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Standard Guide for

Conducting Early Life-Stage Toxicity Tests with Fishes[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 freshwater and saltwater fishes during 28 to 120-day (depending on species) continuous exposure, beginning before hatch and ending after hatch, using the flow-through technique. This guide will probably be useful for conducting early life-stage toxicity tests with some other species of fish, although modifications might be necessary.

1.2 Other modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of many other tests. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting early life-stage toxicity tests with fishes.

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 Guide E1192), leachates, oils, particulate matter, sediments, and surface waters.

1.4 This guide is arranged as follows:

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

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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 6 and 9.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System) (Withdrawn 1997)[3]

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

E943 Terminology Relating to Biological Effects and Environmental Fate

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

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]

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see 13.1). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions:*

3.2.1 For definitions of other terms used in this standard, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to Practice E380.

3.2.2 *antagonism*—a situation which an effect of an exposure to multiple substances is less than would be expected if the known effects of the individual substances were added together.

3.2.3 *synergism*—a situation in which an effect of an exposure to multiple substances is more than would be expected if the known effects of the individual substances were added together.

3.2.4 *confounding*—a situation in which one or more other variables covary with the independent variable, making it impossible to determine the influence of the independent variable on the dependent variable.

4. Summary of Guide

4.1 In each of two or more treatments, embryos and the subsequent larvae of one species of fish are maintained in two or more test chambers in a flow-through system for 28 to 120 days, depending upon species. In each of the one or more control treatments, the embryos and larvae are maintained in dilution water to which no test material has been added in order to provide (*a*) a measure of the acceptability of the test by giving an indication of the quality of the embryos and larvae and the suitability of the dilution water, food, test conditions, handling procedures, and so forth, and (*b*) the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, the embryos and larvae 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 and growth of the embryos and larvae in each test chamber are obtained and analyzed to determine the effect(s) of the test material on the survival and growth 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. An early life-stage toxicity test provides information about the chronic toxicity of a test material to a species of fish. The primary adverse effects studied are reduced survival and growth.

5.2 Results of early life-stage toxicity tests are generally useful estimates of the results of comparable life-cycle tests with the same species (**1**).[4] However, results of early life-stage tests are sometimes under estimative of those obtained with the same species in the longer life-cycle tests (**2**).

5.3 Results of early life-stage toxicity tests might be used to predict long-term effects likely to occur on fish in field situations as a result of an exposure under comparable conditions, except that motile organisms might avoid exposure when possible.

5.4 Results of early life-stage toxicity tests might be used to compare the chronic sensitivities of different fish species and the chronic toxicities of different materials, and to study the effects of various environmental factors on results of such tests.

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

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

5.6 Results of an early life-stage test 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. Most such predictions take into account the results of acute toxicity tests, and so the usefulness of the results of an early life-stage test is greatly increased by reporting also the results of an acute toxicity test (see Guide E729) conducted with juveniles of the same species under the same conditions.

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

5.8 Results of early life-stage toxicity tests will depend on temperature, composition of the dilution water, condition of the test organisms, and other factors.

6. Hazards

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses, and by using dip nets, forceps, or tubes, to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**4**), recommended handling procedures (**5**), and chemical and physical properties of the test material should be studied before a test is begun. (**Warning—**Special procedures might be necessary with radiolabeled test materials (**6**) and with test materials that are, or are suspected of being, carcinogenic (**7**).

6.2 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.

6.3 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. (**Warning—**An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

6.4 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.

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

7. Apparatus

7.1 *Facilities*—Flow-through tanks should be available for culturing brood stock, and for holding and acclimating test organisms. The test chambers should be in a constant-temperature area or recirculating water bath. An elevated headbox might be desirable so dilution water can be gravity-fed into holding, acclimation, and culture tanks, and the metering system (see 7.3). Strainers and air traps should be included in the water-supply system. Headboxes and holding, acclimation, culture, and dilution-water tanks should be equipped for temperature control and aeration (see 8.3). 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) holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. During holding, acclimation, culture, and testing, organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress. A timing device should be used to control the photoperiod (see Appendix X1-Appendix X9). A 15- to 30-min transition period (**8**) might be desirable whenever the lights go on to reduce the possibility of organisms being stressed by large, sudden increases in light intensity. A transition period when the lights go off might also be desirable.

7.2 *Construction Materials*—Equipment and facilities that come in contact with stock solutions, test solutions, or any water into which test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect fish. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water.

7.2.1 *Glass, Type 316 Stainless Steel, Nylon, and Fluorocarbon Plastics*—Use whenever possible to minimize dissolution, leaching, and sorption, except that stainless steel should not be used for tests on metals in salt water.

7.2.2 *Concrete and Rigid Plastics*—May be used for holding, acclimation, and culture tanks and in the water-supply system, but these materials should be soaked, preferably in flowing dilution water, for a week or more before use (**9**). Cast iron pipe should not be used with salt water and probably should not be used in a freshwater-supply system because colloidal iron will be added to the dilution water and strainers will be needed to remove rust particles. A specially designed system is usually necessary to obtain salt water from a natural water source (see Guide E729). Dilution water, stock solutions, or test solutions should not contact brass, copper, lead, galvanized metal, and natural rubber before or during the test. Items made of neoprene rubber or other materials not mentioned above should not be used unless it has been shown that their

use will not adversely affect either survival or growth of embryos and larvae of the test species.

7.3 *Metering System:*

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

7.3.2 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 daily in the morning and afternoon throughout the test. The metering system should be adjusted during the test if necessary.

7.3.3 The flow rate through each test chamber should be at least 5 volume additions per 24 h depending on the test species (see Appendix X1-Appendix X9). It is usually desirable to construct the metering system so that it can provide at least 10 volume additions per 24 h if desired, in case (*a*) the loading is high (see 11.5.4) or (*b*) 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 concentrations of test material (see 11.9.3) remain acceptable.

7.4 *Test Chambers and Incubation Cups:*

7.4.1 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, cups, and so forth, may be used to create two or more compartments within each chamber. Thus, 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 or in an enclosure to keep out extraneous contaminants and to reduce evaporation of test solution and test material. Also, chambers filled to within 150 mm of the top sometimes need to be covered to prevent organisms from jumping out. All chambers (and compartments) in a test must be identical.

7.4.2 Test chambers may be constructed by welding (but not soldering) stainless steel, or gluing double-strength or stronger window glass with clear silicone adhesive. Stoppers and silicone adhesive sorb some organochlorine and organophosphorus pesticides, which are then difficult to remove. Therefore, as few stoppers and as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed for strength, they should be on the outside of chambers rather than on the inside.

7.4.3 Embryos and young fish should be exposed in glass incubation cups constructed by gluing (*a*) stainless steel or nylon screen bottoms to lengths of glass tubing or bottles with the bottoms cut off, or (*b*) nylon or stainless steel screen tubes (collars) to petri dishes. To ensure that test solution regularly flows into and out of each cup, either (*a*) test solution should flow directly into the cups, or (*b*) the cups should be oscillated in the test solution by means of a rocker arm apparatus driven by a 1 to 6 r/min electric motor, or (*c*) the water level in the test chamber should be varied by means of a self-starting siphon. (Clogging of the screens on cups in some treatments might be greater than in others because of bacterial or algal growth caused by differences in the concentration of solvent, intensity of light, etc. If some cups must be changed, all cups in the test should be changed to minimize differences in growth not related to the test material.) The metering system, test chambers, and incubation cups should be constructed so that test organisms remain submerged and are not unacceptably stressed by crowding or turbulence.

7.4.4 Species-specific information on test chambers and incubation cups is given in Appendix X1-Appendix X9. 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.

7.5 *Cleaning*—The metering system, test chambers, incubation cups, 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 washed at least twice with deionized, distilled, or dilution water. (Some lots of some organic solvents might leave a film that is insoluble in water.) A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and the acid, but it might attack silicone adhesive. At the end of the test, all items that are to be used again should be immediately (*a*) emptied, (*b*) rinsed with water, (*c*) 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 (*d*) rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits, and 200 mg of hypochlorite/L is often used to remove organic matter and for disinfection. (A solution containing about 200 mg ClO^-/L may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water.) However, hypochlorite is quite toxic to most fishes **(10)** and is difficult to remove from some construction materials. It is often removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, or by

autoclaving in distilled water for 20 min, or by drying the item and letting it sit for at least 24 h before use. An item cleaned or disinfected with hypochlorite should not be used unless it has been demonstrated at least once that unfed larvae held for at least 48 h in static dilution water in which the cleaned item is soaking, do not show more signs of stress, such as discoloration, unusual behavior, or death, than do unfed larvae held in static dilution water containing a similar item that was not treated with hypochlorite. The metering system, test chambers, and incubation cups should be rinsed with dilution water just before use.

7.6 *Acceptability*—Before an early life-stage test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain dilution water without added test material. Determine before the first test, (*a*) whether embryos and larvae will survive and grow acceptably (see 13.1.8) in the new facilities, (*b*) whether the food, water, handling procedures, etc., are acceptable, (*c*) whether there are any location effects on either survival or growth, and (*d*) the magnitudes of the within-chamber and between-chamber variances.

8. Dilution Water

8.1 *Requirements:*

8.1.1 Besides being available in adequate supply, the dilution water should (*a*) be acceptable to the test organisms, (*b*) be of uniform quality, and (*c*) except as per 8.1.4, not unnecessarily affect results of the test.

8.1.2 To be acceptable to the test organisms, the dilution water must allow satisfactory survival and growth of embryos and larvae of the test species (see 13.1.8).

8.1.3 The quality of the dilution water should be uniform during the test. During a test in fresh water, the range of hardness should be less than 5 mg/L or 10 % of the average, whichever is higher. During a test in salt water, the range of salinity should be less than 2 g/kg or 20 % of the average, whichever is higher.

8.1.4 The dilution water should not unnecessarily affect results of an early life-stage test because of such things as sorption or complexation of test material. Therefore, except as per 8.1.5, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L.

8.1.5 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of an early life-stage test, it will be necessary to use a water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such a water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or conducted in a more usual dilution water to facilitate interpretation of the results in the special water.

8.2 *Source:*

8.2.1 Although reconstituted water (see Guide E729) may be used in early life-stage toxicity tests, its use generally is not practical because of the large volume that is necessary for use.

8.2.2 If a natural dilution water is used, it should be obtained from an uncontaminated, uniform quality source. The quality of water from a well or spring is usually more uniform than that of water from surface water. If surface water is used as a source of fresh or salt water, the intake should be positioned 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 residual chlorine and chlorine-produced oxidants are quite toxic to many fishes **(10)**. 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 **(11)**. Some organic chloramines, however, react slowly with sodium bisulfite **(12)**. In addition to residual chlorine, municipal drinking water often contains unacceptably high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. Excessive concentrations of most metals can usually be removed with a chelating resin **(13)**, but use of a different dilution water might be preferable.

8.3 *Treatment:*

8.3.1 Dilution water should be aerated intensively by such means as air stones, surface aerators, or column aerators **(14, 15)** prior to addition of test material. Adequate aeration will stabilize pH, bring 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 **(16)** to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation by dissolved gases, which might be caused by heating the dilution water, should be avoided to prevent gas-bubble disease **(15, 17)**.

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.

8.3.3 Dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer **(18)** 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 predators.

8.3.5 When necessary, sea salt may be added to prevent excessive decreases in salinity, (see 8.1.2), if the salt has been shown to cause no adverse effects on either survival or growth of embryos and larvae of the test species 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 semiannually for at least two years or if a surface water is used:

8.4.1.1 *All Waters:* pH, particulate matter, Total Organic Carbon (TOC), organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus 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.

