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Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians[1]

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This standard has been approved for use by agencies of the U.S. Department of Defense.

ε[1] NOTE—Sections 3 and 4 were editorially reorganized, and 7.8 and 8.2.1.1 were editorially corrected in February 2023.

1. Scope

1.1 This guide (1)[2] describes procedures for obtaining laboratory data concerning the adverse effects (for example, lethality and immobility) of a test material added to dilution water, but not to food, on certain species of freshwater and saltwater fishes, macroinvertebrates, and amphibians, usually during 2 to 4-day exposures, depending on the species. These procedures will probably be useful for conducting acute toxicity tests with many other aquatic species, although modifications might be necessary.

1.2 Other modifications of these procedures might be justified by special needs or circumstances such as meeting specific study goals, regulatory needs, or to accommodate specific test organism life stages. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual or novel procedures are not likely to be comparable to results of many other tests. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting acute tests.

1.3 This guide describes tests using three basic exposure techniques: static, renewal, and flow-through. Selection of the technique to use in a specific situation will depend on the needs of the investigator and on available resources. Tests using the static technique provide the most easily obtained measure of acute toxicity, but conditions often change substantially during static tests; therefore, static tests should not last longer than 96 h, and test organisms should not be fed during such tests unless the test organisms are severely stressed without feeding over 48 h. Static tests should probably not be conducted on materials that have a high oxygen demand, are highly volatile, are rapidly transformed biologically or chemically in aqueous solution, or are removed from test solutions in substantial quantities by the test chambers or organisms during the test. Because the pH and concentrations of dissolved oxygen and test material are maintained at desired levels and degradation and metabolic products are removed, tests using renewal and flow-through methods are preferable; test organisms may be fed during renewal and flow-through tests. Although renewal tests might be more cost-effective, flow-through tests are generally preferable.

1.4 Acute tests may be performed to meet regulatory data requirements or to obtain time-independent estimates of toxicity.

1.4.1 If the objective is to obtain data to meet regulatory requirements, it may be necessary to limit the number of observation times based on stipulations of the regulatory agency and cost considerations.

1.4.2 If the objective of an acute toxicity test is to determine a time-independent (that is, incipient, threshold, or asymptotic) toxicity level, an appropriate number of observations must be taken over an exposure duration of sufficient length to establish the shape of the toxicity curve or allow the direct or mathematically estimated determination of a time-independent toxicity value (1), or both.

1.5 In the development of these procedures, an attempt was made to balance scientific and practical considerations and to ensure that the results will be sufficiently accurate and precise for the applications for which they are commonly used. A major consideration was that the common uses of the results of acute toxicity tests do not require or justify stricter requirements than those set forth herein. Although the tests may be improved by using more organisms, longer acclimation times, and so forth, the requirements presented herein should usually be sufficient.

1.6 Results of acute toxicity tests should usually be reported in terms of an LC50 (median lethal concentration) or EC50 (median effective concentration) at the end of the test, but it is

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

desirable to provide information concerning the dependence of adverse effects on both time and concentration. Thus, when feasible, flow-through and renewal tests should be conducted so that LC50s or EC50s can be reported from 6 h to an asymptotic (time-independent, threshold, incipient) value, if one exists. In some situations, it might only be necessary to determine whether a specific concentration is acutely toxic to the test species or whether the LC50 or EC50 is above or below a specific concentration.

1.7 This guide is arranged as follows:

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

1.9 *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.

1.10 *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]

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E724 Guide for Conducting Static Short-Term Chronic Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

E943 Terminology Relating to Biological Effects and Environmental Fate (Withdrawn 2023)[4]

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1203 Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology (Withdrawn 2013)[4]

E1563 Guide for Conducting Short-Term Chronic Toxicity Tests with Echinoid Embryos

E1604 Guide for Behavioral Testing in Aquatic Toxicology

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

E1733 Guide for Use of Lighting in Laboratory Testing

E2455 Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels

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 guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see 13.1). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although 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:*

[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.

3.2.1 *acute test, n*—a comparative study in which organisms, that are subjected to different treatments, are observed for a relatively short period usually not constituting a substantial portion of their life span.

3.2.2 *dilution water, n*—non-toxic aqueous exposure media (that is, water) used to reduce the concentration of a test substance in aquatic toxicity tests and is used as the control water.

3.2.3 *reconstituted water, n*—a dilution water that is prepared by adding sea salt or appropriate amounts of reagent-grade salts to water, which is usually prepared using deionization, distillation, 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.

3.2.4 *IC50, n*—a statistically or graphically estimated concentration of test material that is expected to cause a 50 % inhibition of one or more specified biological processes (such as shell growth of saltwater bivalve molluscs in acute shell deposition tests), for which the data are not dichotomous, under specified conditions.

