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

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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 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:*[2]

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

[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] 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.

E943 Terminology Relating to Biological Effects and Environmental Fate
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 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

[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.

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 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 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,

[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.

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. A15 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 the highest concentration, each concentration should be at least 50 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5, five properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of chronic toxicity is particularly nebulous (see 9.3.1), six or seven concentrations might be desirable.

11.1.1.2 If it is necessary only to determine whether a specific concentration reduces survival, growth, or reproduction (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.

11.1.2 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 (**36**). Because test solution can flow from one compartment to another, but not from one test chamber to another (see 6.4.1), 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 a significance test increases. With respect to factors that might affect results within test chambers and, therefore, the results of the test, all chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible 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. 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 effect of the test material on survival, growth, and reproduction cannot be determined accurately if any factor that affects one or more of them is too dissimilar between experimental units. Because survival, growth, and reproduction might be affected by the number of first- and second-generation mysids in the chamber or compartment, and the concentration or amount of available food, the best experimental design is to install randomly selected mysids into chambers or compartments, remove the young daily, and regularly supply food to each chamber or compartment equally to excess. It is common to place several compartments in each test chamber and place one male and female pair within each compartment. Although increasing the number of test chambers per treatment and increasing the number of compartment per chamber both improve the experimental design, statistically the best design is to increase the number of test chambers per treatment (**7**).

11.1.4 Selection of the required number of replicates in a life-cycle toxicity test with saltwater mysids is an important prerequisite in the development of an appropriate experimental design. Of particular concern in this test is the number of replicates required to detect effects of substances on reproduction. A test lacking the statistical power to detect reproductive impairment is unacceptable. Selection of the proper number of replicates requires (*1*) an estimate of the expected coefficient of variation of reproductive success for tests conducted at your laboratory or a default value; (*2*) a selection of the percentage reduction in the number of young/female/reproductive day, relative to controls, that you wish to be able to detect; and (*3*) a selection of the frequency that you are willing to have tests fail because they lack the desired power.

11.1.4.1 An example of how this is accomplished uses a laboratory-specific coefficient of variation (CV) of 0.60 (**7**). The appropriate number of replicates can be selected using Table 1 (or Fig. 1) after choosing the magnitude of the percentage reduction relative to controls you wish to detect, and the proportion of tests you wish to have this detection potential. For example, with a CV of 0.60, if you wish the test to statistically detect an 80 % reduction in the number of young 80 % of the time, eight replicates/treatment are required. Alternatively, the goal might be to design tests that have the power to detect reproductive impairments that population models indicate would compromise population maintenance. In this case, application of the model described by Kuhn et al. (**3**) to databases from 15 life-cycle tests (conducted at the U.S. EPA Atlantic Ecology Division (AED) laboratory at Narragansett, RI), indicate that reproductive impairments of from 23 to 82 % (mean = 58 %) would likely result in population declines. Recognizing the caveats associated with predicting the responses of natural populations using laboratory data, this level of reproductive impairment may be used with Table 1 to bound your selection of an appropriate number of replicates.

TABLE 1 Sample Size Needed to Achieve Selected Power and Percent Reduction of the Control Mean[A]

Reduction of Control Mean, %	Power						
	0.5	0.6	0.7	0.8	0.9	0.95	0.99
50	9	12	15	19	26	32	47
60	7	8	11	14	18	23	33
70	5	6	8	10	14	17	24
80	4	5	6	8	11	13	19
90	4	4	5	7	9	11	15
95	3	4	5	6	8	10	14
99	3	4	5	6	7	9	13

[A] CV = 60 %, $\alpha = 0.05$ (one-sided).

11.1.4.2 The equation for calculating your laboratory-specific replicate requirements (**37**) is as follows:

$$n \geq \frac{(2C)(CV^2)}{f^2} + 0.25Z_{\alpha}^2 \quad (1)$$

where:

n = number of replicates,
CV = coefficient of variation = standard deviation of replicates ÷ control mean × 100 %,
f = percent reduction from control mean you wish to detect,
C = constant that is a function of P (Type I) and P (Type II) acceptable error rate, and
Z_{α} = 1.645 for $\alpha = 0.05$ one-tailed test.

