong-walled containers for shipping. Square bottles, when properly sealed, can be laid on their sides; the square form may help prevent piling or bunching of mussels during shipment. Teflon tape can be wound around the threads of the bottle to help seal the cap of the bottle. Flat (or square) bottom fish-shipping bags also work well for containing mussels. Use of the pleated bag (flat bottom) provides a larger surface area for the mussels to lie on during shipping. For added security, the shipping bag should be doubled bagged. Each bag should be sealed with rubber bands. Zip-lock bags should not be used because these bags may open during shipment. Pure oxygen can be added to the water containing the mussels before sealing the bags or bottles for shipment.

10.4.5.4 Shipping containers should be durable and water tight. Six-pack beverage coolers are well insulated, durable, and work well for shipping bottles or bags containing glochidia or juvenile mussels. The addition of bubble wrap, newspaper or foam peanuts will reduce jostling and keep the bottles or bags more secure in the container. These materials also add an additional layer of insulation. Coolers containing test organisms should be firmly taped shut before shipment.

10.4.5.5 Care should be taken in shipping mussels when outdoor temperatures are reduced or elevated. Insulated shipping containers will help protect from temperature fluctuations during shipping. Ice packs can be used to stabilize the temperature of the shipping container. Small temperature recorders can be used to monitor temperature of the container during shipment. Once received in the laboratory, the temperature of the water and the water quality characteristics of the water containing the mussels should be gradually adjusted (for example, a temperature increase of no more than about 3°C/h). See section A1.4.2.2 for additional guidance on acclimation of test organisms before the start of a toxicity test.

10.5 *Care and Handling of Organisms in the Laboratory:*

10.5.1 Information in the following sections and in section 10.6 summarizes procedures for the culture of mussels.

10.5.2 *Adult Mussels:*

10.5.2.1 In the laboratory, adult mussels can be maintained in aquaria with a substrate of sediment or gravel. Maintaining the physiological condition of adult mussels in the laboratory is difficult because the diet and nutritional requirements for mussels are poorly understood (Cope et al 2004) (**76**). Adult mussels held for up to one month without feeding can produce viable glochidia; however feeding adult mussels algae enhanced survival of adult mussels (Johnson et al 1993 (**79**), Patterson et al 1999 (**80**), Gatenby et al 2000 (**81**)). Holding and maintaining adult mussels in laboratory conditions is necessary to allow for transport acclimation, glochidia development, and in some cases, for reproduction to occur. *Villosa* spp. and *Lampsilis* spp. are particularly easy to maintain in the laboratory when given adequate food quantity and quality (Bishop et al 2005) (**46**). Maintenance of these species results in relatively low mortality and measurable growth, indicating that these individuals are in reasonably good condition. Females of *Villosa*, *Pyganodon*, *Utterbackia*, *Tritogonia*,

Elliptio, and *Pleurobema* have repeatedly become gravid in holding conditions (Bishop et al 2005) (**46**).

10.5.2.2 Adult mussels should be observed daily for signs of stress or mortality. Gaping mussels that do not close when touched with a probe should be discarded. Mussels that never open or do not deposit feces should be discarded. Waste and feces should be siphoned out of the culture systems as needed. Concentrations of glycogen in the adult mussels should also be monitored during the time that the organisms are held in the laboratory (Patterson et al 1999 (**80**), Naimo et al 1998 (**82**), Naimo and Monroe 1999 (**83**), Cope et al 2004 (**76**)).

10.5.2.3 Cope et al (2004) summarizes conditions for holding adult mussels in the laboratory or in ponds and recommends feeding adult mussels 1×10^5 algal cells/mL or 4.0 mg/L dry weight of algae twice daily or 2 to 5×10^4 algal cells/mL or 1.9 mg/L dry weight of algae on a continuous bases (Gatenby et al 2000 (**81**) and Gatenby 2002 (**84**)). The amount of algae required is dependent on the biomass of adult mussels in a particular culture location.

10.5.2.4 Adult *Lampsilis cardium* have been held in the laboratory in aerated 100 to 150-L flow-through aquaria receiving about 20 to 30 L/h containing sand and aerated well water at 10 to 15°C. Adults were fed a commercial shellfish diet[4] at a ration of 1.2 mL/individual/day. To deliver feed, about 80 % of the water was siphoned from the aquaria and the shellfish diet was added (mixed with about 500 mL of well water) and then the tank was filled with water back to volume. Adults were usually fed three times a week and the ration was adjusted accordingly (for example, to get a 7-d supply of food delivered in 3 feedings). Adult *L. cardium* have been held in this manner with few to no mortality for up to one year (Newton et al 2003) (**61**).

10.5.2.5 USGS (2004) held adult mussels containing glochidia in an indoor laboratory setting. Well water (hardness 280 mg/L as $CaCO_3$ at 10 to 17°C) was provided at a rate of about 1 volume addition/h. Mussels were held in 250 to 600-L tanks. Plastic containers (35 by 24 by 23 cm) were placed in the fiberglass tanks and a 10-cm layer of creek gravel (about 0.5 to 2 cm diameter) was used as a substrate in each container. About 10 adult mussels were placed in each container. About 15 mL of two instant algae mixtures (prepared from non-viable microalgae concentrates of *Nannochloropsis* and from a commercial shellfish diet)[4] were added every other day to each container (section A1.4.5 for a description of the process used to prepare these two instant algae mixtures).

10.5.2.6 Adult mussels have been held in a 0.1 hectare pond for more than 1 year in suspended pocket nets or in sediment-filled containers placed on the bottom of the pond (Dick Neves, USGS, Blacksburg, VA; personal communication).

10.5.3 *Glochidia:*

10.5.3.1 During early development, glochidia are carried in the gills of the female mussel. The maturity of the glochidia can be determined by the color of the gills of the female. Gills containing mature glochidia are enlarged and brown in color whereas enlarged beige or white gills may contain immature glochidia (Johnson et al 1993) (**79**). Many short-term brooders have conglutinates that change in color from red to pink as the glochidia mature (Jones et al 2004) (**36**). Visual examination of gill of a female mussel can be done by carefully prying the sides of the shell open.

10.5.3.2 Mature glochidia can be gently flushed from the marsupium of a female mussel into a basin or shallow container using a sterile hypodermic syringe filled with dilution water in which the female mussels are held. The gage of the needle used should be based on the size of marsupium of the mussel (for example, needle about 3.8-mm long, 16 to 20 gauge). Care should be taken not to damage the gill structure within the marsupium. The valves of the adult mussel should be slowly opened with reverse pliers (Gordon 2001) (**78**) or with a small nasal speculum. Opening mussels too quickly or too wide can crack the valves or rip the adductor mussels. The valves can be propped open with a silicon stopper or similar object. Caution should be taken not to damage internal organs, labial palps, or gill structure (Gordon 2001) (**78**). Glochidia have also been isolated by cutting a section of gill from the female mussel and then teasing out the glochidia in water. This latter technique is destructive to the gills of the adult female and should be avoided if possible. No studies were identified where glochidia were isolated for toxicity testing from conglutinates released into the water by female mussels (Kernaghan et al 2005) (**5**).

10.5.3.3 Isolated glochidia can be held in glass chambers before the start of a toxicity test or before the glochidia are used to produce juvenile mussels (section 10.5.4). Glochidia of anodontines may stick together due to byssal thread adhesion. These aggregates of glochidia can be separated by carefully aspirating the aggregates in and out of a pipette. The maturity of glochidia can be determined through microscopic examination. Mature glochidia will be free of embryonic membranes and the shell valves of viable glochidia will open and close sporadically in anodontine species. Viability of glochidia isolated from a female mussel should be evaluated before the start of a toxicity test using a solution of NaCl (section A1.4.8.4).

10.5.3.4 Gravid female mussels are usually collected from the field and held in the laboratory before isolating glochidia to start a toxicity test. Alternatively, Zimmerman and Neves (2002) (**42**) suggested glochidia of some species (including *Villosa iris* and *Actinonaias pectorosa*) could be extracted in the field from a female and transported back to the laboratory in cool water where the glochidia can remain viable for several days without a reduction in ability to successfully attach on a host fish. This procedure may be particularly useful when glochidia of endangered species are extracted in the field, and the female mussels should be immediately returned to their habitat.

10.5.3.5 Before starting an exposure, the viability of glochidia should be evaluated by the response of the glochidia to the addition of a solution of NaCl (section A1.4.8.4). Mature and healthy glochidia will snap shut in response to the addition of a salt solution. Immature glochidia isolated from the

[4] The sole source of supply of the materials known to the committee at this time is Instant Algae 520 McGlincy Lane #9, Campbell, CA 95008. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

marsupium of a female will often be enclosed in an egg membrane and will be fragile and tend to fracture. Toxicity tests are usually started if >90 % viability of the glochidia is observed (Annex A1). If an abundance of immature glochidia are isolated from a female mussel, progeny of this female should not be used to conduct a toxicity test.

10.5.3.6 Exposures are usually started the same day that glochidia are isolated from female mussels without an extended acclimation period in the dilution water before the start of a toxicity test (Table A1.1 and Table A1.3). However, Wang et al (2003) **(85)** observed that the sensitivity of *Lampsilis siliquoidea* glochidia held for 24 h after isolation from a female was similar to newly-released glochidia in exposures to copper. The viability of glochidia isolated from each female should be evaluated before glochidia are pooled together (section A1.4.8.4). Toxicity tests should be conducted by pooling glochidia from at least three female mussels. Toxicity tests can be conducted with glochidia obtained from one female mussel (for example, when a limited number of organisms of an endangered species is available for testing); however, the results of tests conducted with a limited number of mussels should be interpreted with caution. Additional research is needed to determine the minimum number of females that should be sampled to obtain glochidia to start a toxicity test. This research might include an evaluation of the variability in sensitivity of glochidia obtained from individual females using a variety of toxicants (section A1.6).

10.5.4 *Juvenile Mussels:*

10.5.4.1 Toxicity tests with juvenile mussels are typically started within about 5 d after juvenile mussels are released from a fish host (Table A1.4; for example, *in vivo* propagation; Lefevre and Curtis 1912) **(39)**. Alternatively, artificial media has also been used to transform juvenile mussels for use in toxicity testing (for example, *in vitro* propagation; Johnson et al 1993 **(79)**, Clem 1998 **(69)**, Isom and Hudson 1982 **(59)**, Summers 1998 **(86)**, Hudson et al 2003 **(87)**).

10.5.4.2 Bishop et al (2005) **(46)** provides an overview of *in vitro* and *in vivo* methods used to culture juvenile mussels. Juvenile mussels cultured *in vitro* should not be used to conduct toxicity tests unless it has been demonstrated that the sensitivity of the juvenile mussels cultured *in vitro* is similar to the sensitivity of juvenile mussels cultured *in vivo*. Comparisons of physiological conditions of juvenile mussels transformed *in vitro* and *in vivo* indicate that individuals that transform on a fish host tend to be healthier than individuals that transform in artificial culture media. Juvenile mussels transformed with fish exhibited several features that were not present in juvenile mussels transformed *in vitro* (Fisher and Dimock 2002) **(73)**. There was little evidence of lipids and glycogen in the larval mantle cells of the juvenile mussels transformed *in vitro*, whereas the juvenile mussels transformed with fish had numerous lipid droplets and glycogen granules in the basal portions of the cells (Fisher 2002 **(88)**, Hudson et al 2003 **(87)**). Juvenile mussels transformed *in vivo* on fish hosts were less sensitive to thermal and hypoxic stresses compared to juvenile mussels transformed *in vitro* (Fisher 2002) **(88)**. Juvenile *U. imbecillis* transformed *in vitro* were less sensitive compared to juvenile mussels transformed *in vivo* in 24-h exposures to sodium dodecylsulfate; however, sensitivity to cadmium or ammonia was similar between the two groups of juvenile mussels (Summers 1998) **(86)**. Comparisons of toxicity tests conducted with *in vitro*- and *in vivo*-transformed juvenile mussels indicated that juvenile mussels transformed in an artificial medium were more sensitive to copper than the juvenile mussels transformed on a fish host (Warren and Klaine 1994) **(89)**.

10.5.4.3 Table 2 provides a summary of techniques that have been used to transform juvenile mussels (Bishop et al 2005) **(46)**. Most freshwater mussels require a host fish for reproductive success. Freshwater mussels are identified as either generalists, where glochidia can transform on a variety of fish species, or specialists, where only one or two host fish have been identified that successfully metamorphose glochidia to the juvenile life stage. Techniques for determination of fish hosts for a particular species have been reported and used by many researchers for decades, while some unconventional hosts (for example, amphibians) have also been used to transform juvenile mussels. Some freshwater mussels can transform from glochidia directly to juvenile mussels inside the marsupial pouch of the mussel (for example, *Strophitus undulatus*, *Utterbackia imbecillis*, *Obliquaria* spp.; Bishop et al 2005) **(46)**.

10.5.4.4 Common species as well as state and federally listed species are often difficult to transform due to the lack of knowledge of life history complexities and requirements (section 10.1). Glochidial attachment can range from several days to several months depending on the mussel species, fish health, water temperature, and other unknown variables (Bishop et al 2005b) **(46)**. Alternatively, fish survival can be jeopardized by excessive glochidial infestation, limiting gas exchange across the gill lamellae. Maintenance of healthy host fish before and during encystment is critical to the success of transforming juvenile mussels. While 50 to 100 glochidia/gill for fish 15 to 25 cm in length have been reported as adequate, others investigators have directly infested host fish with several thousand and achieved successful transformation and still maintained fish viability (Bishop et al 2005) **(46)**. Transformation of glochidia to juveniles on the fish gill (or in artificial media) may range from 7 to >110 d, depending on mussel species, water temperature, and host fish condition (Bishop et al 2005) **(46)**.

10.5.4.5 Host fish should not be fed for several days before the release of the transformed juvenile mussels. The bottom of the chamber holding the host fish should be kept clean of debris before the release of the newly-transformed juvenile mussels. Bottom-feeding minnows and catostomids may feed on newly-transformed juvenile mussels; therefore these fish should be separated from the bottom of the chamber with fine mesh (Bishop et al 2005) **(46)**. The newly-transformed juvenile mussels can be siphoned from bottom of the chamber holding the host fish and collected using a sieve of appropriate size (for example, 130 µm). A polarized lens attached to the objective lens of a dissecting microscope can be used to reflect, through under stage lighting, only prismatic objects and block out sediment or feces that can make juvenile identification and counting difficult (Watters 1996) **(90)**.

10.5.4.6 Section 10.4 provides guidance on obtaining and shipping juvenile mussels from facilities that culture mussels. The following sections provide examples of approaches used by culture facilities to transform juvenile mussels. Laboratories interested in transforming juvenile mussels in their own facilities are encouraged to obtain the publications cited in the sections below for additional detail. Laboratories interested in transforming juvenile mussels at their own facilities may also want to contact facilities listed in Table 2 for guidance.

10.5.4.7 Techniques for determining fish host suitability include the use of aeration tanks, direct gill placement, and the use of anesthetics to reduce handling stress on the fish (Zale and Neves 1982) (**91**). Aeration tanks have been used when there are viable glochidia with several fish species and cohorts. However, if glochidia are limited or the fish are small, direct gill placement using pipettes is a viable alternative to aeration techniques for attachment onto the gill (Bishop et al 2005b) (**46**). Host suitability trials should include multiple attempts using several individuals of the same host organism with glochidia from different females to assure that metamorphosis occurs in at least two different test trials (Bishop et al 2005) (**46**).

10.5.4.8 The U.S. Fish and Wildlife Service Genoa National Fish Hatchery in Genoa, WI uses the following procedure to encyst glochidia of federally-endangered *Lampsilis higginsi* using largemouth (*Micropterus salmoides*) or smallmouth (*M. dolomieu*) bass as the fish hosts (Tony Brady, Genoa, WI, personal communication; Gordon 2001) (**78**). Glochidia are flushed from the gills of 1 to 3 adult mussels. About 2 mL of glochidia are added to 1 to 2 L of water, and 10 fish are then placed into this solution for about 3 minutes. Host fish should be introduced after the addition of the glochidia to minimize fouling of the chamber with excess feces or mucus. A smaller volume of water allows for more concentrated glochidia when infesting fish. Aeration with an air stone is used to keep the glochidia in suspension. The target infestation is 250 glochidia per fish. Light levels should be reduced as much as feasible to minimize activity of the infested fish.