3.3 For definitions of other terms used in this guide, refer to Terminology E943 and Guide E1203. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 In each of two or more treatments, test organisms of one species are maintained for 2 to 8 days in one or more test chambers. In each of the one or more control treatments, the organisms are maintained in dilution water to which no test material has been added in order to provide (*1*) a measure of the acceptability of the test by giving an indication of the quality of the test organisms and the suitability of the dilution water, test conditions, handling procedures, and so forth, and (*2*) the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, the organisms are maintained in dilution water to which a selected concentration of test material has been added. Data concerning effects on the organisms in each test chamber are usually obtained periodically during the test and analyzed to determine LC50s, EC50s, or IC50s for various lengths of exposure.

4.2 Acute toxicity tests are generally used to determine the concentration of test material that produces a specific adverse effect on a specified percentage of test organisms during a short exposure, relative to the life-cycle of the test organism. Because death is an obviously important adverse effect and is easily detected for many species, the most common acute toxicity test is the acute lethality test. Experimentally, effect on 50 % of a group of test organisms is the most reproducible and easily determined measure of toxicity, and 96 h is often a convenient, useful exposure duration. Therefore, the measure of acute toxicity most often used with fishes, macroinvertebrates, and amphibians is the 96-h LC50. However, because immobilization is a severe effect and is not easy to distinguish from death for some species, the measure of acute toxicity most often used with daphnids and midge larvae is the 48-h EC50 based on death plus immobilization. The terms LC50 and EC50 are consistent with the widely used toxicological terms LD50 (median lethal dose) and ED50 (median effective dose), respectively. The terms LC50 and EC50 should be used whenever results are calculated based on the concentration of test material in dilution water, whereas the terms LD50 and ED50 should be used whenever results are calculated based on the quantity of test material that enters or is applied directly to test organisms. For toxic agents or tests for which neither concentration nor dose is appropriate, such as tests on temperature or with poorly water-soluble materials, the terms LL50 (median lethal level) and EL50 (median effective level) should be used, if the effect is dichotomous. For tests in which the effect is expressed as a percent inhibition compared to the control, for example, a percent inhibition in shell growth in acute 96-h shell deposition tests with saltwater bivalve molluscs (**2**), and not as the percentage of the individual organisms that were affected, the term IC50 should be used to denote the concentration that causes a 50 % inhibition compared to the control.

4.3 Acute toxicity tests in which test organisms are exposed to test solutions containing a test material can be conducted by at least four techniques:

4.3.1 In the static technique, test solutions and organisms are placed in chambers and kept there for the duration of the test.

4.3.2 The recirculation technique is like the static technique except that each test solution is continuously circulated through an apparatus designed to maintain water quality, and possibly remove degraded, but not undegraded, test material by such means as aeration, filtration, and sterilization and then returned to the test chamber.

4.3.3 The renewal technique is like the static technique except that test organisms are periodically exposed to fresh test solution of the same composition, usually once every 24 h or 48 h, either by transferring the organisms from one test chamber to another or by replacing nearly all the test solution.

4.3.4 In the flow-through technique, test solution flows through the test chamber on a once-through basis throughout the test.

4.3.4.1 Two procedures may be used. In the first a large volume of each test solution is prepared before the beginning of the test, and these solutions flow through the respective chambers. In the second and more common procedure, fresh test solutions are prepared every few minutes or hours just before they enter the respective test chambers. In both procedures a metering system controls the flow of test solution, and in the latter procedure the test solutions are prepared by the metering system. Both of the procedures may be used to conduct continuous-flow tests. Many tests conducted using the second procedure, however, are intermittent-flow tests because the metering system cycles and delivers test solution every few minutes or hours.

4.3.5 With any of these techniques a pump or stirrer can be used to create a current in the test chamber to accommodate particular species, but the current will often increase both aeration and volatilization.

4.4 In flow-through tests a “volume addition” is the introduction into the test chamber of a volume of test solution equal to the volume of solution in the chamber.

4.5 For the purposes of 8.4.1, the term“ organophosphorus pesticides” refers to chlorpyrifos, demeton, diazinon, disulfoton, fenitrothion, malathion, methyl parathion, and parathion; the term “organochlorine pesticides” refers to aldrin, chlordane, DDD, DDE, DDT, dieldrin, endosulfan, endrin, heptachlor, heptachlor epoxide, lindane, methoxychlor, mirex, and toxaphene; and the term “chlorinated phenoxy herbicides” refers to the free acids, salts, and esters of 2,4-D, dicamba, silvex, and 2,4,5-T. The term “organic chlorine” refers to chlorine that would be detected if, when samples are prepared for gas chromatographic analysis for polychlorinated biphenyls (PCBs) and the organochlorine pesticides listed above, a chloride detector is used instead of an electron capture detector to measure compounds that elute from just before lindane to just after mirex on the gas chromatograph being used. Organic chlorine does not refer only to chlorine associated with organochlorine pesticides and PCBs; it refers to all chlorine that elutes within the specified period.

