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

This standard is issued under the fixed designation E2455; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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 and effluent exposures (Annex A1). Future revisions to this standard may describe methods for conducting toxicity tests with endpoints of reproduction, behaviors, and biomarkers.

1.2 Freshwater mussels (order Unionida) are one of the most imperiled groups of animals in the world, and environmental contamination has been linked as a contributing factor to the decline of mussel populations (Lydeard et al. 2004 (**1**); Strayer et al. 2004 (**2**); Haag 2012 (**3**); Lopes-Lima et al. 2017 (**4**)).[2] Three critical life stages (glochidia, juvenile mussels, and adults) have been used in toxicity assessments and the toxicity studies are separated according to the medium of exposure (water, sediment, and host fish (Ingersoll et al. 2007 (**5**)). Recent studies on early life stages of mussels have demonstrated that the mussels are among the most sensitive freshwater species to a variety of contaminants, including ammonia, some metals (for example, aluminum, copper, nickel, and zinc), and major ions (for example, chloride, nitrate, potassium, and sulfate) (Bringolf et al. 2007 (**6**); Newton et al. 2007 (**7**); Wang et al. 2007ab, 2010, 2011ab, 2016, 2017ab, 2018abc, 2020ab (**8-20**); Cope et al. 2008 (**21**); Gillis et al. 2008, 2010, 2011, 2021 (**22-25**); Miao et al. 2010 (**26**); Salerno et al. 2020 (**27**)). These studies indicate that environmental guideline values for individual chemicals established for the protection of aquatic organisms may not be adequately protective of sensitive stages of freshwater mussels. For example, when freshwater mussel toxicity data were included in an update to the United States Environmental Protection Agency (USEPA) ambient water quality criteria (WQC) for ammonia, the acute criterion decreased by about a 1.4 fold and the chronic criterion decreased by 2.4 fold (USEPA 2013) (**28**).

1.3 *Summary of Life History of Freshwater Mussels:*

1.3.1 Freshwater mussels are bivalve mollusks belonging to the taxonomic Order Unionida (section 10.1). Like most bivalves, mussels are totally aquatic, relatively sedentary, filter-feeding animals, and spend most of their lives partially or completely burrowed in the substrate of streams, rivers, or lakes. Freshwater mussels have an unusual and complex life cycle that includes a larval stage, the glochidium, that is briefly parasitic on fish (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 (Haag 2012) (**3**). 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. Host fish specificity varies among mussels. While some mussel taxa appear to require a single host species, others can utilize several species of host fish. Encapsulation on the host occurs by overgrowth of host tissue. Within the capsule, glochidia obtain nutrition from the host, continue their development, and metamorphose within days to weeks. Metamorphosis is followed by excapsulation (drop-off) and transition to self-sustained existence as a benthic organism.

1.3.3 Both juvenile and adult mussels live embedded in sediment and feed using ciliary mechanisms to capture fine particles (Haag 2012) (**3**). However, young juvenile mussels (~0.2 mm –10 mm) may bury several centimeters in sediment where they feed and respire from interstitial water, while larger adult mussels can access the water column. Water column and substrate conditions suitable for adult life stages may not be protective of juvenile life stages 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 laboratory toxicity tests with glochidia and juvenile mussels. Recommended test conditions for conducting these laboratory toxicity tests are based on various published methods and are

