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Standard Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids[1]

This standard is issued under the fixed designation E1191; 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.

ε[1] NOTE—Section 13.1.11 and References were editorially corrected in January 2023.

1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the adverse effects of a test material added to dilution water, but not to food, on certain species of saltwater mysids during continuous exposure from immediately after birth until after the beginning of reproduction using the flow-through technique. These procedures will probably be useful for conducting life-cycle toxicity tests with other species of mysids, 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 on new concepts and procedures for conducting life-cycle toxicity tests with saltwater mysids.

1.3 These procedures are applicable to all chemicals, either individually or in formulations, commercial products, or known mixtures, that can be measured accurately at the necessary concentrations in water. With appropriate modifications, these procedures can be used to conduct tests on temperature, dissolved oxygen, and pH and on such materials as aqueous effluents (see also Guide E1192), leachates, oils, particulate matter, sediments, and surface waters.

1.4 This guide is arranged as follows:

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

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

[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Actionand is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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2. Referenced Documents

2.1 *ASTM Standards:*[2]

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate (Withdrawn 2023)[3]

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

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)[3]

IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

3. Terminology

3.1 The words "must," "should,"" may," "can," and "might" have very specific meanings in this guide.

3.1.1 "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).

3.1.2 "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.

3.1.3 "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." Therefore, the classic distinction between may and can is preserved, and might is never used as a synonym for either may or can.

3.2 For definitions of other terms used in this guide, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 In each of two or more treatments, saltwater mysids of one species are maintained in two or more test chambers from immediately after birth until after the beginning of reproduction in a flow-through system. In each of the one or more control treatments, the mysids 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 mysids and the suitability of the dilution water, food, test conditions, and handling procedures and (*2*) the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, the mysids are maintained in dilution water to which a selected concentration of test material has been added. Specified data on the concentration of test material, and the survival, growth, and reproduction of the mysids are obtained and analyzed to determine the effect(s) of the test material on survival, growth, and reproduction of the test organisms.

5. Significance and Use

5.1 Protection of a species requires prevention of unacceptable effects on the number, weight, health, and uses of the individuals of that species. A life-cycle toxicity test is conducted to determine what changes in the numbers and weights of individuals of the test species result from effects of the test material on survival, growth, and reproduction. Information might also be obtained on effects of the material on the health and uses of the species.

5.2 Results of life-cycle tests with mysids might be used to predict long-term effects likely to occur on mysids in field situations as a result of exposure under comparable conditions.

5.3 Results of life-cycle tests with mysids might be used to compare the chronic sensitivities of different species and the chronic toxicities of different materials, and also to study the effects of various environmental factors on results of such tests.

5.4 Results of life-cycle tests with mysids 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 (**1**).[4]

5.5 Results of a life-cycle test with mysids might be useful for predicting the results of chronic tests on the same test material with the same species in another water or with another species in the same or a different water (**2**). Most such predictions take into account results of acute toxicity tests, and so the usefulness of the results from a life-cycle test with mysids is greatly increased by also reporting the results of an acute toxicity test (see Guide E729) conducted under the same conditions.

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

5.7 Results of life-cycle tests with mysids might be useful for predicting population effects on the same species in another water or with another species in the same or a different water (**3**).

6. Apparatus

6.1 *Facilities*—Flow-through or recirculating brood-stock tanks and flow-through, but not recirculating, test chambers should be maintained in constant-temperature areas or recirculating water baths. An elevated headbox might be desirable so dilution water can be gravity-fed into brood-stock tanks and the metering system (see 6.3), which mixes and delivers test

[2] 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.

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

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

solutions to the test chambers. Strainers and air traps should be included in the water supply system. Headboxes and brood-stock tanks should be equipped for temperature control and aeration (see 8.3). 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. 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, the brood-stock tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. During culture and testing, organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress. A timing device should be used to provide either a 14-h light and 10-h dark or a 16-h light and 8-h dark photoperiod. A 15 to 30-min transition period **(4)** should be provided whenever lights go on or off to reduce the possibility of mysids being stressed by instantaneous changes in light intensity. In the natural environment, the normal vertical migration of mysids allows gradual acclimation to light intensity. Under artificial laboratory conditions, some mysids exhibit an escape response to sudden increases or decreases in light intensity resulting in jumping and impingement on the sides of test chambers or compartments.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which mysids will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect mysids. 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, Teflon, and fluorocarbon plastics should be used whenever possible to minimize dissolution, leaching, and sorption. Stainless steel should not be used for tests on metals. Concrete and rigid plastics may be used for brood-stock tanks and in the water supply, but they should be soaked, preferably in flowing dilution water, for a week or more before use **(5)**. Cast iron pipe should not be used with salt water. Specially designed systems are usually necessary to obtain salt water from a natural water source (see Guide E729). 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 mentioned previously should not be used unless it has been shown that their use will not adversely affect either survival, growth, or reproduction of mysids (see 13.1.9 and 13.1.10).