5. Significance and Use

5.1 An acute toxicity test is conducted to obtain information concerning the immediate effects on test organisms of a short-term 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. Bioavailability of the test substance may also differ between real-world exposures and laboratory exposures due to site-specific water quality conditions (see Guides E1192, E1563, and E2455).

5.2 Results of acute toxicity tests might be used to predict acute effects likely to occur on aquatic organisms in field situations as a result of exposure under comparable conditions, except that (*1*) motile organisms might avoid exposure when possible, and (*2*) toxicity to benthic organisms might be dependent on sorption or settling of the test material onto the substrate.

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

5.4 Results of acute toxicity tests might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E1023) or when deriving water quality criteria for aquatic organisms **(3).**

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

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

6. Apparatus

6.1 *Facilities*—Although some small organisms can be held and acclimated in static or 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 gravity fed into holding and acclimation tanks and test chambers. For flow-through tests an elevated headbox is often desirable so that dilution water can be gravity fed into holding and acclimation tanks and into the metering system (see 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. Headboxes and holding, acclimation, culture, and dilution-water tanks should be equipped for temperature control and aeration (see 8.3.1). 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 **(4)**. 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. Organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress during holding, acclimation, culture, and testing. A timing device should be used to provide a 16 h light and 8-h dark photoperiod. A 15 min to 30 min transition period **(5)** 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 (see Guide E1733).

6.2 *Special Requirements*—Some organisms require special conditions during holding, acclimation, and testing. For example, burrowing mayfly nymphs should be provided a substrate suitable for burrowing **(6)** or artificial burrows **(7, 8)**; immature stream insects should be provided with a current **(7)** or mild aeration, or both **(8)**; and amphipods, midge larvae crabs, shrimp, and bottom-dwelling fish should be provided a silica sand substrate. Nylon or stainless steel mesh can also be used to provide a substrate to which amphipods can cling. Because cannibalism might occur among many species of decapod crustaceans or midges, the claws of crabs and crayfish should be banded, or the individuals should be physically isolated by such means as screened compartments or held individually in test chambers during testing.

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 non-fluorocarbon plastics should be used whenever possible to minimize dissolution, leaching, and sorption, except that stainless steel should not be used in tests on metals in salt water. 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 **(8)**. Cast iron pipe should

not be used with salt water and probably should not be used in a freshwater-supply system because colloidal iron will probably be added to the dilution water, and strainers will be needed to remove rust particles. A specially designed system is usually necessary to obtain salt water from a natural water source (**9**). 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 (see 8.1.1.1 and 8.1.1.2) 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 that does not contain the item, or (*2*) their use will not adversely affect survival, growth, or reproduction of a sensitive species.

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 (see 9.3 and 11.9.3.4) in a reproducible fashion. Various metering systems, using different combinations of syringes, “dipping birds”, siphons, pumps, saturators, solenoids, valves, and so forth, have been used successfully to control the concentrations of test material in, and the flow rates of, test solutions (**10**). Proportional diluters (**11**) use an intermittent flow design and various devices for metering the test material (**12**). Continuous-flow metering systems are also available, as are systems that prepare the different test solutions independently of each other (**10**).

6.4.2 The metering system should be calibrated before use, and verified after the test to confirm that the targeted flow rates were met, 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.

6.4.3 The flow rate through each test chamber should be at least five-volume additions per 24 h depending on test species.. 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 (see 11.4) or (*2*) there is rapid loss of test material due to microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization (see 11.9.3.4). In shell deposition tests with saltwater bivalve molluscs (**2**), the minimum necessary flow rate might also depend on the amount of food available in the dilution water (see 10.5.3). At any particular time during the test, the flow rates through any two test chambers should not differ by more than 10 %.

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, and so forth, 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. Also, chambers may need to be covered to prevent organisms from jumping out. 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 organochlorine and organophosphorus pesticides, 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, they 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 minimum acceptable dimensions of test chambers and the minimum depth of test solution depend on the size of the individual test organisms and the loading (see 11.4). The smallest horizontal dimension of the test chambers should be at least three times the largest horizontal dimension of the largest test organism. The depth of the test solution should be at least three times the height of the largest test organism. In addition, the test solution should be at least 150 mm deep for organisms over 0.5 g (wet weight). Use of excessively large volumes of solution in test chambers will probably unnecessarily increase the amount of dilution water and test material used, and, in flow-through tests, increase the average retention time.