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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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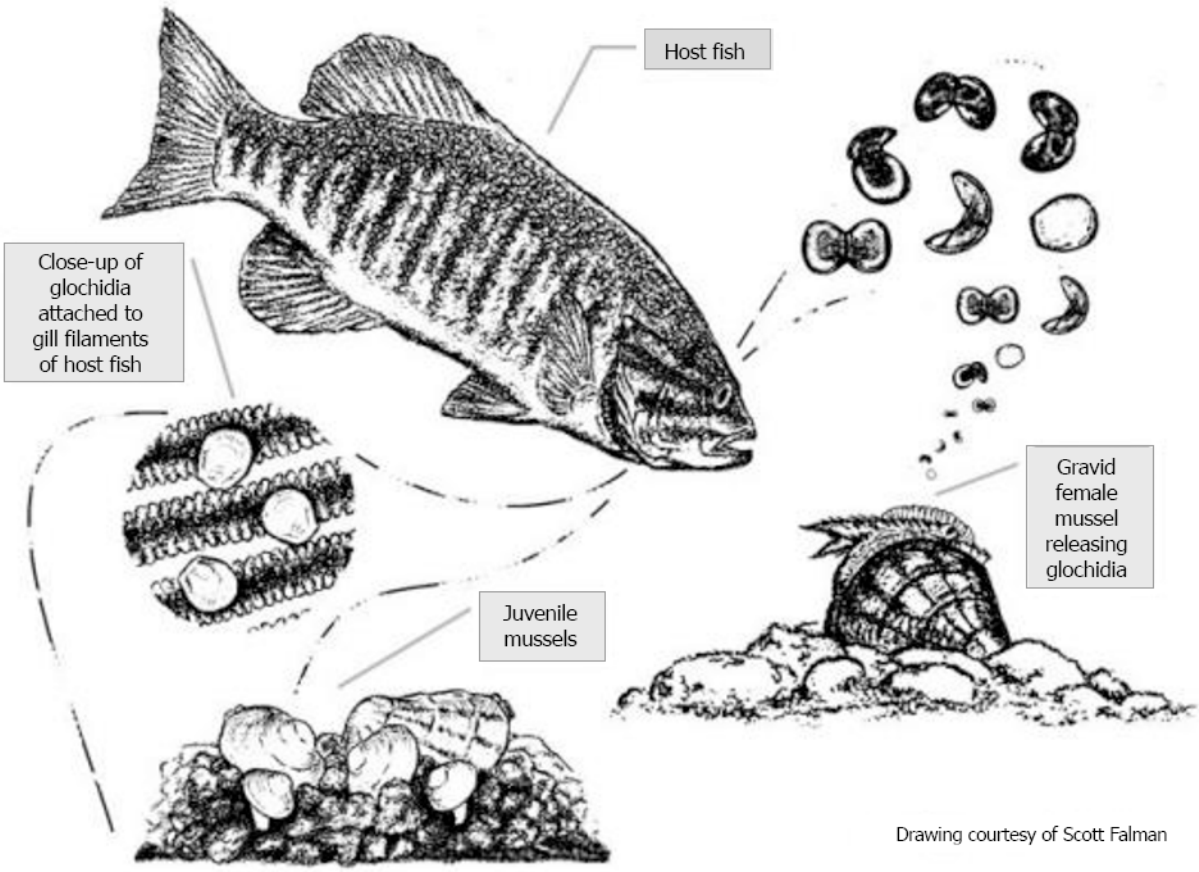

FIG. 1 Life Cycle of a Freshwater Mussel (Scott Faiman, Missouri Department of Conservation, Columbia, MO, USA)

based on the conditions used to conduct an intra- or interlaboratory toxicity test with glochidia and juvenile mussels (section 16.5). Female mussels brooding matured glochidia are only available on a seasonal basis. Section 10 describes procedures for collecting brooding female mussels from the field and holding them in the laboratory to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host fish. Juveniles propagated using host-free (*in vitro*) methods may or may not produce comparable results (section 10.5.4.2).

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 E1706 provides guidance for conducting sediment toxicity tests with juvenile mussels. Guide E2122 provides guidance for conducting *in situ* field exposures with caged mussels.

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 This standard is arranged as follows:

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

1.7 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

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

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

E1733 Guide for Use of Lighting in Laboratory Testing

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[4]

E1850 Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests

E2122 Guide for Conducting In-situ Field Bioassays With Caged Bivalves

IEEE/ASTM SI 10 Standard for Use of the International System of Units (SI) (the Modernized Metric System)

[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.

[4] The last approved version of this historical standard is referenced on www.astm.org.