11.1.4.3 Because mysid life cycle tests must be powerful enough to detect reproductive impairment, a 99 % reduction in reproduction can be used to define a minimally acceptable test. With this as a goal, 3, 6, 7, or 9 replicates would be required if you wish 50, 80, 90, or 95 % of the tests to be expected to be able to statistically detect this magnitude of difference (Table 1). After completion of testing, the minimal significant difference (MSD) should be calculated to determine if the test is acceptable. An acceptable test is one that has the statistical sensitivity to detect a 99 % reduction in reproduction relative to controls. The MSD for a test is as follows:

$$MSD = t_{critical} S(2/n)^{1/2} \quad (2)$$

where:

S^2 = pooled estimate of variance from test run,
n = number of replicates per treatment, and
$t_{critical}$ = is the 95th percentile t-value (one-tailed).

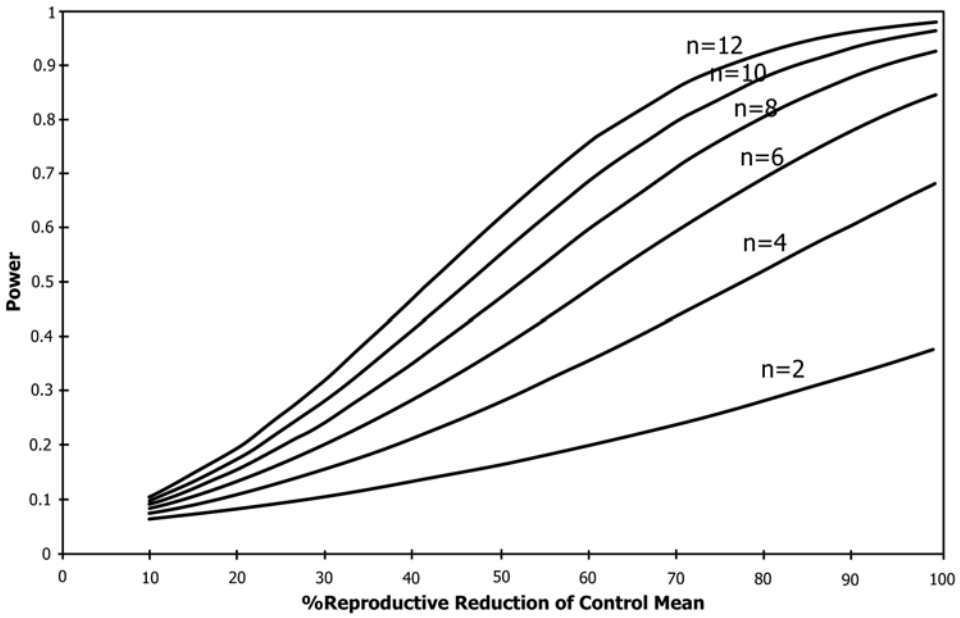

FIG. 1 Relationship Between Power of Test and Percent Control Mean for Various Samples Sizes (CV = 60 %, α = 0.05 (one-sided))

A TEST is acceptable if (MSD ÷ $\bar{x}$ control) × 100 % <99 %. This equation can also be used by an investigator who wishes to demonstrate that a specific experiment had the power to detect a percentage difference other than 99 %.

11.2 *Dissolved Oxygen*—The concentration of dissolved oxygen in each test chamber should be from 60 to 100 % of saturation (**21**) at all times during the test and the time-weighed average measured concentration in each test chamber at the end of the test must be between 60 and 100 % of saturation. Because results are based on measured rather than calculated concentrations of test material, some loss of test material by aeration is not necessarily detrimental and test solutions may be aerated gently. Turbulence, however, should be avoided because it might stress mysids, 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.