6.5.4 For static and renewal tests, organisms weighing more than 0.5 g (wet weight) each are often exposed in 19 L (5 gal) wide-mouth soft-glass bottles (**13**) containing 15 L of solution or in 30 L of solution in 300 mm by 600 mm by 300 mm deep all-glass test chambers. Smaller organisms are often exposed in 500 mL to 1 L glass beakers containing 200 mL to 800 mL of solution. Daphnids, amphipods, midge larvae, juvenile freshwater mussels, and mayflies are often exposed in 100 mL beakers containing 50 mL of solution or 50 mL beakers containing 30 mL of solution.

6.5.5 For flow-through tests, chambers may be constructed by modifying glass bottles, glass canning jars, or beakers to provide screened overflow holes, standpipes, or V-shaped notches. Organisms weighing more than 0.5 g (wet weight) each are often exposed in 30 L of solution in 300 by 600 by 300-mm deep all-glass test chambers. Smaller organisms are

often exposed in 2 L to 4 L of solution. In tests with daphnids and other small species, the test chambers or metering system, or both, should be constructed so that the organisms are not stressed by turbulence (**14**).

6.5.6 Embryos are often exposed in glass cups with stainless steel or nylon screen bottoms or cups constructed by welding stainless steel screen or gluing nylon screen with clear silicone adhesive. The cups should be suspended in the test chambers in such a way as to ensure that the embryos are always submerged and that test solution regularly flows into and out of the cups without creating too much turbulence.

6.6 *Cleaning*—The metering system, test chambers, and equipment used to prepare and store dilution water, stock solutions, and test solutions must be cleaned after use and may need to 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, distilled, 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, distilled, or dilution water. Acid is often used to remove mineral deposits, and 200 mg of hypochlorite (ClO^-)/L is often 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 (**15**) and is difficult to remove from some construction materials. It is often removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, by autoclaving in distilled 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 (see 8.1.1.1 and 8.1.1.2) 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^-.) The metering system and test chambers should be rinsed with dilution water just before use.

6.7 *Acceptability*—The acceptability of new holding, acclimation, and testing facilities should be demonstrated with a sensitive species (see 8.1.1.1 and 8.1.1.2) before use.

7. Hazards

7.1 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 (**16**), recommended handling procedures (**17**), and biological, chemical, and physical properties of the test material should be studied before a test is begun. (See Appendixes X2, X3, and X4 of Guide E1023.) Special procedures might be necessary with radiolabeled test materials (**18**) and with test materials that are, or are suspected of being, carcinogenic (**19**).

7.2 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 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

7.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.5 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 in a fume hood.

7.6 Because dilution water and test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help prevent electrical shocks. Salt water is such a good conductor that protective devices are strongly recommended.

7.7 To protect hands from being cut by sharp edges of shells, cotton work gloves should be worn (over appropriate protective gloves (see 7.1) if necessary) when juvenile and adult bivalve molluscs are handled.

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

7.9 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).

7.10 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). Some regulatory authorities have developed requirements for the management of hazardous waste. As a handler of hazardous materials, it is your responsibility to know and comply with applicable regulations where you are operating (Test Method E1706).

7.11 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 nonlocalized 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 (*1*) be available in adequate supply, (*2*) be acceptable to the test organisms, (*3*) be of uniform quality, and (*4*) except as stated in 8.1.4, not unnecessarily affect results of the test.

8.1.1 The minimal requirement for an acceptable dilution water for acute 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, and reproduce satisfactorily in it.

8.1.1.1 *Fresh Water*—Because daphnids are more acutely 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 acute 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-.The "US Lab" strain of amphipod (*Hyalella azteca*) has an additional requirement for chloride concentrations higher than those present in many natural or reconstituted waters (20). Growth and reproduction of *H. azteca* also declines when chloride concentrations are below 15 mg/L (21). Importantly, the "US Lab" strain of *H. azteca* also showed greater sensitivity to the acute toxic effects of sodium sulfate and sodium nitrate at lower chloride concentrations of <10 mg/L. Special attention to this constituent is required when selecting culture and test water for *H. azteca* (Test Method E1706). Selenium is also recommended as a micronutrient for *C. dubia* culture and testing by adding sufficient sodium selenate (Na_2SeO_4) to provide 2 µg selenium per liter of final dilution water (22).

8.1.1.2 *Salt Water*—Because *Acartia tonsa* (copepod), mysids (less than 24 h post-release from the brood sac), and bivalve mollusc larvae are more acutely sensitive to many test materials than many other saltwater animal species, water in which they will survive for 48 h without showing signs of stress is probably acceptable for acute tests with most saltwater animal species. Water in which *Acartia tonsa* or mysids will survive, grow, and reproduce satisfactorily in a life-cycle test is probably an acceptable dilution water for tests with most saltwater 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. In fresh water, the range of hardness should be less than 10 % of the average. In salt water, the range of salinity should be less than 20 % of the average.

