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

This standard is issued under the fixed designation E729; 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.

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

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 during 2 to 8-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. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual 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. 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 and may last longer than 96 h; 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 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

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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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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:

	Section
Referenced Documents	2
Terminology	3
Summary of Guide	4
Significance and Use	5
Apparatus	6
Facilities	6.1
Special Requirements	6.2
Construction Materials	6.3
Metering System	6.4
Test Chambers	6.5
Cleaning	6.6
Acceptability	6.7
Hazards	7
Dilution Water	8
Requirements	8.1
Source	8.2
Treatment	8.3
Characterization	8.4
Test Material	9
General	9.1
Stock Solution	9.2
Test Concentration(s)	9.3
Test Organisms	10
Species	10.1
Age	10.2
Source	10.3
Care and Handling	10.4
Feeding	10.5
Disease Treatment	10.6
Holding	10.7
Acclimation	10.8
Quality	10.9
Procedure	11
Experimental Design	11.1
Dissolved Oxygen	11.2
Temperature	11.3
Loading	11.4
Beginning the Test	11.5
Feeding	11.6
Duration of Test	11.7
Biological Data	11.8
Other Measurements	11.9
Analytical Methodology	12
Acceptability of Test	13
Calculation of Results	14
Report	15

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

E943 Terminology Relating to Biological Effects and Environmental Fate

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 Static Acute Toxicity Tests with Echinoid Embryos

E1604 Guide for Behavioral Testing in Aquatic Toxicology

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

3. Terminology

3.1 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. 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 growth, 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.

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

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

3.2.2 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.

3.2.3 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, either by transferring the organisms from one test chamber to another or by replacing nearly all the test solution.

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

3.2.4.1 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 continuously or every few minutes 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.

3.2.5 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.

3.3 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.

3.4 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.

3.5 *reconstituted water*—a dilution water that is prepared by adding sea salt or appropriate amounts of selected chemicals 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.

3.6 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."

3.7 *IC50*—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 growth or reproduction), for which the data are not dichotomous, under specified conditions.

3.8 For definitions of other terms used in this guide, refer to Terminology E943 and Guide E1023. For an explanation of units and symbols, refer to Standard 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.

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.

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 **(2).**

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 **(3)**. 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. A15 to 30-min transition period **(4)** 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.

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 **(5)** or artificial burrows **(6, 7)**; immature stream insects should be provided with a current **(6)** or mild aeration, or both **(7)**; and crabs, shrimp, and bottom-dwelling fish should be provided a silica sand substrate. Because cannibalism might occur among many species of decapod crustaceans, the claws of crabs and crayfish should be banded, or the individuals should be physically isolated by such means as screened compartments.

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, 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 **(7)**. 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 **(8)**. 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 **(9)**. Proportional diluters **(10)** use an intermittent flow design and various devices for metering the test material **(11)**. Continuous-flow metering systems are also available, as are systems that prepare the different test solutions independently of each other **(9)**.

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.

6.4.3 The flow rate through each test chamber should be at least five-volume additions per 24 h. 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, 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 filled to within 150 mm of the top sometimes 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) each, and at least 50 mm deep for smaller organisms. 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 **(12)** containing 15 L of solution or in 300 by 600 by 300-mm deep all-glass test chambers. Smaller organisms are often exposed in 500-mL to 1-L glass beakers containing 200 to 800 mL of solution. Daphnids and midge larvae are often exposed in 100-mL beakers containing 50 mL of solution.

6.5.5 For flow-through tests, chambers may be constructed by modifying glass bottles, battery 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 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 **(13)**.

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 should be cleaned before use. New items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid (HCl)), and rinsed at least twice with deionized, 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 **(14)** 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 **(15)**, recommended handling procedures **(16)**, 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 **(17)** and with test materials that are, or are suspected of being, carcinogenic **(18)**.

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.

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.

8.1.1.2 *Salt Water*—Because *Acartia tonsa*, 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 5 mg/L or 10 % of the average, whichever is higher. In salt water, the range of salinity should be less than 2 g/kg or 20 % of the average, whichever is higher.

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 If a reconstituted water is used for tests with freshwater species, the soft reconstituted water described in Table 1 should be used whenever possible, although some problems have been encountered with daphnids in this water. The other reconstituted fresh waters (Tables 1 and 2) may be used for studying the effects of water quality on results of toxicity tests. 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 **(19)**, especially if daphnids are cultured in these waters.

8.2.1.2 If a reconstituted water is used for tests with saltwater species, the reconstituted water described in Table 3 should be used whenever possible. If desired, a reconstituted water may be prepared using a commercially obtained sea salt. 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 µΩ/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:*

[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*.

TABLE 2 Quantities of Reagent Grade[5] Chemicals to Be Added to Aerated Soft Reconstituted Fresh Water to Buffer pH (20)

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

pH^A	Millilitres 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 (21)

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
$SrCl_2·6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2·2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2·6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3·9H_2O$	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 ± 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 **(22)**.