6.3 *Metering System:*

6.3.1 The metering system should be designed to accommodate the type and concentration(s) of test material and the necessary flow rates of test solutions. The system should permit the mixing of the test material with dilution water immediately before entrance to the test chambers (see 11.9.3.4) and permit the supply of selected concentration(s) of test material in a reproducible fashion (see 9.3 and 11.1.1). Various metering systems, using different combinations of syringes, dipping birds, siphons, pumps, saturators, solenoids, and valves have been used successfully to control the concentrations of test material in, and the flow rates of, test solutions (see Guide E729).

6.3.2 The metering system should be calibrated before the test by determining the flow rate through each test chamber and 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 twice daily, in the morning and afternoon, throughout the test. The metering system should be adjusted during the test if necessary and any malfunction or adjustment should be noted in the study records.

6.3.3 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 to provide at least ten volume additions per 24 h in case there is rapid loss of test material due to microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization (see 11.4.2). At any particular time during the test, the flow rates through any two test chambers should not differ by more than 10 %. Flow rates through all test chambers may be equally changed simultaneously during the test as long as the test temperature (see 11.3) and the concentrations of dissolved oxygen and test material (see 11.4.1 and 11.9.3) remain acceptable (see 11.3, 11.9, and 13).

6.4 *Test Chambers:*

6.4.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. However, screens and cups may be used to create two or more compartments within each chamber. Therefore, test solution can flow from one compartment to another within a test chamber, but, by definition, cannot flow from one chamber to another. Because 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 to reduce evaporation of test solution and test material. All chambers and compartments in a test must be identical.

6.4.2 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 that are 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.

6.4.3 Mysids should be exposed in compartments that are placed within test chambers. Compartments that have been used successfully include (*1*) 140-mm inside diameter glass Petri dish bottoms with collars made of 210 or 250-µm mesh nylon screen **(6, 7)**, and (*2*) 110 by 180 by 200-mm deep glass rectangular chambers partitioned into compartments with a 65-mm high, 330-µm mesh nylon collar **(8)**. The compartments

may be removed to a light table (illuminated from the bottom, such as used for viewing slides) for observation, or the test chambers may be permanently located on a light table. To ensure that test solution regularly flows into and out of each compartment, either (*1*) test solution should flow directly into the compartments, (*2*) the compartments should be oscillated in the test solution by means of a rocker arm apparatus driven by a 1 to 6 r/min electric motor (**9**), or (*3*) the water level in the test chamber should be varied by means of a self-starting siphon (**10**). The metering system, test chambers, and compartments should be constructed so that the mysids remain submerged and are not unacceptably stressed by crowding or turbulence. Best survival and reproduction are obtained when the compartment provides a surface area of at least 30 cm^2 per mysid and a solution depth of at least 25 mm (**7**) at all times.

6.4.4 Use of excessively large volumes of solution in test chambers will probably unnecessarily increase the amount of dilution water and test material used, and the average retention time. All glass chambers that are 300 by 450 by 150-mm deep containing a minimum test solution depth of 100 mm and adequate compartments have been successfully used.

6.5 *Cleaning*—The metering system, test chambers, compartments, 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), and at least twice with deionized, distilled, or dilution 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 will 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. The metering system, test chambers, and compartments should be rinsed with dilution water just before use.

6.6 *Acceptability*—Before a life-cycle test is conducted in new test facilities, it is desirable to conduct a nontoxicant test, in which all test chambers contain dilution water with no added test material, to determine before the first test (*1*) whether mysids will survive, grow, and reproduce acceptably (see 13.1.9 and 13.1.10) in the new facilities, (*2*) whether the food, water, and handling procedures are acceptable, (*3*) whether there are any location effects on either survival, growth, or reproduction, and (*4*) the magnitudes of the within-chamber and between-chamber variances.