8.4.1.2 *Fresh Water:* hardness, alkalinity, conductivity, sodium, and chloride.

8.4.1.3 *Salt Water:* salinity or chlorinity.

8.4.1.4 The methods used (see 12.3) should either (*a*) be accurate and precise enough to adequately characterize the dilution water or (*b*) have detection limits below concentrations that have been shown to adversely affect fish **(19)**.

9. Test Material

9.1 *General:*

9.1.1 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:

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

9.1.1.2 Solubility and stability in the dilution water,

9.1.1.3 Acute toxicity to the test species,

9.1.1.4 Measured or estimated chronic toxicity to the test species,

9.1.1.5 Precision and bias of the analytical method at the planned concentration(s) of test material,

9.1.1.6 Estimate of toxicity to humans, and

9.1.1.7 Recommended handling procedures (see 6.1).

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, of the water might be necessary. If the hardness (or salinity) of the dilution water will not be affected, deionized or distilled water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(20)**. 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 or organic amines, sodium or potassium salts of phenols or organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of 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 or growth of the test organisms. Because of its low toxicity to aquatic animals **(21)**, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms. 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.) (**Warning—**Acetone is also quite volatile.)

9.2.4 If a solvent other than water is used, at least one solvent control using solvent from the same batch used to make the stock solution, must be included in the test. If no other solvent other than water is used, a dilution-water control must be included in the test and the survival and growth, or both, of test organisms in the dilution water control must meet test acceptability requirements in order for the test to be considered acceptable (13.1). Using no solvent other than dilution water is the most desirable option (9.2.2) because using any other solvent means that antagonism, synergism, and confounding are possible. Using different concentrations of a solvent at the different concentrations of the test material should be avoided because both the concentration of the solvent and the concentration of the test material vary across the treatments, potentially resulting in confounding. Therefore, it is desirable to test the same concentration of solvent in all of the test solutions.

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

9.2.4.2 If the concentration of solvent is not the same in all test solutions that contain test material, either (*a*) an early life-stage test must be conducted to determine whether survival and growth, or both of the test organisms is related to the concentration of the solvent over the range used in the toxicity test, or (*b*) such an early life-stage test must have been conducted on the solvent using the same dilution water and test species. If survival and growth, or both, is found to be related to the concentration of solvent, an early life-stage test with that species in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival nor growth is found to be related to the concentration of solvent, an early life-stage toxicity test with that same species in that same water may contain solvent concentrations within the tested

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

range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

9.2.4.3 There may be instances when a toxicity test is to be conducted with a species that is not routinely available for testing (for example, such as with an endangered species (Dwyer et al. 2005a,b ; Besser et al. 2005 (**22**,**23**,**24**)). In these instances, the toxicity test used to evaluate potential effects of a solvent outlined in 9.2.4.2 may be conducted with a species in the same family (preferably the same genus) as long as the concentrations of solvent are at least double the concentration of solvent used in the toxicity test on the test material. For example if data were available for a commonly tested species such as rainbow trout (*Oncorhynchus mykiss*) demonstrating no effect of a solvent of interest at 100 ug/L, then the solvent concentration for toxicity test conducted with a species in the same family (for example, the *Oncorhynchus* of interest) can be no higher than 50 ug/L. Testing at least double the concentration of solvent used in the toxicity test would provide some margin of safety in extrapolating results of toxicity tests between species in the same family. For example, Dwyer et al. (2005a,b) (**22**,**23**) and Besser et al. (2005) (**24**) reported the sensitivity of endangered species of fish was within a factor of about 2 of commonly tested surrogate fish species for a variety of organic and inorganic chemicals in acute or chronic toxicity tests. Similarly, USEPA (2003) (**25**) reported similar sensitivity of aquatic species to a variety of organic or inorganic chemicals in toxicity tests conducted within a family.

9.2.4.4 If the test contains both a dilution-water control and a solvent control, the survival and growth, or both, of the organisms in the two controls should be compared (see X10.6). If a statistically significant difference in survival and growth or both is detected between the two controls, only the solvent control may be used for meeting the requirements of 13.1.8 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be pooled for meeting the requirements of 13.1.8 and as the basis for calculation of results.

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

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to provide a good estimate of the highest concentration of test material that will not unacceptably affect survival or growth of the early life stages of the test species (see Section 14), 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) using the same dilution water, test material, and species. If an acute-chronic ratio has been determined for the test material with a species of comparable sensitivity, the result of the acute test can be divided by the acute-chronic ratio. Except for a few materials, acute-chronic ratios with sensitive species are often less than five. Thus, if no other useful information is available, the highest concentration of test material in an early life-stage test is often selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test.

9.3.2 In some (usually regulatory) situations, it is only necessary to determine whether one specific concentration of the test material reduces survival or growth. For example, the specific concentration might be the concentration occurring in a surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When there is 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*—Whenever possible and appropriate, tests should be conducted with species listed in Appendix X1-Appendix X9 mainly because these species have been used successfully in early life-stage tests. With appropriate modification of these procedures, other species can be used. Use of the species listed in the appendices is encouraged to increase comparability of results and availability of much information about a few species rather than a little information about many species. Use of a specific strain should be specified only when it is of special concern. The species used should be determined using an appropriate taxonomic key.

10.2 *Age*—Except possibly with salmonids (see Appendix X1) and with Gulf toadfish (see Appendix X7), tests should be begun with newly fertilized (uneyed) embryos (≤24 h after fertilization) and must be begun with embryos less than 48 h after fertilization so that the exposure encompasses the early stages of organogenesis.

10.3 *Source*—All organisms in a test must be from the same source. Gametes or embryos may be obtained from: (*a*) brood fish cultured in the laboratory: (*b*) commercial, state or federal hatcheries; or (*c*) wild populations from relatively unpolluted areas. Laboratory cultures of species such as fathead and sheepshead minnows and Atlantic and tidewater silversides usually can provide at anytime of the year gametes whose history, age, and quality are known. Whenever salmon or trout are to be used, gametes should be obtained from a hatchery that has been certified disease-free, for example, free of infectious pancreatic necrosis, furunculosis, kidney disease, enteric redmouth, and whirling disease. Requirements for certification vary from state to state and from species to species. Gametes of the other suggested species are usually obtained directly from wild populations (see Appendix X1-Appendix X9 for specific methods regarding care of brood fish and obtaining gametes for testing). Importing and collecting permits might be required by local and state agencies.

10.4 *Brood Stock:*

10.4.1 Brood fish can be obtained from either another laboratory, a commercial, state, or federal hatchery, or a wild population in a relatively unpolluted area. When a brood stock is brought into the laboratory, it should be placed in a tank along with the water in which it was transported. Then the water should be gradually changed to 100 % dilution water over a period of 2 or more days and the temperature should be changed at a rate not to exceed 3°C within 12 h. Also,

whenever brood fish are brought into a facility, they should be quarantined for 14 days or until they appear to be disease-free, whichever is longer. No dip nets, brushes, other equipment, organisms, or water should be transferred from a quarantined tank to any other tank without being sterilized or autoclaved in distilled water.

10.4.2 After quarantine, if mature brood fish are not to be spawned immediately, it might be desirable to hold some species in aquaria equipped with temperature and photoperiod controls so that they are reproductively inactive. Fish requiring substrates for spawning should not have substrates available. Water quality during this period should be equal to that of acceptable dilution water (see Section 8). Water temperature for certain freshwater brood fish during the holding period should be optimum for maintaining adult fish reproductively inactive.

10.4.3 The brood stock should be cared for properly **(26)** so it is not unnecessarily stressed. To maintain fish in good condition and avoid unnecessary stress, they should not be crowded and should not be subjected to rapid changes in temperature or water quality. Fish should not be subjected to more than a 3°C change in water temperature in any 12-h period and preferably not more than 3°C in 72 h. The concentration of dissolved oxygen should be maintained between 60 and 100 % saturation **(16)** and continuous gentle aeration is usually desirable. Supersaturation by dissolved gases should be avoided to prevent gas-bubble disease **(15, 17)**. Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer **(18)** equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.22 µm or less. The un-ionized ammonia concentration in holding and acclimation tanks should be less than 35 µg/L **(27)**.

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

10.4.4 At least once a day, brood fish should be fed a food that will support survival, growth, and reproduction (see Appendix X1-Appendix X9). Analysis of the food for the test material is desirable if the material might be present in the environment.

10.4.5 Tanks should be scraped or brushed as needed. Between use with different groups of fish, tanks should be sterilized by autoclaving or by treatment with an iodophor **(28)** or with 200 mg of hypochlorite/L for 1 h, brushed well once during the hour, and then rinsed well. Although iodophors are not very acutely toxic to fish, hypochloride is (see 7.5 concerning preparation and removal of hypochlorite).

10.4.6 Brood fish should be carefully observed daily during quarantine, holding, acclimation, and culture for signs of stress, physical damage, mortality, disease, and external parasites. Abnormal, dead, and injured individuals should be discarded. If visual examination of the behavior and external appearance indicates that they are not eating or are flashing, flipping, swimming erratically, emaciated, gasping at the surface, hyperventilating, hemorraghing, producing excessive mucus, or showing abnormal color, the cause should be determined and eliminated. If they show signs of disease or external parasites, appropriate action should be taken.

10.4.7 Fish may be chemically treated to cure or prevent some diseases using appropriate treatments (see Guide E729). If they are severely diseased, it is often better to destroy the entire lot immediately. Fish with other diseases should be discarded immediately, because systemic bacterial infections usually cannot be treated efficiently, internal parasites cannot be removed without extensive treatment, and viral diseases cannot be treated. Generally, fish should not be treated during the first 16 h after arrival at a facility because of possible stress or drug treatment during collection or transportation. However, immediate treatment is necessary in some situations, such as treatment of bluegills for columnaris disease during hot weather. Gametes should not be obtained from treated fish for at least 14 days after treatment, and, organisms except for channel catfish embryos, must not be treated during the test.

10.4.8 *Maturation*—Environmental conditions for brood fish during this period should be those optimal for the production of viable gametes (see Appendix X1-Appendix X9). Because optimal temperatures and photoperiods for holding and maturation occasionally differ, changes from one condition to another should be made gradually (see 10.4.3).

10.5 *Handling*—Embryos and fish should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that organisms are not unnecessarily stressed. Organisms that are injured or dropped during handling or that touch dry surfaces should be discarded. Smooth glass tubes are best for handling embryos, whereas dip nets are best for handling fish that weigh over 0.5 g each. Such nets are commercially available, or can be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar knotless material. Nets coated with urethane resin are best for handling catfish. Equipment used to handle fish should be sterilized between uses (see 10.4.5). Hands should be washed before and after handling or feeding fish.

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

11.1.1.1 An early life-stage test intended to allow calculation of an endpoint (see Appendix X10.2) 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), embryos and larvae 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 only necessary to determine whether a specific concentration causes adverse effects on survival or growth (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 (**29**). Because test solution can flow from one compartment to another, but not from one test chamber to another (see 7.4.1), 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, preferably on one level (tier), but they may be on two levels. 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. If chambers are on two levels, a randomized block design must be used.

11.1.3 The minimum desirable number of test chambers, compartments, and test organisms per treatment should be calculated from (*a*) the expected variance within test chambers, (*b*) the expected variance between test chambers in a treatment, and (*c*) either the maximum acceptable width of the confidence interval on a point estimate, or the minimum difference that is desired to be detectable using hypothesis testing (**30**). If such calculations are not made at the beginning of the test, for each treatment (test concentration and control) there must be at least two test chambers and enough embryos to result in at least 40 embryos remaining after thinning in each treatment in which mortality is not attributable to the test material. Replicate test chambers (that is, experimental units) are necessary in order to allow estimation of experimental error (**29**). If each concentration of test material is more than 50 % of the next higher one and if the results are to be analyzed using regression analysis, fewer test organisms per concentration of test material, but not the control treatment(s), may be used. Because of the importance of the controls in the calculation of results, it might be desirable to use more test chambers, compartments, and embryos for the control treatment(s) than for each of the other treatments.