TABLE 1 Quantities of Reagent Grade[5] Chemicals Required to Prepare Reconstituted Fresh Waters (20) and the Resulting Water Qualities

Name	Salts Required, mg/L				pH^A	pH^B	Hardness^C	Alkalinity^C
	$NaHCO_3$	$CaSO_4·2H_2O$	$MgSO_4$	KCl				
Very soft	12	7.5	7.5	0.5	6.7–6.8	6.4–6.9	10–13	10–13
Soft	48	30.0	30.0	2.0	7.3–7.5	7.2–7.6	40–48	30–35
Hard	192	120.0	120.0	8.0	7.8–8.0	7.6–8.0	160–180	110–120
Very hard	384	240.0	240.0	16.0	8.0–8.2	8.0–8.4	280–320	225–245

^{A} Approximate equilibrium pH after aeration.
^{B} Approximate equilibrium pH after aeration and with fish in water.
^{C} Expressed as mg $CaCO_3$/L.

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 metre 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, 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 **(14)**. 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 **(23)**. Some organic chloramines, however, react slowly with sodium bisulfite **(24)**. 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 **(22)**, 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 **(14)**.

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 **(25, 26)** 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 **(27)** 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 **(25, 26, 28)**.

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 **(29)** 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 **(3)**.

8.3.5 Except for shell deposition tests, 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, and

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 **(30)**.

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 (**31**). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than use of the minimum necessary amount of a strong acid or base.

9.2.3 If a solvent other than dilution water is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect the test species. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals (**32**), 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.

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 might be the 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 year class, 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 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. 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 8-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 (**33**). 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, state, and federal hatcheries. Whenever salmon or trout are to be used, they should be

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,*	17
G. pseudolimnaeus	17
Crayfish, *Orconectes* sp., *Cambarus* sp.,	17, 22
Procambarus sp.,	17, 22
Pacifastacus leniusculus	17
Stoneflies, *Pteronarcys* sp.	12
Mayflies, *Baetis* sp., *Ephemerella* sp.	17
Hexagenia limbata, H. bilineata	22
Midges, *Chironomus* sp.	22
Snails, *Physa integra, P. heterostropha, Amnicola limosa, Aplexa hypnorum*	22
Planaria, *Dugesia tigrina*	22
Vertebrates:	
Frog, *Rana* sp.	22
Toad, *Bufo* 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, *Mysidopsis bahia, M. bigelowi, M. 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, C. gigas*	22
Polychaete, *Capitella capitata*	22
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.

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. 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 (**34**) 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 100 % of saturation (**27**) and continuous gentle aeration is usually desirable. Supersaturation by dissolved gases should be avoided to prevent gas bubble disease (**25, 26, 28**). Except when maintaining bivalve molluscs, water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (**29**) equipped with an intensity meter and flow controls, or passed through a filter effective to 0.45 µm or less. The concentration of unionized ammonia in holding and acclimation tanks should be less than 35 µg/L (**35**). See note Note 1.

NOTE 1—The value given is for 15°C at pH = 8.0 to 9.0. Other values might be more appropriate depending on the species, temperature, and pH.

10.4.3 Holding and acclimation tanks should be scraped or brushed as needed. Between use with different groups of test organisms, tanks should be sterilized by autoclaving or by treatment with an iodophor (**36**) or with 200 mg of hypochlorite/L for at least 1 h, brushed well once during the hour, and then rinsed well. Although iodophors are not very acutely toxic to aquatic animals, hypochlorite is (see 6.6 concerning preparation and removal of hypochlorite).

10.4.4 Organisms should be handled as little as possible. When handling is necessary, it should be done carefully, gently, and quickly so that organisms are not unnecessarily stressed. Organisms that are injured or dropped during handling and fish that touch dry surfaces should be discarded. Glass tubes with rubber bulbs and smooth ends are best for handling small organisms, whereas dip nets are best for handling organisms over 0.5 g each. Such nets are commercially available, or may be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting or similar knotless material. Nets coated with urethane resin are best for handling catfish. Equipment used to handle aquatic organisms should be sterilized between uses (see 10.4.3). Hands should be washed before handling or feeding test organisms.

10.4.5 Organisms should be carefully observed daily during quarantine, holding, and acclimation for signs of stress, physical damage, mortality, disease, and external parasites. Abnormal, dead, and injured individuals should be discarded. Open bivalve molluscs that do not close when touched with a probe should be discarded. Bivalve molluscs that never open or do not deposit feces or pseudofeces also should be discarded. If visual examination of the behavior and external appearance of test organisms indicates that they are not eating or are flashing, flipping, swimming erratically, emaciated, gasping at the surface, hyperventilating, hemorrhaging, producing excessive mucus, or showing abnormal color, the cause should be determined and eliminated. If organisms show signs of disease or external parasites, appropriate action should be taken (see 10.6).

10.5 *Feeding:*

10.5.1 At least once a day, organisms should be fed a food that will support normal functioning. Analysis of the food for the test material, if it might be present in the environment, is desirable.

10.5.2 Live brine shrimp nauplii (see Practice E1203) is a good food for many aquatic species.

10.5.3 Bivalve molluscs should be provided enough water containing enough phytoplankton to support survival and growth. If unsterilized and unfiltered natural salt water is used without adding algae, at least 1 L/h/individual is usually a minimum for molluscs for which the distance from the tip of the umbo to the distal valve edge is 40 to 60 mm. If the flow rate or the concentration of food, or both, is too low, a saltwater alga such as *Monochrysis lutheri* or *Isochrysis galbana* may be added to the dilution water.