7. Hazards

7.1 Many materials can adversely affect humans if precautions are inadequate. Therefore, skin contact with all test materials and solutions should be minimized by wearing appropriate protective gloves (especially when washing equipment or putting hands into test solutions), laboratory coats, aprons, and glasses, and by using pipets or dip nets to remove mysids 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 (**11**), recommended handling procedures (**12**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled materials (**13**) and with test materials that are, or are suspected of being, carcinogenic (**14**).

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 Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks because salt water is a good conductor of electricity.

8. Dilution Water

8.1 *Requirements*—The dilution water should (*1*) be in adequate supply, (*2*) be acceptable to saltwater mysids, (*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 dilution water must allow satisfactory survival, growth, and reproduction of saltwater mysids (see 13.1.9 and 13.1.10).

8.1.2 The quality of the dilution water should be uniform during the test. During the test each measured salinity should be between 15 g/kg and 30 g/kg, and the difference between the highest and lowest measured salinities should be less than 5 g/kg and must be less than 10 g/kg. Each measured pH should be between 6.6 and 8.2.

8.1.3 The dilution water should not unnecessarily affect results of a life-cycle test with mysids because of such things as sorption or complexation of test material. Therefore, except as stated in 8.1.4, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L.

8.1.4 If it is desired to study the effect of an environmental factor such as Total Organic Carbon, (TOC), particulate matter, or dissolved oxygen on the results of a life-cycle test with mysids, it will be necessary to use water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such 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 Some reconstituted salt waters prepared from either reagent-grade chemicals or sea salts have been shown to be acceptable for life-cycle toxicity tests with saltwater mysids (**15**). It might be desirable to condition (age) reconstituted salt water by aerating it for two or more days.

8.2.2 If natural salt water is used, it should be obtained from an uncontaminated, uniform quality source. The quality of well water is usually more uniform than surface water. If surface water is used, the intake should be positioned (for example, suspended about 1 m below a float) to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron.

8.2.3 Chlorinated water should not be used as, or in the preparation of, dilution water because chlorine-produced oxidants are quite toxic to mysids. 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 (**16**). Some organic chloramines, however, react slowly with sodium bisulfite (**17**). In addition to residual chlorine, municipal drinking water often contains unacceptably high concentrations of copper, lead, zinc, and fluoride and the quality is often variable. When necessary, excessive concentrations of most metals can usually be removed by a chelating resin (**18**), but use of a different water might be preferable.

8.3 *Treatment:*

8.3.1 Dilution water should be aerated intensively by using air stones, surface aerators, or column aerators (**19**, **20**) before addition of test material. Adequate aeration will bring the pH and 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 (**21**) to help ensure that dissolved oxygen concentrations in the test chambers are acceptable. Supersaturation by dissolved gases, which can be caused by heating the dilution water, should be avoided to prevent gas bubble disease (**20**, **22**).

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

8.3.3 Dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (**23**) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm or less.

8.3.4 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 mysid predators.

8.3.5 When necessary, sea salt may be added to increase salinity (see 8.1.2), if the salt has been shown to cause no adverse effects on either survival, growth, or reproduction of saltwater mysids at the concentration used.

8.4 *Characterization:*

8.4.1 The following items should be measured at least twice each year and more often if such measurements have not been made semi-annually for at least two years or if surface water is used: salinity (or chlorinity), pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides and polychlorinated biphenyls, (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 The methods used (see 12.3) should either (*1*) be accurate and precise enough to adequately characterize the dilution water or (*2*) have detection limits below concentrations that have been shown to adversely affect saltwater mysids (**24**).

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 dilution water.

9.1.3 Acute toxicity to the test species.

9.1.4 A measurement or estimate of chronic toxicity to the test species.

9.1.5 Precision and bias of the analytical method at the planned concentration(s) of the test material.

9.1.6 Estimate of toxicity to humans.

9.1.7 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 in the metering system, but usually it is dissolved in a solvent to form a stock solution that is then added to the dilution water in the metering system. 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, might be necessary. If the 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

[5] *Reagent Chemicals, American Chemical Society Specifications* , American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

solutions of slightly soluble materials (**25**). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such acid or base 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 the use of the minimum necessary amounts of strong acids and bases.