10.5.4.9 Barnhart (2003) (**92**) described a system used to transform juvenile mussels of three species of freshwater mussels: *Lampsilis rafinesqueana*, *L. abrupta*, and *Leptodea leptodon*. A large-scale recirculating system for mussel propagation was developed and used to produce large numbers (14 000 to 375 000) of juvenile mussels. Barnhart (2003) (**92**) also provides a description of procedures that can be used to encyst the glochidia on the fish hosts and maintain the host fish during the transformation of the juvenile mussels. Host fish containing encysted glochidia were held in flow-through raceways and then transferred into low-flow or recirculating tanks during the drop-off period to avoid losing the juvenile mussels. Water supplies at hatcheries often contain a wide variety of zooplankton that are the same size as glochidia or juvenile mussels. Some invertebrates such as flatworms and hydra are predators on juvenile mussels. Other species are the same size range of glochidia or juvenile mussels and are very difficult to separate (for example, cladocerans, ostracods, bryozoans). Efforts to remove invertebrates by pre-filtering water supplies were unsatisfactory. Vacuuming the tanks holding the host fish to remove transformed juvenile mussels was labor intensive and missed a large proportion of the juvenile mussels that dropped from the fish host. The recirculating propagation system (RPS) developed by Barnhart (2003) (**92**) was designed to hold several hundred host fish and recover glochidia or juvenile mussels continuously from the recirculating flow of water (Figure 1 to 7 in Barnhart 2003 (**92**)). The RPS consists of: *(1)* 2 conical-bottom 1000-L tanks each with a double stand pipe to contain the host fish, *(2)* a sump containing a biological filter to maintain water quality, *(3)* recovery filters to recover juvenile mussels from each tank, and *(4)* a pump to recirculate water. Host fish can be held in the RPS during the entire encystment period or the fish can be moved to the RPS shortly before drop-off of the juvenile mussels. Host fish are not fed for several days in advance of the drop-off of the juvenile mussels. The RPS system eliminates most problems with zooplankton because these organisms do not enter the system. Vacuuming debris from the bottom of the tank is also eliminated because recirculation of water is used to recover the juvenile mussels by moving them to a filtration system. The juvenile mussels can be removed from the filters to facilitate counts and expedite handling for use in culture or toxicity testing.

10.5.4.10 Newton et al (2003) (**61**) used *in vivo* infestation to obtain about 2000 juvenile *Lampsilis cardium* from largemouth bass (*Micropterus salmoides*). Glochidia were combined from at least three female mussels and used to infest four, 8 to 15-cm long largemouth bass. Glochidia were isolated from a female mussel by flushing the gill with about 30 mL of well water (delivered three times via a 10 mL syringe). The water containing isolated glochidia was placed into a glass dish and glochidia viability was determined on a subsample and then glochidia isolated from all of the female mussels were composited into one dish. Four fish were placed into a 19-L bucket with about 9.5 L of vigorously aerated well water followed by the addition of the glochidial solution. After 10 min, one fish was randomly removed and placed into a separate 19 L bucket with 9.5 L of well water and 1.0 g MS-222. Once the fish became lethargic, the gills were checked for level of glochidial infestation (the target was about 400 to 500 glochidia/fish). If the infestation was low, the fish was put back into the bucket containing glochidia for about 2 to 5 min and re-checked to evaluate infestation. Once the encystment was complete, the fish were transferred into 38-L flow-through aquaria (about 500 mL/min) containing dechlorinated well water at 22°C. Temperature, dissolved oxygen, and flow rate were measured daily and tank bottoms were siphoned daily. At this temperature, juvenile mussels began to excyst in about 17 to 19 d. Encysted fish were fed rainbow trout, *Oncorhynchus mykiss*, until about 7 to 10 d before the expected release of juvenile mussels. About 3 d before the expected release of juvenile mussels, fish were consolidated into 2 aquaria using a plastic baffle to separate the fish. To determine post-excystment age, water was siphoned from the aquaria bottoms daily through a 153-µm sieve and the contents was examined under a microscope. Juvenile mussels from a given day were transferred into 4.4-cm inner diameter glass cylinders fitted with a 153-µm mesh bottom and suspended in 38-L flow-through aquaria at

22°C until use in toxicity testing. This procedure has been used to produce juveniles for conducting more than 10 toxicity tests and has resulted in acceptable survival of both host fish and juvenile mussels.

10.6 *Feeding:*

10.6.1 *Adult Mussels*—See 10.5 for a description of procedures for feeding adult mussels held in the laboratory.

10.6.2 *Glochidia*—Glochidia isolated from female mussels are not fed in culture or in toxicity tests.

10.6.3 *Juvenile Mussels:*

10.6.3.1 The following sections summarize information on general feeding requirements of juvenile mussels. Examples of procedures used by facilities to culture newly-transformed juvenile mussels are also presented. Bishop et al (2005) (**46**) also describes procedures for rearing juvenile mussels caged in rivers and describes case studies where facilities have propagated and reintroduced juvenile mussels into the environment.

10.6.3.2 Little is known about the survival, growth, and reproduction of naturally produced mussels once the juvenile mussels excyst from the host organisms. Growth of juvenile mussels during the first year is variable among species and consequently, collection from the wild and assessment of these young individuals is difficult. Certain species of juvenile mussels may only grow a few millimeters to centimeters in a typical year. Percentage of juvenile survival that results in reproductively-viable adults for most species is unknown (Bishop et al 2005) (**46**) ; however, some information is available for some European species of freshwater mussels (Bauer and Wachtler 2000) (**93**).

10.6.3.3 The addition of sediment fines as a substrate has been shown to increase growth rates of juvenile mussels of some species in the laboratory (Hudson and Isom 1984 (**56**), Gatenby et al 1997 (**25**), O'Beirn et al 1998 (**26**)). Juvenile mussels can use the organic matter that coats small sediment particles. While some juvenile mussels do well in fine sediment, juvenile mussels of other species (typically riffle-dwelling species) do poorly in fine sediment (Neves 2004) (**3**). Sediment used to culture juvenile mussels is typically sieved to remove larger particles and autoclaved to remove invertebrate predators and fungal growth that may kill juvenile mussels. Hudson et al (2003) (**87**) report that sediment pretreated with low concentrations of bentonite clay or EZ mud[5] clears the suspension of the finest clay particles, resulting in better survival of juvenile mussels. This indicates that finer particles may impair gill function of juvenile mussels (Bishop et al 2005) (**46**).

10.6.3.4 Nutrition in juvenile and adult mussels is important for the survival, growth, and reproduction of mussel populations. However, little is known about the quantity or quality of food source that provides conditions for sustaining populations in the wild or in the laboratory (Gatenby et al 2003 (**94**), Christian et al 2004 (**95**)). A diversity of algae reportedly improves growth of juvenile mussels (Hudson and Isom 1984 (**56**); Gatenby et al 1997 (**25**), 1999a (**96**) ; Beck and Neves 2003 (**21**)). Algae containing higher levels of lipids (for example, *Neochloris oleoabundans*) promoted the best growth of juvenile mussels (Gatenby et al 1997, 2003) (**25, 94**).

10.6.3.5 Barnhart (2005) (**97**) described a compact recirculating system for rearing newly-transformed juvenile freshwater mussels. The system consisted of nested buckets that partition a volume of 18 L of culture water into an upper and lower compartment. A small submersible pump is used to move water from the lower compartment to the upper compartment, and the water then returns to the lower compartments through cylindrical screen-capped chambers that contain juvenile mussels. The design minimizes space requirements and facilitates the isolation, containment, and handling of juvenile mussels. Newly-transformed juvenile mussels of 8 species were held in these systems for several months and fed continuously by drip with a monoculture of algae (*Neochloris oleoabundans*). River water filtered to remove particles >30 µm was used to culture juvenile mussels to provide a natural community of microorganisms which may aid in digestion. Survival rates were higher than most previous reports for captive juvenile mussels. Survival of newly-transformed *Lampsilis siliquoidea* and *L. reeveiana* exceeded 95 % over 2 months. Changes in shell length in these two species were about linear ranging from 4.2 to 12.5 µm/day at 22°C. These growth rates are similar to or higher than previous reports of growth of juvenile mussels in recirculating systems. The bucket rearing system may be particularly useful for conducting studies feeding studies with juvenile mussels. This recirculating system might also be adapted for conducting chronic toxicity tests with juvenile mussels.

10.6.3.6 Henley et al (2001) (**98**) described two air-driven recirculating water systems for culturing juvenile mussels. An 8-L system was used to hold newly-transformed juvenile mussels for about 10 weeks. Juvenile mussels were then transferred to polyvinyl chloride (PVC) trays place into a larger 350-L system for grow out. The 8-L system consisted of two interconnected polypropylene containers. One container served as the juvenile rearing tank and the other as a reservoir. Water entered the rearing tank via an airlift through silicone tubing from the reservoir and exited the rearing tank through a stand pipe. The rearing tank was designed to have some algal settling for juvenile mussels at a pedal-feeding stage of development. Juvenile mussels were fed periodically to maintain an algal cell density of about 30 000 cells/mL in the water column (Neves 2004) (**3**). *Scenedesmus*, *Nannochloropsis*, and *Neochloris* were genera of algae that are suitable for the diet of juvenile mussels (Neves 2004) (**3**). The 350-L system consisted of an interconnected polyethylene feed trough, a polyethylene drum and a polyvinyl chloride airlift and return tubes. A series of air stones were used to suspend algae in the trough containing the trays with juvenile mussels and in the drum and were used to recirculate water from the drum to the trough. Juvenile mussels were placed in PVC trays (0.2 m by 1.2 m by 20 mm; bottom area about 0.25 m^2) containing about 10 mm of course sand and silt substrate. Algal rations were added to the trough through an algal recirculating system. Similar types of juvenile

[5] The sole source of supply of the apparatus known to the committee at this time is BAROID Industrial Drilling Products. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

mussel systems using electrical pumps to recirculate water were described by O'Beirn et al (1998) (**26**), Jones and Neves (2002) (**99**).

10.6.3.7 Jones and Neves (2002) (**99**) also described a static system for culturing juvenile mussels in 6 cm square and 5 cm deep plastic containers. Juvenile mussels were placed in containers containing 50 mL of water, 50 mL of an algal suspension, and about 0.5 mL of a fine sediment (particle size <105 µm). The sediment was autoclaved to kill predators such as flatworms and diptera larvae before placement into the containers (Jones et al 2004) (**36**). The water, algae, and sediment were exchanged every 2 d. Better survival of juvenile *Cyprogenia stegaria* was observed in the static system compared to a recirculating system; however, the density of algae in the static system was higher than the algae in the recirculating system (Jones and Neves 2002) (**99**).

10.6.3.8 Beaty and Neves (2004) (**100**) described a flow-through culture system using natural river water to maintain newly-transformed juvenile *Villosa iris* for about 90 days. Juvenile mussels were placed in containers partially filled with sieved river sediment, providing both a food source and some protection from physical disturbance. Most of the juvenile mussels were found in a loose, flocculent layer of sediment brought into the containers by the river water. Survival and growth of juvenile mussels was best when cultures were started in June compared to cultures started in August or September, perhaps due to warmer temperatures earlier in the summer.

10.6.3.9 USGS (2005a,b) (**8, 9**) conducted a 28-d feeding study with 2-month-old juvenile *Lampsilis siliquoidea* that compared the influence of various sources of algae, concentrations of algae, and the presence of sediment on survival or growth of juvenile mussels. Juvenile mussels were fed three species of live algae (*Neochloris oleoabundans*, *Pseudokirchneriella subcapitata (formerly Selenastrum capricornutum)*, or *Nannochloropsis oculata*) at three feeding concentrations or two combinations of commercial Instant Algae[4] brand non-viable microalgae concentrates [*Nannochloropsis* or a combination of *Nannochloropsis* and Shellfish Diet; Reed Mariculture, Campbell, CA]) at three feeding concentrations: *(1)* amount recommended by the food providers, *(2)* two times the recommended amount, and *(3)* three times the recommended amount. The feeding study was conducted in a flow-through system with about 60-mL additional water added to each chamber once every 4 h. Juvenile mussels were fed twice a day right after the addition of the new water. By the end of 28-d experiment, the mean survival (n=2) of controls (no-food or sediment-only) ranged 25 to 35 %. Survival of juvenile mussels fed with various foods at the recommended feeding rates ranged from 70 to 90 %. Higher feeding rates generally did not increase the survival of juvenile mussels. The better survival rates (≥85 %) were observed in feeding treatments with the two microalgae concentrates. The results of this feeding study indicate that 28-d chronic toxicity tests starting with 2-month-old juvenile *L. siliquoidea* might be conducted with a control survival of over 80 % using a diluter system and Instant Algae[4] brand microalgae concentrates. Survival of *Villosa iris* was ≥85 % in a subsequent 28-d feeding study using this combined diet of Instant Algae[4] brand microalgae concentrates (USGS 2005b) (**9**).

10.6.3.10 Water hardness concentrations ranging from 250 to 350 mg/L (as $CaCO_3$) have been shown to support the long-term maintenance of juvenile mussels (Bishop et al 2005) (**46**). Others have found that water hardness concentrations as low as 180 mg/L provide adequate levels of calcium and magnesium to support juvenile and adult survival (Farris et al 1998) (**101**). A daily ration of about 30 000 cells/mL of *Neochloris oleoabundans* or *Nannochloropsis oculata* (small-celled species with high lipids) provided adequate nutrition for survival and growth of juvenile mussels for several weeks or months (Henley et al 2001 (**98**), Bishop et al 2005 (**46**)). Holding juvenile mussels in recirculating water system provides a continuous assortment of fine sediments used as a food source and provides more consistent water quality compared to static systems. Juvenile mussels held in recirculating systems for several weeks increased in size by 7 to 12-fold from the newly-transformed juvenile mussels (Milam et al 2000) (**60**).

10.6.3.11 Hudson and Isom (1984) (**56**) observed an 18-fold increase in growth of juvenile *Utterbackia imbecillis* mussels held in raceways over a 74-d period using river water supplemented with sediment and plankton under static conditions. Mussels cultured at 30°C exhibited a slight increase in growth compared to mussels cultured at 23°C. Hudson and McKissick (1999) (**102**) raised artificially-transformed juvenile mussels in a static system for 93 d and observed a 10-fold increase in growth in sediment from the Conasauga River, TN. Although juvenile mussels can survive and grow in static systems, water should be renewed to reduce waste products or the build up of bacteria or fungus (Michaelson and Neves 1995 (**103**), Layzer et al (1993) (**104**). Hanlon (2000) (**105**) reported 82 % survival of juvenile *Lampsilis fasciola* held in concrete raceways for 90 d using recirculating water with sediment fines added as a substrate.

10.6.3.12 Most investigators have observed high mortality of juvenile mussels about 4 to 6 weeks after transformation (as reviewed by Kernaghan et al 2005 (**5**)). As a result of this problem, the duration of toxicity tests started with newly-transformed juvenile mussels is less than 14 d, with survival or growth measured at the end of the exposures (Table A1.4). Food (mixtures of different species of algae) and sediment have been added to test chambers. Some investigators have found that newly-transformed juvenile mussels will survive for at least 14 d without the addition of food (Table A1.4). The high mortality of newly-transformed juvenile mussels in toxicity tests conducted for >14 d is likely related to a lack of an understanding of the nutritional requirements of mussels at this life stage (section 10.5.4).

10.6.3.13 Newly-transformed juvenile mussels depend on pedal-feeding to obtain food (cilia on the foot are used to move food into the juvenile mussel; see 10.1). Juvenile mussels gradually begin to use a combination of pedal- and suspension-feeding to obtain food until the mussels eventually depend on suspension-feeding to obtain food by about 6 months in laboratory cultures supplied with a silt-clay sediment substrate. However, in the field, juvenile mussels probably depend on a combination of suspension-, deposit- and pedal-feeding in

coarser substrates. Research is ongoing to improve culturing methods for propagation, holding, and feeding of newly-transformed juvenile mussels (Keller and Zam 1990 (**66**) ; Gatenby et al 1996 (**106**), 1997 (**25**); Henley et al 2001 (**98**); Jones and Neves 2002 (**99**); Jones et al 2004 (**36**) ; Bishop et al 2005 (**46**)). Once developed, these culturing methods should help to refine methods for conducting chronic exposures with juvenile mussels.

10.6.3.14 Valenti et al (2005) (**107**) conducted toxicity tests starting with 2-month-old juvenile mussels of *Villosa iris* and observed control survival >90 % in 21-d exposures. Juvenile mussels were held in a small amount of sediment and were fed algae (*Neochloris*) and survival and growth were the endpoints. USGS (2005a,b) (**8, 9**) and Bringolf et al (2005) (**108**) conducted toxicity tests starting with 2- to 4-month old juvenile *Actinonaias ligamentina*, *Lampsilis siliquoidea* or *Villosa iris* and observed control survival >88 % in 21- to 28-d exposures when algae was used as a food source (Table A1.4). The size of the algal cells used to feed the juvenile mussels should meet the dietary requirements of the species (for example, usually <10 µm; Gatenby et al 2003 (**94**)), but can be species specific. The algae should be high in polyunsaturated fats (Gatenby et al 2003) (**94**). Addition of a small amount of sediment substrate improves survival and growth of some species of newly-transformed juvenile mussels (Neves 2004) (**3**).

10.7 *Disease Treatment:*

10.7.1 Whenever adult mussels are brought into a facility, these organisms should be quarantined until use for 14 d or until these organisms appear free of disease and a record of the general health of the mussels should be made at least weekly. If a group of mussels is severely diseased, it is often best to destroy the entire group immediately. Although little is known about diseases of freshwater mussels inhabiting North America, there is a potential for pathogen transmission among mussels and fish (Cope et al 2004) (**76**). Disease transmission between mussels and fish may be particularly problematic when mussel culturing facilities are co-located with fish hatcheries. Cope et al (2004) (**76**) recommend establishing a pathogen and disease monitoring plan for adult mussels similar to approaches used for hatchery-reared fish. For example, Newton et al (2001) (**109**) certified that adult mussels collected from the upper Mississippi River were free of bacterial and viral agents based on inspections conducted by the U.S. Fish and Wildlife Service Fish Disease Control Center in Onalaska, WI.