10.6 *Disease Treatment*—Fish may be chemically treated to cure or prevent some diseases using appropriate treatments (see Table 5). If they are severely diseased, however, it is often better to destroy the entire lot immediately. Fish with other diseases and all other diseased animals should be discarded immediately, because (*1*) systemic bacterial infections usually cannot be treated effectively, (*2*) internal parasites cannot be removed without extensive treatment, (*3*) viral diseases cannot be treated, and (*4*) diseased invertebrates can rarely be treated effectively. Generally, organisms should not be treated during the first 16 h after arrival at the test facility because of possible stress or drug treatment during collection or transportation. However, immediate treatment is necessary in some situations, such as treatment of bluegills for columnaris disease during hot weather. Tests must not be begun with treated organisms for at least 10 days after treatment, and organisms must not be treated during the test.

10.7 *Holding*—Small organisms may be held in aerated, constant temperature static or renewal systems. Most species, however, should be held in uncontaminated, aerated water of constant temperature and quality in a flow-through system with a flow rate of at least two volume additions per day. For bivalve molluscs for which the distance from the tip of the umbo to the

TABLE 5 Prophylactic and Therapeutic Treatments for Freshwater Fish[A]

Disease	Chemical	Concentration, mg/L	Application
External bacteria	Benzalkonium chloride (Hyamine 1622™)	1–2 AI[B]	30–60 min[C]
	Nitrofurazone (water mix)	3–5 AI	30–60 min[C]
	Neomycin sulfate	25	30–60 min[C]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30–60 min[C]
Monogenetic trematodes, fungi, and external protozoa[D]	Potassium permanganate	2–6 15 000–30 000	30–60 min[C] 5–10 min dip[C]
	Sodium chloride	2000–4000	[E]
	Dexon™ (35 % AI)	20	30–60 min[C]
Parasitic copepods	Trichlorfon (Masoten™)	0.25 AI	[F]

[A] This table is merely an attempt to indicate the order of preference of treatments that have been found to be the most generally effective. Before a treatment is used, additional information should be obtained **(37)**. This list does not imply that these treatments have been cleared or registered for these uses. Appropriate state and federal regulatory agencies should be consulted to determine if the treatment in question may be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish might be altered by temperature or water quality. Treatments should be tested on small lots of fish before large scale applications. Prevention of disease is preferred.

[B] AI = active ingredient.

[C] Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or dosing **(38)** technique.

[D] One treatment is usually sufficient except for "Ich," which must be treated daily or every other day until no sign of the protozoan remains. This may take 4 to 5 weeks at 5 to 10°C and 11 to 13 days at 15 to 21°C. A temperature of 32°C is lethal to Ich in 1 week.

[E] Minimum of 24 h, but may be continued indefinitely.

[F] Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for longer than 4 weeks and should not be used above 27°C.

distal valve edge is 40 to 60 mm, the flow rate should be at least 1/L/h/organism. When possible, the organisms should be held in the dilution water and at the temperature at which they are to be tested. The temperatures given in Table 4 are generally good temperatures at which to hold the listed species. During long holding periods, however, it is generally easier and safer to hold fish at temperatures lower than those given in Table 4 because the metabolic rate and the number and severity of disease outbreaks are reduced.

10.8 *Acclimation*—Precautions should be taken to prevent test organisms from being stressed by an instantaneous change in water quality or temperature when they are placed in the test chambers.

10.8.1 Freshwater amphipods, caddisflies, daphnids, burrowing mayflies, and midge larvae and saltwater mysids should be reared in dilution water at the test temperature.

10.8.2 Several days before the beginning of tests with other species, an appropriate number of similar-sized individuals should be transferred from a holding tank to an acclimation tank. For flow-through tests the acclimation tank should have a flow rate of at least 1 L/h/organism for bivalve molluscs for which the distance from the tip of the umbo to the distal valve edge is 40 to 60 mm, and at least two volume additions per day for other species. The water in the acclimation tank should be gradually changed from 100 % holding water to 100 % dilution water over a period of 2 or more days. Similarly, the water temperature should be changed at a rate not to exceed 3°C within 72 h until the test temperature is reached. The test organisms must be maintained in the dilution water at the test temperature for at least the last 48 h before they are placed in the test chambers. Complete acclimation, which has not been adequately experimentally defined, might take considerably longer; therefore, organisms should be maintained in the dilution water at the test temperature for more than 48 h whenever possible.

10.8.3 Young amphibian larvae and fish that have been actively feeding for less than about 20 days, freshwater amphipods, daphnids, and midge larvae and saltwater mysids must be fed, and all other insects may be fed, up to the beginning of the test. All other amphibian larvae and fish over 0.5 g each must not be fed for 48 h, and all other invertebrates over 0.5 g each must not be fed for 24 h, before the beginning of the test. If adult amphipods or daphnids are isolated before the beginning of the test for the collection of young, the adults must be fed.

10.9 *Quality:*

10.9.1 Analysis of the test organisms for the test material, if it might be present in the environment, and other chemicals to which major exposure probably occurred is desirable.