11.2 *Dissolved Oxygen*—The concentration of dissolved oxygen in each test chamber should be between 60 and 100 % of saturation (**17**) at all times during the test, and the time-weighted 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 test organisms, resuspend fecal matter, and greatly increase volatilization. Because aeration readily occurs at the surface, efficient aeration can be achieved with minimum turbulence by using an air lift to transfer solution from the bottom to the surface. Aeration should be the same in all test chambers, including the control(s), at any particular time during the test.

11.3 *Temperature:*

11.3.1 Test temperature depends upon the species used (see Appendix X1-Appendix X9). Other temperatures may be used to study the effect of temperature on survival and growth of embryos and larvae of the test species, or to study the effect of temperature on the results of an early life-stage test on the test material.

11.3.2 The upper or lower 95% confidence limit on the individual temperatures measured in the test chambers through the test must not be more than 2°C above or below the mean of the time-weighed average measured temperature for the individual test chambers. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. The upper or lower 95% confidence limit on the individual temperatures measured in the test chambers through the test must not be more than 2°C above or below the mean of the time-weighed average measured temperature for the individual test chambers. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C. Uniform temperature is important to maintain in a test because survival or growth or both of the test organisms can be influenced by temperature. The stated requirements are necessary to prevent confounding and unnecessary large variance.

11.4 *Beginning the Test:*

11.4.1 After test solutions have been flowing through the chambers long enough 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 embryos 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 concentration of test material in the solution flowing into the test chamber will indicate whether the cause is in the metering system or 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, volatilization, and a faster flow rate is

probably desirable (see 7.3.3). Measurement of degradation and reaction products is also desirable (see 11.9.3.2).

11.4.3 The test begins when embryos (or gametes) are first placed in test solution.

11.4.4 A representative sample of embryos must be either (*a*) impartially distributed among the cups by adding to each cup no more than 20 % of the number of embryos to be placed in each cup and repeating the process until each cup contains the desired number of embryos, or (*b*) assigned either by random assignment of one embryo to each cup, random assignment of a second embryo to each cup and so forth, or by total randomization. It might be convenient to assign embryos to cups in dilution water and then randomly assign the cups to the test chambers.

11.4.5 The embryonic stage at the beginning of the exposure should be determined as precisely as possible (**31**).

11.5 *Thinning:*

11.5.1 Successful fertilization and survival through hatching and larval development can vary widely among species and among various batches of eggs and sperm. Although it is desirable to have test organisms in which fertilization and control survival are 100 %, such success is rarely achievable. Although some species usually provide very good fertilization and control survival, other species, certain seasons, and necessary procedures may provide less than optimum fertilization and survival under control conditions; this can occur even with state-of-the-art practices.

11.5.1.1 Because of the uncertainties of fertilization success, control survival, and to provide an adequate number of organisms for a statistically valid test, it is often necessary to begin a test with numbers of eggs or embryos greater than the number of fry needed.

11.5.1.2 Where fertilization is the area of uncertainty, a large number of eggs can be started in each test chamber and then randomly thinned to a desired number of embryos within each test chamber at such time as noticeable embryonic development, and the handling of the embryos will not damage them. This procedure is possible with salmonids, northern pike, fathead minnow, white sucker, and bluegill.

11.5.1.3 Thinning of embryos is impractical with channel catfish and is unnecessary with gulf toadfish, sheepshead minnows, and silversides because fertilization success is not an area of uncertainty. Therefore, thinning of these species to desired numbers should be done with newly hatched fry.

11.5.1.4 Regardless of when thinning occurs, percent survival from the start of the test to the time of thinning must be noted. Overall test survival for each test chamber is calculated as the product of percent survival to the time of thinning times percent survival from thinning through the end of the test.

11.5.2 It is best to determine the maximum number of organisms that can be in each test cup or test chamber without causing a reduction in growth due to crowding (loading) and reducing to a number lower than the maximum at thinning. This maximum number would have to be determined for the species, temperature, flow rate, chamber or cup size, food, feeding regime, and so forth used in the test.

11.5.3 In some apparatus designs (for example, incubation cups) several groups of embryos or larvae are held separately within a test chamber. Because these groups are not considered as separate treatment replicates, it is permissible to transfer organisms among such groups within a test chamber in order to achieve the desired number of organisms in each test chamber and have reasonably even distribution of organisms among groups. It is never permissible to transfer organisms from one test chamber to another because this would violate the statistical assumption of independence of test chambers, a necessary assumption of both regression analysis and hypothesis testing.

11.5.4 The number of embryos or fry left in each test chamber after thinning should not be so high that the larvae will be crowded in the test chamber at the end of the test if they all survive and grow acceptably. Thus the number should be determined based on the expected size of the larvae at the end of the test, the volume of solution in the test chamber, and the flow rate of test solution through the test chamber. Generally, at the end of the test, the loading (grams of organisms; wet weight; blotted dry) in each test chamber should not exceed 0.5 g/L of solution passing through the chamber in 24 h and should not exceed 5 g/L of solution in the chamber at any time.

11.6 *Feeding*—Recommended food, ration, and method and frequency of feeding larvae are contained in Appendix X1-Appendix X9. Larvae of most test species grow acceptably on good quality live brine shrimp nauplii (see Practice E1203). The food used should be analyzed for the test material if it might be present in the environment.

11.7 *Duration of Test*—The test begins when embryos (or gametes) are first placed in test solution (Day 0) and continues for the minimum duration specified in the pertinent appendix. The test should be extended, however, if previously unaffected fish are adversely affected near the intended end of the test.

11.8 *Biological Data:*

11.8.1 Unfertilized eggs and dead embryos are often discerned from living embryos by a change in coloration or opacity. In embryos of some species, heartbeat and movement can be seen through the chorion. For non-salmonid species, death of embryos should be recorded daily and dead embryos removed when discovered to prevent the spread of fungal infection. For salmonid species, dead embryos in the pre-eyed stage should be removed at intervals dependent upon the species (Appendix X1), age of the embryo, extent of embryo mortality, and severity of any resultant fungal infection. Extreme care should be used when removing dead salmonid embryos because healthy embryos are very sensitive to disturbance and might be damaged.

11.8.2 When hatching commences, the number of embryos hatched and the number of physically abnormal fish (or embryos) in each incubation cup should be recorded daily. A written or photographic record of all deformed larvae should be kept throughout the entire post-hatch exposure.

11.8.3 Fish should be observed daily; dead young fish should be counted, recorded, and removed when observed. The criteria for death of young fish are usually immobility (especially absence of respiratory movement in older individuals) and lack of reaction to gentle prodding.

11.8.4 At the end of the test, all surviving fish must be weighed as specified (see pertinent Appendix). Individual

weight of each fish is preferred, but if fish are especially small, they may be weighed in groups. Dry weights (dried at 60°C for 24 to 48 h or to constant weight) are preferable to wet weights (blotted dry) especially if the fish are edematous. Measurements of individual lengths (to the nearest 0.5 mm) is optional. Either standard, fork or total length may be measured. However, if caudal fin rot or fin erosion occurs, standard lengths should be measured. It might be desirable to determine the size of fish that die during the test.

11.8.5 Fish should be carefully observed regularly during the test for abnormal development and abberant 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 (see 11.7).

11.8.6 Morphological examination of fish alive at the end of the test in each treatment, before they are dried, might be desirable (**32**). Biological and histological examination and measurement of test material in exposed fish will probably not be possible unless additional embryos and larvae are exposed specifically for such purposes.

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

11.9 *Other Measurements:*

11.9.1 *Water Quality*—If a freshwater dilution water is used, its hardness, alkalinity, conductivity, and pH should be measured at the beginning and end of the test and at least weekly in the control treatment(s). If a saltwater dilution water is used, its salinity (or chlorinity) should be measured at least daily, and pH should be measured at the beginning and end of the test and at least weekly in the control treatment(s). Alkalinity (in fresh water only) and pH should also be measured in the highest test concentration at least once to determine whether these are affected by the test material. The dissolved oxygen concentration must be measured in at least one test chamber in each treatment containing live test organisms (*a*) at the beginning and end of the test and at least weekly during the test, (*b*) whenever there is an interruption of the flow of test solution, and (*c*) whenever the behavior of the test organisms 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 un-ionized ammonia, particulate matter, TOC (or chemical oxygen demand (COD) in fresh water) 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 as concurrently as possible 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 during the test 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 that could alter the concentration of the test material occurs in the metering system, water samples must be taken immediately from affected test chambers and analyzed as soon as possible. If the test organisms are probably being exposed to substantial concentrations of one or more impurities, degradation, or reaction products (see 11.4.2), measurement of the impurities and product 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 test material might be lost due to sorption onto the walls of the sample container, the container and siphon or pipette should be rinsed with test solution before collecting the sample. Water samples should be collected directly into appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber, it may be desirable to collect and analyze additional water samples from selected areas of the chamber(s) to further characterize the exposure. Analysis of additional samples after filtration or centrifugation to determine the percentage of test material that is not dissolved or is associated with particulate matter is desirable, especially if the concentration of particulate matter present 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 greater 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 Methodology

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 or 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 by 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 (**33**) 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 (**34**). The concentration of un-ionized ammonia may be calculated from the pH, temperature, and concentration of total ammonia (**35**).

12.4 Methods used to analyze food (see 10.4.4 and 11.6) or fish (see 10.6 and 11.8.6) should be obtained from appropriate sources (**36**).

12.5 The precision and bias of each analytical method used should be determined in an appropriate matrix, for example, in water samples from culture tanks or control test chambers, in food, and in test organisms. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

13. Acceptability of Test

13.1 An early life-stage test should usually be considered unacceptable if one or more of the following occurred.

13.1.1 All 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, the concentration of solvent in the range used affected survival or growth of the test organisms (see 9.2.4.2, 10.4, and 10.6).

13.1.4 The test was started using organisms older than specified in the appendix for the test species.

13.1.5 The test organisms were not impartially or randomly assigned to test chambers or compartments.

13.1.6 The test was terminated before the duration specified in the appropriate appendix for the test species.

13.1.7 Data on survival and growth were not obtained as specified in 11.8.1, 11.8.2, and 11.8.4.

13.1.8 The control organisms did not survive or grow as specified in the appendix for the test species.

13.1.9 Temperature, dissolved oxygen, and concentration of test material were not measured as specified in 11.9.

13.1.10 The time-weighted, average-measured dissolved-oxygen concentration at the end of the test for any test chamber was not between 60 and 100 % of saturation.

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

13.1.12 The upper or lower 95% confidence limit on individual temperatures measured in the test chambers throughout the test must not be more than 2°C above or below the mean of the time-weighted average measured temperature for the individual test chambers.

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

13.1.14 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.

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

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

14. Calculation of Results

14.1 The primary data to be analyzed from an early life-stage test are those on (*a*) survival in each treatment (may be analyzed as embryo survival, fry survival, and overall survival), (*b*) weight of the survivors in each treatment, and (*c*) the concentration of test material in the test solutions in each treatment.

14.2 The variety of procedures that can be used to calculate results of early life-stage tests can be divided into two categories: those that test hypotheses, and those that provide point estimates. No procedure should be used without careful consideration of (*a*) the advantages and disadvantages of various alternative procedures and (*b*) appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of 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 early life-stage tests are discussed in Appendix X10.