10.7.2 Zimmerman et al (2003) (**110**) described a procedure for control of predatory flatworms in culturing juvenile mussels. Newly-transformed juvenile mussels did not survive in concentrations of formalin required to kill flatworms. Therefore, Zimmerman et al (2003) (**110**) recommend treatment of host fish with formalin before these fish are used to transform mussels.

10.7.3 Adult mussels collected from the field should be inspected for the presence of zebra mussels (*Dreissena polymorpha*). Soft brushes should be used to remove attached zebra mussels. The adult mussels should be held in a quarantined area for at least one month to determine whether additional zebra mussels are present (Gatenby et al 2000 (**94**), Newton et al 2001 (**109**), Cope et al 2004 (**76**)). The equipment used in mussel cultures suspected to be infested with *D. polymorpha* should be treated with 25 to 250 mg/L hypochlorite and effluent water from the mussel cultures should treated to a concentration of at least 5 mg/L hypochlorite. Additional guidance on handling or control of zebra mussels is describe in:

(1) Gatenby et al (1999b, 2000) (**111, 81**), Newton et al 2001 (**109**) and Cope et al (2004) (**76**)

(2) http://sgnis.org/publicat/papers/zmr_2_06.pdf

(3) http://nas.er.usgs.gov/zebra.mussel/

(4) http://www.clo2.com/reading/Subject_Papers/zebra-mussel-control.htm

(5) http://ag.ansc.purdue.edu/EXOTICSP/732_articles_related_to_ZM.htm

10.8 *Acclimation*—Section A1.4.2.2 provides information of acclimation of test organisms before the start of a toxicity test.

10.9 *Quality*—Section 11 provides information on quality assurance and quality control for the culture and testing of test organisms.

11. Quality Assurance and Quality Control

11.1 *Introduction:*

11.1.1 Developing and maintaining a laboratory Quality Assurance (QA) program requires an ongoing commitment by laboratory management and also includes the following: *(1)* appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program, *(2)* preparation of a Quality Assurance Project Plan with Data Quality Objectives, *(3)* preparation of written descriptions of laboratory Standard Operating Procedures (SOPs) for test organism culturing, testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, and *(4)* provision of adequate, qualified technical staff and suitable space and equipment to ensure reliable data (Guide E1391).

11.1.2 Quality Assurance (QA) practices within a testing laboratory should address all activities that affect the quality of the final data, such as: *(1)* sample sampling and handling, *(2)* the source and condition of the test organisms, *(3)* condition and operation of equipment, *(4)* test conditions, *(5)* instrument calibration, *(6)* replication, *(7)* use of reference toxicants, *(8)* record keeping, and *(9)* data evaluation.

11.1.3 Quality Control (QC) practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to testing, see Guide E1391 and Test Method E1706).

11.2 *Performance-based Criteria:*

11.2.1 The USEPA Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards for chemical analytical methods (Test Method E1706). Performance-based methods were defined by EMMC as a monitoring approach which permits the use of appropriate methods that meet preestablished demonstrated performance standards. Minimum required elements of

performance, such as precision, reproducibility, bias, sensitivity, and detection limits should be specified and the method should be demonstrated to meet the performance standards.

11.2.2 No single method is required for collection or culture of mussels used conduct a toxicity test. Success of a test relies on the health of the culture from which organisms are taken for testing. Having healthy organisms of known quality and age for testing is the key consideration relative to culture methods. Therefore, a performance-based criteria approach is the preferred method through which individual laboratories can evaluate culture health rather than requiring all laboratories to use the same culturing procedure. Performance-based criteria are used in ASTM standards dealing with toxicity testing to allow each laboratory to optimize culture methods while providing organisms that produce reliable and comparable test results (for example, Test Methods E1367 and E1706). See Table A1.3 and Table A1.5 in Annex A1 for a listing of performance criteria for culturing and testing of organisms.

11.3 *Facilities, Equipment, and Test Chambers:*

11.3.1 Separate areas must be maintained for culturing and testing organisms to avoid loss of cultures because of cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or toxicity testing areas, and from toxicity testing laboratories and sample preparation areas into culture areas.

11.3.2 Equipment for temperature control should be adequate to maintain recommended test-water temperatures. Recommended materials should be used in the fabrication of the test equipment which comes in contact with the dilution water (that is, water or sediment).

11.3.3 Before a toxicity test is conducted in a new facility, a "non-contaminant" test should be conducted in which all test chambers contain control water. This information is used to demonstrate that the facility, control water, and handling procedures provide acceptable responses of test organisms.

11.3.4 *Water*—Quality of water used for organism culturing and testing is extremely important. Water used to conduct toxicity tests and water used to culture organisms should be uniform in quality. Acceptable water should allow satisfactory survival or growth of the test organisms. Organisms should not show signs of disease or apparent stress (for example, discoloration, unusual behavior). See Section 8 for additional details.

11.4 *Test Conditions*—Temperatures should be maintained within the limits specified for each test. Dissolved oxygen, alkalinity, water hardness, conductivity, ammonia, and pH in toxicity tests should be checked in accordance with Annex A1.

11.5 *Quality of Test Organisms:*

11.5.1 Test organisms should appear healthy, behave normally, and have low mortality in cultures, during holding, and in test controls (for example, <20 % for 48 h before the start of a juvenile mussel toxicity test).

11.5.2 Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$, see 16.4). Data from these reference-toxicant tests can be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.

11.5.3 All organisms in a test must be from the same source. The supplier of organisms should also certify the species identification of the organisms, and provide the taxonomic references, or name(s) of the taxonomic expert(s) consulted.

11.6 *Quality of Food*—Problems with the nutritional suitability of the food will be reflected in the survival or growth of the test organisms in cultures or in toxicity tests.

11.7 *Test Acceptability*—Table A1.3 and Table A1.5 in Annex A1 outline requirements for acceptability of tests. An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the toxicity test (see test condition summaries in Table A1.1 and Table A1.4). The acceptability of a test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications should be noted when reporting data from a test.

11.8 *Analytical Methods:*

11.8.1 All routine chemical and physical analyses for culture and testing water, food, and sediment should include established quality assurance practices (Guide E1391).

11.8.2 Reagent containers should be dated when received from the supplier and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

11.9 *Calibration and Standardization:*

11.9.1 Instruments used for routine measurements of chemical and physical characteristics such as pH, dissolved oxygen, temperature, and conductivity should be calibrated before use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see Test Method E1706 for a listing of USEPA Methods) Calibration data should be recorded in a permanent log.

11.9.2 Known-quality water should be included in the analyses of each batch of water samples (for example, water hardness, alkalinity, conductivity). It is desirable to include certified standards in the analysis of water samples.

11.10 *Replication and Test Sensitivity*—Sensitivity of toxicity tests will depend in part on the number of replicates/treatment, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of a test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

11.11 *Demonstrating Acceptable Performance:*

11.11.1 Before conducting tests with chemicals of interest, it is strongly recommended that the laboratory conduct the toxicity test with control water alone. Results of these preliminary studies should be used to determine if the use of the control water and other test conditions result in acceptable performance in the toxicity test as outlined in Annex A1.

11.11.2 Section 16.4 provides a summary of techniques to evaluate acceptable laboratory performance (for example, reference-toxicity tests, variance associated with intra-laboratory toxicity tests, variance associated with inter-laboratory toxicity tests). Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$, see 16.4).

11.12 *Record Keeping*—Section 14.1 outlines recommendations for recorded keeping (that is, data files, chain-of custody).

12. Experimental Design

12.1 Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and numbers of test chambers and organisms per treatment should be based on the purpose of the test and the type of procedure that is to be used to calculate results (Section 14). One of the following two types of experimental design will probably be appropriate in most cases.

12.1.1 A toxicity test intended to allow calculation of an LC50, EC50, IC50, NOEC, or LOEC usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the dilution-water or solvent control(s), or both (section 9.3), organisms are exposed to dilution water to which no test material has been added. Except for the control(s) and the highest concentration, each concentration should be at least 50 to 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5 to 0.6, five properly chosen concentrations will often provide LC50s, EC50s, IC50s, NOECs, and LOECs for several durations (Annex A1) and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of toxicity is particularly uncertain (section 9.3), six or seven concentrations might be desirable. If it is desirable to provide extensive information concerning the dependence of adverse effects on time or concentration, or both, seven or more appropriately spaced concentrations might be desirable to cover the range from effects on almost all organisms at quite short times to effects on few organisms at quite long time.

12.1.2 If it is only necessary to determine *(a)* whether a specific concentration is acutely toxic to the test species or *(b)* whether the LC50, EC50, or IC50 is above or below a specific concentration (section 9.3), only that concentration and the control(s) are necessary. Two additional concentrations at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

12.1.3 If an endpoint near the extremes of toxicity, such as an LC5 or LC95, is to be calculated, at least one concentration of test material should have killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the LC, EC, or IC is to be calculated. This requirement might be met in a test to determine an LC50, EC50, or IC50, but a special test with appropriate test concentrations and more test organisms per treatment will usually be necessary.

12.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned. Because test solution can flow from one compartment to another, but not from one test chamber to another (section 6.5), the test chamber is the experimental unit. As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases and, therefore, the width of the confidence interval on a point estimate decreases and the power of a hypothesis test increases. With respect to factors that might affect results within the test chambers and the results of the test, all chambers in the test should be treated as similarly as practical. For example, the temperature in all test chambers should be as similar as practical unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or a rectangle) is preferable to a completely randomized design.

12.3 The minimum desirable number of test chambers and organisms per treatment should be calculated from *(a)* the expected variance within test chambers, *(b)* the expected variance between test chambers within a treatment, and *(c)* the maximum acceptable width of the confidence interval on the LC50, EC50, or IC50 (Guide E729). Organisms in each treatment should be divided between two or more test chambers in order to allow estimation of experimental variation. If the controls are important in the calculation of results, such as because of correction for spontaneous mortality using Abbott's formula or because the results are calculated as a percent reduction from the controls, it might be desirable to use more test chambers and test organisms for the control treatment(s) than for each of the other treatments (Guide E729).

12.4 The shape of the concentration-effect curve is critical for the determination of time-independent toxicity levels, and observations of dead and affected organisms should be with sufficient frequency to facilitate the estimation of a time-independent value, either directly or mathematically. Depending on the objectives of the test, a design should be selected that includes sufficient observations to determine the desired endpoint. If regulatory or cost factors are a consideration, observations may be made in acute toxicity tests at 24, 48, and 96 h or as stipulated by the regulatory guideline. Depending on the shape of the toxicity curve, more observations will typically be desirable (for example, 3, 6, 12, and 24 h and twice daily thereafter) to provide a sound measurement of a time-independent toxicity value. For chronic toxicity tests, ideally, survival should be measured weekly during the exposures. It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

13. Analytical Methodology

13.1 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, the samples should be handled and stored to minimize loss of test material by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

13.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources (Guide E729). The concentration of un-ionized ammonia may be calculated from the pH, temperature, and concentration of total ammonia (Guide E729).

13.3 Methods used to analyze food or test organisms for chemicals of interest should be obtained from appropriate sources (Guide E729).

13.4 The precision and bias of each analytical method used should be determined in an appropriate matrix, for example, in water samples from a control test chamber or brood-stock tank, in food, and in test organisms. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

14. Calculation of Results

14.1 *Data Recording*—Quality assurance project plans with data quality objectives and standard operating procedures should be developed before starting a test. Procedures should be developed by each laboratory to verify and archive data (Guide E1391). A file should be maintained for each toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); control data sheets for reference toxicants; detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions used; and results of reference-toxicant tests. Original data sheets should be signed and dated by the laboratory personnel performing the toxicity tests and archived. Electronic copies of data should also be archived.

14.2 *Data Analysis:*

14.2.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies in data (Guide E1241). The data display and statistical techniques most commonly used to achieve these goals are *(a)* preliminary and diagnostic graphical displays, *(b)* pairwise comparison techniques such as t-tests and 2 by 2 contingency table tests, *(c)* analysis of variance (ANOVA) and corresponding contingency table tests, *(d)* multiple comparison techniques for simultaneous pairwise comparison of other treatment groups with control groups, *(e)* concentration-effect curve analyses, and *(f)* multiple regression. If used correctly, each of these techniques can provide useful information about the results of an acceptable toxicity test. The three kinds of data that can be obtained from toxicity tests are dichotomous or categorical (for example, mortality), and continuous (for example, length or weight). Statistical methods for analyzing dichotomous and other categorical data are directly analogous to those for analyzing count and continuous data. However, for technical reasons and because they arose from different application areas, different terminologies and computing tools were developed for analyzing the three kinds of data.

14.2.2 *Endpoint*—The endpoint determined in toxicity tests generally has been defined in terms of whether differences from control organisms are statistically significant at the 5 % level (that is, analysis of variance followed by mean separation; Guide E1241). One of the main conceptual problems with such a definition of the endpoint is that the notions of biological importance and statistical significance are logically distinct. Effects of considerable biological importance might not be statistically significant if sample sizes are small or if effects are extremely variable or both. Conversely, biologically trivial effects might be highly statistically significant if sample sizes are large or effects are very reproducible. An endpoint based solely on statistical significance might depend as much or more on sample sizes as on the magnitudes of the effects. An alternative is to define the endpoint in terms of a specified absolute or relative amount of difference in a biological attribute from the control treatment(s). A regression-type model would be fitted to the data and the concentration associated with a specified amount of difference from the control treatment(s) would be estimated using the model. For example, the concentration resulting in a specified percent decrease in survival or shell length might be estimated along with confidence limits on the estimated concentration. The result of a toxicity test would then be reported as a point estimate, preferably with confidence limits, of the concentration expected to cause an amount of effect that had been pre-selected as being biologically unacceptable.

14.2.2.1 In general, an endpoint defined in terms of a statistically significant difference is calculated using analysis of variance, contingency tables, or other hypothesis testing procedures. An endpoint defined in terms of a specified amount of effect is calculated using regression analysis, concentration-effect curve analysis, and other point estimation procedures. Regardless of the procedure used, sufficient data should be presented in reports to permit calculation of endpoints other than those chosen by the investigator and to allow other uses, such as modeling.

14.2.3 For each set of data the LC50, EC50, IC50 and its 95 % confidence limits or NOEC and LOEC should be calculated on the basis of *(a)* the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and *(b)* the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LCs, ECs, or ICs are calculated, their 95 % confidence limits should also be calculated (Guide E729, Guide E1241).

14.2.4 Most acute toxicity tests produce quantal or dichotomous data, that is, counts of the number of organisms in two mutually exclusive categories, such as alive or dead. A variety of methods summarized in Guide E729 and Test Method E1706 can be used to calculate an LC50 or EC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or affected is between 0 and 100. The method used should appropriately take into account the number of test chambers per treatment and the number of test organisms per

chamber. When fewer than two concentrations kill or affect between 0 and 100 %, the binomial test can usually be used to obtain statistically sound information about the LC50 or EC50. The binomial test does not provide a point estimate of the LC50 or EC50, but it does provide a range within which the LC50 or EC50 should lie. If desired, an interpolation procedure may be used to obtain an approximate LC50 or EC50.

14.2.5 Although they generally require more effort to obtain, quantitative data on individual organisms, such as time-to-death or shell length, contain more information per organism than do quantal data. Quantitative data can usually be analyzed to calculate an IC50. For each test chamber in each treatment other than the control treatment(s), the percent inhibition (%*I*) should usually be calculated as follows:

$$\%I = 100(M - X)/M \tag{1}$$

where:

M = average value for the control test chambers, and
X = value for a test chamber in any other treatment.

14.2.5.1 The %*I* for each test chamber should be plotted against the corresponding concentration of test material after transformation of %*I* or concentration, or both, if appropriate. The IC50 can then be obtained from a line of best fit by determining the concentration corresponding to %*I* = 50. If possible, the 95 % confidence limits on the IC50 should be calculated, appropriately taking into account the number of test chambers per treatment, the number of test organisms exposed in each chamber, the range of concentrations tested, and the variance within each treatment, especially in the controls. Alternatively, an appropriate linear or nonlinear inverse regression technique can be used to calculate the IC50 and its 95 % confidence limits (Guide E729). If the percent inhibition covers an appropriate range, such as at least 37 to 63 %, a variety of regression models will usually give nearly the same IC50 from a set of data. However, only the correct model, which is not known to be available at this time, will appropriately take into account the variance between the test chambers in the control treatment(s) and give the correct confidence limits.

14.2.6 The values for *X* may be plotted against the corresponding concentrations of test material, after transformation of *X* or concentration, or both, if appropriate, and the IC50 determined by graphical or statistical interpolation to the concentration of test material at which a line of best fit = *M*/2.

14.2.7 An endpoint near an extreme of toxicity, such as an LC5 or LC95, should not be calculated unless at least one concentration of test material killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the LC, EC, or IC is to be calculated. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment(s) or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are usually more reliable than reporting a calculated result such as an LC5 or LC95 unless several percent killed or affected were obtained close to 5 or 95 %.

14.2.8 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material killed or affected a statistically significant number of the exposed organisms. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and tests of heterogeneity. Then a pair wise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test. See Guide E1241, Practice E1847, and Test Method E1706 for additional detail on hypothesis testing.