11.3 *Temperature:*

11.3.1 Tests with *M. bahia* should be conducted at 27°C. The test temperature selected for another species should be representative of its natural reproductive period. Other temperatures may be used to study the effect of temperature on saltwater mysids or to study the effect of temperature on the chronic toxicity of a material to mysids.

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 with 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 *Beginning the Test:*

11.4.1 After test solutions have been flowing through the chambers long enough so that the concentration(s) of test material have probably reached steady state, two sets of water samples should be taken at least 24 h apart. The analyses should verify that the concentration(s) of test material have reached steady state before organisms are placed in test chambers.

11.4.2 The measured concentration of test material in each treatment should be no more than 30 % higher or lower than its nominal concentration. If the difference is more than 30 %, the cause should be identified. Measurement of the test material concentration 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, and a faster flow rate is probably desirable (see 6.3.3). Measurement of degradation and reaction products is also desirable (see 11.9.3.2).

11.4.3 The test begins when mysids, less than 24-h post release from the brood sac, are first placed in the test solution.

11.4.4 The mysids should be transferred using a wide bore (larger than the largest mysid) glass pipet with a smooth tip and handled gently to avoid injuries. Bubbles should be avoided in the pipet because they might impinge mysids on the side of the pipet. A representative sample of mysids must be either (*1*) impartially distributed among the compartments by adding to each compartment no more than 20 % of the number of mysids

to be placed in each compartment and repeating the process until each compartment contains the desired number of mysids or (*2*) assigned either by random assignment of one mysid to each compartment, etc., random assignment of a second mysid to each compartment, or by total randomization. It might be convenient to assign the mysids to compartments in dilution water and then randomly assign the compartments to the test chambers.

11.5 *Feeding*—At least once daily, the mysids should be fed live brine shrimp nauplii to excess and a supplement if desired (see 10.5). The food ration for each compartment should be proportional to the number of mysids in order to ensure comparable growth data.

11.6 *Cleaning:*

11.6.1 Dead brine shrimp and other debris in the test chambers and compartments should be removed daily before new nauplii are added to the compartments.

11.6.2 Clogging of screens on compartments in some treatments might be greater than in others as a result of the test material or a solvent on microbial or algal growth. Screens on all compartments should be brushed daily on the outside only to remove extraneous material. If excessive fouling occurs, it might be desirable to replace compartments periodically. If some compartments are replaced, all compartments in the test should be replaced concurrently.

11.7 *Duration of Test*—A complete life-cycle test would end when the last first-generation mysid (mysid used to start the test) died. However, growth rate is greatest before sexual maturity and the first brood is the greatest contribution of a female to population growth **(38)**. Therefore, shorter tests will probably provide adequate data on the effect of a test material on the survival, growth, and reproduction of saltwater mysids. A test with *Mysidopsis bahia* at 27°C must not be terminated before seven days past the median time of first brood release in the control treatment(s) to allow for delays in first brood release by mysids exposed to the test material. The test should be extended, however, if previously unaffected mysids in any treatment are adversely affected near the intended end of the test.

11.8 *Biological Data:*

11.8.1 The criteria for death of mysids are opaque white coloration, immobility (especially absence of movement of respiratory and feeding appendages), and lack of reaction to gentle prodding. Dead first-generation mysids must be counted, recorded, sexed (if mature), and removed daily. In each test chamber, live animals must be counted at the beginning of the test, throughout the test, and daily to account for cannibalism or death resulting from impingement on the sides of test compartments. Missing or impinged animals should be recorded. The number of live females should be recorded daily for determination of available female reproductive days (AFRD).

11.8.2 The live young in each compartment must be counted, recorded, and removed daily and the day of brood release recorded.