8.1.3 The dilution water should not unnecessarily affect the results of an acute test because of such things as sorption or complexation of test material. Except as in accordance with 8.1.4, it is desirable for the purpose of reducing interlaboratory variability that the concentrations of both total organic carbon (TOC) and particulate matter should be less than 20 mg/L for shell deposition tests with saltwater bivalve molluscs (see 10.5.2) and less than 5 mg/L for all other tests.

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 an acute 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 Reconstituted water used for tests with freshwater species is generally prepared by adding specified amounts of reagent-grade salts to deionized water (Tables 1 and 2). However, the buffers used in Table 2 might react chemically with some test materials. Because these waters might be deficient in some trace nutrients, addition of about 2 µg of selenium(IV) and 1 µg of crystalline vitamin B_{12}/L might be desirable (**23**), especially if daphnids are cultured in these waters. Examples of other reconstituted fresh waters that have been used successfully for toxicity testing in some laboratories are also available ((**24**), (**25**), Test Method E1706 Section 7.1.3.5).

8.2.1.2 If a reconstituted water is used for tests with saltwater species, the reconstituted water described in Table 3

TABLE 1 Quantities of Reagent Grade[5] Chemicals Added to Deionized Water to Prepare Reconstituted Fresh Waters (23) and the Resulting Water Qualities

	Reagent grade salts Required, mg/L				pH[A]	Hardness[B]	Alkalinity[B]
	$NaHCO_3$	$CaSO_4{\cdot}2H_2O$	$MgSO_4$	KCl			
Very soft	12	7.5	7.5	0.5	6.7–6.8	10–13	10–13
Soft	48	30	30	2.0	7.3–7.5	40–48	30–35
Moderately hard	96	60	60	4.0	7.4–7.8	80–100	57–64
Hard	192	120.0	120.0	8.0	7.8–8.0	160–180	110–120
Very hard	384	240.0	240.0	16.0	8.0–8.2	280–320	225–245

[A] Approximate equilibrium pH after aeration.
[B] Expressed as mg $CaCO_3$/L.

TABLE 2 Quantities of Reagent Grade[5] Chemicals to Be Added to Aerated Soft Reconstituted Fresh Water to Buffer pH (26)

NOTE 1—The solutions should not be aerated after addition of these chemicals.

pH[A]	Milliliters of Solution to Add to 15 L of Soft Water		
	1.0 *N* NaOH Solution	1.0 *M* KH_2PO_4 Solution[B]	0.5 *M* H_3BO_3 Solution[B]
6.0	1.3	80.0	...
6.5	5.0	30.0	...
7.0	19.0	30.0	...
7.5	...	...	...
8.0	19.0	20.0	...
8.5	6.5	...	40.0
9.0	8.8	...	30.0
9.5	11.0	..	20.0
10.0	16.0	...	18.0

[A] Approximate equilibrium pH with fish in water.
[B] Buffers containing ions such as phosphate and borate should not be used when conducting tests on metals unless it has been shown that the buffers do not affect the toxicity of the metal to the test species.

TABLE 3 Reconstituted Salt Water (27)

Add the following reagent grade[5] chemicals in the amounts and order listed to 890 mL of water. Each chemical must be dissolved before the next is added.[A]

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
Na_4EDTA[B]	1 mg
$NaHCO_3$	200 mg

[A] If the resulting solution is diluted to 1 L, the salinity should be 34 g/kg ± 0.5 g/kg and the pH should be 8.0 ± 0.2. The desired test salinity is attained by dilution at time of use.
[B] Tetrasodium ethylenediaminetetraacetate. This should be omitted when toxicity tests are conducted on metals. When tests are conducted with fish or bivalve mollusc larvae, zooplankton, or crustaceans, the EDTA should be omitted and the reconstituted salt water stripped of trace metals (28).

should be used whenever possible. If desired, a reconstituted water may be prepared using a commercially obtained "bioassay grade" sea salt. that does not contain EDTA or sodium thiosulfate. However, because quality may differ among commercial brands, tests to determine the acceptability of the water (8.1.1) may be necessary. The reconstituted water should be used at a salinity of 34 g/kg and pH = 8.0 for tests with true marine stenohaline species, and at a salinity of 17 g/kg and pH = 7.7 with euryhaline species. Other salinities may be used for studying the effects of water quality on results of toxicity tests. It might be difficult to provide saltwater bivalve molluscs with an adequate amount of acceptable food (see 10.5.3) if reconstituted water is used for shell deposition tests.