10.9.2 A group of organisms should not be used for a test if more than about 5 % of the individuals show signs of disease or stress, such as discoloration, unusual behavior, or death, during the 48 h immediately preceding the test. If the percentage is greater than about 5 % all individuals should be either discarded or treated, held an additional 10 days, and reacclimated if necessary.

10.9.3 Reference toxicants might be useful for assessing the quality of test organisms. Characteristics that might be evaluated when selected a reference toxicant include water solubility, stability, mode of action, chemical similarity to the test substance, use hazards, and available toxicity data for the test (or related) species. Many chemicals have been used or evaluated as reference toxicants **(39)**, but none has been proven to be a reliable indicator of the overall quality of any species or test results. Antimycin has been found useful for detecting stressed freshwater fish in some situations **(40)**. A reference toxicant is more likely to be useful when used in conjunction with tests on materials that have the same mode of action as the reference toxicant. No one reference toxicant is likely to be able to detect all problems.

11. Procedure

11.1 *Experimental Design:*

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

11.1.1.1 An acute test intended to allow calculation of an LC50, EC50, or IC50 usually consists of one or more control

treatments and a geometric series of at least five concentrations of test material. In the dilution-water or solvent control(s), or both, (see 9.2.4 – 9.2.6), organisms are exposed to dilution water to which no test material has been added. Except for the control(s) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.6, five properly chosen concentrations will often provide LC50s, EC50s, or IC50s for several durations (see 11.8.3) and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of acute toxicity is particularly uncertain (see 9.3.1), six or seven concentrations might be desirable. If it is desirable to provide extensive information concerning the dependence of adverse effects on time or concentration, or both, seven or more appropriately spaced concentrations might be desirable to cover the range from effects on almost all organisms at quite short times to effects on few organisms at quite long times.

11.1.1.2 If it is only necessary to determine (*1*) whether a specific concentration is acutely toxic to the test species or (*2*) whether the LC50, EC50, or IC50 is above or below a specific concentration (see 9.3.2), only that concentration and the control(s) are necessary. Two additional concentrations at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

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

11.1.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned. Because test solution can flow from one compartment to another, but not from one test chamber to another (see 6.5.1), the test chamber is the experimental unit. As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases and, therefore, the width of the confidence interval on a point estimate decreases and the power of an hypothesis test increases. With respect to factors that might affect results within the test chambers and the results of the test, all chambers in the test should be treated as similarly as practical. For example, the temperature in all test chambers should be as similar as practical unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or a rectangle) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of test chambers and organisms per treatment should be calculated from (*1*) the expected variance within test chambers, (*2*) the expected variance between test chambers within a treatment, and (*3*) the maximum acceptable width of the confidence interval on the LC50, EC50, or IC50 (**41**). If such calculations are not made, at least ten organisms should be exposed to each treatment in static and renewal tests, and at least 20 organisms in flow-through tests. If each test concentration is more than 60 % of the next higher one, fewer organisms per concentration of test material, but not the control treatment(s), may be used. Organisms in each treatment should be divided between two or more test chambers in order to allow estimation of experimental variation (**42**). If the controls are important in the calculation of results, such as because of correction for spontaneous mortality using Abbott's formula or because the results are calculated as a percent reduction from the controls, it might be desirable to use more test chambers and test organisms for the control treatment(s) than for each of the other treatments.

11.1.4 The shape of the concentration-effect curve is critical for the determination of time-independent toxicity levels, and observations of dead and affected organisms should be with sufficient frequency to facilitate the estimation of a time-independent value, either directly or mathematically. Depending on the objectives of the test, a design should be selected that includes sufficient observations to determine the desired end point. If regulatory or cost factors are a consideration, observations may be made at 24, 48, and 96 h or as stipulated by the regulatory guideline. Depending on the shape of the toxicity curve, more observations will typically be desirable (for example, 3, 6, 12, and 24 h and twice daily thereafter) to provide a sound measurement of a time-independent toxicity value.

11.1.5 It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

11.2 *Dissolved Oxygen:*

11.2.1 For static tests the concentration of dissolved oxygen in each test chamber must be from 60 to 100 % of saturation (**27**) during the first 48 h of the test and must be between 40 and 100 % of saturation after 48 h. For renewal and flow-through tests the concentration of dissolved oxygen in each test chamber must be between 60 and 100 % of saturation at all times during the test.

11.2.2 Test solutions may be gently aerated during static and renewal tests if the concentration of test material in the aerated test chamber at the end of the test is not more than 20 % lower than that in a comparable unaerated test chamber. Test solutions may be gently aerated during flow-through tests if the concentrations of test material are measured according to 11.9.3.4. Turbulence, however, should be avoided because it might stress test organisms, resuspend fecal matter, and greatly increase volatilization. Because aeration readily occurs at the surface, efficient aeration can be achieved with minimum turbulence by using an air lift to transfer solution from the bottom to the surface. Aeration should be the same in all test chambers, including the control(s), throughout the test. If

aeration is used, it might be desirable to conduct a simultaneous test without aeration to determine if aeration affects the results of the test.