15. Documentation

15.1 The record of the results of an acceptable early life-stage test should include the following information either directly or by reference to available documents:

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

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

15.1.3 Source of the dilution water, its chemical characteristics, a description of any pretreatment, and results of any demonstration of the ability of a species to survive, grow and reproduce in the water.

15.1.4 Source, history and age of test organisms, scientific name (and strain for salmonids when appropriate), name of person who identified the organisms and the taxonomic key used, history, and age; if a brood stock was used, observed diseases, disease treatments, holding, acclimation, and culture procedures (if appropriate), and number of males and females or number of nests and substrates used if natural spawning was used. If hormonal injections were used, report the number of

males and females used as well as type of hormone and frequency and timing of injections.

15.1.5 Description of the experimental design and test chambers (and compartments), the depth and volume of solution in the chambers, number of organisms and test chambers (and compartments) per treatment, procedure used for thinning, loading and lighting, a description of the metering system, and the flow rate as volume additions per 24 h.

15.1.6 Source and composition of food, concentrations of test material and other contaminants, feeding methods, frequency, and ration.

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

15.1.8 Range and time-weighted average measured test temperature and the methods 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 of test material, impurities, and reaction and degradation products, including validation studies and reagent blanks.

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

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 include enough information to clearly identify the procedures used and the quality of the results.

16. Keywords

16.1 aquatic; chronic toxicity; culture techniques; early life-history; *Morone saxatilis*; striped bass

APPENDIXES

(Nonmandatory Information)

X1. SALMON, TROUT, AND CHAR

X1.1 *Obtaining Embryos*—Embryos of salmon (*Oncorhynchus* sp.), trout (*Salmo* sp.) and char (*Salvelinus* sp.) can be obtained in several ways: (*a*) collecting ripe wild fish at the time of spawning and taking the amount of eggs and semen required; (*b*) procuring gametes or embryos from brood stock resident at or returning to public or private hatcheries; or (*c*) providing fish with the proper conditions for spawning in the laboratory. The latter procedure has been used successfully for brook trout **(37, 38)**, but gametes and embryos of other species are usually obtained from hatcheries. The investigator should obtain and report all pertinent data regarding brood stock, gametes, and embryos obtained from outside facilities. This information should include the strain or geographic race of the brood stock, number of males and females used, age, and their recent temperature, feeding and disease history.

X1.1.1 Removal of eggs usually is preceded by killing salmon or by anesthetizing trout or char in about 100 mg MS-222/L **(40)**. Stripping of eggs from live fish should be conducted by experienced personnel. Eggs are forced from the vent by manual or air pressure techniques. The air pressure technique, although requiring two persons, is the preferred method because the possibility of damaging eggs and the anesthetized brood fish is reduced. One person holds the female fish diagonally, with the vent pointed down, over a round-bottom receiving vessel and the other inserts an 18 to 20 gauge needle into the body cavity posterior to the pectoral fins and along the median ventral line. The body of the fish should be gently dried with a towel prior to inserting the needle to prevent any water from dripping into the receiving vessel. Avoid inserting the needle so deeply that internal organs are damaged (usually ≤1.3 cm is satisfactory); inserting the needle at an angle of about 30° to the ventral surface of the fish and in an anterior direction will greatly reduce the chance of damaging the internal organs. The needle is connected to a regulated air supply at 13.8 to 20.7 MPa (2 to 3 psi), and the air pressure forces all loose eggs out the vent into the receiving vessel. The fish holder can gently shake the brood fish to facilitate the expulsion of ripe eggs.

X1.1.2 Ripe female salmon are usually killed with a blow to the head and bled by slashing the caudal peduncle or slitting the isthmus just anterior to the pectoral fins. An incision is then made along the median ventral line from the vent to near the pectoral fins. Care should be taken to keep the eggs free from mucus and blood. Only loose eggs which fall freely from the incision are taken; eggs still adhering to the ovaries should not be taken **(39)**.

X1.1.3 Eggs from at least three females should be completely mixed and then fertilized with sperm from at least three males. The embryos (fertilized eggs) from all brood fish should be completely mixed to ensure randomness prior to allocating embryos to test chambers. Brood fish should not be fed 48 h prior to taking eggs to allow them to evacuate their lower gastrointestinal tract.

X1.1.4 Male salmon, trout, and char can be anesthesized with a solution of about 100 mg MS-222/L to facilitate

handling. Semen is collected by applying slight but firm pressure to the sides of a ripe male while aiming the vent at a beaker or directly onto the eggs. The male should be gently dried with a towel to help prevent water from dripping onto the eggs or into the semen collecting beaker. Semen can also be collected with a "dual-tube suction apparatus" (**40**). Semen can be held on ice if collected prior to egg taking. Eggs and semen should not be exposed to water before fertilization. Tests with coho salmon and steelhead trout indicate that fertility exceeds 90 % when at least 0.25 mL of semen is used per 50 mL of eggs. Eggs and semen should be gently and thoroughly mixed to ensure maximum fertilization. Enough water to cover the eggs may be added after mixing eggs and semen. Eggs, semen, and water should sit for two to five minutes after mixing and then the semen, broken eggs, and any blood clots should be rinsed from the eggs.

X1.1.5 Embryos may be shipped, but they can be killed by rough handling. Eggs should be water hardened for one h prior to shipping and kept at cool temperatures, generally less than 10°C (**39**). Severe jolts should be avoided and the eggs should not be packed too deeply. If the shipping period exceeds a few h, it is advisable to pack the eggs with ice (made from unchlorinated water).

X1.1.6 Unfertilized eggs and semen can be transported for a period of 24 h after stripping if they are kept in plastic bags free of air. Unfertilized eggs may be chilled on ice. Using a 0.75 % (water/volume) saline solution when mixing the eggs and semen may facilitate fertilization and reduce egg rupturing (**39**).

X1.1.7 It is preferable to begin tests with embryos within 96 h of fertilization. Tests may be begun with older embryos if the embryos are in the test chambers for at least seven days before hatching. Because few data are available comparing results of tests begun with newly fertilized embryos and eyed embryos, whenever an early life-stage test is begun with newly fertilized embryos, it might be desirable to begin a simultaneous comparable test with eyed embryos.

X1.2 *Early Life-Stage Toxicity Test*—Incubation cups should be of sufficient size that embryos are not stacked upon each other. This allows easy removal of dead embryos with minimal chance of disturbing remaining embryos. Cups can hold one to three embryos per cm^2 depending on embryo size. Sixty embryos per treatment are recommended at the beginning of the test. Embryos should be incubated at 10°C (except lake trout at 7°C) under dim incandescent lighting (≤20 fc) or total darkness (**339**), although eggs and embryos may be inspected daily with a flashlight or brighter incandescent or fluorescent source. Embryos must not be subjected to prolonged exposure to direct sunlight, fluorescent lighting, or high intensity incandescent lighting.

X1.2.1 The time required for hatching depends on species, egg size, and water temperature. At 10°C, hatching occurs by about 52 days for coho (**41**), 56 days for chinook (**42**), 41 days for brown trout (**39**), 44 days for brook trout (**43**), and 31 days for rainbow trout (**39**). Lake trout hatch in about 72 days at 7°C (**39**).

X1.2.2 Jarring, moving, or otherwise shocking embryos should be avoided during incubation, especially during the sensitive pre-eyed stage. The sensitive period for handling coho salmon and steelhead trout embryos occurs from about 7 days to the eyed stage at 16 days after fertilization (**40**). Specific data for other species are not available and it is strongly recommended that salmonid embryos not be disturbed during the pre-eyed stage except for the very gentle removal of infertile eggs and dead embryos.

X1.2.3 Infertile eggs, dead embryos, and dead alevins should be recorded and carefully removed with a pipette whose bore slightly exceeds the egg diameter. Stage of development or absence of development can be determined after clearing the eggs in an appropriate clearing agent such as Stockard's Solution (an 85:6:5:4 mixture of water, glycerin, formalin, and glacial acetic acid). Periodically, unfertilized eggs and extra control embryos can be cleared for comparison of development. If more than about a third of the embryos in the control treatment die within the first 48 h of the test, it will usually be cost-effective to restart the test because the chances for a successful test are small. In addition, if concentration-related effects occur in the first 48 h, it will probably be cost-effective to restart the test because all of the concentrations will probably cause adverse effects during the test.

X1.2.4 In actual practice, ≥80 % survival of control embryos from fertilization to hatching is frequently achieved when gametes are obtained by experienced personnel, embryos are maintained under controlled conditions, and tests are initiated within a few h after the fish are stripped. However, a number of factors such as age and condition of brood stock, methods used, time elapsed in transporting gametes prior to fertilization, and handling and transport of embryos can cause considerably lower survival of embryos, particularly during the pre-eyed stage. Therefore, when the eyed stage is first discernible, all dead embryos should be counted and discarded. At this time, surviving embryos should be randomly thinned to the desired number per treatment (see 11.5).

X1.2.5 Alevins should be counted and released to the larger exposure chamber at swim-up. Post swim-up alevins should be fed ad libitum at least four times daily (two times daily over weekends is acceptable) with live brine shrimp nauplii or a starter-grade commercial salmon or trout food or both. Generally, moist diets are more palatable than dry diets at this stage of development. If desired, food size or quantity or both may be increased during the test on the basis of average fish size. Excessive quantities of uneaten food and feces should not be allowed to accumulate and should be carefully siphoned from the test aquaria at least twice a week. Although many procedures have been used for feeding fish in toxicity tests in which growth is measured, the following are recommended for early life-stage tests with salmonids:

1) feed at a relatively high rate, such as >4 % food (dry weight)/fish (wet weight)/day:

a) base the ration for each test chamber on the product of the mean weight of control fish and the number of fish in the test chamber; this provides a constant ration per individual fish.

b) if appropriate for the diet, it is convenient to weigh out a week's ration for each chamber and simply estimate daily feeding allotments.

c) adjust ration quantity at least weekly for fish numbers and at least semi-monthly for fish weight.

d) mean control fish weight can be estimated by: 1) periodic destructive sampling of several control fish; 2) periodic nondestructive sampling of five to ten control fish; or 3) estimation of control fish weight based on historic growth rate, food conversion efficiency, and water temperature data.

2) An alternative to weighing or growth rate estimation is to feed a constant amount of food per fish throughout the required 30 days of feeding; this is acceptable only if the initial ration is sufficiently high (>8 %). In effect, the initial feeding level of 8 % gradually decreases to about 2 % by 30 days, as the fish double in weight approximately every 2 weeks. If this procedure is used, feed >8 % of the mean weight of the water-hardened embryos at the start of the test. This weight approximates the weight of the swim-up stage alevin (minus yolk).

X1.2.6 Salmonids do not normally feed prior to swim-up and a delay in swim-up may be a result of the experimental variable. When the first group of fish (usually controls) attains swim-up and feeding begins, food should be offered daily to fish in all treatments to determine their readiness to feed. The fish should not be fed during the 24 h preceding termination of the test.

X1.2.7 Mortalities and abnormalities should be recorded during the hatching period. The monitoring of mortalities may be coordinated with the feeding schedule after hatching is complete and the experimental fish reach swim-up. The test should last at least 30 days post swim-up. At termination, weight of all survivors should be determined (weight to the nearest 0.01 g); measurement of standard length (to the nearest 0.5 mm) may be desirable. If edema is a possible toxicant effect, both wet and dry weights (dried at 60°C for 24 h) should be determined.