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," 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. A listing of the common and scientific names of freshwater mussels in North America can be found in AFS (1998) (**29**). For an explanation of units and symbols, refer to Standard IEEE/ASTM SI 10.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *acute test, n*—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 h exposures for glochidia; 96 h exposures for juvenile mussels).

3.3.2 *chronic test, n*—a comparative study in which organisms that are subjected to different treatments are observed for a relatively long period or a substantial portion of their life span (for example, 28 d to 84 d exposures for juvenile mussels). 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.

3.3.3 *short-term chronic test, n*—a comparative study in which organisms that are subjected to different treatments are observed for a short period (7 d exposures). The short-term chronic 7 d test with survival and growth endpoints provides a more direct estimate of the safe concentrations of toxicants or effluents than acute tests by evaluating lethal and sublethal (that is, growth) endpoints, at a lower level of effort compared to chronic 28 d toxicity test.

3.3.4 *EC50/EC20, n*—a statistically or graphically estimated concentration that is expected to cause one or more specified effects in 50%/20% of a group of organisms under specified conditions.

3.3.5 *IC50/IC20, n*—a point estimate of the toxicant concentration that would cause a 50 %/20 % reduction in a non-quantal measurement such as fecundity or growth.

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3.3.6 *LC50/LC20, n*—a statistically or graphically estimated concentration that is expected to be lethal to 50 %/20 % of a group of organisms under specified conditions.

3.3.7 *lowest-observed-effect concentration (LOEC), n*—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.

3.3.8 *no-observed-effect concentration (NOEC), n*—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.

3.3.9 *reconstituted water, n*—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.

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

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

3.3.12 *Unionoidea, n*—the taxonomic order of freshwater mussels, comprising six families, two of which (Unionidae and Margaritiferidae) occur mainly in the northern hemisphere. Unionidae comprises 98 % of North American species including representatives of 6 taxonomic tribes: Anodontini, Amblemini, Pleurobemini, Lampsilini, Quadrulini, and Gonideini (Graf and Cummings 2007) (**30**).

3.3.13 *unionoid, n*—any mussel species in the order Unionida.

3.3.14 *unionid, n*—any mussel species in the family Unionidae.

3.3.15 *margaritiferid, n*—any mussel species in the family Margaritiferidae.

3.3.16 *bradytictic, adj*—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).

3.3.17 *tachytictic, adj*—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).

3.3.18 *glochidia (*sing. *glochidium), n*—bivalve larvae of unionid mussels which are generally parasitic on the gills of fish.

3.3.19 *marsupium, n*—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 and are based on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels (section 16.5). Female mussels brooding mature glochidia are only available for a limited time on a seasonal basis. Section 10 describes procedures for collecting brooding mussels from the field to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host fish.

4.1.1 Acute toxicity tests with glochidia should be conducted at 20 °C and acute toxicity tests with juvenile mussels should be conducted at 20 °C or 23 °C. Chronic tests should be conducted at 23 °C and short-term chronic 7 d tests should be conducted at 25 °C. A 16L:8D photoperiod should be used at an illuminance of about 100 lux to 1000 lux (Guide E1733).

4.1.2 Toxicity tests with glochidia should start within 4 h after glochidia are isolated from the gills of the female mussels. The endpoint measured in toxicity tests with glochidia is 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. The duration of toxicity exposures conducted with glochidia should be 24 h . Control viability (adjusted for initial viability; section A1.2.2) must be ≥90 % at the end of 24 h toxicity tests. Longer duration toxicity tests with glochidia (for example, 48 h) can be conducted as long as control viability >90 % is achieved. Glochidia are not fed during the toxicity test.

4.1.3 Acute toxicity tests with juvenile mussels should start with <10 d-old newly transformed juveniles. Juveniles recovered from the host fish during 2 d peak of drop-off period (ages within 2 d) should be pooled and shipped to laboratories for culture and testing. Acute toxicity tests with juvenile mussels should conduct for 96 h. Juvenile mussels are not fed during acute toxicity tests. The endpoint is survival. Control survival must be ≥90 % at the end of an acute toxicity test.