11.3 *Temperature:*

11.3.1 Except for some species noted in Table 4, the test temperature should be selected from the series 7, 12, 17, 22, 27, and 32°C. This series was selected because it provides temperatures that better suit more species and is more convenient for use by investigators than any other series. The temperatures listed in Table 4 should be used as the selected test temperatures for the listed species whenever possible. Other temperatures from the series may be used for studying the effect of temperature on the results of toxicity tests. Because some life stages of some aquatic invertebrates have rather narrow temperature requirements, the selected test temperature should be within about 5°C of the temperature of the water from which they were obtained. They should be exposed at the temperature listed in Table 4 if it is within this range; otherwise they should be exposed at the temperature from the series 7, 12, 17, 22, 27, and 32°C that is closest to the listed temperature and is within 5°C of the temperature of the water from which they were obtained.

11.3.2 For each individual test chamber in which temperature is measured, the time-weighted average measured temperature at the end of the test should be within 1°C of the selected test temperature. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Each individual measured temperature must be within 3°C of the mean of the time-weighted averages. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C.

11.4 *Loading:*

11.4.1 The grams of organism (total soft tissue of bivalve molluscs, whole body of other species; wet weight, blotted dry) per litre of solution in the test chambers should not be so high that it affects the results of the test. Therefore, the loading should be limited to ensure that (*1*) the concentrations of dissolved oxygen and test material do not fall below acceptable levels, (*2*) concentrations of metabolic products do not exceed acceptable levels, and (*3*) the test organisms are not stressed because of aggression or crowding. In the shell deposition test, for which the flow-through technique must be used, the maximum loading depends mostly on the flow rate and the amount of food in the dilution water (see 10.5.3).

11.4.2 In static and renewal tests with species listed in Table 4, the loading in each test chamber should not exceed 0.8 g of organism/L at or below the lower of (*1*) 17°C and (*2*) the temperature(s) listed for the species in Table 4, and should not exceed 0.5 g of organism/L at higher temperatures. If necessary, a lower loading should be used to keep the concentration of dissolved oxygen from (*1*) falling below 60 % of saturation during the first 48 h of a static test and below 40 % of saturation thereafter in any chamber containing live test organisms, and (*2*) falling below 60 % of saturation during a renewal test in any chamber containing live test organisms.

11.4.3 In flow-through tests with species listed in Table 4, the loading in each test chamber should not exceed 1 g of organism/L of solution passing through the chamber in 24 h and should not exceed 10 g of organism/L of solution in the chamber at any time at or below the lower of (*1*) 17°C and (*2*) the temperature(s) listed in Table 4. At higher temperatures, the loading should not exceed 0.5 g of organism/L/day or 5 g of organism/L. If supplementary saltwater algae are not added to the dilution water, for bivalve molluscs for which the distance from the tip of the umbo to the distal valve edge is 40 to 60 mm, the loading in the test chamber should not exceed one organism per litre of solution passing through the chamber in 1 h. For all species a lower loading or higher flow rate, or both, should be used, if necessary, to meet the following three criteria at all times during the test in each chamber containing live test organisms: (*1*) the concentration of dissolved oxygen should be at least 60 % of saturation (see also 11.2.2); (*2*) the concentration of un-ionized ammonia should not exceed 35 µg/L (**35**) (see Note 1); and (*3*) the concentration of test material should not be lowered more than 20 % because of uptake by the test organisms.

11.4.4 A lower loading should be used if aggression occurs.

11.4.5 Comparable loadings should be used for other species.

11.5 *Beginning the Test:*

11.5.1 The test begins when the test organisms are first placed in test solutions containing test material.

11.5.2 A representative sample of the test organisms must be either (*1*) impartially distributed among the test chambers by adding to each chamber no more than 20 % of the number of test organisms to be placed in each chamber and repeating the process until each chamber contains the desired number of test organisms, or (*2*) assigned by random assignment of one organism to each chamber, random assignment of a second organism to each chamber, or by total randomization. It might be convenient to assign organisms to other containers, and then add them to the test chambers all at once. Caution must be exercised to minimize the transfer of dilution water with the test organism to the chambers, particularly in higher test concentrations and for tests using small volumes.

11.5.3 Static and renewal tests should begin by placing test organisms in the chambers within 30 min after the test material was added to the dilution water. If the test material forms a film on the surface of the test solution, static and renewal tests may begin by placing test material in the test chambers 18 to 24 h after the test organisms were added. In the alternative procedure, the dilution water with organisms in it may be gently aerated in the chambers, but aeration must be stopped before addition of test material, except as in accordance with 11.2.2.

11.5.4 Flow-through tests should begin by either (*1*) placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of test material to have reached steady state, or (*2*) activating the metering device in the metering system several days after organisms were placed in test chambers that had dilution water flowing through them. This second alternative requires the addition of a "spike," that is, an aliquot of test material sufficient to establish the desired test concentration in the test chamber at the time of activation of the metering

device. The first alternative (*1*) allows the investigator to study the properties of the test material (see 11.9.3.4) and the operation of the metering system immediately prior to the test, whereas the second alternative (*2*) allows the organisms to partially adjust to the chambers before the beginning of the test.

11.6 *Feeding*—Organisms should not be fed during an acute toxicity test or for a time before the test, when possible (see 10.8.3), because fecal matter and uneaten food will decrease the dissolved oxygen concentration and the biological activity of some test materials. These problems are most severe with the static technique, but are sometimes important with the renewal and flow-through techniques. If cannibalistic organisms cannot be physically restrained or separated, however, minimal feeding is necessary. Because saltwater mysids less than 24-h post-release from the brood sac are severely stressed if not fed within 48 h, they should be fed before and during acute tests.