11.8.3 The dry weight (dried at 60°C for 72 to 96 h or to constant weight) of each individual first-generation mysid alive at the end of the test must be determined to the nearest microgram. Males and females must be weighed and recorded separately to observe possible sex-specific effects of the test material **(39)**. Before drying, mysids should be rinsed with deionized water to remove salt, and separated by sex. Any brine shrimp nauplii caught in female brood sacs should be removed using a fine glass or stainless steel needle. Wet weight is not acceptable due to large variations resulting from retention of water in female brood sacs. It might be desirable to dry and individually weigh, immediately after death, each first-generation mysid that dies before the end of the test.

11.8.4 Measurement of total body length (total midline body length from the anterior tip of the carapace to the posterior margin of the endopod of the uropod, excluding setae) **(39)** is desirable for mysids alive at the end of the test, but is not possible for preserved mysids due to the resultant body curvature. During or at the end of the test, total body lengths of mysids can be photographically recorded through a stereo microscope with appropriate scaling information **(40)**.

11.8.5 Both first- and second-generation mysids should be carefully observed regularly during the test for abnormal development and aberrant behavior, such as inability to maintain position in the water column, uncoordinated swimming, and cessation of feeding. Although developmental and behavioral effects are often difficult to quantify and might not provide suitable endpoints, they might be useful for interpreting effects on survival and growth and for deciding whether the test should be extended beyond the minimum duration **(41)** (see 11.8).

11.8.6 Morphological examination of first-generation mysids alive at the end of the test in each treatment, before they are dried, might be desirable. Biological and histological examination and measurement of the test material in exposed mysids will probably not be possible unless additional mysids are exposed specifically for such purposes.

11.8.7 It might be desirable to obtain data on the effect of the test material on survival, development, and behavior of some second-generation mysids for four or more days.

11.8.8 All mysids used in a test should be destroyed at the end of the test.

11.9 *Other Measurements:*

11.9.1 *Water Quality*—Salinity or chlorinity should be measured daily during the test. The pH should be measured at the beginning and end of the test and at least weekly during the test in the control treatment(s). The pH should also be measured in the highest test concentration at least once to determine whether it is affected by the test material. The concentration of dissolved oxygen must be measured in at least one test chamber in each treatment containing live test organisms (*1*) at the beginning and end of the test and at least weekly during the test, (*2*) whenever there is an interruption of the flow of test solution, and (*3*) whenever the behavior of the mysids indicates that the dissolved oxygen concentration might be too low. If a measured dissolved oxygen concentration is less than 60 % of saturation, corrective action should be taken and measurements must be performed at least daily until 60 % is reached. Weekly determinations of particulate matter, TOC, and total dissolved gas are desirable.

11.9.2 *Temperature*—Throughout the test in at least one test chamber, either temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily. In addition, near the beginning, middle, and end of the test, temperature must be measured concurrently in all test chambers.

11.9.3 *Test Material:*

11.9.3.1 The concentration of test material in each treatment must be measured frequently enough to establish its average and variability. If the test material is an undefined mixture, such as a leachate or complex effluent, direct measurement is probably not possible or practical. Concentrations of such test materials will probably have to be monitored by such indirect means as turbidity or by measurement of one or more components.

11.9.3.2 The concentration of test material must be measured at least weekly in each treatment, including the control(s), in which live test organisms are present. If a malfunction occurs in the metering system that could alter the concentration of test material, water samples must be taken immediately from affected test chambers and analyzed as soon as possible before and after the malfunction is corrected. Affected test chambers should be tested after the malfunction is corrected but after sufficient time to allow for new test solutions to run through the system, approximately one full turn-over. If the mysids are being exposed to substantial concentrations of one or more impurities, degradation, or reaction products (see 11.4.2), measurement of the impurities and products is desirable.

11.9.3.3 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 the test material might be lost due to sorption onto the walls of the sample container, the container and siphon or pipet 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 throughout the test chamber, a large volume of the solution flowing into the test chamber 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. Analysis of additional samples after filtration or centrifugation is desirable to determine the percentage of test material that is not dissolved or is associated with particulate matter, especially if the concentration of particulate matter in the test solution is greater than 5 mg/L.