X1.2.8 An early life-stage test with a salmon, trout, or char is unacceptable if survival of the controls is less than 70 % from thinning of the embryos (see 11.5) to test termination.

X2. NORTHERN PIKE

X2.1 *Obtaining Embryos*—Gametes of northern pike *(Esox lucius)* must be obtained from wild adults collected in the field at their spawning time. Several states, such as Minnesota, conduct collection programs soon after "ice-out" in the spring and might help in obtaining embryos for experimental purposes. Adults should be held in suitably large tanks that are covered to minimize disturbance and to prevent fish from jumping out. Adults are generally quiescent unless disturbed.

X2.1.1 Egg viability is increased by acclimating adults to 12°C for at least 24 h before they are stripped. This temperature is near the optimum for normal hatch (**44**). Females should be checked for ripeness daily by exerting pressure on their abdomen in an anterior to posterior direction with fish held vertically. Injection of hormones usually is not necessary.

X2.1.2 Sperm collection is somewhat difficult in the case of escocidae and is best accomplished by sacrificing the fish. A few drops of milt should be obtained by stroking the belly of prospective males. After microscopic confirmation of sperm viability (motility), the chosen fish are then heavily anesthetized or killed, the testes removed through a long incision in the fish's side, placed in a double layer of clean cheesecloth, and squeezed to extrude milt. Care should be taken to avoid contaminating the sperm with urine, which severely reduces viability. Sperm collected in this manner can be stored under refrigeration for up to five days. This and a non-destructive sperm collection procedure are described by Sorenson et al. (**45**).

X2.1.3 Eggs from at least three females should be combined and dry-fertilized by gentle but thorough mixing with milt from at least three males for three minutes. Then about 170 mL of water should be added and stirred for three more minutes.

X2.2 *Early Life-Stage Toxicity Test*—Early life-stage tests with northern pike should begin three to four min after fertilization and must begin within 48 h after fertilization. After distribution of embryos to the incubation cups, they should remain motionless for four h. A rocker arm apparatus driven by a slow speed motor assembly (**46**) should be used to slowly oscillate the incubation cups. However, mortality will occur if embryos are jostled or rolled. The test begins when the embryos are distributed to the incubation cups and is terminated 32 days later. During the test, the temperature should be held at 15 ± 1°C and 16 h photoperiod.

X2.2.1 The test should be started with about 60 embryos per treatment, which should be randomly thinned to the desired number per treatment at 48 h, after removal of dead and fungused embryos (see 11.5). If more than about a third of the embryos in the control treatment are discarded within the first 48 h of the test because they are dead or heavily fungused, it will usually be cost-effective to restart the test because the chances for a successful test are small. In addition, if concentration-related effects on embryos occur in the first 48 h, it will probably be cost-effective to restart the test because all of the concentrations are likely to cause adverse effects during the test.

X2.2.2 Hatching will occur in about 6 days at 15°C. Larvae should be retained in the incubation cups until day ten and then counted and transferred to growth chambers. Live larvae that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal in either swimming behavior or physical appearance may be counted, but must not be discarded. The day hatching begins and ends in each cup should be recorded.

X2.2.3 Dead fish should be removed and recorded when observed. Hatched fish should be counted at least during the transfer from the incubation cups to the test chambers and on days 18, 25, and 32, after the beginning of the test. Fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic and grossly abnormal in either swimming behavior or physical appearance should also be counted.

X2.2.4 Feeding of live brine shrimp nauplii (≤24-h post hatch) should commence at the first indication of swim-up or at about day 12. Larvae should be fed a minimum of three times daily in order to maintain an excess of live food in growth chambers. This is especially important toward the end of the test period because northern pike become increasingly cannibalistic if live brine shrimp nauplii are not available.

X2.2.5 Fish should not be fed for the last 24 h prior to termination on day 32. At termination, the weight of each surviving fish should be determined. If the fish exposed to test material appear to be edematous compared to control fish, both dry weight (dried at 60°C for 24 h) and wet weight of all live fish should be determined.

X2.2.6 An early life-stage test with northern pike is unacceptable if (*a*) the average survival of the controls from 48 h to 32 days is less than 70 %; or (*b*) survival in any control chamber from 48 h to 32 days is less than 60 %.

X3. FATHEAD MINNOW

X3.1 *Obtaining Embryos*—Embryos of the fathead minnow *(Pimephales promelas)* may be obtained from a brood stock maintained in a pond when the water temperature is above 22°C, but culture in the laboratory is usually preferable because embryos can be available throughout the year and are less likely to be diseased. If brood stock are obtained as embryos, they can develop into sexually mature fish in 3 to 6 months when maintained at 25°C, dissolved oxygen concentrations of between 75 and 100 % saturation, flow rate of 2 to 10 water volume additions per 24 h, and a 16-h photoperiod. Live brine shrimp (*Artemia* sp) nauplli ≤24-h old are acceptable food for 2 to 30-day old fathead minnows; fish over 20 days old may be fed frozen adult brine shrimp. Mature fathead minnows can be sexed by the method of Flickinger (**47**).

X3.1.1 Fathead minnows readily deposit embryos on the underside of half-round ceramic substrates with an inside diameter of 75 mm and a length of 75 mm (**48**). Such substrates can be placed on the bottom of a pond. In the laboratory, embryos can be obtained by placing three mature males and five mature females in a 300 by 600 by 300 mm-deep glass chamber containing 150 mm of water and 4 to 8 substrates. Alternatively, the chamber can be divided into quadrants with stainless steel screen and one male, one female, and one or two substrates placed in each quadrant.

X3.1.2 Embryos should be rolled off the substrate with a gentle circular motion of an index finger (**49**) and visually examined using a dissecting scope or magnifying viewer. If more than 50 % of the eggs on a substrate are not fertile or are heavily fungused, all embryos on that substrate probably should be discarded. Embryos from remaining substrates can then be combined. It is preferable to test single embryos with no fungus or partial shells attached, although embryos with partial shells and clumps of two or three embryos (with or without separation) have been used successfully.

X3.2 *Early Life-Stage Toxicity Test*—Early life-stage tests with fathead minnows should be conducted at 25°C with a 16-h photoperiod and last at least 28 days duration beyond the mean day to hatch of the controls. Several test chambers have been used routinely, including: (*a*) Twenty fish have been tested in a chamber that is 160 by 440 by 180 mm deep with a 160 by 180 mm 40-mesh stainless steel screen 60 mm from one end, with a water depth of 128 mm and with a flow rate of 190 mL/minute. (*b*) Fifteen fish have been tested in a chamber that is 65 by 180 by 90 mm deep with a 65 by 90 mm 40-mesh stainless steel screen 25 mm from one end, with a water depth of 45 mm and with a flow rate of 12 mL/minute. Water level is controlled by a standpipe in the smaller screened compartment with test solution entering at the other end of the chamber. A cylindrical screen over the drain can be used instead of a screened compartment to prevent loss of fry. Incubation cups can be made from glass cylinders about 45 mm inside diameter and about 70 mm high, by gluing 40-mesh nylon or stainless steel screen to the bottom with silicone adhesive. The cups should be suspended in the test chambers so as to ensure that the organisms are always submerged and that test solution regularly flows into and out of the cup without agitating the organisms too vigorously. Self-starting siphons and a rocker arm apparatus driven by a 2-r/min motor and having a vertical-travel distance of 25 to 40 mm, have been used successfully to create flow into and out of incubation cups. The bottoms of the cups might need to be cleaned periodically to allow flow of test solution.

X3.2.1 The test should begin with at least 60 embryos per treatment. The embryos should be between 2 and 24-h old and must be less than 48-h old. With the incubation cups standing in dilution water within 3°C of the test temperature, the embryos should be randomly assigned to the incubation cups. The cups should then be randomly placed in the test chambers. All floating embryos should be gently squirted with water so that they sink.

X3.2.2 On test days 1 and 2, the embryos should be counted, examined by placing the cups under a dissecting scope or magnifying viewer, and dead or heavily fungused embryos counted and discarded. The remaining embryos must not be removed from the test solution. If the cups are not designed to hold the solution, a small container must be placed under the cup prior to removal from the test chamber for examination. Embryos remaining after day 2 should be randomly thinned to the desired number (see 11.5). The cup should be returned to the test chamber and subsequent handling of the cup should be minimized and done as gently as possible. Dead embryos may

be removed daily from the cup, but live embryos, even if fungused, must not be removed.

X3.2.3 If more than about a third of the embryos in the control treatment are discarded within the first 48 h, it will usually be cost-effective to restart the test because the chances for a successful test are small. In addition, if concentration-related effects occur in the first 48 h, it is likely that all concentrations will cause effects, and it might be cost-effective to restart the test.

X3.2.4 In each treatment, when hatching is about 90 % complete or 48 h after first hatch in that treatment and at a minimum on days 11, 18, 25, and 32, the live young fish should be counted. The live fish that, to an unaided eye, are lethargic or grossly abnormal in swimming behavior or physical appearance may be counted, but must not be discarded. All live fish should be released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The range of time-to-hatch (to the nearest day) in each cup should be recorded.

X3.2.5 In each treatment on days 2 to 5 after first hatch and at least 5 days a week thereafter, each test chamber containing live fish should be fed live newly-hatched brine shrimp (<24-h old) at least either (*a*) 2 times a day at least 6 h apart or (*b*) 3 times a day about 4 h apart. They must be fed at least once a day on all other days.

X3.2.6 The fish should not be fed for the last 24 h prior to termination on day 32. At termination, the weight of each surviving fish should be determined. If the fish exposed to test material appear to be edematous compared to control fish, both dry (24 h at 60°C) weight and wet weight of all live fish should be determined.

X3.2.7 An early life-stage test with the fathead minnow is unacceptable if the average survival in any control chamber from 48 hours to 32 days is less than 70 %.

X4. WHITE SUCKER

X4.1 *Obtaining Embryos*—Little information is available on the environmental conditions necessary to promote gonadal development of the white sucker *(Catostomus commersoni)* in the laboratory. Therefore, brood fish must be obtained in the spring just prior to natural spawning. The exact time of spawning will differ regionally; dates should be obtained from local fisheries experts. The fish should be netted or trapped as they move up tributary streams to spawn from March to June. Two- to three-pound adults should be selected and placed in large flow-through holding tanks, which are shaded to minimize disturbance and covered with screen to prevent the fish from jumping out. Water is maintained at the optimum hatching temperature of 15°C **(50)** and a 16-h photoperiod is desirable.

The males can be separated from the females by observing a series of tubercles on the first anal fin ray. The fish are checked routinely for ripeness by applying pressure (anterior to posterior) to the abdomen and watching for the presence of gametes. When the fish become ripe, eggs from at least three females are combined and fertilized with milt from at least three males according to the dry technique described by Hokanson et al. **(44)**. The embryos are then placed in flowing water at 15°C.

X4.2 *Early Life-Stage Toxicity Test*—Early life-stage toxicity tests with white suckers should be conducted at 15°C with a 16-h photoperiod and last 32 days. Several test chambers have been used routinely, including: (*a*) Twenty fish have been tested in a chamber which is 160 by 440 by 180-mm deep with a 160 by 180-mm 40-mesh stainless steel screen 60 mm from one end, with a water depth of 128 mm and with a flow rate of 190 mL/minute. (*b*) Fifteen fish have been tested in a chamber which is 65 by 180 by 90-mm deep with a 65 by 90-mm 40-mesh stainless steel screen 25 mm from one end, with a water depth of 45 mm and with a flow rate of 12 mL/minute. Water level is controlled by a standpipe in the smaller screened compartment with test solution entering at the other end of the chamber. A cylindrical screen over the drain can be used instead of a screened compartment to prevent loss of fry. Incubation cups can be made from glass cylinders about 45 mm inside diameter and about 70 mm high by gluing a 40-mesh nylon or stainless steel screen to the bottom with clear silicone adhesive. The cups should be suspended in the test chambers so as to ensure that the organisms are always submerged and that test solution regularly flows into and out of the cup without agitating the organisms too vigorously. Self-starting siphons and a rocker arm apparatus driven by a 2-r/min motor and having a vertical-travel distance of 25 to 40 mm have been used successfully to create flow into and out of incubation cups. The bottoms of the cups might need to be cleaned periodically to allow flow of test solution.