4.1.4 Chronic toxicity tests should start with 2 week-old to 6 week-old juvenile mussels. All organisms in a test should be uniform in age (ages within 4 d, preferably within 2 d) and size. Chronic toxicity tests should be conducted for at least 28 d. Juvenile mussels are fed with algae during a chronic toxicity test. The endpoints are survival and growth (that is, length, dry weight), and biomass as described in Annex A1. The average survival of control mussels must be ≥80 % at the end of a chronic test.

4.1.5 Short-term chronic 7 d toxicity test should start with 1 week-old to 3 week-old juvenile mussels (ages within 2 d) and conduct for 7 d. Juvenile mussels are fed with algae during the test. The endpoints are survival and growth (that is, length, dry weight), and biomass as described in Annex A1). The average survival of control mussels must be ≥80 % at the end of a short-term chronic toxicity test.

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 species

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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 environmental guideline values for toxicants.

5.5 An acute toxicity test is conducted to obtain information concerning the immediate effects on mussels of a short exposure to a test material under specific experimental conditions. An acute toxicity test does not provide information about whether delayed effects will occur, although a post-exposure observation period, with appropriate feeding, if necessary, might provide such information (Guide E729).

5.6 Results of chronic (at least 28 d) toxicity tests with mussels might be used to predict chronic or partial chronic effects on species in field situations as a result of exposure under comparable conditions.

5.7 Short-term chronic toxicity tests are conducted for 7 d, a complementary test duration in the USEPA shot-term methods for estimating the chronic toxicity of effluents and receiving waters to fathead minnow (*Pimephales promelas*; USEPA 2002) **(31)** and provides a more direct estimate of the safe concentrations of effluents and receiving waters than acute toxicity tests, at a slightly lower level of effort compared to chronic 28 d toxicity test.

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

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

5.10 *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.

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

5.10.2 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.

5.10.3 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.

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

5.10.5 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. Methods for conducting sediment toxicity tests with juvenile mussels are included in Guide E1706.

5.10.6 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 d 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 (section 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 and Guide E1733).

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 example, creek gravel of ~0.2 cm to 1.5 cm diameter) for burrowing or juvenile mussels should be provided a thin layer of silica sand (~100 µm to 400 µm particles) in test chambers during short-term chronic 7 d and chronic 28 d toxicity tests.

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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 of solutes from containers. 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.

6.4.3 The frequency of water addition to 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 (Wang et al. 2007a) (**8**)). In 28 d exposures with juvenile mussels, about 4 volume additions/d were delivered to each test chamber (Wang et al. 2007b (**9**); 2018c (**18**); 2020a,b (**19, 20**)).

6.4.4 Speciation of some metals (for example, lead or copper) and perhaps other test materials is not instantaneous and may change over 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 (**32**); Besser et al. 2005a (**33**)).

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 or 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. Chambers should be covered to keep out extraneous contaminants and, especially in static and renewal tests, to reduce evaporation of test solution and test material. 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

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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 plain liquid household chlorine bleach (nominally 5 %-6% ClO^-, without additives) 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 (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.

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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 workplace 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).

7.7 *Biosecurity*—Appropriate measures and practices should be implemented to prevent the spread of non-target species when acquiring or transferring the target test species. Additionally, bio-secure practices should be utilized when working with either non-localized or non-native species to prevent escapement into local watersheds and potentially altering or negatively influencing existing ecosystems.

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 section 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 tested 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 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

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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

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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) (**34, 35**) and Besser et al. (2005b) (**36**) 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) (**37**) 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 A1.2 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 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 an acute 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. 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.

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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 or chronically toxic to the test species, or *(b)* whether the acute effect concentration (for example, LC50, EC50, or IC50) or chronic effect concentration (for example, LC20, EC20, or IC20) 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 effect concentration.