11.9.3.4 In each treatment the measured concentration of test material must not be less than 50 % of the time-weighted average measured concentration for more than 10 % of the duration of the test. In addition, the measured concentration must not be more than 30 % higher than the average concentration for more than 5 % of the duration of the test. The variability of both 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 these requirements are not violated just because of sampling or analytical variability.

12. Analytical Methology

12.1 The methods used to analyze water samples for test material might determine the usefulness of the test results because all results are based on measured concentrations. For example, if the analytical method measures any impurities, reaction, or degradation products along with the parent test material, results can be calculated only for the whole group of materials, and not for the parent material itself, unless it is demonstrated that such impurities and products are not present.

12.2 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(42)** to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

12.3 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 **(43)**.

12.4 Methods used to analyze food (see 10.5) and mysids (see 10.8) should be obtained from appropriate sources **(44)**.

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

13. Acceptability of Test

13.1 A life-cycle test with saltwater mysids should usually be considered unacceptable if one or more of the following occurred, except that if, for example, temperature was measured numerous times, a deviation of more than 3°C (see 13.1.15) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature had been measured more often.

13.1.1 All test chambers and compartments were not identical.

13.1.2 Treatments were not randomly assigned to test chamber locations.

13.1.3 A required dilution-water control or solvent control was not included in the test or, if the concentration of solvent was not the same in all treatments that contained test material, the concentration of solvent in the range used affected survival, growth, or reproduction of the test species.

13.1.4 The test was started with mysids from more than one brood stock or from a brood stock that had been obtained after sexual maturity or had been maintained for less than 14 days in the laboratory using the same food, water, temperature, and salinity as used in the test.

13.1.5 The test was started with mysids older than 24-h post release from the brood sac.

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

13.1.7 The test was terminated before the minimum duration specified in 11.8.

13.1.8 Data on survival, growth, and reproduction were not obtained as specified in 11.9.

13.1.9 More than 30 % of the first-generation control mysids died between the start and the end of the test.

13.1.10 More than 25 % of the first-generation females in the control(s) failed to produce young or the average number of young produced by the first-generation females in the control(s) was less than three.

13.1.11 Salinity, dissolved oxygen, temperature, and concentration of test material were not measured as specified in 11.10.

13.1.12 The highest and lowest measured test salinities differed by more than 10 g/kg.

13.1.13 The time-weighted average measured dissolved oxygen concentration at the end of the test for any test chamber was not from 60 to 100 % of saturation.

13.1.14 The difference between the time-weighted average measured temperatures for any two test chambers was more than 1°C.

13.1.15 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.

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

13.1.17 The measured concentration of test material in any treatment was less than 50 % of the time-weighted average measured concentration for more than 10 % of the duration of the test.

13.1.18 The measured concentration of test material in any treatment was more than 30 % higher than the time-weighted average measured concentration for more than 5 % of the duration of the test.

13.2 An assessment should be made of the significance of concentrations of test material in the water, in the control treatment(s), in the food (see 10.5), and in the brood stock (see 10.8).

14. Calculation

14.1 The primary data to be analyzed from a life-cycle test with saltwater mysids are those on (*1*) survival of first-generation mysids, (*2*) the number of live young produced by each first generation female, (*3*) the dry weight of each first-generation mysid alive at the end of the test, and (*4*) the concentration of test material in the test solutions in each treatment.

14.2 The variety of procedures that can be used to calculate the results of life-cycle tests can be divided into two categories: (*1*) those that test hypotheses and (*2*) those that provide point estimates. No procedure should be used without careful consideration of (*1*) the advantages and disadvantages of various alternative procedures and (*2*) appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of the results should be appropriate to the experimental design (see 11.1). The major alternative procedures and points to be considered when selecting and using procedures for calculating results of life-cycle tests with saltwater mysids are discussed in Appendix X1.