8.2.1.3 Reconstituted water is prepared by adding a sea salt or specified amounts of reagent grade[5] chemicals to highquality water with (*1*) conductivity less than 1 µS/cm and (*2*) 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, distillation, 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 a surface water, TOC or COD should be measured on each batch. The reconstituted water should be intensively aerated before use, except that the buffered soft fresh waters (Table 2) should be aerated before, but not after, addition of buffers. Problems have been encountered with some species in some fresh and salt 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 a 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 fresh or salt 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 For shell deposition tests with saltwater bivalve molluscs (**2**), it might be desirable to position the intake to maximize the amount of phytoplankton that will support growth and survival (see 10.5.3).

8.2.2.3 The hardness, salinity, pH, and so forth, of a water may be adjusted, if desired, by addition of appropriate reagent grade[5] chemicals, sea salt, acid, base, distilled or deionized water, and so forth, if desired. When necessary, sea salt may be added to salt water to prevent excessive decreases in salinity (see 8.1.2), if the salt has been shown to cause no adverse effects on the test species at the concentration used.

8.2.3 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 (**15**). 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 (**29**). Some organic chloramines, however, react slowly with sodium bisulfite (**30**). 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

[5] *Reagent Chemicals, American Chemical Society Specifications* , Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory U.K. Chemicals*, BDH Ltd., Poole, Dorset, and the *United States Pharmacopeia*.

usually be reduced with a chelating resin (28), but use of a different dilution water might be preferable. If dechlorinated water is used as dilution water or in its preparation, during the test either (*1*) it must be shown that a sensitive aquatic species (see 8.1.1.1 and 8.1.1.2) will survive, grow, and reproduce acceptably in it, or (*2*) it must be shown at least three times each week on nonconsecutive days that in fresh samples of dilution water either (*a*) *Acartia tonsa*, mysids (less than 24-h post-release from the brood sac), bivalve mollusc larvae, or 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, or (*b*) the concentration of residual chlorine in fresh water is less than 11 µg/L or the concentration of chlorine-produced oxidants in salt water is less than 7.5 µg/L (15).

8.3 *Treatment:*

8.3.1 Except as stated in 8.2.1.3, dilution water should be aerated intensively by such means as air stones, surface aerators, or column aerators (31, 32) 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 (33) to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation by dissolved gases, which might be caused by heating the dilution water, should be avoided to prevent gas bubble disease (31, 32, 34).

8.3.2 For shell deposition tests with bivalve molluscs, unfiltered, unsterilized natural salt water is often used in order to provide as much natural planktonic food as possible (see 10.5.3).

8.3.3 Except for shell deposition tests, filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low (see 8.1.3) and as a pretreatment before ultraviolet sterilization or filtration through a finer filter.

8.3.4 Except for shell deposition tests, dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (35) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 m or less. Water that might be contaminated with *Aphanomyces daphniae* should be autoclaved if it is to be used for culturing or testing daphnids (4).

8.3.5 Except for shell deposition tests (2), salt water from a surface water source should be passed through a filter effective to 15 µm or less to remove parasites and larval stages of predators.

8.4 *Characterization*—The following items should be measured at least twice each year, or more often (*1*) if such measurements have not been made semiannually for at least two years, or (*2*) if a surface water is used:

8.4.1 *All Waters*—pH, particulate matter, TOC, organophosphorus 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.

8.4.2 *Fresh Water*—Hardness, alkalinity, conductivity, sodium, and chloride.

8.4.3 *Salt Water*—Salinity or chlorinity.

8.4.4 For each analytical method used (see 12.2) the detection limit should be below either (*1*) the concentration in the dilution water or (*2*) the lowest concentration that has been shown to unacceptably affect the test species (36).

9. Test Material

9.1 *General*—The test material should be reagent grade[5] or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material,

9.1.2 Solubility and stability in the dilution water,

9.1.3 Measured or estimated acute toxicity to the test species,

9.1.4 Precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentrations are to be measured,

9.1.5 Estimate of toxicity to humans, and

9.1.6 Recommended handling procedures (see 7.1).

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 (or salinity) of the dilution water will not be affected, deionized or distilled water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials (37). 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. Additional guidance is available for difficult to test substances (38).

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 (**39**), low volatility, and high ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms and acetone is also very volatile (see 7.3). If an organic solvent is used, it should be reagent grade[5] or better and its concentration in any test solution must not exceed 0.5 mL/L. 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 no solvent other than water is used, (*1*) a dilution-water control must be included in the test, and (*2*) the percentage of organisms in the control that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 10 % or less.

9.2.5 If a solvent other than water is used and the concentration of solvent is the same in all test solutions that contain test material, (*1*) at least one solvent control, containing the same concentration of solvent and using solvent from the same batch used to make the stock solution, must be included in the test, and (*2*) a dilution-water control should be included in the test. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 10 % or less in the solvent control and should be 10 % or less in the dilution-water control, if one is included in the test.