15. Report

15.1 The record of the results of an acceptable toxicity test should include the following information either directly or by referencing available documents:

15.1.1 Name of test and investigator(s), name and location of laboratory, and dates of start and end of test.

15.1.2 For sediment testing, source of control or test sediment, method for collection, handling, shipping, storage, and disposal of sediment.

15.1.3 Source of test material, lot number if applicable, composition (identities and concentrations of major ingredients and impurities if known), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

15.1.4 Source and characteristics of dilution water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

15.1.5 Source, history, and age of test organisms; culture procedures; and source and date of collection of organisms from the field, scientific name, name of person who identified the organisms and the taxonomic key used, age or life stage, means and ranges of shell length, observed diseases or unusual appearance, treatments, holding, and acclimation procedures.

15.1.6 Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency, and ration.

15.1.7 Description of the experimental design and test chambers, volume water in the chambers, lighting, number of test chambers and number of test organisms/treatment, date and time test starts and ends, temperature measurements, dissolved oxygen concentration (as percent saturation), and any aeration used before starting a test and during the conduct of a test.

15.1.8 Methods used for physical and chemical characterization of water or sediment samples.

15.1.9 Definition(s) of the effects used to calculate LC50 or EC50s, biological endpoints for tests, and a summary of general observations of other effects.

15.1.10 Methods used for statistical analyses of data: *(a)* summary statistics of the transformed or raw data as applicable (for example, mean, standard deviation, coefficient of variation, precision and bias); *(b)* hypothesis testing (raw data, transformed data, null hypothesis, alternate hypothesis, target

Type I and II error rates, statistics used (including calculation of test statistic)), decision rule used (for example, approach used to establish the rejection of the null hypothesis), calculated test statistic and decision rule result, achieved Type I and II error rates (for some discrete tests, achieved error rates only approximate the target rates); *(c)* results of regression analyses (parameters of regression fit, uncertainty limits on the regression parameters, correlation coefficient).

15.1.11 Summary of general observations on other effects or symptoms.

15.1.12 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

15.2 Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

16. Precision and Bias

16.1 *Determining Precision and Bias:*

16.1.1 Precision is a term that describes the degree to which data generated from replicate measurements differ and reflects the closeness of agreement between randomly selected test results. Bias is the difference between the value of the measured data and the true value and is the closeness of agreement between an observed value and an accepted reference value (Practices E177 and E691). Quantitative determination of precision and bias in toxicity testing of aquatic organisms is difficult or may be impossible in some cases, as compared to analytical (chemical) determinations. This is due, in part, to the many unknown variables which affect organism response. Determining the bias of a toxicity test using field samples is not possible since the true values are not known. Since there is no acceptable reference material suitable for determining the bias of toxicity tests, bias of the procedures described in this standard has not been determined (section 16.2).

16.1.2 Toxicity tests exhibit variability due to several factors. Test variability can be described in terms of two types of precision, either single laboratory (intra-laboratory or repeatability; see 16.5.1) precision or multi-laboratory (inter-laboratory or reproducibility; see 16.5.2) precision (also referred to as round-robin or ring tests). Intra-laboratory precision reflects the ability of trained laboratory personnel to obtain consistent results repeatedly when performing the same test on the same organism using the same toxicant. Inter-laboratory precision is a measure of how reproducible a method is when conducted by a large number of laboratories using the same method, organism, and toxic sample. Generally, intra-laboratory results are less variable than inter-laboratory results (Test Method E1706).

16.1.3 A measure of precision can be calculated using the mean and relative standard deviation, or percent coefficient of variation (CV % = standard deviation/mean × 100) of the calculated endpoints from the replicated endpoints of a test. However, precision reported as the CV should not be the only approach used for evaluating precision of tests and should not be used for the no-observed-effect concentrations (NOECs) derived from statistical analyses of hypothesis testing. The CVs may be very high when testing extremely toxic or nontoxic samples. For example, if there are multiple replicates with no survival and one with low survival the CV may exceed 100 %, yet the range of response is actually quite consistent. Therefore, additional estimates of precision should be used, such as range of responses and minimum detectable differences (MDD) compared to control survival or growth (Test Method E1706). Several factors can affect the precision of the test, including test organism age, condition, sensitivity, handling, and feeding of the test organisms, overlying water quality, and the experience in conducting tests. For these reasons, it is recommended that trained laboratory personnel conduct the toxicity tests in accordance with the procedures outlined in Annex A1. Quality assurance practices should include: *(a)* single laboratory precision determinations that are used to evaluate the ability of the laboratory personnel to obtain precise results using reference toxicants for each of the test organisms and *(b)* preparation of control charts (Figure 16 in Test Method E1706) for each reference toxicant and test organism. The single laboratory precision determinations should be made before conducting routine toxicity tests.

16.1.4 Intra-laboratory precision data are routinely calculated for test organisms using water-only acute exposures to a reference toxicant such as NaCl or $CuSO_4$. Intra-laboratory precision data should be tracked using a control chart. Each laboratory's reference-toxicant data will reflect conditions unique to that facility, including dilution water, culturing, and other variables (Section 11). However, each laboratory's reference toxicant CVs should reflect good repeatability.

16.1.5 Results of one intra-laboratory toxicity study and one inter-laboratory (round-robin) study using 24 and 48-h toxicity tests with glochidia and 48 and 96-h toxicity tests with juvenile mussels are reported in section 16.5.

16.2 *Bias*—Bias of toxicity tests cannot be determined since there is no acceptable reference material. The bias of the reference-toxicant tests can only be evaluated by comparing test responses to control charts.

16.3 *Replication and Test Sensitivity*—Sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the probability levels (alpha and beta), and the type of statistical analysis. For a specific level of variability, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

16.4 *Demonstrating Acceptable Laboratory Performance:*

16.4.1 Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$). Bringolf et al (2005) (**108**) reported 24-h EC50s ranging from 0.55 to 3.3 g NaCl/L for glochidia of five species of mussels and 96-h EC50s ranging from 4.0 to 6.3 g NaCl/L for 5 species of juvenile mussels in reference-toxicity tests. USGS (2005b) reported 24-h EC50s ranging from 10 to >100 µg Cu/L for glochidia of 11 species of mussels and 96-h EC50s ranging from 6.8 to 60 µg Cu /L for 7 species of juvenile mussels in reference-toxicity tests (hardness 170 mg/L as $CaCO_3$). Test conditions for conducting

reference toxicity tests should follow the recommended conditions for conducting toxicity tests with glochidia outlined in Table A1.1 and with juvenile mussels outlined in Table A1.4.

16.4.2 Intra-laboratory precision, expressed as a coefficient of variation (CV), of the range for each type of test to be used in a laboratory can be determined by performing multiple toxicity tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (for example, the same test duration, type of water, age of test organisms, feeding), and same data analysis methods. A reference-toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical.

16.4.3 A control chart can be prepared for each combination of reference toxicant and test organism. Each control chart should include the most current data. Endpoints from five tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the values (X_i) from successive tests with a given reference toxicant (Figure 16 in Test Method E1706), and the endpoint (LC50, NOEC, ICp) are examined to determine if these endpoints are within prescribed limits. Control charts as described in Test Method E1706 are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits (±2 SD) are recalculated with each successive test result.

16.4.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified using control charts. With an alpha of 0.05, one in 20 tests would be expected to fall outside of the control limits by chance alone. If 2 of 20 reference-toxicant tests fall outside the control limits, the toxicity tests conducted during the time in which the second reference-toxicant test failed are suspect, and should be considered as provisional and subject to careful review.

16.4.5 A toxicity test may be acceptable if specified conditions of a reference-toxicant test fall outside the expected ranges. Specifically, a toxicity test should not be judged unacceptable if the LC50 for a given reference-toxicant test falls outside the expected range or if control survival in the reference-toxicant test is less that the acceptability requirement outlined in Annex A1. All the performance criteria outlined in Annex A1 should be considered when determining the acceptability of a toxicity test. The acceptability of the toxicity test would depend on the experience and judgment of the investigator and the regulatory authority.

16.4.6 If the value from a given test with the reference toxicant falls more than two standard deviation (SD) outside the expected range, the sensitivity of the organisms and the overall credibility of the test system may be suspect (Test Method E1706). In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

16.4.7 Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ±2 SD, by definition, will be exceeded 5 % of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not an outlier is to be rejected. This determination should be made by the regulatory authority evaluating the data.

16.4.8 The recommended reference-toxicant test consists of a control and five or more concentrations in which the endpoint is an estimate of the toxicant concentration which is lethal to 50 % of the test organisms in the time period prescribed by the test. The LC50 is determined by an appropriate procedure, such as the trimmed Spearman-Karber Method, Probit Method, Graphical Method, or the Linear Interpolation Method (Section 14 and Test Method E1706).

16.4.9 The point estimation analysis methods recommended in this standard have been chosen primarily because point estimates are well-tested, well-documented, and are applicable to most types of test data. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis for toxicity data.

16.5 *Precision of Toxicity Tests Conducted with Glochidia or Juvenile Mussels:*

16.5.1 *Intra-laboratory Precision*—Table 3 summarizes the results of intra-laboratory toxicity tests conducted with glochidia of *Actinonaias ligamentina* and *Lampsilis siliquoidea* (USGS 2004) (**112**) and juvenile mussels of *L. siliquoidea* (USGS 2005b (**9**)). Test conditions for conducting the toxicity tests with glochidia were in accordance with the recommended test conditions outlined in Table A1.1 and all of the toxicity tests met the test acceptability requirements outlined in Table A1.3 (**112**). The dilution water was reconstituted hard water (160-180 mg/L as $CaCO_3$; Guide E729). Survival of glochidia

TABLE 3 Intra-laboratory Precision of EC50s (expressed as the Coefficient of Variation; CV) from Toxicity Tests with Glochidia or Juveniles of *Actinonaias ligamentina* or *Lampsilis siliquoidea* (USGS 2004, 2005b) (9)(112)

Test Organism	Life Stage	Exposure Duration	Copper (µg/L)			Ammonia (mg NIL)[A]			Chlorine (µg/L)		
			N	EC50	CV(%)	N	EC50	CV(%)	N	EC50	CV(%)
A. ligamentina	Glochidia	24 h	4	53	25	4	8	25	3	91	17
A. ligamentina	Glochidia	48 h	4	26	22	4	5	36	3	47	13
L. siliquoidea	Glochidia	24 h	6	35	15	5	13	20	5	77	27
L. siliquoidea	Glochidia	48 h	6	23	25	5	11	20	5	66	38
L. siliquoidea	Juvenile	48 h	4	40	26						
L. siliquoidea	juvenile	96 h	4	22	13						

[A] At about pH 8.3

TABLE 4 Inter-laboratory Precision of EC50s in Copper Toxicity Tests (µg Cu/L and 95 % Confidence Intervals) with Glochidia and Juveniles of *Lampsilis siliquoidea* (USGS 2004) (112)

Lab	Glochidia		Juvenile	
	24-h EC50	48-h EC50	48-h EC50	96-h EC50
1. CERC	29 (28-31)	13 (12-14)	29 (23-36)	18 (15-22)
2. NCSU	33 (32-35)	24 (22-25)	48 (40-59)	18 (16-20)
3. OSU	27 (25-29)	26 (24-28)	47 (40-54)	41 (35-47)
4. UMESC	38 (35-41)	21 (20-23)	34 (26-45)	21 (17-25)
5. WSLH	32 (31-34)	20 (19-21)	36 (24-54)	19 (12-30)
Mean EC50 (µg/L)	32	21	39	23
SD	4.2	5.0	8.3	9.9
Coefficent of variation (%)	13	24	22	42
H/L EC50	1.4	2.0	1.7	2.3

(based on valve closure in response to a solution of NaCl) was measured at 24 and 48 h. Survival of juvenile mussels (based on movement of the foot) was measured at 48 and 96 h. The variability of EC50s for glochidia toxicity tests conducted with copper, ammonia, or chlorine over two exposure periods, expressed as the coefficient of variation (CV), ranged between 13 and 36 % for toxicity tests conducted with glochidia of *A. ligamentina* and between 15 and 38 % for toxicity tests conducted with glochidia of *L. siliquoidea* (Table 3). The variability of EC50s for toxicity tests conducted with juvenile mussels and copper at 48 and 96 h, expressed as the CV, ranged from 13 to 26% (Table 3). These measures of intra-laboratory precision were similar to previous measures of intra-laboratory precision for tests conducted using commonly-tested species and reference toxicants (i.e., Lewis and Weber 1985, USEPA 1993,**113,114**).

16.5.2 *Inter-laboratory Precision*—Table 4 summarizes the results of an inter-laboratory toxicity test conducted with glochidia and juvenile mussels of *Lampsilis siliquoidea* (USGS 2004) (**112**). Test conditions for conducting the toxicity tests with glochidia were in accordance with the recommended test conditions outlined in Table A1.1 and test conditions for conducting the toxicity tests with juvenile mussels were in accordance with the recommended test conditions outlined in Table A1.4. Survival of glochidia (based on valve closure in response to a solution of NaCl) was measured at 24 and 48 h. Survival of juvenile mussels (based on movement of the foot) was measured at 48 and 96 h. The dilution water was reconstituted hard water (160-180 mg/L as $CaCO_3$; Guide E729). One laboratory prepared the dilution water, the high concentration of test water, and supplied each laboratory with the testing equipment. A separate facility produced the glochidia (about <24-h old at the start of the toxicity tests) and juvenile mussels (about 4-d old at the start of the toxicity tests). Test organisms were shipped overnight at about 10°C to five laboratories participating in the inter-laboratory toxicity test. The testing laboratories included 2 federal facilities and 3 university facilities. All of the laboratories met the test acceptability requirements outlined in Table A1.3 for glochidia toxicity tests and met the test acceptability requirements outlined in Table A1.5 for juvenile mussel toxicity tests (USGS 2004) (**112**). Control survival across all of the testing laboratories was >92 % at 24 and 48 h in the glochidia toxicity tests and was >95 % at 48 and 96 h in the juvenile toxicity tests. The variability of EC50s for glochidia, expressed as the CV, was 13 % for the 24-h EC50s and was 24 % for the 48-h EC50s (Table 4). The 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 (Table 4). The ratio of the high to low EC50 was less than 2.3 for all of the toxicity tests conducted. These measures of inter-laboratory precision in glochidia or juvenile mussel toxicity tests were similar to the variation reported for previous inter-laboratory studies in water-only exposures (for example, Lewis and Weber 1985, USEPA 1993 (**113,114**)) or in sediment exposures, (for example USEPA 2000 (**115**), Test Method E1706) using commonly-tested organisms.

17. Keywords

17.1 acute toxicity test; bivalve; chronic toxicity test; freshwater; glochidia; juvenile mussels; Margaritiferidae; Margaritiferid mussels; mollusc; mollusk; mussels; sediment; Unionidae; Unionid mussels; Unionoidea

ANNEX

(Mandatory Information)

A1. GUIDANCE FOR CONDUCTING WATER-ONLY TOXICITY TESTS WITH EARLY LIFE STAGES OF FRESHWATER MUSSELS

A1.1 *Significance*

A1.1.1 Many factors are cited as potentially contributing to the decline of freshwater mussel populations in North America. Of the nearly 300 taxa of freshwater mussels in North America, 70 species (23 %) are listed as endangered or threatened and another 40 species (14 %) are candidates for possible listing (Williams et al 1993 (**1**); Neves 1997, 2004 (**2, 3**)). Habitat alteration, introduction of exotic species, over-utilization, disease, predation and pollution are considered causal or contributing factors in many areas of the United States (Neves et al 1997) (**4**). Numerous laboratory toxicity studies have been conducted with freshwater mussels in an attempt to understand the role of contaminants in the decline of mussel populations in the field. Kernaghan et al (2005) (**5**) provides a review of over 75 toxicity studies conducted with a variety of freshwater species of mussels and contaminants in laboratories worldwide. Three critical life stages (glochidia, juvenile mussels, and adults) have been used in these toxicity assessments. Toxicity studies are separated according to the medium of exposure (water, sediment, and host fish; Kernaghan et al 2005 (**5**)). In these studies, early life stages of mussels of several species are highly sensitive to some metals and ammonia in water exposures when compared to many of the most sensitive species of other invertebrates, fish, or amphibians that are commonly used to establish U.S. Environmental Protection Agency Water Quality Criteria (WQC; Augspurger et al 2003 (**6**), Keller et al 2005 (**7**); USGS (2005a,b)(**8, 9**) section 1.5). Importantly, results of these previous studies indicate WQC for individual chemicals established for the protection of aquatic organisms may not be adequately protective of sensitive stages of freshwater mussels.

A1.1.2 Short-term 24-h exposures with glochidia may be useful for screening of chemicals, but response of juvenile mussels may be more ecologically relevant (A1.4.2,A1.5.2, and A1.5.3). Use of glochidia to screen the relative sensitivity of a particular mussel species to 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.

A1.1.3 In the field, mussels may be exposed to contaminants in water, sediment, or food. Annex A1 only addresses effects associated with exposure of mussels to contaminants in water.