14.3 *Survival*—First-generation mysids are counted from the beginning of the test until the end of the test. Missing or impinged animals are counted as dead.

14.4 *Number of Young Released per Reproductive Day*—For each female, the young released per reproductive day can be calculated by dividing the cumulative number of young released by the number of reproductive days. For each female, the number of reproductive days is the number of days that the female was alive from the day of first brood release of any female in the test to the end of the test (**7**). The average young per female reproductive day provides an index that corrects for varying numbers of reproductive days per treatment and female mortality.

14.5 It may be desirable to analyze data for day of first brood release of each female.

15. Documentation

15.1 The record of the results of an acceptable life-cycle test with saltwater mysids 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, and a description of any pretreatment.

15.1.4 Source of the brood stock, scientific name, name of person who identified the organisms and the taxonomic key used, acclimation and culture procedures used, observed diseases, and age of mysids at the beginning of the test.

15.1.5 Description of the experimental design, test chambers, compartments, and covers, the depth and volume of solution in the chambers, number of mysids, test chambers, and compartments per treatment, lighting, and a description of the metering system and the flow rate as volume additions per 24 h.

15.1.6 Source of the brine shrimp used, concentrations of test material and other contaminants in the brine shrimp, feeding method, frequency, and ration.

15.1.7 Range and time-weighted average measured concentration of dissolved oxygen (as percent of saturation) for each treatment and a description of any aeration performed on test solutions before or during the test.

15.1.8 Range and time-weighted average measured test salinity, temperature, and the method(s) of measuring or monitoring, or both.

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

15.1.10 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.

15.1.11 A table of data on survival, growth, and reproduction of mysids in each test chamber and compartment in each treatment, including the control(s), in sufficient detail to allow independent statistical analysis.

15.1.12 Methods used for, and results of, statistical analyses of data.

15.1.13 Summary of general observations on other effects.

15.1.14 Results of all associated acute toxicity tests.

15.1.15 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 flow-through test; life cycle; mysids; toxicity test

APPENDIX

(Nonmandatory Information)

X1. STATISTICAL GUIDANCE

X1.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies in data. The data display and statistical techniques most commonly used to achieve these goals are (*1*) preliminary and diagnostic graphical displays, (*2*) pairwise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (*3*) analysis of variance (ANOVA) and corresponding contingency table tests, (*4*) multiple comparison techniques for simultaneous pairwise comparison of other treatment groups with control groups, (*5*) concentration-effect curve analyses, and (*6*) multiple regression. If used correctly, each of these techniques can provide useful information about the results of an acceptable life-cycle toxicity test with mysids.

X1.1.1 The three kinds of data that can be obtained from toxicity tests are dichotomous or categorical (for example, mortality), count or enumeration (for example, number of young), and continuous (for example, weight). Statistical methods for analyzing dichotomous and other categorical data are directly analogous to those for analyzing count and continuous data. However, for technical reasons and because they arose from different application areas, different terminologies and computing tools were developed for analyzing the three kinds of data. The corresponding procedures are considered together herein.

X1.2 *Endpoint*—The endpoint of life-cycle toxicity tests with mysids generally has been defined in terms of whether differences from control mysids are statistically significant at the 5 % level. One of the main conceptual problems with this definition is that the notions of biological importance and statistical significance are logically distinct. Effects of considerable biological importance might not be statistically significant if sample sizes are small or if effects are extremely variable, or both. Conversely, biologically trivial effects might be highly statistically significant if sample sizes are large or if effects are very reproducible. An endpoint based solely on statistical significance might depend as much or more on sample sizes as on the magnitudes of the effects.

X1.2.1 An alternative is to define the endpoint in terms of a specified absolute or relative amount of difference in a biological attribute from the control treatment(s). A regression-type model would be fitted to the data and the concentration associated with a specified amount of difference from the control treatment(s) would be estimated using the model. For example, the concentration resulting in a specified percent decrease in survival or weight might be estimated along with confidence limits on the estimated concentration. The result of a life-cycle test would then be reported as a point estimate, preferably with confidence limits, of the concentration expected to cause an amount of effect that had been preselected as being biologically unacceptable.