9.2.6 If a solvent other than water is used and the concentration of solvent is not the same in all test solutions that contain test material, both a solvent control, containing the highest concentration of solvent present in any other treatment and using solvent from the same batch used to make the stock solution, and a dilution-water control must be included in the test. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 10 % or less in the solvent control and in the dilution-water control. When possible, it is desirable for each test concentration to have the same solvent concentration so that any effect from the solvent is consistent throughout the concentration-response.

9.2.7 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 the possible effects of the solvent on the results of the test.

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 (see 11.1.1.1) 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 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. The greater the similarity between the range-finding test and the definitive test, the more useful the range-finding test will be.

9.3.1.1 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 some (usually regulatory) situations, it is necessary only to determine (*1*) whether a specific concentration of test material is acutely toxic to the test species, or (*2*) whether the LC50, EC50, or IC50 is above or below a specific concentration. For example, the specific concentration occurring in a 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 (see 11.1.1.2), and it is not necessary to actually determine the LC50, EC50, or IC50.

10. Test Organisms

10.1 *Species*—If an objective of the test is to increase the comparability of results or increase information about a few commonly used species, or both, the test should be conducted with a species listed in Table 4. These species were selected on the basis of availability; commercial, recreational, and ecological importance; past successful use; and ease of handling in the laboratory. Their use is encouraged to increase the comparability of results and availability of much information about a few species rather than a little information about many species. If a desired species is unavailable, a species from a listed genus should be used. A special strain should be used only when that strain is of specific concern. The species used should be identified using an appropriate taxonomic key.

10.2 *Age*—All organisms in a test should be uniform in age and size.

10.2.1 *Fish*—Use of fish weighing between 0.1 and 5.0 g each is usually desirable. Unless data on another life stage are specifically desired, tests should be conducted with juvenile fish, that is, post-larval or older and actively feeding, but not sexually mature, spawning, or spent. Tests may be conducted with newly hatched fish, which are sometimes more sensitive than older stages, and embryos if appropriate precautions are taken. All fish in a test should be from the same cohort, and the standard (tip of snout to end of caudal peduncle), fork, or total length of the longest fish should be no more than twice that of the shortest fish.

10.2.2 *Invertebrates*—Except for shell deposition tests with bivalve molluscs and tests with copepods, immature organisms should be used whenever possible, because they are often more sensitive than older individuals of the same species. Among freshwater invertebrates, daphnids should be less than 24 h old; amphipods, mayflies, and stoneflies in an early instar; and

TABLE 4 Species and Test Temperatures

Species[A]	Test Temperature, °C[B]
Freshwater:	
Invertebrates:[B]	
Daphnids, *Daphnia magna, D. pulex, D. pulicaria,*	20[C]
Ceriodaphnia dubia	25
Amphipods, *Gammarus lacustris, G. fasciatus, Hyalella azteca*	17
G. pseudolimnaeus	17, 20, 23, 25
Crayfish, *Faxonius* sp., *Cambarus* sp.,	17, 22
Procambarus sp.,	17, 22
Pacifastacus leniusculus	17
Stoneflies, *Pteronarcys* sp.	12
Mayflies, *Baetis* sp., *Neocloeon triangulifer,Ephemerella* sp.	17
Hexagenia limbata, H. bilineata	22
Midges, *Chironomus* sp.	22
Snails, *Physella integra, P. heterostropha, Amnicola limosa, Aplexa hypnorum Lymnaea stagnalis*	22
Mussels, *Lampsilis siliquoidea, L. fasciola, Villosa iris*	20, 23
Planaria, *Dugesia tigrina*	22
Vertebrates:	
Frog, Lithobates sp.	22
Toad, Anaxyrus sp.	22
Coho salmon, *Oncorhynchus kisutch*	12
Rainbow trout, *Oncorhynchus mykiss*	12
Brook trout, *Salvelinus fontinalis*	12
Goldfish, *Carassius auratus*	17, 22
Fathead minnow, *Pimephales promelas*	25[C]
Channel catfish, *Ictalurus punctatus*	17, 22
Bluegill, *Lepomis macrochirus*	17, 22
Green sunfish, *Lepomis cyanellus*	17, 22
Saltwater:	
Invertebrates:[B]	
Copepods,	
Acartia clausi	12
Acartia tonsa	22
Shrimp, *Penaeus setiferus, P. duorarum, P. aztecus*	22
Grass shrimp, *Palaemonetes pugio, P. intermedius,*	22
P. vulgaris	22
Sand shrimp, *Crangon septemspinosa*	17
Shrimp, *Pandalus jordani, P. danae*	12
Bay Shrimp, *Crangon nigricauda*	17
Mysid, *Americamysis bahia, A. bigelowi, A. almyra*	27[C]
Blue crab, *Callinectes sapidus*	22
Shore crab, *Hemigrapsus* sp., *Pachygrapsus* sp.	12
Green crab, *Carcinus maenas*	22
Fiddler crab, *Uca* sp.	22
Oyster, *Crassostrea virginica, Magellana gigas*	22
Polychaete, *Capitella capitata*	22
Polychaete, *Neanthes arenaceodentata*	20
Vertebrates:	
Sheepshead minnow, *Cyprinodon variegatus*	22
Mummichog, *Fundulus heteroclitus*	22
Longnose killifish, *Fundulus similis*	22
Silverside, *Menidia* sp.	22
Threespine stickleback, *Gasterosteus aculeatus*	17
Pinfish, *Lagodon rhomboides*	22
Spot, *Leiostomus xanthurus*	22
Shiner perch, *Cymatogaster aggregata*	12
Tidepool sculpin, *Oligocottus maculosus*	12
Sanddab, *Citharichthys stigmaeus*	12
Flounder, *Paralichthys dentatus, P. lethostigma*	22
Starry flounder, *Platichthys stellatus*	12
English sole, *Parophrys vetulus*	12
Herring, *Clupea harengus*	12