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 either survival, growth, or reproduction of saltwater mysids. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals (**26**), low volatility, and high ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, dimethylformamide (DMF) (**27**), and acetone may also be used, but they might stimulate undesirable growths of microorganisms, and acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[5] or better and its concentration in any test solution should not exceed 0.1 mL/L. A surfactant should 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 of a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution.)

9.2.4 If a solvent other than water is used (*1*) at least one solvent control, 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. If no solvent other than water is used, a dilution-water control must be included in the test.

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

9.2.4.2 If the concentration of solvent is not the same in all test solutions that contain test material, either (*1*) a solvent test must be conducted to determine whether either survival, growth, or reproduction of the test species is related to the concentration of solvent over the range used in the toxicity test or (*2*) such a solvent test must have already been conducted using the same dilution water and test species. If either survival, growth, or reproduction is found to be related to the concentration of solvent, a life-cycle test with that species in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival, growth, nor reproduction is found to be related to the concentration of solvent, a life-cycle toxicity test with that same species in that same water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

9.2.4.3 If the test contains both a dilution-water control and a solvent control, the survival, growth, and reproduction of the mysids in the two controls should be compared (see Appendix X1.4). If a statistically significant difference in either survival, growth, or reproduction is detected between the two controls, only the solvent control may be used for meeting the requirements of 13.1.9 and 13.1.10 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of 13.1.9 and 13.1.10 and as the basis for calculation of results.

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

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to provide a good estimate of the highest concentration of test material that will unacceptably affect neither survival, growth, nor reproduction of the test species, the test concentrations (see 11.1.1.1) should bracket the best prediction of that concentration. Such a prediction is usually based on the results of a flow-through acute toxicity test (see Guide E729) on the test material using the same dilution water and mysids of the same age as at the start of the life-cycle test (less than 24-h post release from the brood sac). If an acute to chronic ratio has been determined for the test material with a species of comparable sensitivity, the result of the acute test with the test species can be divided by the acute to chronic ratio. Except for a few materials (**28**), acute to chronic ratios determined with saltwater mysids are often less than five. Therefore, if no other useful information is available, the highest concentration of test material in a life-cycle test with mysids is often selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test.

9.3.2 In some (usually regulatory) situations, it is only necessary to determine whether one specific concentration of test material reduces survival, growth, or reproduction. For example, the specific concentration might be the concentration occurring in surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When there is only interest in a specific concentration, it is often only necessary to test that concentration (see 11.1.1.2).

10. Test Organisms

10.1 *Species*—The test species is usually selected on the basis of geographical distribution, availability, ease of handling in the laboratory, and past successful use. Both *Mysidopsis bahia* (**6, 7, 29, 30, 31**) and *Mysidopsis bigelowi* (**6**) have been successfully cultured and tested using these procedures. Other species of mysids, such as *Mysidopsis almyra* (**32**), might also be used satisfactorily. The species used should be identified using an appropriate taxonomic key (**33**).

NOTE 1—Mysids are often incorrectly referred to as shrimp.

NOTE 2—*Mysidopsis bahia* has been redescribed as *Americamysis bahia*, *Mysidopsis bigelowi* has been redescribed as *Americamysis bigelowi*, and *Mysidopsis almyra* has been redescribed as *Americamysis almyra* by Price et al., 1999 (**33**).

10.2 *Age*—Life-cycle tests with saltwater mysids must be started with individuals less than 24-h post release from the

brood sac. Use of the youngest possible mysids of a consistent age is recommended to ensure that data on delays in first brood release are accurate.

10.3 *Source*—All mysids used in a test must be from the same brood stock. The mysids used to start a test must have been obtained from adults either (*1*) hatched and raised in the laboratory or (*2*) brought into the laboratory before sexual maturity and held for at least 14 days using the same food, water, temperature, and salinity as will be used in the life-cycle test. The first method is preferable because it will not only acclimate the mysids, but will also demonstrate the acceptability of the food, water, and handling procedures before the test is begun.

10.4 *Brood Stock:*

10.4.1 Brood stock may be obtained from another laboratory, a commercial source, or a wild population from an unpolluted area. When brood stock is brought into the laboratory, it should be placed in a tank along with the water in which it was transported. The temperature should then be changed at a rate not to exceed 3 °C within 12 h and the salinity should be changed at a rate not to exceed 3 g/kg within 12 h.