A1.1.4 Sections 12 and A1.5 provide guidance on experimental design of toxicity tests with glochidia or juvenile mussels. Section A1.2 provides guidance for conducting water-only toxicity tests with glochidia isolated from adult mussels. Section A1.3 provides guidance for conducting water-only toxicity tests with juvenile mussels. Refinement of these methods may be described in future versions of this standard after additional laboratories have used these methods (section A1.5). Results of tests using procedures different from the procedures described in section A1.2 or A1.3 may not be comparable. Comparisons of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting toxicity tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in this standard, additional tests are required to determine comparability of results (section 1.4).

A1.2 *Test Conditions for Conducting Water-only Toxicity Tests with Glochidia of Freshwater Mussels*

A1.2.1 Test conditions used by investigators to conduct toxicity tests with glochidia are summarized in Table A1.1. Selection of specific test conditions and decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Sections 12 and 16). When variability remains constant, the statistical sensitivity of a test increases as the number of replicates increase.

A1.2.2 Table A1.1 also provides a list of recommended test conditions for conducting toxicity tests with glochidia. The list of recommended test conditions is based on the various methods outlined in Table A1.1 and is based on the conditions used to conduct an inter-laboratory toxicity test with glochidia (section 16.5). Toxicity tests with glochidia should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux (Table A1.1). Toxicity tests are typically started within 2 h after glochidia are isolated from the gills of the female mussels; however, some toxicity tests have been started with glochidia isolated from female mussels for about 24 h before the start of a toxicity 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 (KCl has also been previously been used, but this standard recommends use of NaCl in order to have more consistency between laboratories). 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 (for example, 48 h) can be conducted as long as control survival >90 % is achieved. Toxicity tests conducted for >24 h with glochidia might be used for species for which juvenile mussels

are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates).

A1.2.3 Glass test chambers should be used to conduct toxicity tests with glochidia. Test chambers should be a minimum of volume of 100 mL containing a minimum of 75 mL of dilution water. Static, renewal, or flow-through conditions can be used depending on the chemical being tested. Glochidia are not fed during the toxicity test and aeration of dilution water is not necessary unless dissolved oxygen is below acceptable concentrations (section A1.4.9.3). Dilution water should be a source of water that has been demonstrated to support survival of glochidia for the duration of the toxicity test. For site-specific evaluations, the characteristics of the dilution water should be as similar as possible to the site of interest.

A1.2.4 The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. A minimum of 3 replicates should be tested, each replicate containing about at least 500 glochidia (preferably 1000 glochidia/replicate if survival is to be evaluated in subsamples of glochidia collected during the toxicity test). Survival can be determined throughout the toxicity test by subsampling each replicate (for example, by subsampling about 100 glochidia at 6 and 24 h and then placing these organisms into one well of a multi-well plate to determine survival with the addition of a salt solution; Wang et al 2003 (**85**) and A1.4.8.4). Water-quality characteristics of the dilution water (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of the exposures in at minimum the high and medium test concentrations and in the control. Requirements for test acceptability for toxicity tests conducted with glochidia are summarized in Table A1.3.

A1.2.5 Toxicity tests with glochidia have been conducted for up to 144 h, but 24 and 48-h exposures are most often used (Table A1.1). The relatively short duration of toxicity tests with glochidia is based on the relatively short duration between release of glochidia into the water column and encystment on the host and is based on the relatively short survival time of glochidia after isolation from the female mussel (Table A1.2). If the life history of a particular species is not known (for example, the host required for encystment or how long glochidia released from a female mussel can remain in the water column before encysting on a host), it might be appropriate to conduct toxicity tests with glochidia for longer than 24 h as long as 90 % control survival can be achieved at the end of the test.

A1.2.6 The time between the release of glochidia from the marsupium of the female mussel to attachment of these glochidia on a host may only take a few seconds for some species (10.1.4), but hours are required for the gill tissue of a fish to migrate to form a cyst around the glochidia. During that time, the glochidia may be exposed to water-borne toxicants. Many anodontinae species release glochidia into water column that remain viable for days before infesting a host fish. Therefore, a prolonged glochidial test would have ecological relevance for these species. Other species release glochidia in mucus strands that coat the bottom or remain suspended on vegetation, waiting for their hosts to swim by and still other species release glochidia packaged in conglutinates that serve as a lure to host fish. Hence, glochidia of these species may also be in water for extended periods of time; however, it is not known how exposure to water-borne contaminants would be influenced by the mucus or conglutinate surrounding the glochidia. Toxicity tests conducted for 24 h with glochidia may not be as ecologically relevant in some cases as toxicity tests conducted with juvenile mussels, but may be useful for some purposes such as deriving concentrations of a chemical that may be protective of the species. Use of glochidia to evaluate the relative sensitivity of a particular mussel species to chemicals would be particularly useful when evaluating species where only a limited number of adult mussels are available for methods development or a limited number of adults are available for producing 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.

A1.3 *Test Conditions for Conducting Water-only Toxicity Tests with Juvenile Freshwater Mussels*

A1.3.1 Test conditions used by investigators to conduct toxicity tests with juvenile mussels are summarized in Table A1.4. Selection of specific test conditions and decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Sections 12 and 14). When variability remains constant, the statistical sensitivity of a test increases as the number of replicates increase.

A1.3.2 Table A1.4 also provides a list of recommended test conditions for conducting toxicity tests with juvenile mussels. The list of recommended test conditions is based on the various methods outlined in Table A1.4 and is based on the conditions used to conduct an inter-laboratory toxicity test with juvenile mussels (section 16.5). Toxicity tests with juvenile mussels should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux (Table A1.4). Toxicity tests are typically started with newly-transformed juvenile mussels <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 toxicity tests conducted for 10 to 28 d with juvenile mussels (Table A1.4).

A1.3.3 In acute static tests, glass test chambers should be a minimum of volume of 50 mL containing a minimum of 30 mL

of dilution water. In chronic tests or in flow-through tests, glass chambers should be a minimum volume of 300 mL containing a minimum volume of 200 mL of dilution water. Static, renewal, or flow through conditions can be used depending on the chemical being tested. Juvenile mussels are not typically fed during acute toxicity tests. Algae have been used as a food source in toxicity tests conducted for 10 to 28 d. Aeration of dilution water is not necessary unless dissolved oxygen is below acceptable concentrations (section A1.4.9.3). Dilution water should be a source of water that has been demonstrated to support survival of juvenile mussels for the duration of the toxicity test. For site-specific evaluations, the characteristics of the dilution water should be as similar as possible to the site of interest.

A1.3.4 The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. In 96-h toxicity tests, a minimum of 20 organisms should be exposed to each concentration (for example, 4 replicates each containing a minimum of 5 juvenile mussels). It may be desirable to test only 5 juvenile mussels in each replicate when a limited number of test organisms are available or when test organisms are relatively small (for example, when juvenile mussels are small, it may be difficult to observe more than about 5 test organisms simultaneously in a replicate test chamber under the microscope). However, some investigators have tested 10 to 20 juvenile mussels in each replicate. In chronic toxicity tests, a minimum of 3 replicates should be tested, each replicate containing a minimum of 10 juvenile mussels. Water-quality characteristics of the dilution water (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of the acute exposures and at least weekly in chronic exposures in the high and medium test concentrations and in the control as live organisms are present. Requirements for test acceptability for toxicity tests conducted with juvenile mussels are summarized in Table A1.5.

A1.4 ***Conducting a Toxicity Test***

A1.4.1 Procedures for constructing and maintaining exposure systems are outlined in Section 6 and in section A1.4.3. Hazards associated with conducting the toxicity tests are outlined in Section 7. Procedures for preparing dilution water are outlined in Section 8. Procedures for preparation and delivery of the test material to test chambers are outlined in Section 9 and in section A1.4.3. Procedures for obtaining test organisms are outlined in Section 10. Procedures for addressing quality assurance and quality control associated with a toxicity test are outlined in Section 11 and in Section 16. Considerations of experimental design for a toxicity test are outlined in Section 12 and in A1.5. Procedures for analysis of test materials are outlined in Section 13. Procedures for analyzing data generated from a toxicity test are outlined in Section 14. Reporting requirements for a toxicity test are outlined in Section 15.

A1.4.2 *Beginning the Test:*

A1.4.2.1 Section 10.5 provides information on obtaining glochidia or juvenile mussels to start a toxicity test.

A1.4.2.2 *Acclimation*—Glochidia should be acclimated to a 50 to 50 mixture of culture to dilution water for about 2 h before the start of a toxicity test. Juvenile mussels should be acclimated to the dilution water for at least 24 h before the start of a toxicity test (for example, by holding juvenile mussels for 2 h in a 50 to 50 mixture of culture water to dilution water, then for 2 h in a 25 to 75 mixture of culture water to dilution water, followed by a transfer into 100 % dilution water until the start of the toxicity test). The temperature of the water used to acclimate test organisms and the water quality characteristics of the water should be gradually adjusted over the acclimation period (for example, increase by no more than about 3°C /h). Glochidia and newly-transformed juvenile mussels are not fed during the acclimation period; however, older juvenile mussels should be fed during the acclimation period (A1.4.5).

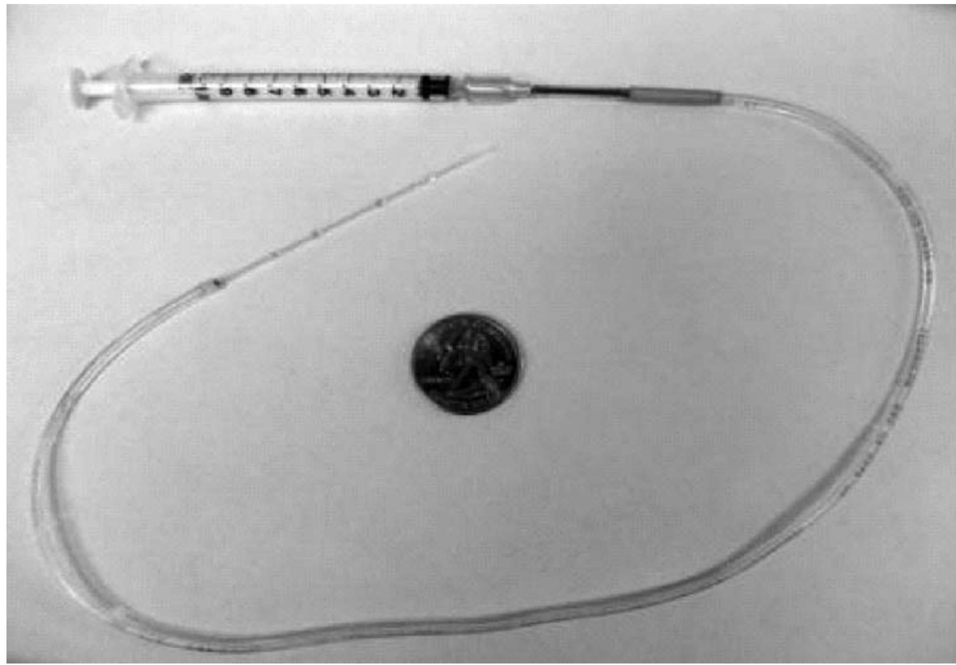
A1.4.2.3 *Placing Test Organisms in Test Chambers*—The test begins when the test organisms are first placed in dilution water containing test material. Section A1.4.8.4 provides information on establishing the viability of glochidia at the start of a toxicity test. Only active juvenile mussels should be used to start a toxicity test (that is, with foot movement).

A1.4.2.4 A representative sample of the test organisms must be impartially distributed among the test chambers. Caution should be exercised to minimize the transfer of dilution water with the test organism to the chambers. Test organisms should be handled as little as possible. Test organisms should be introduced into the test water below the air-water interface. A pipette or syringe can be used to place organisms directly into the test water. Fig. A1.1 illustrates a syringe system used to transfer newly-transformed juvenile mussels into test water (Wang et al 2003) (**85**). This syringe system consists of a glass capillary tube (1.17-mm inner diameter), connected to vinyl tubing (1.0-mm inner diameter), connected to a 2.5-cm, 16-gauge needle; that is connected to a 1-mL syringe. For 2- to 4-month old juveniles, a larger system should be used (for example, 2.2-mm inner diameter glass capillary tube connected to a 2.3 mm inner diameter vinyl tube, connected to a 5-mL syringe). If the shell of a juvenile mussel is broken, this organism should not be used in a toxicity test. A subsample of about 30 juvenile mussels should be archived at the start of chronic toxicity tests for subsequent length measurements (section A1.4.8.3). This information can be used to determine consistency in the size of the juvenile mussels used to start a test.

A1.4.3 *Static, Renewal, and Flow-through Exposure Systems:*

A1.4.3.1 Section 6 provides a description of procedures for constructing exposure systems.

A1.4.3.2 Static and renewal tests should begin by placing test organisms in the chambers within 30 min after the test material was added to the dilution water. Flow-through tests should begin by either *(a)* placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of test material to have reached steady state or *(b)* activating the metering device in the metering system several days after organisms were placed in test chambers that had dilution water flowing through them. This second alternative requires the addition of a "spike"



NOTE 1—The tubing is secured to the needle with a small piece of tape.

FIG. A1.1 Syringe Used to Transfer Juvenile Mussels (Wang et al, 2003) (85)

that is, an aliquot of test material sufficient to establish the desired test concentration in the test chamber at the time of activation of the metering device. The first alternative *(a)* allows the investigator to study the properties of the test material and the operation of the metering system immediately before the test, whereas the second alternative *(b)* allows the organisms to partially adjust to the chambers before the beginning of the test.

A1.4.3.3 In flow-through tests with glochidia or juvenile mussels, where there may be turbulence with each addition of dilution water, it is desirable to place a stainless-steel baffle in the test chamber to reduce turbulence. Specifically, Wang et al (2003) (**85**) placed stainless-steel mesh screen (4 by 15 cm; 300-µm opening) bent over the surface of the water in a 300-mL beaker used in flow-through tests to reduce the turbulence of water. Each of these beakers contained 200 mL of test water and had a 2.5-cm hole in the side covered with stainless-steel mesh screen (300-µm opening; Wang et al 2003 (**85**)). A description of the flow-through exposure system used by Wang et al (2003) (**85**) to conduct toxicity tests with glochidia and juvenile mussels can be found in USEPA (2000) (**115**),Figure A.5. Survival of glochidia in 48-h toxicity tests and survival of juvenile mussels in 10-d toxicity tests with copper and ammonia were similar in static or renewal exposure systems compared to flow-through exposure systems (Wang et al 2003) (**85**).

A1.4.3.4 Alternative test chambers that have been used to conduct toxicity tests with glochidia are multi-well (6 or 12 well) polystyrene (or other types of plastic) tissue-culture plates containing about 4 to 12 mL of water and a specific number of glochidia/chamber (Table A1.1). Larger glass test chambers have also been used to conduct toxicity tests with glochidia (for example, 250- to 400-mL beakers). A difficulty in using small multi-well plates is that there is a limited volume of water available for conducting water quality or chemical analyses. Jacobson (1990) (**116**) suggested that subsampling of glochidia from a smaller test chamber (for example, 12-well plates) may result in a biased sampling of glochidia. Wang et al (2003) (**85**) exposed groups of about 1000 glochidia in 200-mL glass chambers in about 100 to 150 mL of exposure water. Survival was then evaluated with the addition of a solution of NaCl at 6, 24, and 48 h to subsamples of glochidia (that is, about 100 glochidia in about 2 mL of exposure water placed into one well of a multi-well plate; see A1.4.8.4). Use of larger test chambers permits easier sampling of water quality and chemical concentrations during the exposures (Wang et al 2003) (**85**). In addition, exposures in larger chambers can be conducted using water-renewal systems (for example, Zumwalt et al 1994 (**117**), Brunson et al 1998 (**118**)). Similar survival of glochidia from several species was observed when glochidia were held under control conditions in multi-well plates or in larger chambers under static, renewal, or flow-through conditions (Wang et al 2003) (**85**). Wang et al (2003) (**85**) also observed that concentrations of copper in the multi-well plates substantially decreased during 48-h exposures; whereas, the concentration of copper in larger glass chambers remained relatively consistent over this time period.

A1.4.3.5 Alternative test chambers used to conduct toxicity tests with juvenile mussels have included multi-well tissue-culture plates for short-term exposures or larger chambers for longer exposures (Table A1.4). Investigators have also exposed juvenile mussels in glass cylinders with a mesh bottom placed inside larger test chambers (Dimock and Wright 1993 (**48**); Wade et al 1993 (**119**); McKinney and Wade 1996 (**120**); Farris et al 1994, 1995 (**121, 122**)).

A1.4.4 *Loading*—Table A1.1 outlines the number of glochidia added to each replicate test chamber and Table A1.4 outlines the number of juvenile mussels added to each test chamber. Loading should be limited to ensure that *(a)* the concentrations of dissolved oxygen and test material do not fall below acceptable levels, *(b)* concentrations of metabolic products do not exceed acceptable levels, and *(c)* the test organisms are not stressed because of crowding. Guides E729 and E1241 provide additional guidance on loading of organisms used in acute or chronic toxicity tests.

A1.4.5 *Feeding:*

A1.4.5.1 Glochidia are not fed during toxicity tests.