10. Test Organisms

10.1 *Life History of Freshwater Mussels:*

10.1.1 Freshwater mussels are bivalve mollusks belonging to the Order Unionida. Adults are sedentary animals living partially burrowed in the bottoms of streams, rivers, or lakes. Both juvenile and adult mussels are filter feeders, using ciliary mechanisms to capture microorganisms and fine particle of detritus ((Fuller 1974) (**38**), Strayer et al. 2004 (**2**)). Young juvenile mussels are very small and live and feed within interstitial spaces in sediments, where they are exposed mainly to pore water and deposited particles (Yeager et al. 1994 (**39**); Hyvärinen et al. 2021 (**40**)). Larger juveniles and adult mussels mainly filter the water column but may also ingest deposited particles. Evidence suggests that detritus, bacteria, and phytoplankton are all important food sources (Silverman et al. 1997 (**41**), Nichols and Garling 2000 (**42**), Gatenby et al. 1997 (**43**), O'Beirn et al. 1998 (**44**), Parker et al. 1998 (**45**). The extent of particle selectivity exhibited by mussels is poorly understood and is likely to vary by species (Baker and Levington 2003 (**46**); Beck and Neves 2003 (**47**)).

10.1.2 Unionid mussels have an unusual and complex life cycle, which for nearly all species includes a brief, obligatory parasitic stage on fish (Barnhart et al. 2008 (**48**); Fig. 1). Freshwater mussels are typically dioecious, but a few species may be hermaphroditic (Watters 2007 (**49**)). During the breeding season, males release sperm aggregates (spermatozeugmata) into the water column which drift downstream to be captured by females. The eggs are presumed to be fertilized in the suprabranchial chambers of the ctenidia and then transported to the marsupial water tubes, where they are brooded until released as mature glochidia by the thousands to millions (Fig. 2)

10.1.3 The timing of spawning, brooding, and glochidia release varies among mussel taxa. Spawning (the release of gametes) takes place in the spring for most Quadrulini, Pleurobemini, and Margaritiferidae, and these taxa generally release glochidia as soon as they mature (tachytictic or short-term brooders). In contrast, most Anodontini and Lampsilini spawn in the late summer and brood the mature glochidia for several months, releasing them in the following spring (bradytictic or long-term brooders). A few taxa release glochidia in the fall or winter and remain attached to the host until spring (Watters 2007) (**49**).

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 (Barnhart et al. 2008) (**48**). 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 encapsulated, glochidia may be relatively protected from *in situ* exposure contaminants in water (Jacobson et al. 1997) (**51**). 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 (**52**), Barnhart and Roberts 1997 (**53**)). 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 encloses large numbers of glochidia (Hartfield and Hartfield 1996) (**54**). Conglutinates may resemble prey items of the host fish; the host fish are infested with glochidia when fish attempt to eat conglutinates (Chamberlain 1934 (**55**); Barnhart and Roberts 1997 (**53**); Jones et al. 2004 (**56**)).

10.1.5 Glochidia range in size from about 50 to 400 µm (Hoggarth 1999 (**57**); McMahon and Bogan 2001 (**58**); Wachtler et al. 2001 (**59**); Barnhart et al. 2008 (**48**)). After release from the female mussels, glochidia must be encapsulated on the gills or the fins of a compatible host to complete development (Zimmerman and Neves 2002) (**60**). Although glochidia may survive for months during brooding in the female mussel, glochidia typically remain viable for only a few hours or days after release unless they reach a compatible host (Fritts et al. 2014) (**61**). The only visible behavior of which 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 (**62**); Arey 1921 (**63**)). The sharp valves cut into the epithelium of the host, enclosing and compressing the tissue (LeFevre and Curtis 1912 (**62**); Arey 1932 (**64**)). In nature, glochidia attach indiscriminately to the gills or the fins of fish upon contact but