X4.2.1 Early life-stage tests with white suckers should begin with embryos that have water hardened for at least 3 h at 15°C and must be begun within 48 h after fertilization. The embryos should be gently separated from each other, gently mixed with those of other spawnings, and randomly distributed to the incubation cups. The cups are then randomly placed in the exposure chambers so there are about 60 embryos per treatment. All floating embryos should be gently squirted with water so that they sink.

X4.2.2 On test days 1 and 2, the embryos should be counted, examined by placing the cups under a dissecting scope or magnifying viewer, and dead or heavily fungused embryos counted and discarded. The remaining embryos must not be removed from the test solution for examination; if the cups are not designed to hold a small volume of test solution, a small container must be placed under the cup prior to the removal from the test chamber for examination. Embryos remaining on day 2 should be randomly thinned to the desired number (see 11.5). The cup should be returned to the test chamber and subsequent handling of the cup should be minimized and done

as gently as possible. Dead embryos may be removed daily from the cup, but live embryos, even if fungused, must not be removed.

X4.2.3 If more than about a third of the embryos in the control treatment are discarded within the first 48 h, it will usually be cost-effective to restart the test because the chances for a successful test are small. In addition, if concentration-related effects occur in the first 48 h, it is likely that all concentrations will cause effects and it might be cost-effective to restart the test.

X4.2.4 Hatching occurs in about 7 days at 15°C. Live young fish should be counted in each treatment when hatching is about 90 % complete or 48 h after first hatch in that treatment. Thereafter, at a minimum fish should be counted on days 11, 18, 25, and 32. The live fish that, to the unaided eye, are lethargic or grossly abnormal in either swimming behavior or physical appearance should be counted, but must not be discarded. All live fish must be released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The days on which the first and last embryos hatch in each cup should be recorded.

In each treatment on days 7 and 8 after first hatch and at least 5 days a week thereafter, each chamber containing live fish should be fed live newly hatched brine shrimp (≤24-h old) at least either (*a*) 2 times a day at least 6 h apart or (*b*) 3 times a day about 4 h apart. They should be fed at least once a day on all other days.

X4.2.5 The fish should not be fed for the last 24 h prior to termination on day 32. At termination, the weight of each fish should be determined. If the fish exposed to test material appear to be edematous compared to control fish, both dry (24 h at 60°C) weight and wet weight of all live fish should be determined.

X4.2.6 An early life-stage test with white sucker is unacceptable if the average survival of the controls from 48 h to 32 days is less than 80 % and if survival in any control chamber is less than 70 %.

X5. CHANNEL CATFISH

X5.1 *Obtaining Embryos*—Embryos of channel catfish *(Ictalurus punctatus)* can be obtained from commercial, federal, or state fish hatcheries. If embryos less than 24-h old are needed, it might be necessary to obtain them from laboratory cultures. Channel catfish can be induced to spawn naturally in the laboratory by controlling day length and temperature (**51**) or they may be stimulated to spawn by injection of human chorionic gonadotropin (300 IU/454 g bodyweight) (**52**). To achieve natural spawning adult (2- to 3-year old) channel catfish should be obtained or reared to maturity in the laboratory. They should be maintained at 26°C and 15 to 16-h day length. As they approach sexual maturity, males should be separated from females to avoid aggressive behavior. When the males become aggressive, it is necessary to separate them from each other. At this time, each male to be used for spawning should be provided a suitable spawning substrate, such as a 19-L stainless steel milk can. It may also be necessary to divide females into groups of 2 to 3 individuals of approximately equal size if aggressive behavior occurs. Mature male channel catfish can be distinguished from females by the somewhat broader and flatter conformation of their head. Confirmation of the sex of channel catfish should be obtained by gentle insertion of a blunt probe into the urogenital papilla. With the female, the probe should encounter no resistance, whereas it will be directed anteriorly in the male.

X5.1.1 When individual females are judged to be ready to spawn (that is, distinguished by flaccid abdomen and blood-gorged urogenital papilla), they should be individually paired with males of approximately equal size. Each pair of fish should be closely observed each day for aggressive behavior as the female will often be killed if the pairing is not successful. The tank should be checked for spawn each day, disturbing the fish as little as possible. One channel catfish spawn will contain 5000 to 20 000 eggs.

X5.1.2 If gonadal maturation must be induced by hormonal injection, females can be injected once every 3 or 4 days with 300 IU human chorionic gonadotropin per 454 g of body weight (**52**). The last few layers of eggs will be of poor quality in many cases, but no problems should be encountered with the rest of the spawn. Attempts to induce fish that have failed to spawn naturally will usually be unsuccessful.

X5.1.3 Separating the embryos from the substrate and from each other should be done with extreme care. The embryo mass can be freed from the inner surface of the can with a bevelled-edged dry wall joint knife. Because channel catfish embryos are in an adhesive gelatinous matrix, the mass should first be split into four to eight clumps of embryos for distribution in the incubation cups. Each hatching cup should receive embryos from each clump, in order to avoid the possibility of an incubation cup receiving embryos from only one location in the egg mass. Attempts to separate individual embryos will result in many damaged embryos. Instead, the embryos should be pulled apart into groups of four to ten embryos.

X5.2 *Early Life-Stage Toxicity Test*—Early life-stage toxicity tests with channel catfish should be conducted at 25°C with a 16-h photoperiod and 32-days duration. Twenty larvae have been successfully tested in a chamber which is 160 mm wide by 270 mm long with a water depth of 200 mm, and receiving approximately 100 mL/minute of test solution.

X5.2.1 The test is begun by placing 20 embryos in each of 2 incubation cups per test chamber. Tests should be begun within 24 h after fertilization of the eggs and must be begun within 48 h after fertilization. The cups should be aerated at a rate sufficient to provide adequate water exchange and to vigorously roll the embryo masses and keep them suspended in the water column. Because the embryos of channel catfish are

held together by an adhesive gelatinous matrix, no attempt should be made to remove dead embryos. If fungus appears in any treatment, all embryos should be treated with malachite green at a concentration of 60 mg/L for 3 min once each day until embryos are eyed. As soon as hatching begins (6 to 7 days at 25°C), the air flow must be decreased to a gentle stream so as not to damage newly hatched larvae. Upon completion of hatching, the successfully hatched larvae should be counted, randomly thinned (see 11.5) to the desired number, and transferred to the test chambers. Newly hatched larvae can be carefully handled and transferred with a 6-mm inside diameter glass tube with a soft suction bulb and should be fed newly hatched brine shrimp nauplii (≤24-h old) at least 3 times per day beginning immediately after hatch. This ensures that food will be available as soon as the fry begin feeding. Starting at swim-up, fry should be fed a commercial catfish food at least 2 times per day ad libitum in addition to the brine shrimp.

X5.2.2 Dead fish should be removed and recorded when observed. The fish should not be fed for the last 24 h prior to termination on day 32. Individual fish should be weighed and measured to the nearest 0.5 mm (fork length). If the fish exposed to the test material appear edematous compared to the control fish, both dry (dried at 60°C for 24 h) weight and wet weight of all surviving fish should be determined.

X5.2.3 An early life-stage test with channel catfish is unsuccessful if the average survival of the controls from the beginning to the end of the test is less than 65 %.

X6. BLUEGILL

X6.1 *Obtaining Embryos*—Techniques for consistent, successful spawning of the bluegill *(Lepomis macrochirus)* in the laboratory are not presently available. Therefore, both natural and artificial methods for obtaining eggs are described.

X6.1.1 Using the natural method, bluegills approaching completion of their second year, or older, should be provided with concrete substrates such as those described by Eaton (**53**). When possible, use brood stock for which historical records relating age and sexual maturity are available. No more than three substrates and male fish should be placed in water a ft or so deep and in a water volume of 500 L. Even if the depth is greater, several square feet of bottom space should be provided around each substrate. It might also be necessary to screen males visually from one another if interaction between them becomes too vigorous. The number of fish per tank should be no more than a 2:3 ratio of males to females. Because the embryos are difficult to see, substrates should be examined carefully during the morning and evening hours by gently rubbing a finger over the surface of the substrate.

X6.1.2 The artificial method developed by Banner and Van Arman (**54**) has the advantage of being able to provide eggs from several females simultaneously. The procedure consists of seven steps:

(1) Fish are collected in the field and returned to the laboratory, acclimated to captivity, and sexed as well as possible on the basis of morphology, coloration, and behavior (**55**).

(2) Males are kept at 16-h photoperiod at 26°C, with as many as 20 fish per 2.9 kL aquarium. Ripening requires up to two months for fish collected outside the spawning season.

(3) Females are kept isolated from males and given a 16-h photoperiod at a temperature of 26 to 28°C. The production of ripe eggs might require up to three months.

(4) Females are injected intraperitoneally with two mg carp pituitary every other day, and egg development is checked daily.

(5) When a sufficient number of females has developed premature eggs, 20 to 30 such females are selected and injected intramuscularly with 1 mg carp pituitary every four hours. Eggs are sampled at each injection interval and examined for size, appearance, adhesiveness, and fertilization-ability until several females have ripe eggs simultaneously.

(6) Females which produced eggs with greater than 50 % fertilization at the previous sampling interval are stripped and eggs fertilized with milt from four or more males.

(7) Females and then males are stripped into a shallow tray or pan (large enough to accommodate eggs in a single layer) containing an in. or less of water.

X6.1.2.1 Bluegill embryos can be removed from substrates by dislodging them with a paint brush (**56**) or by sucking them into a tipless pipet (**52**, **53**).

X6.2 *Early Life-Stage Toxicity Test*—The early life-stage toxicity test with bluegills should be conducted at 28 ± 1°C with a 16-h photoperiod and its duration should be 32 days. At the beginning of the test, the embryos should be between 2 and 24-h old and must be less than 48-h old. With the incubation cups standing in dilution water within 3°C of the test temperature, the embryos must be randomly assigned to the incubation cups. The cups should then be randomly placed in the test chambers so that there are about 60 embryos per treatment. Both oscillating and stationary incubation cups have been used successfully.

X6.2.1 On test days one and two, the embryos should be counted, examined by placing the cups under a dissecting scope or magnifying viewer, and dead or heavily fungused embryos should be counted and discarded. The remaining embryos must not be removed from the test solution. If the cups are not designed to hold some solution, a small container must be placed under the cup prior to removal from the test chamber for examination. After 48 hours, embryos remaining on day 2 should be randomly thinned to the desired number per treatment (see 11.5). The cups should be returned to the test chambers and subsequent handling of the cup should be minimized and done as gently as possible. Dead embryos should be removed daily from the cup, but live embryos, even if fungused, must not be removed.

X6.2.2 Live young fish should be counted in each treatment when hatching is about 90 % complete or 48 h after first hatch

in that treatment (approximately 30 h at 28°C). Thereafter, at a minimum, fish should be counted on days 11, 18, 25, and 32. The live fish that, to the unaided eye, are lethargic or grossly abnormal in either swimming behavior or physical appearance may be counted, but must not be discarded. All live fish must be released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The range of time-to-hatch (to the nearest day) in each cup should be recorded.