A1.4.5.2 Juvenile mussels are not typically fed during an acute toxicity test (for example, ≤ 96 h) or for a time before the test because fecal matter and uneaten food can decrease the dissolved oxygen concentration and can influence the bioavailability of some test materials. Toxicity tests with juvenile mussels have been conducted for 10 d without feeding juvenile mussels (USGS 2004) **(112)**. The acute toxicity of copper was determined in 48-h tests with juvenile *Lampsilis siliquoidea* and *L. rafinesqueana* that had been held for 10 d under control conditions (for example, with the replacement of dilution water, but without the addition of food; USGS 2004 **(112)**). Similar 48-h EC50s were observed in tests conducted with juvenile mussels held for 10 d before testing compared to tests started with newly-transformed juvenile mussels. Results of these tests indicate that the sensitivity of juvenile mussels did not change over the 10-d exposure without feeding.

A1.4.5.3 In 10 to 28-d toxicity tests, algae have been used as a source of food (Table A1.4). USGS (2005b) **(9)** described a procedure for conducting 28-d toxicity tests starting with 2-month-old juvenile *Villosa iris*. In this 28-d toxicity test, juvenile mussels were fed 4-mL of an instant algae mixture twice daily. The instant algae mixture was prepared from commercial Instant Algae[4] brand non-viable microalgae concentrates (Reed Mariculture, Campbell, CA) by adding 1 mL of a *Nannochloropsis* concentrate and 2 mL of a Shellfish Diet (a mix of four marine microalgae [*Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii*]) to 1.8 L of well water. Control survival of the juvenile mussels was 88 % in a 28-d copper toxicity test and was 100 % in a 28-d ammonia toxicity test (USGS 2005b **(9)**.) Additional information on feeding of juvenile mussels in culture or in toxicity tests is included in 10.6.3

A1.4.6 *Monitoring a Test*—Operation of the exposure system should be monitored daily. A microscope is needed to determine survival of test organisms. Therefore, survival of juvenile mussels typically monitored only periodically during a toxicity test (for example, at 48 and 96 h in an acute test and at 4, 7, 10, 14, 21, or 28 d in a chronic test).

A1.4.7 *Duration of Test*—Toxicity tests with glochidia are typically conducted for at least 24 h (Table A1.1; section A1.2.5), A 48-h toxicity test with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates). Acute toxicity test with juvenile mussels are typically conducted for 96 h, and chronic toxicity tests starting with 2- to 4-month-old juvenile mussels have been conducted for 21 to 28 d (Table A1.4). The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 24 h after the end of a 24-h glochidia toxicity test and control survival should be >90% for 96 h after the end of a 96-h juvenile toxicity test). At the end of the test it may be desirable to place the live test organisms for 1 to 2 d in dilution water that does not contain any added test material to determine whether delayed effects occur (Guide E729). It may also be desirable to maintain all test chambers with surviving organisms until at least 10% mortality occurs in each chamber.

A1.4.8 *Biological Data:*

A1.4.8.1 Endpoints measured in the toxicity tests with glochidia include survival (that is, measured as viability of glochidia at 6 and 24 h; Table A1.1). Endpoints measured in toxicity tests with juvenile mussels include survival (measured at 48 and 96 h in acute tests and about weekly in chronic tests) and growth (measured at the end of a chronic test; Table A1.4). Newton et al (2003) **(61)** observed small reductions in growth of juvenile mussels in 96-h toxicity tests.

A1.4.8.2 *Measurement of Juvenile Survival*—The endpoint typically measured in juvenile mussel toxicity tests is survival based on movement of the foot. However, ciliary activity on the foot, heartbeat, or vital staining has also been used to establish survival of juvenile mussels at the end of a toxicity test (Table A1.4). Survival of juvenile mussels in each replicate should be determined using a microscope to observe movement of the foot of each juvenile mussel within a 5-min period. Laboratories may also want to evaluate other measures of survival such as heart beat or cilia movement on the foot. In order to observe the juvenile mussels under the microscope during a test, it may be necessary to remove some of the water from the test chamber (for example, it is easier to observe foot movement of a juvenile mussel with a microscope if there is less than about 1 cm of water in the test chamber). Gently swirling the test chamber will create a slight vortex in the water, concentrating the juvenile mussels in a small area in the chamber, making it easier to see all of the organisms simultaneously in the field of view under the microscope.

A1.4.8.3 *Measurement of Juvenile Growth*—Growth of juvenile mussels has been measured at the maximum shell length parallel to the hinge or at the maximum shell height perpendicular to the hinge. These measurement provide comparable results, but the maximum shell height is somewhat easier to measure; (Teresa Newton, USGS, LaCrosse, WS, personal communication). Subsamples of about 30 juvenile mussels at the start of a toxicity test and juvenile mussels surviving at the end of the toxicity test can be preserved for subsequent growth measurements. Juvenile mussels can be placed in a small glass vial and preserved in 70 % ethanol until growth is measured.

Alternatively, juveniles can be placed in neutral buffered formalin for 24 h and then transferred to 70 % ethanol until growth is measured (Newton et al 2003) (**61**). Growth can be measured using a microscope interfaced with a digitizing system (for example, Newton et al 2003 (**61**)).

A1.4.8.4 *Evaluation of Viability of Glochidia*—Percent survival (viability) of glochidia should be calculated from the proportion of glochidia that close with the addition of a saturated salt solution (NaCl). Specifically, survival of glochidia should be calculated as: Survival (%) = 100 (# of closed glochidia after adding salt solution − # of closed glochidia before adding salt solution) / (# of open and closed glochidia after adding salt solution). A subsample of 100 to 200 glochidia isolated from each female mussel should be evaluated at the beginning of a toxicity test to confirm the viability of the glochidia from that female using a saturated salt solution. Readings of percent viable glochidia should be made about 1 min after the addition of the saturated salt solution. The saturated solution of NaCl can be prepared by adding about 12 g of reagent-grade NaCl to 50 mL of deionized water. About 1 drop of this saturated salt solution should be added to about 2 mL of a water sample containing glochidia. If viability is >80 % (preferably >90 %), the rest of glochidia collected from that female can be used for toxicity testing. Glochidia with >80 % (preferably >90 %) viability from at least three female mussels should be composited into in a large chamber before the start of a toxicity test or before a host is infested with glochidia to produce juvenile mussels. The recommendation to record the response of the glochidia 1 min after addition of a specific amount of NaCl is based on the observations that after addition of a saturated salt solution, glochidia sometimes closed slowly (USGS 2004) (**112**) or initially close then reopen after several minutes (for example, *Utterbackia imbecillis*; Bringolf et al 2005 (**108**)).

A1.4.9 *Other Measurements:*

A1.4.9.1 *Water Quality*—Water-quality characteristics (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of an acute toxicity test and at least weekly in chronic toxicity tests in a minimum of the high and medium test concentrations and in the control (as long as live organisms are present). Measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable in the dilution water. It may be necessary to composite water samples from individual replicates. The pipette used to collect water samples should be checked to make sure no organisms are removed during sampling of water. Water quality should be measured for each new batch of water prepared for the test.

A1.4.9.2 *Temperature*—Toxicity tests should be conducted at 20°C. In static and renewal tests, either *(a)* in at least one test chamber temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily or *(b)* if the test chambers are in a water bath or a constant-temperature room or incubator, the temperature of the water or air must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured at least daily. In addition, temperature must be measured concurrently near both the beginning and end of the test in all test chambers or in various parts of the water bath, room, or incubator. In flow-through tests, in at least one chamber either temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily. In addition, near both the beginning and end of the test, temperature must be measured concurrently in all test chambers. Uniform temperature is important to maintain in a test because survival or growth of test organisms can be influenced by temperature. The stated requirements are necessary to prevent confounding and unnecessary large variance in temperature. Table A1.3 and Table A1.5 summarize acceptable variation in temperature during a toxicity test.

A1.4.9.3 *Dissolved Oxygen*—Dissolved oxygen (and pH and conductivity) can be measured directly in the overlying water with a probe. If a probe is used to measure dissolved oxygen, it should be rinsed between samples to minimize cross contamination. Concentrations of dissolved oxygen should be maintained above 4 mg/L during the test. Sparks and Strayer (1998) (**50**) observed effects on behavior of juvenile *Elliptio complanata* at dissolved oxygen concentrations of 2 to 4 mg/L. Gentle aeration can be used if dissolved oxygen in the test water is below 4 mg/L (that is, about 1 bubble/second from a glass pipette in the test water). Turbulence should be avoided because it might stress test organisms or increase volatilization of the test material. Aeration should be the same in all test chambers, including the control(s), throughout the test.

A1.4.10 *Test Material:*

A1.4.10.1 If the test material is uniformly dispersed throughout the test chamber, water samples should be taken by using a pipette or by siphoning water through glass or fluorocarbon plastic tubing from a point midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides (Guide E729). If test material might be lost due to sorption onto the walls of the sample container, the container and the siphon or pipette should be rinsed with test solution before collecting the sample. Water samples should be collected into appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber in static and renewal tests, the whole volume of solution in the test chamber should be *(a)* used as the sample or *(b)* treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to uniformly distribute the test material before a sample is taken. If the test material is not uniformly dispersed in the test chamber in flow-through tests, a large volume of the solution flowing into the test chambers should be collected and used as the sample or treated appropriately to uniformly distribute the test material in the sample before a subsample is taken.

A1.4.10.2 If some of the test material is not dissolved, measurement of the concentration of dissolved test material in each treatment might be desirable.

A1.4.10.3 In acute tests, the concentration of test material in the exposure chambers should be measured in the control and high, medium and low concentrations of test material at least at beginning and end of a test. In chronic tests, concentration of test material in the exposure chambers should be measured at

the beginning and weekly in the control and high, medium and low concentrations of test material. It is desirable to measure the concentration of test material in all of the test concentrations. Measurement of degradation products might be desirable. Whenever a serious malfunction is detected in the metering system, the test material in the test chambers should be measured. Guides E729 and E1241 provide additional guidance on calibration of flow-through systems before the start of a toxicity test and on monitoring concentrations during a toxicity test.

A1.5 ***Additional Information on Experimental Design and Interpretation of Data Generated in Toxicity Tests Conducted with Glochidia or Juvenile Mussels***

A1.5.1 Kernaghan et al (2005) (**5**) addressed several questions that have been raised regarding the experimental design or interpretation of data from toxicity tests conducted with glochidia or juvenile mussels. Glochidia and juvenile mussels of several genera are highly sensitive to some metals and ammonia in water exposures compared to many of the more sensitive genera of other invertebrates, fish, or amphibians that are commonly tested (for example, Augspurger et al 2003 (**6**), Keller et al 2005 (**7**); section 1.5). However, concerns have been expressed regarding the use of toxicity data generated with glochidia or juvenile mussels in the derivation of U.S. Environmental Protection Agency Water Quality Criteria; (Kernaghan et al 2005) (**5**). These concerns mainly include: *(1)* the duration of the toxicity tests conducted with glochidia, *(2)* the quality of organisms at the start of a test, and *(3)* test acceptability criteria. The following section summarizes information presented in Kernaghan et al (2005) (**5**) that addresses these concerns. Future research needs identified throughout the standard are highlighted in section A1.6.

A1.5.2 How long should a toxicity test be conducted with glochidia? There are nearly 300 species of freshwater mussels in North America and the length of time that glochidia remain viable after release from the marsupium of a female into the environment depends on the life history of the species and the temperature of the water (Table A1.2; section 10.1). Longevity of glochidia after release and before attachment to a host may exceed one week and may be dependent on temperature (Zimmerman and Neves 2002) (**42**); however, some reports are anecdotal (Murphy 1942 (**123**), Matteson 1948 (**124**), Tedla and Fernando 1969 (**125**)). Glochidia of some species released in conglutinates remain viable for days or weeks after release into the environment (Kernaghan et al 2005) (**5**). Glochidia of several species, including *Anodonta* spp., remain viable while free in the environment for 7 to 14 d (Howard and Anson 1922 (**126**), Mackie 1984 (**127**), Huebner and Pynnonen 1992 (**128**), Pynnonen 1995 (**129**)).

A1.5.2.1 Table A1.2 provides a summary of laboratory studies that have evaluated survival times of glochidia after removal from the marsupium of the female or survival time based on results reported in toxicity tests conducted with glochidia. For example, Zimmerman and Neves (2002) (**42**) report that the viability of glochidia of *V. iris* was >75 % for 8 d at 10°C and 2 d at 25°C and viability of glochidia of *A. pectorosa* was >75 % for 13 d at 10°C and 5 d at 25°C (Table A1.2). Similarly, glochidia of *Utterbackia imbecillis* may survive up to 19 d, but exhibit 50 % mortality within 13.5 d (Fisher and Dimock 2000) (**73**). Survival of isolated glochidia from many species listed in Table A1.2 is typically >90 % after 2 to 3 d; however, the viability of glochidia for a particular species should be determined before the start of an exposure. For example, glochidia of *Lampsilis teres* and *Epioblasma capsaeformis* were viable for only 4 to 6 h, glochidia of *Megalonaias nervosa* and *Quadrula quadrula* were viable for 1 d after removal from the marsupium of the female (Table A1.2). Therefore, 24 h is a reasonable time period to conduct toxicity tests with glochidia of many species at 20°C, although shorter or longer tests might be needed for a particular species depending on glochidia survival time and the life history characteristics of the species (that is, survival of glochidia in the control must be >90 % at the toxicity test Table A1.3).

A1.5.3 Short-term exposures with glochidia may be useful for screening of chemicals, but response of juvenile mussels would be more ecologically relevant (Kernaghan et al 2005) (**5**). Use of glochidia to screen the relative sensitivity of a particular mussel species to 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.

A1.5.4 How long can glochidia survive and still be able to attach to a host? Glochidia of some species can still attach to a host for several days after release from a female depending on temperature (Kernaghan et al 2005) (**5**). The maximum time at which >50 % of *Utterbackia imbecillis* metamorphosed in a tissue culture medium was 9 d after isolation from a female (Fisher and Dimock 2002) (**73**). Zimmerman and Neves (2002) (**42**) reported that glochidia can successfully attach to a host 1 to 2 weeks after isolation from a female. A future research project could be to conduct a series of toxicity tests to determine if there is a change in sensitivity over time after glochidia have been released into the environment. Sensitivity of *Lampsilis siliquoidea* glochidia held for 24 h after isolation from a female was similar to newly-released glochidia in exposures to copper (Wang et al 2003) (**85**). The sensitivity of glochidia held in an extra piece of the marsupium in a refrigerator overnight was similar to the sensitivity of glochidia tested immediately after isolation from a female in toxicity tests conducted with zinc or copper (Kernaghan et al 2005) (**5**). Ultimately, it is more practical to base duration of exposure on survival of control organisms in the laboratory rather than on an estimate of the length of time glochidia can survive and still attach to a host (for example, Table A1.2).

A1.5.5 What life stage should be used to start acute or chronic toxicity tests with juvenile mussels? Toxicity tests have been started with newly-transformed juvenile mussels that have either been transformed on a host or have been transformed with the use of an artificial medium (Table A1.4). Glochidia, newly-transformed juvenile mussels, and 2- to 4-month-old juvenile mussels have been successfully shipped via overnight carriers to other laboratories for use in toxicity

testing (for example, section 16.5, USGS 2004 (**112**), Bringolf et al 2005 (**108**)). Toxicity tests have been successfully conducted for 10 to 14 d starting with newly-transformed juvenile mussels (Table A1.4), but exposures conducted for longer periods of time have resulted in high mortality in controls at about 4 to 6 weeks, probably due to nutritional limitations of the diet (for example, Newton et al 2003) (**61**). Valenti et al (2005) (**107**) conducted 21-d exposures with 2-month old juvenile *Villosa iris* held in a small amount of sediment and fed algae (Neochloris). USGS (2005a,b) (**8, 9**) and Bringolf et al (2005) (**108**) conducted toxicity tests starting with 2- to 4-month-old juvenile *Actinonaias ligamentina*, *Lampsilis siliquoidea*, or *Villosa iris* and observed control survival >88 % in 21- to 28-d exposures when algae was used as a food source.

A1.5.6 Are there data that indicate that effect concentrations do not change very much during the last half of a toxicity test conducted with glochidia (that is, does the EC50 at 6, 24, 48, or 96 h differ)? There are limited studies with glochidia that have compared changes in toxicity over this timeframe. The toxicity of copper (Jacobson et al 1997 (**31**), Wang et al 2003 (**85**)), ammonia (Wang et al 2003) (**85**), and chlorine (Wang et al 2003) (**85**) decreased over 48 to 96-h exposures. In contrast, no change in the toxicity of several pesticides was observed in 24 to 48-h exposures (Keller and Ruessler 1997 (**58**), Bringolf et al 2005 (**108**)). If glochidia for a particular species are able to survive for more than 24 h, then a 24-h toxicity test should be considered. Importantly, researchers are encouraged to design studies that generate toxicity data throughout the exposure period (for example, reporting 6, 24, and 48-h responses; Guide E729). However, generating data for a 6–h exposure period is logistically difficult in an 8-h day.

A1.5.7 How should death of juvenile mussels be determined at the end of a toxicity test? Lack of foot or shell movement, lack of ciliary activity on the foot, lack of a heart beat, or a wide gaped valve have been used to establish death in toxicity tests with juvenile mussels (Table A1.4). Lack of movement of the foot of a juvenile mussel is the primary endpoint recommended in this standard (section A1.4.8).