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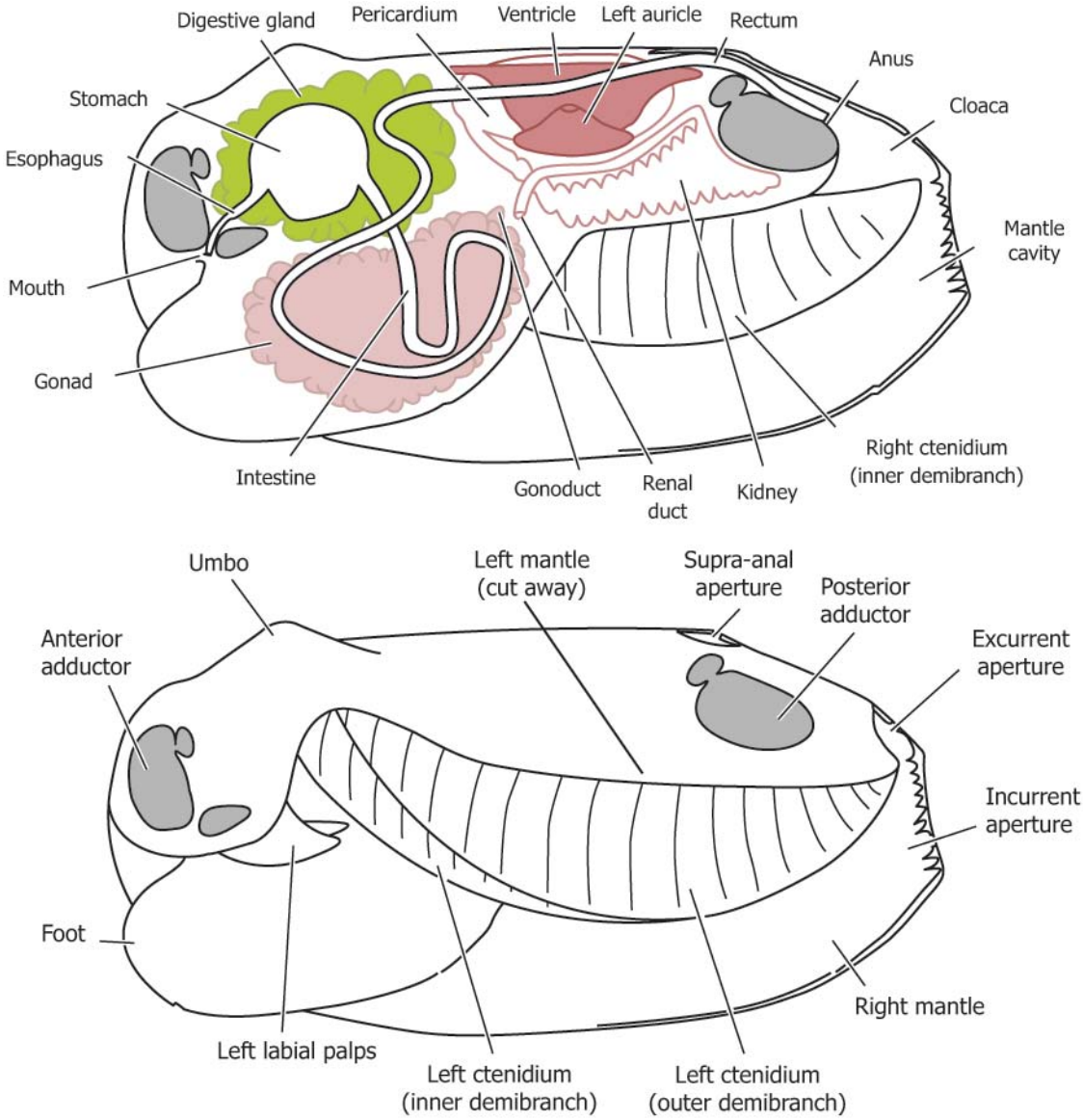

FIG. 2 General Internal Anatomy of Soft Tissues of a Unionid Mussel; adapted from Cummings and Graf, Academic Press, 2010 (50) Copyright Academic Press

subsequent encapsulation and survival is host-specific (Barnhart et al. 2008) (**48**).