X1.2.2 In general, an endpoint defined in terms of a statistically significant difference is calculated using analysis of variance, contingency tables, or other hypothesis testing procedures. An endpoint defined in terms of a specified amount of effect is calculated using regression analysis, concentration-effect curve analysis, or other point-estimation procedures. Regardless of the procedure used, sufficient data should be presented in reports to permit calculation of endpoints other than those chosen by the authors and to allow other uses of the data, such as modeling.

X1.3 *Preliminary Data Analysis:*

X1.3.1 *Graphical Displays*—These should be performed every time data for any biological attribute are analyzed using either regression analysis or hypothesis testing (**45**). Preliminary scatter plots are desirable because they might provide insights into the structure of the data and reveal the presence of unanticipated relations or anomalies. Every time a regression-type model is fitted to data, a graph of predicted and observed values should be examined to assess the goodness of fit of the model. A graph of the residuals from the fit should be examined to assess departures from the model. Histograms are useful for

examining the distribution of the data before hypothesis testing. Statistical computing packages, for example, Minitab, SAS, BMDP, and SPSS (46), have made preparation of graphs both easy and inexpensive. Feder and Collins (47) illustrate the use of various types of preliminary and diagnostic graphical displays in analysis of data from chronic toxicity tests.

X1.3.2 *Outlier Detection Procedures*—Data that do not appear to be in conformance with the substantial majority are often referred to as outliers. Outliers might be due to random variation or to clerical or experimental errors. Statistical outlier detection procedures are screening procedures that indicate whether a datum is extreme enough to be considered not due only to random variation. Barnett and Lewis (48) describe many outlier detection procedures and Feder and Collins (47) illustrate use of several outlier detection procedures with aquatic toxicological data. If outliers can be shown to be due to clerical or experimental error, they should be either corrected or deleted from the data prior to analysis. If outliers are not known to be erroneous values, the question of how to deal with them is a matter of judgment. Data analysis should usually be performed with and without questionable values in order to assess their importance, because one or a few extreme outliers can sometimes greatly affect the outcome of any analysis.

X1.3.3 *Data Transformations*—Many standard statistical procedures such as regression analysis and ANOVA are based on the assumption that experimental variability is homogeneous across treatments. This assumption typically does not hold for certain kinds of data. If data displays or tests of heterogeneity demonstrate that variability is not homogeneous across treatments, variance stabilizing transformations of the data might be necessary. The arc sine, square root, and logarithmic transformations are often used on dichotomous, count, and continuous data, respectively (49). The question of whether to transform raw data should be decided on a case-by-case basis after studying data displays, tests of heterogeneity, and similar data from previous tests. In reality, ANOVA and regression are not very sensitive to departures from normality and small deviations from this assumption are not prohibitive.

X1.4 *Comparison of Solvent Control and Dilution-Water Control*—If both solvent and dilution-water controls are included in the test, they should be compared using a *t*-test for count and continuous data and Fisher's Exact Test or a 2 by 2 contingency table test for categorical data (50). Adjustments for chamber-to-chamber heterogeneity might be necessary. The use of a large alpha level (for example, 0.25) will make it more difficult to accept the null hypothesis when it should not be accepted. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

X1.5 *Analysis of Variance and Contingency Table Analyses*—ANOVA tests are often appropriate for untransformed continuous data and for transformed count and categorical data. Contingency table tests are usually appropriate for untransformed categorical data. If evidence of chamber-to-chamber heterogeneity is found, standard contingency table analyses might be inappropriate for categorical data. In this case it might be appropriate to apply an arc sine variance stabilizing transformation to the proportion dead within each experimental unit and perform an ANOVA on the transformed proportions. Feder and Collins (47) illustrate transformation of data before use of a contingency table test.