A1.5.8 How should the quality of glochidia be determined at the start of a toxicity test? Is the use of a solution of NaCl (or KCl) to determine the percentage of glochidia exhibiting valve closure an appropriate method to judge the acceptability of glochidia used to start a toxicity test? Does the response of glochidia to a solution of NaCl (or KCl) relate to the ability of glochidia to attach to a host? Is there an independent way of determining if glochidia are alive or healthy at the start (or end) of a toxicity test? Valve closure is an ecologically-relevant endpoint that is a critical for glochidia to successfully transform on the host. If glochidia do not snap shut, the glochidia should be considered ecologically dead (Huebner and Pynnonen 1992 (**128**), Goudreau et al 1993 (**130**), McMann 1993 (**131**), Jacobson et al 1997 (**31**)). The response of glochidia in toxicity tests was similar when either KCl or fish plasma was used to make glochidia close at the end of an exposure (Huebner and Pynnonen 1992) (**128**). Decreased response to KCl was considered an indication of reduced glochidia viability and thus reduced capability to attach to the fish host (Pynnonen 1995) (**129**). A significant correlation was observed between the response of glochidia to KCl and ability of glochidia of *Utterbackia imbecillis* to metamorphose to the juvenile life stage (Fisher and Dimock 2002) (**73**). Zimmerman and Neves (2002) (**42**) reported a correspondence between the response of glochidia of *Villosa iris* and *A. pectorosa* to NaCl and the ability to infest a host fish. Jacobson et al (1997) (**31**) reported glochidia of *Villosa iris* that responded to the addition of NaCl following an exposure to copper were able to attach to a host fish with no impairment of subsequent metamorphosis to juvenile mussels. Results of these studies indicate that addition of a solution of NaCl or KCl can be used to estimate the condition of glochidia. While either a solution of salt or fish plasma could be used to determine the percentage of organisms closing, it is easier to work with NaCl compared to KCl or fish plasma.

A1.5.9 Should there be a holding time for glochidia after harvesting but before application of a salt solution to determine if glochidia that are initially closed might open? Mature glochidia are not typically closed after being isolated from a female mussel. Glochidia that are closed after isolation from a female may reopen after being held in clean water a few hours (Goudreau et al 1993 (**130**)).

A1.5.10 Will immature, stressed, or unhealthy glochidia close when exposed to a salt solution? Could glochidia be alive and successfully attach to a host but not close when exposed to a salt solution? Are broken glochidia frequently observed at the start of a test? Would the presence of broken glochidia be indicative of stress during harvesting? Immature glochidia that are free of an egg membrane or mature and healthy glochidia will close when exposed to a salinity challenge. However, immature glochidia are generally enclosed in an egg membrane and are fragile and tend to fracture, thus should not be used for toxicity testing. The best approach for avoiding the use of immature glochidia in toxicity testing is to sample female mussels at a time of the year when the organisms would be expected to be releasing mature glochidia (Kernaghan et al 2005) (**5**). Stressed or unhealthy glochidia could either be opened or closed before the start of a test. If stressed or unhealthy glochidia were to close when exposed to a salinity challenge, then these individuals would be used in a toxicity test. Measurement of the viability of glochidia in the control at the end of a toxicity test would help to identify stressed or unhealthy glochidia. Results of reference-toxicant tests should also be used to evaluate the health of the glochidia used to conduct the test (section 16.4). Broken glochidia have not been observed at the start of a test (Kernaghan et al 2005) (**5**). The presence of broken glochidia may indicate that the glochidia are immature and should not be used for testing.

A1.5.11 Should glochidia be rinsed before use in a toxicity test? Would rinsing glochidia before the start of a test be stressful to the organisms? Glochidia should be rinsed with culture or dilution water after removal from marsupia to: *(1)* eliminate tissues or excess mucus from the excised glochidia that have a high potential for fungal growth and subsequently could affect the survival (toxicity tests) or transformation of glochidia (propagation) and *(2)* reduce the number of protozoans that may be present in the excised gill that could also affect

glochidia survival or transformation (10.5). Rinsed glochidia have been observed to successfully transform on fish or in artificial media and high control survival in toxicity tests has been reported using glochidia that have been rinsed (Huebner and Pynnonen 1992 (**128**), Johnson et al 1993 (**79**), Myers-Kinzie 1998 (**132**), Bishop et al 2005 (**46**)).

A1.5.12 Should glochidia be acclimated to test conditions before the start of a toxicity test? Glochidia are not typically acclimated to the water-quality characteristics of the dilution water before the start of a toxicity test (Table A1.1). Most of these exposures are started the same day that glochidia are isolated from marsupia of the females. Therefore, minimal time is available to acclimate glochidia to the dilution water before the start of a test. In order to maintain organisms in good condition and avoid unnecessary stress, Guide E729 recommends that organisms should not be subjected to rapid changes in temperature or water quality before the start of a test. Wang et al (2003) (**85**) acclimated glochidia in a mixture of 50 % culture water and 50 % test water and gradually adjusted the temperature to the test temperature within about 2 h before the start of an exposure (A1.4.2.2). Investigators have held adult mussels under test conditions before isolation of glochidia (for example, Huebner and Pynnonen 1992 (**128**)) which would result in acclimating glochidia to the selected exposure temperature in the toxicity test. However, brooding glochidia in the marsupium are in contact with the hemolymph of the female that is physically isolated from direct contact with water (Silverman et al 1987) (**133**). In addition, glochidia are typically released instantaneously into the surrounding water from the marsupium of the female mussel. Therefore, holding the female mussels in the dilution water before isolating glochidia for toxicity testing would probably have a minimal influence on the ability of glochidia to acclimate to the conditions of the dilution water.

A1.5.13 What criteria should be used to judge acceptability of a toxicity test conducted with glochidia? Survival (measured as viability) of glochidia at the end of the exposure should be the primary endpoint to establish the acceptability of a toxicity test. Most investigators report >90 % survival of glochidia after 24 h (Tables A1.1 and A1.2). Therefore, setting test acceptability at >90 % survival seems appropriate for 24-h toxicity tests conducted with glochidia. Survival of glochidia was improved at cooler temperatures (Zimmerman and Neves 2002) (**42**) and may be different for short- versus long-term brooders (Kernaghan et al 2005) (**5**). Other investigators have observed inherently lower survival of some species (for example, Lasee 1991 (**134**); Keller and Ruessler 1997 (**58**); McMahon and Bogan 2001 (**29**); Table A1.2). Importantly, the viability of the glochidia should be established before the start of a toxicity test and the duration of the exposure should be established based on these data. For example, there are some species that exhibit <90 % survival for about 24 h after isolation from the female; therefore, toxicity tests with glochidia from these species should not be conducted for longer than this time period.

A1.5.14 What criteria should be used to judge acceptability of a toxicity test conducted with juvenile mussels? Survival of juvenile mussels at the end of the exposure is the primary endpoint to establish the acceptability of toxicity tests conducted for up to 14 d. Investigators have reported >90 % survival of newly-transformed juvenile mussels after the end of exposures conducted for up to 14 d (Table A1.4); however, additional research is needed to improve survival in tests conducted for >14 d with newly-transformed juvenile mussels including research on dietary requirements of juvenile mussels (10.5). Additional research is also needed with additional species to determine if tests started with juvenile mussels >2- to 4-months old will improve survival in chronic exposures. USGS (2005 a,b (**8**,**9**)) and Bringolf et al (2005) conducted toxicity tests starting with 2- to 4- month old juvenile *Actinonaias ligamentina, Lampsilis siliquoidea* or *Villosa iris* and observed control survival > 88% in 21– to 28–d exposures when algae was used as a food source (10.6.3.14). Klaine et al (1997) (**135**) report that shell length of newly-transformed juvenile mussels of *Utterbackia imbecillis* increased by 22 to 35 % in tests conducted from 5 to 15 d. Therefore, growth in should also be evaluated in future studies as a criterion to judge the acceptability of a toxicity tests conducted with juvenile mussels.

A1.6 ***Future Research***—The methods outlined in Table A1.1 and Table A1.4 provide reliable estimates of toxicity of chemicals to glochidia and juvenile mussels in water-only exposures. The following list of research topics have been identified throughout the standard and in Kernaghan et al (2005) (**5**) for improving the reliability of results of toxicity tests conducted with glochidia or juvenile mussels. Results of this research may be included in future revisions of this standard.

A1.6.1 Further evaluate the influence of handling, holding, and acclimation on adult, glochidia, or juvenile mussels used to conduct toxicity tests (section 10.5).

A1.6.2 Determine the minimum number of female mussels that should be sampled to obtain glochidia or juvenile mussels used to start a toxicity test. These studies might include an evaluation of the variability in the sensitivity of glochidia or juvenile mussels obtained from individual females using a variety of chemicals with different toxic modes of action (section A1.4.9).

A1.6.3 Further evaluate the influence of contaminant exposure on immature glochidia developing within the marsupium of the female mussel (section 10.5.3.6).

A1.6.4 Establish methods for improving the performance of juvenile mussels in chronic toxicity tests (for example, test conducted for >14 d), focused on establishing feeding requirements for a variety of mussel species. Additional research is also needed with additional species to determine if tests started with juvenile mussels 2- to 4-months old will improve survival in chronic exposures. Ongoing research to improve culturing methods for propagation, holding, and feeding of newly-transformed juvenile mussels will hopefully provide additional information that can be adapted to establish methods for conducting chronic toxicity tests with juvenile mussels (section 10.6.3).

A1.6.5 Conduct additional intra- and inter-laboratory toxicity tests to evaluate variability in control and toxic responses of mussels to a variety of chemicals with different toxic modes of action (section 16.5).

A1.6.6 Further develop endpoints for establishing effects in toxicity tests with juvenile mussels (for example, behavior, biomarkers).

A1.6.7 Develop standard methods for conducting toxicity tests with *(1)* adult freshwater mussels and *(2)* contaminated sediments using various life stages of freshwater mussels.

A1.6.8 Evaluate the relative sensitivity of glochidia, newly-transformed juvenile mussels, older juvenile mussels, and adult mussels to a variety of different chemicals in acute or chronic toxicity tests.

A1.6.9 Compare the response of various species of mussels to the response of other surrogate species (for example, trout, cladocerans, *Corbicula*) in toxicity tests conducted using a variety of different chemicals.

A1.6.10 Compare the response of different populations of a species collected from different geographic regions to a variety of chemicals in laboratory toxicity tests.

A1.6.11 Compare the response of mussels tested in laboratory toxicity tests to the response of mussels exposed in the field (either using *in-situ* exposure containers or in a natural habitat).

TABLE A1.1 Summary of Test Conditions Used to Conduct Toxicity Tests with Glochidia of Freshwater Mussels (adapted from Kernaghan et al, 2005) (5)[A]

NOTE 1—The last column provides a summary of recommended conditions that can be used to conduct toxicity tests with glochidia.

	Conditions	Johnson et al (1990, 1993) (136, 79)	Lasee (1991) (134)	Huebner and Pynnonen (1992) (128)[B]	Goudreau et al (1993) (130)	Jacobson et al (1997) (31)	Keller and Russeler (1997) (58)	McCann (1993) (131)	Klaine et al (1997) (135)	USGS (2004) (112)	Recommended Test Conditions
1	Species tested	*Utterbackia imbecillis*[C]	*Lampsilis cardium*[D]	*Anodonta cygnea, A. anatina*	*Villosa iris*	Multiple species[E]	Multiple species[F]	*Villosa iris*	*Utterbackia imbecillis*	Mulitple species[G]	NA[H]
2	Test type	Static	Static	Static	Renewal	Static	Static	Static	Static	Static, renewal, flow-through	Static, renewal, or flow-through (depending on chemical tested)
3	Test duration (h)	24	48	24, 48, 72, 144	24	24, 48	4, 24, 48	24	24, 48	6, 24, 48	6, 24 (up to 48 depending on viability of glochidia)
4	Temperature, °C	20	21	13	22	10 to 25	25	20	25	20	20
5	Light quality	Ambient lab light	NR[H]	NR	NR	NR	NR	NR	Ambient lab light	Ambient lab light	Ambient lab light
6	Light intensity	NR	NR	NR	NR	NR	NR	NR	NR	200 lux	100 to 1000 lux
7	Photoperiod	16L:8D	24D	Natural regime	16L:8D	16L:8D	12L:12D	NR	16L:8D	16L:8D	16L:8D
8	Test chamber	100-mL beaker	250-mL crystallizing dish	400-mL beaker	Basket of mesh netting in 4-L chamber	12-well plate	6-well plate	12-well plate	12-well plate	200-mL crystallizing dish	100-mL glass chamber (minimum)
9	Test solution volume (mL)	50	200	200	NR	3.5	NR	5	3.5	100	75 (minimum)
10	Glochidia collection	shake piece of cut gill in water	flush gills with syringe	cut gills and press out glochidia using forceps	flush gills with syringe	cut gills and separate glochidia from marsupia	NR	flush gills with syringe	flush gills with syringe	flush gills with syringe	flush gills with syringe
11	Age of test organisms (h)	NR	NR	3 to 24	NR	NR	NR	<2	NR	<2 to <24	<24
12	No. organisms per test chamber	10	10	1000-3000	Several hundreds	50-75	50-100	40	50-100	about 1000	about 500 (1000 for repeated sampling during a toxicity test)
13	No. replicate chambers per treatment	2	3	2, counting 3 samples with about 100 glochidia	2, counting 3 samples with about 100 glochidia	3	3 or 4	3	3	3, counting a subsample with about 100 glochidia from each replicate	3, counting a subsample with about 100 glochidia from each replicate
14	Feeding	None	None	None	None	None	None	None	None	None	None
15	Aeration	None	None	Yes	None	None	NR	NR	NR	None	None, if dissolved oxygen is maintained above acceptable concentration
16	Dilution water	Reconstituted water, hardness 40-50 mg/L as $CaCO_3$	Hardness 150 mg/L as $CaCO_3$	Tap water	Dechlorinated effluent water	Dechlorinated tap water or Clinch River water, VA	Reconstituted water, hardness 47-76 mg/L as $CaCO_3$	Sinking Creek water, VA	Hardness 99-107 mg/L as $CaCO_3$	Reconstituted water, hardness 170 mg/L as $CaCO_3$	Depends on experimental design
17	Water quality	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	pH, Ca, Cu, Zn	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity

TABLE A1.1 *Continued*

	Conditions	Johnson et al (1990, 1993) (**136**, **79**)	Lasee (1991) (**134**)	Huebner and Pynnonen (1992) (**128**)[B]	Goudreau et al (1993) (**130**)	Jacobson et al (1997) (**31**)	Keller and Russeler (1997) (**58**)	McCann (1993) (**131**)	Klaine et al (1997) (**135**)	USGS (2004) (**112**)	Recommended Test Conditions
18	Endpoint	Survival (valve closure with culture medium)	Survival (valve closure with NaCl)	Survival (valve closure with KCl)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)	Survival (valve closure with salt solution)	Survival (valve closure with saline solution)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)
19	Control survival (%)	>95	>90	>80	80	>90	>80	>80	80	>90	>90 (must)

[A] Reprinted with permission of Kernaghan et al (2005) (**5**). Copyright Society of Environmental Toxicology and Chemistry (SETAC).

[B] See also Pynnonen (1995) (**129**), Hansten etal (1996) (**137**).

[C] Formerly *Anodonta imbecillis*. See also Weinstein (2001) (**138**).

[D] Formerly *Lampsilis ventricosa*.

[E] *Villosa iris*, *Actinonaias pectorosa*, *Pyganodon grandis*, *Lampsilis fasciola*, *Medionidus conradius*. See also Jacobson (1990) (**116**), Cherry et al (2002).

[F] *Villosaosa lienosa*, *Villosa villosa*, *Utterbackia imbecillis*, *Megalonaias nervosa*, *Lampsilis teres*, *Lampsilis siliquoidea*. See also Jacobson (1990) (**116**); McCann (1993) (**125**), *Villosa iris*, *Actinonaias pectorosa*, *Medionidus conradius*.

[G] *Actinonaias ligamentina*, *Alasmidonta heterodon*, *Epioblasma capsaefotmis*, *Lampsilis siliquoidea*, *L. fasciola*, *L. abrupta*, *L. rafinesqueana*, *Potamilus ohiensis*, *Pleurobema plenum*, *Quadrula quadrula*, *Q. pustulosa*, *Leptodea fragilis*, *L. leptodon*, *Venustaconcha ellipsiformis*, *Villosa iris*.

[H] NA: not applicable. NR: not reported.