11.7 *Duration of Test:*

11.7.1 Whenever possible, the exposure duration should be sufficient to ensure that a time-independent toxicity level can be determined or estimated mathematically. In any case, daphnids and larvae of midges and phantom midges should be exposed to the test material for 48 h. All other species should be exposed for at least 96 h. When renewal or flow-through tests are conducted with organisms that will not be substantially affected by starvation for at least 8 days, the test should last for at least 8 days to determine whether additional organisms are affected or killed after 96 h.

11.7.2 At the end of the test it might be desirable to place the live test organisms in dilution water that does not contain any added test material for 2 to 8 days and feed them to determine whether delayed effects occur.

11.8 *Biological Data:*

11.8.1 Death is the adverse effect most often used for the calculation of results of acute toxicity tests with aquatic organisms. The criteria for death are usually lack of movement, especially the absence of respiratory movements in fish and shrimp, and lack of reaction to gentle prodding. If polychaetes break into two parts, the anterior portion should be checked for death. Because death of some invertebrates is not easily distinguished from immobilization, an EC50 is usually determined rather than an LC50. For daphnids and midge larvae, the EC50 should be based on death plus immobilization, defined as the lack of movement except for minor spontaneous, random activity of appendages. For crabs, crayfish, and shrimp, the EC50 should be based on death plus immobilization, defined as lack of movement and lack of response to gentle prodding. Because juvenile and adult bivalve molluscs can close their valves for extended periods of time, acute lethality tests should not be conducted with them. An EC50 based on reduction in shell deposition can be determined with bivalve molluscs, especially oysters, under conditions that promote rapid growth (**43**). An EC50 based on death plus incomplete shell development can be determined with bivalve mollusc larvae, but special procedures must be used (see Guide E724). In order to account for the total severe acute adverse impact of the test material on the test organisms, it is desirable to calculate an EC50 based on death plus immobilization, plus loss of equilibrium, defined as the inability to make coordinated movement and to maintain a normal upright position. Other effects, such as behavior (see Guide E1604), can be used to determine an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, excitability, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, cessation of burrowing by crabs and shrimp, and cannibalism should be reported.

11.8.2 Live test organisms should not be stressed in an attempt to determine whether they are dead, immobilized, or otherwise affected. Prodding of organisms and movement of test chambers during tests should be done very gently. Fish exposed to some organophosphorus compounds seem to be very sensitive to lights being turned on.

11.8.3 The number of dead and affected organisms in each test chamber should be counted every 24 h after the beginning of the test. If the dependence of adverse effects on time is to be defined, counts should be performed more often; a suggested schedule is to count the number of dead and affected organisms in each chamber, 3, 6, 12, and 24 h after the beginning of the test and twice a day thereafter to the end of the test. In some cases, more frequent observations may provide additional useful information. If test solutions are opaque, it might be necessary to insert a partition into the test chamber at the observation periods to move the test organisms to one end where they can be seen. If such a procedure is necessary, great care should be taken not to stress or damage live organisms or to cross-contaminate treatments. In some cases, for example, under conditions of extreme turbidity and in tests with burrowing organisms, the only way to obtain accurate counts before the end of the test is to terminate separate replicate test chambers whenever counts are desired, but such a procedure is usually not worth the effort.

11.8.4 If it can be done without stressing live organisms, dead organisms should be removed at least once every 24 h.

11.8.5 Except for very small organisms such as young daphnids and mysids, the weights of the test organisms should be determined by weighing and discarding either (*1*) a representative group of organisms before the test, or (*2*) the control organisms that are alive at the end of the test. For organisms such as adult daphnids and mysids, the dry weight (dried at 60°C for 72 to 96 h or to constant weight) should be measured, and for juvenile and adult bivalve molluscs the wet weight (blotted dry) of the total soft tissue should be measured. The wet weight (blotted dry) of other species should be measured. Except for such species as daphnids and mysids, length should be measured. The standard (see 10.2.1), fork, or total length of fish should be measured.

11.8.6 All organisms used in the test should be destroyed at the end of the test.

11.9 *Other Measurements:*

11.9.1 *Water Quality:*

11.9.1.1 *Static Tests*—If a freshwater dilution water is used, its hardness, alkalinity, conductivity, and pH should be

measured, and measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable. If a saltwater dilution water is used, its salinity and pH should be measured. In both waters, measurement of ammonia, particulate matter, total dissolved gas, and TOC (or COD in fresh water) is desirable. The dissolved oxygen concentration must be measured at the beginning and end of the test and at least every 48 h in between in the control and the high, medium, and low test concentrations as long as live organisms are present. The pH should be measured at the beginning and end of the test in the control and the high, medium, and low concentrations of test material.