X6.2.3 In each treatment, each test chamber containing swim-up fish should be supplied live newly hatched (≤24-h old) brine shrimp 3 times a day about 4 h apart. Food should be available to the larvae continuously. After larvae have grown noticeably, brine shrimp older than 24-h old may be used. The amount of food provided to each chamber may be proportional to the number and size of fish in the chamber, but each chamber must be treated in a comparable manner.

X6.2.4 The fish should not be fed for the last 24 h prior to termination on day 32. At termination, each live fish should be weighed. If the fish exposed to test material appear to be edematous compared to control fish, both dry (24 h at 60°C) weight and wet weight of all live fish should be determined.

X6.2.5 An early life-stage test with the bluegill is unacceptable if the average survival of the controls from 48 h to 32 days is less than 75 % and if survival in any control chamber is less than 65 %.

X7. GULF TOADFISH

X7.1 *Obtaining Embryos*—Gulf toadfish *(Opsanus beta)* embryos can be collected from estuaries and coastal waters from Cape Sable, FL to Yucatan. This species spawns in 20 to 30°C water from April to August in cavities in shells, and on rocks or man-made objects. Drain tiles placed as toadfish habitats prior to the spawning season provide a spawning site and place of attachment. Male toadfish guard the nest which is removed intact by divers and returned to the laboratory submerged in water obtained at the collection site. The embryos are gently dislodged and examined for developmental stage. The test should begin within 24 h of collection and must begin within 48 h of collection. More than one nest may be needed to obtain sufficient numbers of embryos of a similar stage of development to begin the test.

X7.2 *Early Life-Stage Toxicity Test*—Tests have been completed in which embryos are placed in test solutions within incubation cups constructed by attaching a 9-cm high collar of 2000-µm nylon screen to the inside walls of a 9-cm I.D. glass petri dish bottom with clear silicone adhesive (**57**). Exchange of water within the cup should be ensured by furnishing each test chamber with a self-starting siphon that removes greater than 50 % of the volume of the test solution from the chamber each h. Test conditions used successfully include 12-h photoperiod, 25 and 30°C, and a salinity of 20 g/kg. Tests can probably be completed by placing embryos in incubation cups on a rocker arm apparatus similar to that in Appendix X3.

X7.2.1 At least 60 embryos, no more than 15 per cup, should be used in each treatment at the beginning of the test; their stage of development must be determined. Time to hatch will vary with developmental stage at test initiation and test temperature. Debris and dead embryos must be removed daily to prevent fouling of the cup and spread of fungus from dead to developing embryos. After all embryos hatch, fry may be randomly thinned to the desired number (see 11.5) and either retained in the incubation cup or released into the test chamber. The hatched yolk-sac fry require no feeding during the greater-than 1-week period before yolk utilization is complete and the attachment disk is shed. The duration of embryonic development and yolk-sac larval stage, and the number of disks shed each day in each replicate should be recorded.

X7.2.2 Live young fish should be counted in each test chamber when hatching is about 90 % complete or 48 h after first hatch in that treatment. Thereafter, at a minimum fish should be counted on days 14, 21, 28, 35, and 42.

X7.2.3 Juvenile toadfish should be fed live brine shrimp nauplii (≤24-h old) twice daily to excess after the yolk seems to be absorbed and the attachment disk is dropped. Care should be taken not to overfeed (as evidenced by a large number of nauplii from the previous feeding remaining) to prevent a decrease in dissolved oxygen and water quality. The fish should not be fed during the 24 h preceding termination of the test.

X7.2.4 The test is terminated at the end of the 42nd day of exposure. Individual fish should be weighed; it might also be desirable to measure the standard length of each fish to the nearest 0.5 mm.

X7.2.5 An early life-stage test with the gulf toadfish is unacceptable if the average hatch of the control embryos from the beginning to the end of the test is less than 75 % and overall survival at the end of the test is less than 70 %.

X8. SHEEPSHEAD MINNOW

X8.1 *Obtaining Embryos*—The sheepshead minnow *(Cyprinodon variegatus)* can be cultured to maturity in the laboratory or obtained from Gulf and Atlantic coast estuaries during most months of the year. Feral brood stock are preferred to minimize inbreeding. Fish can be reared to maturity in the laboratory within 3 to 5 months after hatching if they are held in flowing seawater at 25 to 30°C and at a salinity of 10 to 35 g/kg, with a photoperiod of 12 to 14 h. At a standard length of approximately 27 mm, males begin to exhibit sexual dimorphism and initiate territorial behavior.

X8.1.1 Gametes can be obtained by hormonal injection or by natural spawning. If hormonal injections are used, each female should be injected interpertioneally with 50 IU of human chorionic gonadotropin (HCG) on 2 consecutive days. Two days following the second injection, eggs are stripped from females and mixed with sperm derived from excised macerated testes. Usually, at least ten females and five males should be used to ensure that there are a sufficient number of genetically variable embryos. The early life-stage test can be started as soon as fertilization is microscopically verified (1 to 2 h). Embryos should not be agitated excessively.

X8.1.2 If embryos are obtained from natural spawning in the laboratory, a 200 by 350 by 220 mm-deep spawning chamber is recommended (**58**). To obtain sufficient embryos for an early life-stage test, four or more spawning chambers are recommended, each containing five or more females and three males.

X8.2 *Early Life-Stage Toxicity Test*—Embryos are placed in test solutions within incubation cups constructed by attaching a 90-mm high collar of 450-µm nylon screen to the inside walls of a 90-mm I.D. glass petri dish bottom with clear silicone adhesive (**59**). Alternatively, incubation cups can be installed on a rocker arm apparatus similar to those described in Appendix X3. If a rocker arm apparatus is not used, each chamber must have a self-starting siphon to ensure exchange of water in the incubation cups. The self-starting siphons should remove greater than 50 % of the volume of the test solution from the cups each hour.

X8.2.1 Embryos to be used in the test should be selected with the aid of a dissecting microscope and at least 60 embryos should be used in each treatment; the embryos should be less than 24-h old and must be less than 48-h old, and their stage of development should be microscopically determined. No more than 20 randomly assigned embryos should be added to each cup. Embryos should hatch in 4 days at 30°C, and in 7 days at 25°C. After all fish hatch, they may be randomly thinned to the desired number (see 11.5) and either retained in the incubation cup or released into the test chamber.

X8.2.2 Live young fish should be counted in each treatment when hatching is about 90 % complete or 48 h after first hatch in that treatment. Thereafter, at a minimum, fish should be counted on days 11, 18, 25, and 28.

X8.2.3 Hatched sheepshead minnows should be fed live brine shrimp nauplii (≤24-h old) to excess, beginning at hatching and at least daily thereafter. Care should be taken not to overfeed (as evidenced by a large number of nauplii remaining from the previous feeding) to prevent a decrease in dissolved oxygen and water quality.

X8.2.4 The test is terminated at the end of the 28th day of exposure. Fish should not be fed for the last 24 h before termination. Individual fish are weighed and it might also be desirable to measure the standard lengths of fish (to the nearest 0.5 mm). If the fish exposed to test material appear to be edematous compared to control fish, both dry (24 h at 60°C) weight and wet weight of all live fish should be determined.

X8.2.5 An early life-stage test with the sheepshead minnow is unacceptable if less than 75 % of the control embryos hatch, less than 80 % of the hatched control fish survive to the end of the test, and overall control survival from the beginning to the end of the test is less than 65 %.

X9. SILVERSIDES

X9.1 *Obtaining Embryos*—Sexually mature Atlantic silversides *(Menidia menidia)* are generally available in Atlantic estuaries from March through August, depending upon the latitude. Recent evidence indicates that this estuarine fish is a cyclic spawner (**60**). The optimal time for collecting ripe fish is just prior to high tides occurring between 8:00 a.m. and noon, when pre-spawning schools move into the upper intertidal zone.

X9.1.1 The tidewater silverside *(Menidia peninsulae)* is an estuarine species typically found at salinities of about 20 g/kg. It ranges from Daytona Beach, FL, to Horn Island, MS, and from Galveston Bay, TX, to Tamiahua, Mexico (**61**). Sexually mature fish can be collected near shore in shallow waters during the late spring and summer months.

X9.1.2 For either species, females that do not readily release ripe eggs when gently squeezed should not be used. Eggs may be stripped from fish either at the collection site or at the laboratory. If the time required to transport the fish to the laboratory is greater than one h, or if severe environmental conditions are likely during transport, field-stripping is recommended. Eggs are stripped into a glass culture dish containing salt water or onto a nylon screen (450 to 1000 µm-mesh), then placed in a vessel containing salt water from the collection site. Milt from several males can then be stripped into the culture dish or vessel and mixed. Upon contact with salt water, adhesive threads on mature eggs uncoil, making enumeration and separation difficult. If the culture dish is used, one end of a nylon string can be dipped into the dish and gently rolled so the threads can adhere. If the nylon screen is used, embryos can

be separated and counted directly. Embryos on nylon strings or screens may be shipped by placing them in insulated glass bottles filled with salt water at the approximate temperature and salinity of fertilization. Fish from which gametes were obtained should be preserved for taxonomic confirmation of the species used.

X9.1.3 Adult Atlantic and tidewater silversides can be spawned in the laboratory on a year-round basis. Procedures described by Middaugh and Takita **(62)** and by Middaugh and Hemmer **(63)** provide for maintenance of a brood stock of 30 to 50 fish at a sex ratio of 1:1 in a 1.3-m diameter circular holding tank which is part of a recirculating saltwater system. The photoperiod should be 13 h at 300 to 1700 lx, and water temperature should be maintained at 22 to 25°C. The fish should be fed 8 g of a flake food[6] of equivalent nutritional value each morning and afternoon, and 1 L of concentrated brine shrimp nauplii (hatch obtained from approximately 15 mL of cysts after 48 h of incubation at 25°C) in mid-afternoon. Excess food should be siphoned from the holding tanks at least weekly. Filter medium (activated charcoal) located in a reservoir tray should be changed immediately after siphoning the holding tank.

X9.1.4 Decreases in circulation current velocity in the holding tanks (8 decreasing to 0 cm/s) should be provided twice daily by turning off the saltwater circulation pump from midnight to 1:00 a.m. and noon to 1:00 p.m. Atlantic silversides will spawn in response to decreased current velocities during daytime (noon to 1:00 p.m.) and tidewater silversides in response to decreased current velocities during darkness (midnight to 1:00 a.m.). A spawning substrate of spun polyester aquarium-filter fiber (approximately 100 by 100 by 200 mm) should be suspended just below the surface of the water and in contact with the side of the holding tank. Spawning fish will deposit eggs on this substrate. Each morning or afternoon, eggs may be teased from the substrate and utilized in toxicity tests. Typical egg production ranges from 300 to 1200 per spawn. Fish usually will spawn 3 or 4 days or nights each week.

X9.1.5 It is essential that light-tight curtains surround the holding tanks. These curtains should remain closed except during periodic feedings, tank cleaning, and during removal and replacement of spawning substrates.

X9.2 *Early Life-Stage Toxicity Test*—The 28-day test should begin within 36 h after fertilization and must be begun no later than 48 h after fertilization. Test conditions used successfully include 12-h photoperiod, 25 ± 2°C, and salinity between 15 and 27 g/kg **(64, 65)**. After fertilization, embryos should be gently teased apart and threads cut prior to randomly placing them in incubation cups that are constructed by gluing a 100-mm high, 363-µm nylon mesh tube inside a 90-mm I.D. glass petri dish bottom. At least 60 embryos should be used in each treatment. Hatching usually occurs within eight days after fertilization. After hatching is completed, fry may be randomly thinned to the desired number (see 11.5). Live young fish should be counted in each treatment when hatching is about 90 % complete or 48 h after first hatch in that treatment. Thereafter, at a minimum, fish should be counted on days 11, 18, 25, and 28.