[A] The species should be identified using an appropriate taxonomic key.
[B] Freshwater amphipods, daphnids, and midge larvae should be cultured and tested at test temperature. Some life stages of some aquatic invertebrates have rather narrow temperature requirements and so they should be held and tested within 5°C of the temperature of the water from which they were obtained. They should be tested at the listed test temperature if it is within this range; otherwise they should be tested at the temperature from the series 7, 12, 17, 22, 27, and 32°C that is closest to the listed test temperature and is within 5°C of the temperature of the water from which they were obtained.
[C] These species survive, grow, and reproduce acceptably at these temperatures and they are conveniently cultured and tested at these temperatures.

midges in the second or third instar. The term "daphnid" refers to all species in the family Daphnidae. Saltwater mysids should be less than 24 h post-release from the brood sac, but no more than 5 days post release. Since life-cycle tests with mysids must start with organisms less than 24 h old to maximize exposure prior to reproduction (Guide E1191), acute tests that may serve as preliminary tests to a chronic study should also use mysids less than 24 h old. The same is true if comparisons will be made with other studies starting with mysids of similar age or if required by a regulatory guideline. Since mysids less than 24 h old may not be more sensitive to all test materials, juveniles (less than 5 days old) may be used to start acute tests. Ovigerous decapod crustaceans and polychaetes with visible developing eggs in the coelom should not be used.

10.2.3 *Amphibians*—Young larvae should be used whenever possible.

10.3 *Source*—All organisms in a test should be from the same source, because organisms of the same species from different sources might have different acute sensitivities. Laboratory cultures of test species usually can provide organisms whose history, age, and quality are similar throughout the year. Freshwater amphipods, caddisflies, daphnids, burrowing mayflies, midge larvae, mosquito larvae, and saltwater polychaetes should be cultured in the testing facility (**40**). Commercial suppliers for commonly used toxicity testing organisms may also be available. Daphnids from cultures in which ephippia are being produced should not be used. Small fishes such as fathead and sheepshead minnows can also be raised in laboratory cultures. Usual sources of other freshwater fishes are commercial, and government hatcheries. Whenever salmon or trout are to be used, they should be obtained from a hatchery that has been certified disease-free, for example, free of infectious pancreatic necrosis, furunculosis, kidney disease, enteric redmouth, and whirling disease. Requirements for certification vary from state to state and from species to species. Other suggested species are usually obtained directly from wild populations in relatively unpolluted areas, although some amphibian species are also available from commercial suppliers. Importing and collecting permits might be required by local and state agencies. Organisms captured by electroshocking, chemical treatment, and gill nets should not be used.

10.4 *Care and Handling*—Organisms should be cared for and handled properly (**41**) so they are not unnecessarily stressed.

10.4.1 Whenever aquatic animals are brought into a facility, they should be quarantined (*1*) until used or (*2*) for 14 days or until they appear to be disease-free, whichever is longer. Dip nets, brushes, other equipment, organisms, or water should not be transferred from a quarantined tank to any other tank without being autoclaved in distilled water or sterilized.

10.4.2 To maintain aquatic animals in good condition and avoid unnecessary stress, they should not be crowded or subjected to rapid changes in temperature or water quality. In general, organisms should not be subjected to more than a 3 °C change in water temperature in any 12 h period and preferably not more than 3 °C in any 72 h period. The concentration of dissolved oxygen should be maintained between 60 % and