10.4.2 Mysids have been cultured in reconstituted salt water and filtered natural salt water in recirculating and flow-through systems (**6**, **7**, **28**, **29**, **30**, **31**, **33**). Cultures have been maintained for several generations in 76-L (20-gal) glass aquaria containing natural salt water (filtered through a 15-µm filter) at a flow rate of 100 mL/min. If outflows are at the top of the tanks, no screen is needed to retain mysids and food washout is minimal. Under-gravel filters, with a 1-in. deep dolomite substrate prewashed in deionized or distilled water, provide gentle aeration and a current conducive to feeding.

10.4.3 The brood stock should be cared for properly so it is not unnecessarily stressed. To maintain mysids in good condition and avoid unnecessary stress, they should not be subjected to rapid changes in temperature, photoperiod, or water quality. Mysids should not be subjected to more than a 3 °C change in temperature or a 3 g/kg change in salinity in any 12 h period. The concentration of dissolved oxygen should be maintained between 60 % and 100 % of saturation (**21**) and continuous gentle aeration is usually desirable. A 15 min to 30 min transition period (**4**) should be provided when lights go on or off.

10.4.4 Reproduction might be depressed when culture density is above 20 mysids/L (**7**). Therefore, when cultures are not being used for supplying test organisms, enough adults should be removed at least every 2 weeks to stimulate reproduction. Mysid generator systems (**32**) may be used to provide constant cropping and to obtain age-standardized subsamples for tests or new cultures. Brood-stock tanks should be kept free of other animals, such as hydroids and worms, by scraping the sides and siphoning the bottoms every one or two weeks. Salinity and temperature should be appropriate for the particular species and consistent with the specified test conditions (see 8.1.2 and 11.3).

10.5 *Food*—At least once daily, saltwater mysids in brood-stock tanks and in test chambers should be fed live brine shrimp nauplii (see Practice E1203) in excess, in order to (*1*) maintain live nauplii in the chambers at all times to prevent cannibalism of the young and (*2*) support adequate survival, growth, and reproduction. The ration should be adjusted in accordance with mysid density. A ration of 150 brine shrimp nauplii per mysid per day has been used successfully (**6**). Mysid growth and reproduction might be improved by feeding twice a day (75 brine shrimp per mysid per feeding) rather than once a day. A batch of brine shrimp nauplii should not be fed to mysids in a culture or test until it has been shown that the batch will support survival, growth, and reproduction of the species for at least three generations. It might be desirable to supplement brine shrimp nauplii with an alga (for example, *Skeletonema costatum*) a rotifer (for example, *Brachionus plicatilus* or SELCO, a commercial nutrient enrichment product) (**34**). The food(s) used should be analyzed for the test material, if it might be present in the environment.

10.6 *Handling*—Mysids should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that the mysids are not unnecessarily stressed. Dip nets are best for removing gravid female mysids from brood-stock tanks. Such nets are commercially available or can be made from 350 µm mesh nylon netting, silk bolting cloth, plankton netting, or similar knotless material. Mysids that touch dry surfaces or are dropped or injured should be discarded. Equipment used to handle mysids should be sterilized between uses by autoclaving or by treatment with an iodophor (**35**) or with 200 mg of ClO^-/L for at least 1 h. Although iodophors are not acutely toxic to mysids, hypochlorite is.

10.7 *Harvesting Young*—Test organisms may be obtained by using mysid generators (**32**) or by transferring gravid females from brood-stock tanks to separate chambers and allowing an overnight period for brood release (**29**). The number of females needed varies with the size, age, food, temperature, and salinity.

10.8 *Quality*—Mysids less than 24 h post-release from the brood sac should be acceptable for starting a life-cycle test if they were obtained from a brood stock in which more than half of the adult females were producing young. Representative mysids from the brood stock should be analyzed for the test material, if it might be present in the environment.

11. Procedure

11.1 *Experimental Design:*

11.1.1 Decisions concerning aspects of experimental design, such as the dilution factor, number of treatments, and numbers of test chambers, compartments, and pairs of mysids 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 A life-cycle test intended to allow calculation of an endpoint (see X1.2) 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 9.2.3) mysids are exposed to dilution water to which no test material has been added. Except for the control(s) and