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 (**65**); Kemble et al. 2020 (**66**); Hyvärinen et al. 2021 (**40**)).

10.1.7 Newly transformed juvenile mussels feeding from interstitial spaces likely use different food types than older juveniles and adults that access the water column. Given that the parasitic glochidia and interstitial 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. Reproduction and recruitment of mussels are dependent both on the presence of host fish and on microhabitat conditions experienced by post-metamorphic juveniles. 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 2007) (**49**).

10.1.8 Photographs of lures and conglutinates that mimic prey items of the host fish can be found at the following

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websites: http://unionid.missouristate.edu/default.htm and https://molluskconservation.org/. Additional information on the life history and propagation techniques for freshwater mussels can be found in Gordon and Layzer (1989) (**67**), Parmalee and Bogan (1998) (**68**), Bishop et al. (2007) (**69**), and Watters (1995, 2007) (**70, 49**), Patterson et al. 2018 (**71**).

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) (**58**) provide an overview of the basis anatomy and physiology of 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 2007) (**49**). However, anecdotal observations suggest that certain freshwater species are routinely found near the sediment-water interface (for example *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) (**72**) 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*). More recently, the acute and chronic effects of temperature have been evaluated in a variety of mussel species and life stages in water and sediment exposures (Pandolfo et al. 2010 (**73**); Archambault et al. 2014 (**74**); Ganser et al. 2013 (**75**); Khan et al. 2019 (**76**)). Pandolfo et al. (2010) (**73**) tested the glochidia of 8 species of mussels and 7 of the same species also were tested as juveniles. They showed that when exposed to a range of common and extreme water temperatures (20–42 °C) in standard acute laboratory tests that the average median lethal temperature (LT50) among species in 24 h tests with glochidia was 31.6 °C and ranged from 21.4 to 42.7 °C. The mean LT50 in 96 h juvenile tests was 34.7 °C and ranged from 32.5 to 38.8 °C. Based on comparisons of LT50s in their study, thermal tolerances differed among species for glochidia, but not for juveniles. Similarly, Khan et al. (2019) (**76**) reported 24 h median lethal temperatures (LT50) for glochidia ranging from 26.9 to 36.4 °C. They also found that thermal tolerances differed significantly among and within species, and by season. 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) (**77**) summarizes oxygen consumption by 9 species of freshwater mussels. Sparks and Strayer (1998) (**78**) 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 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) (**79**).

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. 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 A1.3). 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. 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, Wang et al. 2017a (14) and Keller et al. 2007) (**80**).

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 is

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yields meaningful data will depend on how well the test performance characteristics meet the data quality objectives of the study. 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) Different life stages of the same species (for example, glochidia versus juvenile mussels) require different exposure durations in order to obtain meaningful test endpoints. As a general rule, acute toxicity tests should conducted for 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 (section 10.5.3.6). 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 5.10.6).

(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 analyses for an effect concentration such as LC50 or EC20, test reliability and data quality of objectives are best stated in terms of the range of the 95 % confidence limit around the effect concentration; the tighter the confidence intervals of the effect concentration, 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 effect concentration) 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, Table A1.3, 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). Mussels at collection sites should be abundant and apparently healthy (for example, stable population with multiple age classes), and reproduction and recent recruitment are evident. 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 2021. Transformation of juvenile mussels has been reported for many species using either fish hosts (*in vivo*) or artificial media (*in vitro*; Bishop et al. 2007 **(69)**; section 10.5). Additionally, Watters (1994) **(81)** compiled reports of 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.

10.4.3 Bishop et al. (2007) **(69)** 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 (Lampsilini and Anodontini) 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). The temperature of the mussels themselves should not be less than 4 °C. Therefore, there should be some insulation around the ice bags or ice packs. Cope et al. (2004) **(82)** 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) **(83)** and Gordon (2001) **(84)** 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 be better

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