X1.5.1 Both contingency table tests and ANOVA F-tests are overall tests that do not assume any particular form for the relation between effects and concentrations. Therefore they are not designed to be particularly sensitive to the one-sided, monotone trends characteristically observed in toxicity tests. Specialized tests have been designed to be more sensitive to relations of this type. Some of these tests are the one-sided measure of association tests, the Cochran-Armitage test for categorical data, and tests based on linear or polynomial regression models for continuous data (51).

X1.5.2 ANOVA tests are based on normal distribution theory and assume (*1*) that the data within treatments are a random sample from an approximate normal distribution and (*2*) that the error variance is constant between treatments. As a part of the ANOVA, residuals should be plotted against concentration to determine whether there are any obvious violations of the assumptions of normality and homoscedasticity, that is, constant error variance. When results of an ANOVA are reported, the ANOVA model and table, the F-statistic and its significance level, the minimum detectable difference, and the power of the test should be presented.

X1.6 *Multiple Comparison Procedures*—The usual approach to analyzing data from life-cycle tests is to compare data for each concentration of the test material to data for the control(s). In Fisher's Protected Test, which should be used only if the ANOVA F-test is significant (52), each concentration of test material is compared to the control(s) using the *t*-test. If the investigator desires to set an experiment-wise α, rather than a comparison-wise α, Dunnett's procedure (52, 53) can be used without the ANOVA *F* -test. Williams's procedure (52, 54) also tests the control(s) versus each concentration, but makes the addition assumption that the true mean follows a monotonic relation with increasing concentration. The latter procedure is more powerful if the assumption is correct. Alternatively, Tukey's (55) No Statistical Significance of Trend (NOSTASE) test can be used with the same assumptions as Williams' procedure. Shirley (56) has developed a nonparametric equivalent for Williams' test and Williams (57) has modified and corrected Shirley procedure to increase its power. Care must be taken when using any of these procedures that an appropriate estimate of variability is used, incorporating any chamber-to-chamber variation that is present. Presentation of results of each comparison should include the test statistic, its significance level, the minimum detectable difference, and the power of the test.

X1.7 *Regression Analysis and Concentration-Effect Curve Estimation* —An alternative to tests for statistically significant differences is to fit concentration-effect models or multiple regression models to the data and estimate the concentration that corresponds to a specified amount of difference from the control treatment(s) (58). Concentration-effect curve models, such as probit and logit, are commonly used to describe trends

in dichotomous data on survival. Linear and quadratic polynomial regression models are commonly used to describe trends in quantitative data on growth and reproduction. Toxicity tests should be designed to avoid the need for extrapolation, because it can introduce biases into estimates.

X1.7.1 Point estimates, such as the EC10, EC25, and EC50, are examples of endpoints calculated using regression analysis. Whenever a point estimate is calculated, its 95 % confidence interval should also be calculated. Finney (**59**) discusses the probit model in considerable detail, and Draper and Smith (**60**) and Neter, Wasserman, and Kutner (**61**) discuss most practical aspects of multiple regression analysis. Feder and Collins (**47**) discuss use of these techniques in aquatic toxicology.

X1.7.2 When a regression model or concentration-effect curve model is fitted, data for each experimental unit are plotted against concentration. If necessary, transformation of the effect data or concentration data, or both should be performed to stabilize the variance across treatments and to produce a smooth trend. For example, if effects or concentrations cover a range of one or more orders of magnitude, a logarithmic transformation of either concentration or effect or both might be appropriate. On the basis of preliminary graphs, a regression model should be postulated and fitted to the data using a linear or nonlinear regression-fitting technique. Residuals from the model should be calculated and plotted against appropriate variables. Any systematic structure in the residuals indicates lack of fit of the model and the model should be modified and the procedure repeated. This cycling should continue until there is no further structure in the residuals to be explained. Presentation of results of regression or concentration-effect curve analysis should include the intercept or other point estimate and the slope and their 95 % confidence limits.

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