TABLE A1.2 Survival Time of Glochidia after Removal from Female Unionid Mussels (Kernaghan et al 2005) (5)[A]

Species	Temperature C	Duration of Viability Day (% Survival)	Reference
Actinonaias ligamentina	20	7 (>90): 8 (>75); 9 (>50)	USGS (2004) (**112**)
Actinonaias pectorosa	10	13 (>75)	Zimmerman and Neves (2002) (**42**)
	25	5 (>75)	Zimmerman and Neves (2002) (**42**)
	20	>2 (>90)*[B]	Jacobson et al (1997) (**31**)
Alasmidonta heterodon	20	2 (>90): 2 (>75): 2 (>50)	USGS (2004) (**112**)
Anodonta anatina	13	>3 (>90)	Huebner and Pynnonen (1992) (**128**)
Anodonta cataracta	10	>14 (>90)	Jacobson (1990) (**116**)
Anodonta cygnea	13	>3 (>90)	Huebner and Pynnonen (1992) (**128**)
Anodonta grandis	10	>14 (>90)	Jacobson (1990) (**116**)
Elliptio complanata	5	7 NR[C]	Matterson (1948) (**124**)
	20	<1 (>90); 3 (>75)	Bringolf et al (2005) (**108**)
Elliptio dilatata	20	<1 (>90); 1 (>75);<2 (>50)	Bringolf et al (2005) (**108**)
Epioblasma capsaeformis	20	0.3 (>90)	Wang et al (2003) (**85**)
Lampsilis abrupta	20	2 (>90): 5 (>75): 7 (>50)	USGS (2004) (**112**)
Lampsilis cardium	21	>2 (>90)*	Lasee (1991) (**134**)
Lampsilis fasciola	20	6 (>90); 7 (>75): 8 (>50)	Wang et al (2003) (**85**)
	20	>2 (>90)*	Jacobson et al (1997) (**31**)
	20	1 (>90); 2 (>75); 3 (>50)	Bringolf et ai. (2005) (**108**)
	20	2 (>90; 4 (>75); 5 (>50)	Bringolf et al (2005) (**108**)
Lampsilis rafinesqueana	20	6 (>90); 6 (>75); 6 (>50)	USGS (2004) (**112**)
Lampsilis siliquoidea	10	9 NR	Tedla and Fernado (1969) (**125**)
	20	8 (>90); 9 (>75); 10 (>50)	Wang et al (2003) (**85**)
	25	>2 (>80)*	Keller and Ruessler (1997) (**58**)
	20	1 (>90); 3 (>75); 4 (>50)	Bringolf et al (2005) (**108**)
Lampsilis teres	25	0.2 (>80)	Keller and Ruessler (1997) (**58**)
Leptodea fragilis	20	1 (>90); 3 (>75); 4 (>50)	Wang et al (2003) (**85**)
Leptodea leptodon	20	1 (>90): 2 (>75)	Bringolf et al (2005) (**108**)
Leptodea leptodon	20	0.25 (>90); 1 (>75); 2 (>50)	USGS (2004) (**112**)
Margaritifera falcata	11	11 NR	Murphy (1942) (**123**)
Medionidus conradicus	20	>2 (>90)*	Jacobson et al (1997) (**31**)
Megalonaias nervosa	25	1 (>80)*	Keller and Ruessler (1997) (**58**)
Potamilus alatus	20	6 (>90) 6 (>75); 6 (>50)	Wang et al (2003) (**85**)
Potamilus ohiensis	20	5 (>90), 6 (>75); 7 (>50)	Wang et al (2003) (**85**)
Pyganodon grandis	20	>1 (>90)'	Jacobson etal. (1997) (**31**)
Quadrula quadrula	20	1 (>90); 1 (>75); 2 (>50)	Wang et al (2003) (**85**)
Quadrula pustulosa	20	<1 (>90): 1 (>75); 1 (>50)	Wang et al (2003) (**85**)
Utterbackia imbecillis	21	10 (>80); 14 (>50)	Fisher and Dimock (2000) (**73**)
	25	>2 (>80)'	Keller and Ruessler (1997) (**58**)
	25	>2 (>80)*	Klaineetal. (1997) (**135**)
	20	>1 (>90)*	Johnson et al (1990, 1993) (**136**, **79**)
Venustaconcha ellipsiformis	20	2 (>90)l 3 (>75); 3 (>50)	Wang et al (2003) (**85**)
Villosa iris	10	8 (>75)	Zimmerman and Neves (2002) (**42**)
	20	5 (>90); 5 (>75): 6 (>50)	Wang et al (2003) (**85**)
	25	2 (>75)	Zimmerman and Neves (2002) (**42**)
	22	>1 (>80)*	Goudreau et al (1993) (**130**)
	20	>1 (>80)*	Scheller (1997) (**139**)
	20	>2 (>90)*	Jacobson et al (1997) (**31**)
Villosa lienosa	25	>2 (>80)*	Keller and Ruessler (1997) (**58**)
Villosa nebulosa	20	>2 (>90)*	Jacobson (1990) (**116**)
Villosa villosa	25	>2 (>80)*	Keller and Ruessler (1997) (**58**)

[A] Reprinted with permission of Kernaghan et al (2005) (**5**). Copyright Society of Environmental Toxicology and Chemistry (SETAC).
[B] An asterik indicates a value based on control survival in 24- or 48-h toxicity tests.
[C] NR: not reported.

TABLE A1.3 Test Acceptability Requirements for Toxicity Tests Conducted with Glochidia Isolated from Freshwater Mussels

A. It is recommended for conducting 24-h toxicity tests with glochidia isolated from adult mussels that the following performance criteria be met:

1. Age of glochidia should be less than 24-h old at the start of the toxicity test. Viability of glochidia isolated at the beginning of a toxicity test must be greater than or equal to 80 % (preferably greater than or equal to 90 %).
2. Average survival of glochidia in the control at the end of a test must be greater than or equal to 90 %.
3. Hardness, alkalinity, and pH in the dilution water should not vary by more than ±10 % during the exposure and dissolved oxygen should be maintained above 4 mg/L.
4. The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 24 h after the end of a 24-h glochidia toxicity test).

B. Performance-based criteria for culturing and handling of glochidia or adult mussels include the following:

1. Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$, section 16.4). Data from these reference-toxicant tests can be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.
2. Laboratories should track survival of adult mussels in the cultures. Records should also be kept on procedures used to collect and hold adult mussels.
3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly: pH, hardness, alkalinity, and ammonia. Dissolved oxygen in the cultures should be measured weekly. Temperature in the cultures should be recorded daily.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.

C. Additional requirements:

1. All organisms in a test must be from the same source and should be acclimated for about 2 h to the dilution water before the start of a toxicity test. It is desirable to combine samples of glochidia obtained from at least three female mussels to start a toxicity test.
2. All test chambers (or compartments) should be identical and should contain the same amount of dilution water. Individual test organisms should be impartially assigned to test chambers (or compartments). Treatments should be randomly assigned to individual test chamber locations.
3. Negative-control and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms (section 9.2.4). The concentration of an organic solvent used in the preparation of a test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a test solution.
4. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C. The upper or lower 95 % confidence limit on individual temperatures measured in the test chambers throughout the test must not be more than 2°C above or below the mean of the time-weighted average measured temperature for the individual test chambers.
5. Calculation of an LC50 or EC50 should usually be considered unacceptable if, *(1)* no treatment other than a control treatment killed or affected less than 37 % of the organisms or, *(2)* no treatment killed or affected more than 63 % of the organisms.

TABLE A1.4 Summary of Test Conditions Used to Conduct Toxicity Tests with Juvenile Freshwater Mussels (adapted from Kemaghan et al, 2005[A]) (5)

NOTE 1—The last column provides a summary of recommended conditions that can be used to conduct toxicity tests with juvenile mussels. In the last column, acute tests are tests conducted for up to 96 h and chronic tests are tests conducted for at least 21 d.

	Conditions	Johnson et al (1990, 1993) (136, 79)	Jacobson (1990) (116), Jacobson et al (1993) (75)	Lasee (1991) (134)	Keller and Zam (1991) (67)	Klaine et al (1997) (135)	Scheller (1997) (138)	Myers-Kinzie (1998) (132)	Dimock and Wright (1993) (48)	Newton et al (2003) (61)	Lasee (1991) (134)	Wade et al (1993) (119)[B]	Jacobson (1990) (116)	Valenti et al (2005) (107)	USGS (2004) (112)	USGS (2005b) (9)	Recommended Test Conditions
1	Species tested	*Utterbackia imbecillis*[C]	*Villosa nebulosa*, *V. iris*, *Anodonta grandise*[D]	*Lampsilis cardium*[E]	Mulitple species[F]	*Utterbackia imbecillis*	*Villosa iris*	*Lampsilis siliquoidea*	*Utterbackia imbecillis*, *Pyganodon cataracta*	*Lampsilis cardium*	*Lampsilis ventricosa*	*Utterbackia imbecillis*	*Villosa nebulosa*	*Villosa iris*	Mulitple species[G]	*Villosa iris*[H]	NA[I]
2	Test type	Renewal	Static	Static	Static	Static	Static	NR	Static	Flow through	Renewal	Renewal	Artificial stream	Renewal	Renewal, flow through	Flow through	Static, renewal or flow-through (depending on duration of exposure and chemical tested)
3	Test duration (d)	2	1	2	1-4	1-4	4	1, 2, 4	1-4	4. 10	7	9	14	21	2, 4, 10	28	Acute:≤4 Chronic:21 to 28
4	Temperature °C	20	20	21	22, 25, or 32	25	25	24	20	21	21	24	20	20	20	20	20
5	Light quality	Ambient lab light	NR[I]	NR	NR	NR	NR	NR	NR	Fluorescent	NR	NR	NR	NR	Fluorescent	Fluorescent	Ambient lab light
6	Light intensity	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	200 lux	200 lux	100 to 1000 lux
7	Photo period	16L:8D	16L:8D	24 D	12L:12D or 16L:8D	16L:8D	NR	NR	NR	16L:8D	24D	24D	16L:8D	12L:12D	16L:8D	16L:8D	16L:8D
8	Test chamber	125-mL beaker	12-well plate	Covered 250-mL crystallizing dish	Petri dish	Petri dish	12-well plate	Petri dish	120-mm diam. tub with mesh bottom in 4-L chamber	132 by 90 by 130 mm chamber	Covered 250-mL crystallizing dish	50-mm diam. glass tub with mesh bottom in 250-mL chamber	Dish covered with mesh	30-mL beakers submerged in a 1-L glass beaker	50- or 300-mL beaker	300-mL beaker	Static or renewal: 50-mL beakers (minimum) Flow-through: 300 mL beakers (minimum)
9	Test solution volume (mL)	100	3.5	NR	15	15	5	10	NR	1200	NR	200	150	950	30 or 200	200	Static or renewal: 30 (minimum) Flow-through: 200 (minimum)
10	Procedure for obtaining juveniles	Artificial media	Fish host	Fish host	Fish host or artificial media	Fish host or artificial media	Fish host	Artificial media	Artificial media	Fish host	Fish host	Artificial media	Fish host	Fish host	Fish host	Fish host	Fish host
11	Age of test organisms (day)	1-10	1-3	0, 7, 14	1-2	1-3	<3, 5, 9	<10	7-10	3-5	0	6-10	1-3	60	3-5, 60	60	Acute:<5 Chronic:60 to 120

TABLE A1.4 *Continued*

	Conditions	Johnson et al (1990, 1993) (136, 79)	Jacobson (1990) (116), Jacobson et al (1993) (75)	Lasee (1991) (134)	Keller and Zam (1991) (67)	Klaine et al (1997) (135)	Scheller (1997) (138)	Myers-Kinzie (1998) (132)	Dimock and Wright (1993) (48)	Newton et al (2003) (61)	Lasee (1991) (134)	Wade et al (1993) (119)[B]	Jacobson (1990) (116)	Valenti et al (2005) (107)	USGS (2004) (112)	USGS (2005b) (9)	Recommended Test Conditions
12	No. organisms per test chamber	10	10	10	10-20	1	5	NR	10	20	50	15	15	5	5	10	Acute:<5 (minimum) Chronic:10 (minimum)
13	No. replicate chambers per treatment	2	2 or 3	3	2-4	10	4	NR	3	6	2	3	3	4	4	4	Acute:4 (minimum) Chronic:3 (minimum)
14	Feeding	None	None	None	None	None	None	None	None	None	Lab cultured phytoplankton	Algae and silt	Algae	Algae and sediment	None	Instant algae mixture[J]	Acute:none Chronic: Algae
15	Aeration	None	None	Yes	NR	None	NR	NR	Yes	Yes	None	None	None	Yes	None	None	None, if dissolved oxygen a maintained above acceptable concentration
16	Dilution water	Reconstituted water, hardness 40-50 mg/L as $CaCO_3$	Clinch River water, VA	Hardness 150 mg/L as $CaCO_3$	Reconstituted water, hardness 47-76 mg/L as $CaCO_3$	Reconstituted water, hardness 99-107 mg/L as $CaCO_3$	Sinking Creek water, VA	Hardness 100 or 200 mg/L as $CaCO_3$	NR	Hardness 133 mg/L as $CaC0_3$	Hardness 150 mg/L as $CaC0_3$	Tennessee River water	Clinch River water, VA	Reconstituted water, hardness 100 mg/L as $CaCO_3$	Reconstituted water, hardness 170 mg/L as $CaCO_3$	Reconstituted water-hardness 170 mg/L as $CaCO_3$	Depends on experimental design
17	Water quality	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	pH, hardness	NR	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	NR	NR	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity
18	Endpoints	Survival (movement)	Survival (gaped valves, foot activity or stained with neutral red)	Survival (foot or ciliary movement)	Survival (activity and heart-beat)	Survival (gaped valves with foot and ciliary activity)	Survival (heartbeat and ciliary action)	Survival (foot or valve movement)	Survival (foot, valve or ciliary activity, heartbeat)	Survival, growth, ratio of stressed to alive	Survival (foot or ciliary movement), growth (length and height)	Survival (Ciliary action)	Survival (extruded foot and gaping valves)	Survival, growth	Survival (foot or shell movement) and growth (shell length)	Survival (fool or shell movement) and growth (shell length)	Survival (foot movement), growth (shell length)
19	Control survival (%)	>95	100	96	NR	>90	>80	99	>90	>95	97	>90	100	90	>90	>88	Acute:>90 (must) Chronic:>80 (should)

[A] Reprinted with permission of Kernaghan et al (2005) (5). Copyright Society of Environmental Toxicology and Chemistry (SETAC).
[B] See also Masnado et al (1995) (140), McKinney and Wade (1996) (120), Keller et al (1999) (141).
[C] Formerly *Anodonta imbedllis.*
[D] See also McCann (1993) (131) for 2- to 4-d exposures with *Villosa iris*, *Actinonaias pectomsa*, *Medionidus conradius.*
[E] Formerly *Lampsilis ventricosa.*
[F] *Anodonta imbecillis*, *Villosa lienosa*, *V. villosa*, *Utterbackia imbecillis*, *Lampsilis straminea daibomensis*, *L. subangulata*, *Elliptic icterina*. See also Keller 1993 (142), Keller and Ruessler 1997 (58).
[G] *Villosa iris*, *Epioblasma capsaeformis*, *Lampsilis fasciola*, *L. siliquoidea*, *L. abrupta*, *L. rafinesqueana*, *Leptodea leptodon.*
[H] Bringotf et al (2005) (108) and USGS (2005a) (8) have adapted this method to conduct 21- to 28-d toxicity tests with 2- to 4-month old juvenile *Actinonaias ligamentina* or *Lampsilis siliquoidea.*
[I] NA: not applicable. NR: not reported.
[J] See section A1.4.5.3 for a description of the procedure used to prepare this instant algae mixture.

TABLE A1.5 Test Acceptability Requirements for Toxicity Tests Conducted with Freshwater Juvenile Mussels

A. It is recommended for conducting toxicity tests with juvenile mussels that the following performance criteria be met:

1. Average survival of juvenile mussels in the control at the end of a 96-h test must be greater than or equal to 90 %. An insufficient number of tests have been conducted with juvenile mussels for 10 or more days to provide specific guidance on control survival in longer-term tests. However, a limited number of toxicity tests have reported control survival greater than 80 % in tests conducted with juvenile mussels for 10 to 28 d. Therefore, average survival of juvenile mussels in the control at the end of a test conducted for 10 to 28 d should be greater than or equal to 80 %.
2. Hardness, alkalinity, and pH in the dilution water should not vary by more than ±10 % during the exposure and dissolved oxygen should be maintained above 4 mg/L.
3. The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 96 h after the end of a 96-h juvenile toxicity test).

B. Performance-based criteria for culturing and handling of juvenile or adult mussels include the following:

1. Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$, section 16.4). Data from these reference-toxicant tests can be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.
2. Laboratories should track survival of juvenile and adult mussels in the cultures. Records should also be kept on procedures used to collect and hold juvenile and adult mussels.
3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly: pH, hardness, alkalinity, and ammonia. Dissolved oxygen in the cultures should be measured weekly. Temperature in the cultures should be recorded daily.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.

C. Additional requirements:

1. All organisms in a test must be from the same source and should be acclimated to the dilution water for at least 24 h before the start of a toxicity test.
2. All test chambers (or compartments) should be identical and should contain the same amount of dilution water. Individual test organisms should be impartially assigned to test chambers (or compartments). Treatments should be randomly assigned to individual test chamber locations.
3. Negative-control and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms (section 9.2.4). The concentration of an organic solvent used in the preparation of a test solution should not exceed 0.5 mL/L in 96-h tests or 0.1 mL/L in longer-term tests. A surfactant should not be used in the preparation of a test solution.
4. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C. The upper or lower 95 % confidence limit on individual temperatures measured in the test chambers throughout the test must not be more than 2°C above or below the mean of the time-weighted average measured temperature for the individual test chambers.
5. Calculation of an LC50 or EC50 should usually be considered unacceptable if, *(1)* no treatment other than a control treatment killed or affected less than 37 % of the organisms or, *(2)* no treatment killed or affected more than 63 % of the organisms.

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