11.9.1.2 *Flow-Through Tests*—Certain measurements should be performed at least once every 30 days or at the beginning of the test, if data are available to show that the quality of the dilution water is constant, and daily if such data are not available. If a freshwater dilution water is used, its hardness, alkalinity, conductivity, and pH should be measured, and measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable. If a saltwater dilution water is used, its salinity and pH should be measured. In both waters, measurement of ammonia, particulate matter, total dissolved gas, and TOC (or COD in fresh water) is desirable. The dissolved oxygen concentration must be measured at the beginning and end of the test and at least every 48 h in between in the control and the high, medium, and low test concentrations as long as live organisms are present. The pH should be measured at the beginning and end of the test in the control and the high, medium, and low concentrations of test material.

11.9.2 *Temperature:*

11.9.2.1 Throughout acclimation, either temperature should be measured or monitored at least hourly or the maximum and minimum temperatures should be measured daily.

11.9.2.2 In static and renewal tests, either (*1*) in at least one test chamber temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily, or (*2*) if the test chambers are in a water bath or a constant-temperature room or incubator, the temperature of the water or air must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured at least daily. In addition, temperature must be measured concurrently near both the beginning and end of the test in all test chambers or in various parts of the water bath, room, or incubator.

11.9.2.3 In flow-through tests, in at least one chamber either temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily. In addition, near both the beginning and end of the test, temperature must be measured concurrently in all test chambers.

11.9.3 *Test Material:*

11.9.3.1 If the test material is uniformly dispersed throughout the test chamber, water samples should be taken by pipetting or siphoning through glass or fluorocarbon plastic tubing from a point midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides. If test material might be lost due to sorption onto the walls of the sample container, the container and the siphon or pipette should be rinsed with test solution before collecting the sample. Water samples should be collected directly into appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber in static and renewal tests, the whole volume of solution in the test chamber should be (*1*) used as the sample or (*2*) treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to uniformly distribute the test material before a sample is taken. If the test material is not uniformly dispersed in the test chamber in flow-through tests, a large volume of the solution flowing into the test chambers should be collected and used as the sample or treated appropriately to uniformly distribute the test material in the sample before a subsample is taken.

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

11.9.3.3 In static and renewal tests, the concentration of test material should be measured, if possible, in at least the control and the high, medium, and low concentrations of test material at the beginning of the test (see 14.1). Measurement of degradation products might be desirable.

11.9.3.4 *Flow-Through Tests:* (*1*) The concentration of test material in the test chambers should be measured as often as practical during the test. The concentration of test material should be measured in (*1*) all chambers concurrently at least once during the test, preferably near the beginning of the test; (*2*) except for the control treatment, each test chamber (especially for those concentrations closest to the LC50, EC50, or IC50) at least one additional time during the test on a schedule designed to give reasonable confidence in the concentration of test material in the test chambers, taking into account the flow rate and the number of independent metering devices; and (*3*) at least one appropriate chamber whenever a malfunction is detected in any part of the metering system.

(*2*) In each treatment the highest measured concentration obtained during the test divided by the lowest should be less than 1.5. The variability of the sampling and analytical procedures should be determined before the beginning of the test to determine how many samples should be taken and analyses performed at each sampling point to ensure that the limit of 1.5 is not violated just because of sampling or analytical variability.

(*3*) If the measured concentration of test material in any chamber is more than 30 % higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the metering system, the cause should be identified. Measurement of the concentration of the material in the solution flowing into the test chamber will indicate whether the cause is in the metering system or in the test chamber. If the concentration in the test chamber is too high, the stock solution might have been prepared incorrectly or the metering system might not have been calibrated correctly. If the concentration is too low, additional possible causes are microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization. A faster flow rate might be desirable (see 6.4.3). If the test organisms are probably being exposed to substantial

concentrations of one or more impurities or degradation or reaction products, measurement of the impurities or products is desirable.

12. Analytical Methodology

12.1 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(44)** to minimize loss of test material by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

12.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources **(45)**. The concentration of un-ionized ammonia may be calculated from the pH, temperature, and concentration of total ammonia **(46)**.

12.3 Methods used to analyze food (see 10.5.1) or test organisms (see 10.9.1) should be obtained from appropriate sources **(47)**.

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

13. Acceptability of Test

13.1 An acute toxicity test should usually be considered unacceptable if one or more of the following occurred:

13.1.1 All test chambers (and compartments) were not identical.

13.1.2 Treatments were not randomly assigned to individual test chamber locations.

13.1.3 A required dilution-water or solvent control was not included in the test.

13.1.4 The test was begun with organisms within ten days after treatment for a disease or the organisms were treated during the test.

13.1.5 The test organisms were not maintained in the dilution water at the test temperature for at least the last 48 h before they were placed in test chambers.

13.1.6 Individual test organisms were not impartially or randomly assigned to test chambers or compartments.

13.1.7 More than 10 % of the organisms in any required control treatment showed signs of disease or stress, such as discoloration, unusual behavior, or death, during the test.

13.1.8 Dissolved oxygen and temperature were not measured as specified in 11.9.

13.1.9 Any measured dissolved oxygen concentration was not between 60 and 100 % of saturation during a renewal or flow-through test or during the first 48 h of a static test or was not between 40 and 100 % of saturation after 48 h in a static test.

13.1.10 The difference between the time-weighted average measured temperatures for any two test chambers, from the beginning to the end of the test, was greater than 1°C.

13.1.11 Any individual measured temperature in any test chamber was more than 3°C different from the mean of the time-weighted average measured temperatures for the individual test chambers. The test may be acceptable if the temperature was measured numerous times and a deviation of 3°C is inconsequential. However, if the temperature was measured only a minimal number of times, a deviation of more than 3°C might indicate that more deviations would have been detected if measurements had been taken more frequently.

13.1.12 At any one time, the difference between the measured temperatures in any two test chambers was more than 2°C.

13.1.13 The concentration of an organic solvent used in the preparation of a stock or test solution exceeded 0.5 mL/L.

13.1.14 A surfactant was used in the preparation of a stock or test solution.

13.2 Calculation of an LC50 or EC50 should usually be considered unacceptable if either or both of the following occurred:

13.2.1 No treatment other than a control treatment killed or affected less than 37 % of the test organisms exposed to it.

13.2.2 No treatment killed or affected more than 63 % of the organisms exposed to it.

14. Calculation of Results

14.1 For each set of data the LC50, EC50, or IC50 and its 95 % confidence limits should be calculated on the basis of (*1*) the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and (*2*) the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LCs, ECs, or ICs are calculated, their 95 % confidence limits should also be calculated.

14.2 Most acute toxicity tests produce quantal or dichotomous data, that is, counts of the number of organisms in two mutually exclusive categories, such as alive or dead. A variety of methods **(48)** can be used to calculate an LC50 or EC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or affected is between 0 and 100. The method used should appropriately take into account the number of test chambers per treatment and the number of test organisms per chamber. When fewer than two concentrations kill or affect between 0 and 100 %, the binomial test can usually be used to obtain statistically sound information about the LC50 or EC50. The binomial test does not provide a point estimate of the LC50 or EC50, but it does provide a range within which the LC50 or EC50 should lie. If desired, an interpolation procedure may be used to obtain an approximate LC50 or EC50.

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

$$\%I = 100(M - X)/M$$

where:

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

The %I for each test chamber should be plotted against the corresponding concentration of test material after transformation of %I or concentration, or both, if appropriate. The IC50 can then be obtained from a line of best fit by determining the concentration corresponding to %I = 50. If possible, the 95 % confidence limits on the IC50 should be calculated, appropriately taking into account the number of test chambers per treatment, the number of test organisms exposed in each chamber, the range of concentrations tested, and the variance within each treatment, especially in the controls. Alternatively, an appropriate linear or nonlinear inverse regression technique (**49**) can be used to calculate the IC50 and its 95 % confidence limits. If the percent inhibition covers an appropriate range, such as at least 37 to 63 %, a variety of regression models will usually give nearly the same IC50 from a set of data. However, only the correct model, which is not known to be available at this time, will appropriately take into account the variance between the test chambers in the control treatment(s) and give the correct confidence limits.

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

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

14.5 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material killed or affected a statistically significant number of the exposed organisms. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and tests of heterogeneity. Then a pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test.

15. Report

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

15.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of test.

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

15.1.3 Source of the dilution water, its chemical characteristics, a description of any pretreatment, and results of any demonstration of the acceptability of the water to an aquatic species.

15.1.4 Source of the test organisms, scientific name (and strain for salmonids, when appropriate), name of person who identified the organisms and the taxonomic key used, age, life stage, means and ranges of weights and lengths, observed diseases, treatments, holding and acclimation procedures, and food.

15.1.5 Description of the experimental design, test chambers (and compartments) and covers, the depth and volume of solution in the chambers, the method of beginning the test, numbers of test organisms and chambers (and compartments) per treatment, the loading and lighting, and for flow-through tests a description of the metering system and the flow rate as volume additions per 24 h.

15.1.6 The average and range of the measured concentration of dissolved oxygen (as % of saturation) for each treatment and a description of any aeration performed on test solutions before or during the test.

15.1.7 The averages and ranges of the acclimation and test temperatures and the method(s) of measuring or monitoring, or both.

15.1.8 Schedule for obtaining samples of test solutions and the methods used to obtain, prepare, and store the samples.

15.1.9 Methods used for, and results (with standard deviations or confidence limits) of, chemical analyses of water quality and concentration(s) of test material, impurities, and reaction and degradation products, including validation studies and reagent blanks.

15.1.10 Definition(s) of the effect(s) used for calculating LC50s, EC50s, or IC50s, and a summary of general observations on other effects.

15.1.11 A table of data in sufficient detail to allow independent statistical analyses of the number of test organisms exposed and killed or otherwise affected at various times throughout the test in each test chamber in each treatment, including the control(s).

15.1.12 For all species, the LC50s, EC50s, or IC50s and their 95 % confidence limits at each scheduled observation interval, and the method used to calculate them; the shape of the toxicity curve and a direct or mathematically estimated time-independent toxicity level (when applicable); and the highest concentration of test material that killed or affected no greater a percentage of the test organisms than did the control treatments. Specify whether results are based on measured or unmeasured concentrations of the test material. For formulations and commercial products, specify whether results are based on whole mixture or active ingredient.

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

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

16. Keywords

16.1 acute toxicity test; amphibians; copepods; EC50; fish; flow-through test; freshwater fishes; freshwater invertebrates; LC50; macroinvertebrates; mollusks; mysids; renewal test; saltwater fishes; saltwater invertebrates; static test; test design; test materials

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