X9.2.1 Silverside larvae are injured easily, and are particularly susceptible to impingement on the mesh tube of incubation cups. Therefore, water flow into and out of the cups should be at a slow rate and larvae should never be netted. Successful tests have been conducted by using a metering system that delivered 1 L to each test chamber every 15 min through splitter boxes. Two 2-mm inside diameter capillary tubes which drained the water from the splitter boxes delivered 500 mL of salt water to each test chamber. Water depth in each chamber (I.D. = 210 by 220 mm) was varied between 45 and 65 mm in approximately a 1-min period by the action of a 4-mm I.D. self-starting siphon. Prior to removing cups containing larvae from the chambers, the water level in the chamber should be lowered slowly with a siphon to the top of the petri dish. Cups should not be removed from the chamber for five days following initial hatching.

X9.2.2 Silverside larvae should be fed the rotifer *Branchionus plicatilis* **(66)** for the first week post-hatch and live brine shrimp nauplii (≤24-h old) during the remainder of the test except for a 2-day feeding transition period when both rotifers and brine shrimp should be provided. Feeding (three times per day for the first seven days after hatching, two times per day thereafter) should begin on the day of hatch. Successful tests have been conducted by using approximately 30 000 rotifers per cup per feeding and approximately 12 000 brine shrimp nauplii per cup per feeding.

X9.2.3 The test should be terminated at the end of the 28th day of exposure. Fish should not be fed for the last 24 h prior to termination. Individual fish should be weighed and it might be desirable to measure standard lengths of fish (to the nearest 0.5 mm). If the fish exposed to the test material appear to be edematous compared to the control fish, both dry (24 h at 60°C) weight and wet weight of all live fish should be determined.

X9.2.4 An early life-stage test with the Atlantic or tidewater silverside is unacceptable if average control embryo survival is less than 80 %, average control survival from the beginning to the end of the test is less than 60 %, and average wet weight for control *M. peninsulae* is less than 12 mg/fish or for *M. menidia* is less than 15 mg/fish.

[6] Tetramin, available from Tetra Werke, Dr., D452, Melle, West Germany, has been found suitable for this purpose.

X10. STATISTICAL GUIDANCE

X10.1 *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: (*a*) preliminary and diagnostic graphical displays, (*b*) pairwise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (*c*) analysis of variance (ANOVA) and corresponding contingency table tests, (*d*) multiple comparison techniques for simultaneous pairwise comparison of other treatment groups with control groups, (*e*) concentration-effect curve analyses, and (*f*) multiple regression. If used correctly, each of these techniques can provide useful information about the results of an acceptable early life-stage toxicity test.

X10.1.1 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.

X10.2 *Endpoint*—The endpoint of early life-stage toxicity tests generally has been defined in terms of whether differences from control organisms are statistically significant at the 5 % level. One of the main conceptual problems with such a definition of the endpoint 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 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.

X10.2.1 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 an early life-stage 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.

X10.2.2 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, and 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, such as modelling.

X10.3 *Preliminary Data Analysis:*

X10.3.1 *Graphical Displays*—These should be performed every time data for any biological attribute are analyzed using either regression analysis or hypothesis testing **(67)**. Preliminary scatterplots 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. The advent of modern computers and statistical computing packages,[7] **(68)**, has made preparation of graphs both easy and inexpensive. Feder and Collins **(69)** illustrate the use of various types of preliminary and diagnostic graphical displays in analysis of data from chronic toxicity tests.

X10.3.2 *Outlier Detection Procedures* —Data that do not appear to be in conformance with the substantial majority are often referred to as "outliers," and 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 just to random variation. Barnett and Lewis **(70)** describe many outlier detection procedures, and Feder and Collins **(69)** illustrate the 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 analyses 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 an analysis.

X10.3.3 *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

[7] Statistical computer packages such as Minitab, 3081 Enterprise Drive, State College, PA, Statistical Analysis System, (SAS), SAS Institute, Box 8000, Cary, NC, Biomedical Department Package, (BMDP), Statistical Software, Inc., 1440 Sepulveda Blvd., Suite 316, Los Angeles, CA, and Statistical Package for the Social Sciences, (SPSS), Suite 3000, 44 N Michigan Ave., Chicago, IL, have been found suitable for this purpose.

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 (71). 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.

X10.4 *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 (72). 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.

X10.5 *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 (69) illustrate transformation of data before use of a contingency table test.

X10.5.1 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. They are thus 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 such tests are the one-sided measure of association test, the Cochran-Armitage test for categorical data, and tests based on linear or polynomial regression models for continuous data (73).

X10.5.2 ANOVA tests are based on normal distribution theory and assume (*a*) that the data within treatments are a random sample from an approximately normal distribution and (*b*) 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.

X10.6 *Multiple Comparison Procedures*—The usual approach to analyzing data from early life-stage tests is to compare data for each concentration of the test material to data for the control(s). In Fisher's Protected Test, that should be used only if the ANOVA *F*-test is significant (74), each concentration of the 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 (74, 75) can be used without the ANOVA *F*-test. Williams' procedure (74, 76) also tests the control(s) versus each concentration, but makes the additional 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 (77) No Statistical Significance of Trend (NOSTASOT) test can be used with the same assumptions as Williams' procedure. Shirley (78) has developed a nonparametric equivalent for Williams' test and Williams (79) has modified and corrected Shirley's procedure to increase its power to detect the alternative hypothesis. 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 corresponding significance level, the minimum detectable difference, and the power of the test.

X10.7 *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) (80). 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, which can introduce biases into estimates.

X10.7.1 Point estimates, such as the EC10, EC25, and EC50, are examples of endpoints derived by using regression. Whenever a point estimate is calculated, its 95 % confidence interval should also be calculated. Finney (81) discusses the probit model in considerable detail, and Draper and Smith (82) and Neter, Wasserman, and Kutner (83) discuss most practical aspects of multiple regression analysis. Feder and Collins (69) discuss use of these techniques in aquatic toxicology.

X10.7.2 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 variability across treatments and to produce a smooth trend. For example, if effects or concentrations differ by 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, the slope, and their 95 % confidence limits.

X11. STRIPED BASS

X11.1 *Preliminary Note*—Striped bass *(Morone saxatilis)* embryos and larvae are difficult to work with and typically require learned expertise. Handling and testing techniques described in this guide might not be readily adaptable to all laboratories. Further, an inherent variability is probably associated with the quality of the gametes which will limit the success of a specific test (see test acceptability benchmarks in X11.4). The allowable survival in controls is due to inherent biological factors and may limit the applicability of the test; methods to improve survival should be considered.

X11.2 *Obtaining Embryos*—Embryos of striped bass can be obtained from commercial, federal, and state fish hatcheries. Adult striped bass for spawning can be collected with nets or electrofishing gear from estuaries, rivers, and lakes from March through June. There is a maximum 1-h window between complete ovulation and overripeness when the eggs must be fertilized for maximum success (**84**, **85**). Several references are available on the culture of striped bass (**84**, **85**, **86**, **87**, **88**, **89**). Female broodfish can be injected with human chorionic gonadotropin (HCG) shortly after capture to discourage reabsorption of eggs and to encourage ovulation (**84**). Dosages of 125 to 150 International Units (I.U.) of HCG per pound of fish are usually divided among two or more injections and given along both sides of the spine between the lateral line and dorsal fin (**85**, **86**). Captured fish should be transported in a tank large enough to allow complete freedom of movement. To reduce stress and incidence of infections, fish should be transported in 2 to 5 g/kg salinity (from NaCl, sea water, or sea salts) containing an antibiotic approved by the U.S. Food and Drug Administration. Chlorine-free ice should be added if necessary during transport so that water temperature does not exceed 20°C; however, salinity should be not allowed to drop below 2 g/kg.

X11.2.1 Handling of fish in the hatchery should be kept to a minimum. Females should be checked for ripeness by taking a sample of eggs and checking for development at 20 to 28 h (perhaps sooner) after hormone injection (**85**). This can be done by inserting a 3-mm OD (2-mm ID) glass catheter through the urogenital opening (**84**). The time of ovulation can be estimated after microscopic examination (**85**, **86**). Ovulation is complete when eggs flow freely and separately under slight manual palpitation to the abdominal region.

X11.2.2 The viability (motility) of the sperm should be confirmed by microscopic examination prior to spawning. Fresh sperm should be placed on a glass slide and covered with a coverslip. A drop of water should be added to one side of the cover only so that it infiltrates the sperm by capillary action. Good sperm motility is defined by active swimming away from the intruding water for a minimum of 30 s.

X11.2.3 Wet-fertilization should begin by stripping eggs from one or two ripe females into a clean plastic dish pan. 1 to 2 L of water should be added and mixed well for 30 s. Milt from two or three males should then be added and mixed by hand for 3 min.

X11.2.4 Following fertilization, embryos can be put into 7-L plexiglass McDonald jars, at a density not to exceed 100 000 per jar (**85**). (McDonald jars might not work well with highly buoyant eggs such as those from fish collected in Chesapeake Bay. For highly buoyant eggs, circular tanks with a center drain pipe equipped with a large funnel covered with 500-µm mesh polypropylene or nylon net can be used.) A continuous flow of oxygenated water sufficient to keep embryos suspended at 18 ± 1°C should be introduced to the bottom of the jar. Dead embryos will usually overflow out of the jar or can be carefully siphoned off. Embryos should be checked for normal development 24 h after fertilization. Egg batches with less than 50 % fertilization should not be used for testing. Embryos remaining alive and developing normally (**84**) after 24 to 26 h may be used for testing, either as embryos or as yolk-sac larvae (prolarvae). Hatching occurs from 40 to 48 h after incubation at a temperature of 17 to 19°C. The larvae will swim over the lip of the jar into rearing chambers (**86**). Those not able to swim over the lip should be considered weak and not used for testing. Initial larval density in the chambers should not exceed 2000 per L. Water flow through the perforated bottom of the rearing chamber should be sufficient to keep the larvae suspended and water quality good (**85**, **86**). The larvae can be kept in the chambers until initiation of toxicity tests and density should not exceed 100 per L when larvae are 13 days posthatch.

X11.2.5 Embryos may be shipped at 26 h after fertilization provided that they are packaged in insulated containers with oxygen and transport does not exceed 24 h. Larvae have been shipped successfully at less than 1 day or greater than 5 days posthatch when transport does not exceed 12 h.

X11.3 *Early Life-Stage Toxicity Test*—Tests are begun with embryos 12 to 24 h prehatch (see X11.3.1) or with prolarvae up to 9 days posthatch (see X11.3.2). Tests which begin exposure with prolarvae are not entire early life-stage tests as defined in this guide (see 10.2). Tests which begin exposure with prolarvae should be called larval tests as opposed to early life-stage tests. Regardless, a description of when exposure began and ended should be clearly indicated in the test documentation. The test is terminated at day 45. Successful tests have been conducted at 16 to 19°C with a 16-h photoperiod and low light intensity. Dilution waters with a hardness and alkalinity of approximately 120 to 140 mg/L $CaCO_3$ are recommended (**85**, **90**) though other water qualities may be used (**91**). Salinity of 2 to 10 g/kg has been shown to increase survival (**88**, **90**, **91**)

and this can be achieved with the addition of sea water or sea salts.

X11.3.1 *Embryos*—Embryos are exposed beginning 12 to 24 h before hatching. At least 200 embryos should be used in each treatment. Embryos are best transferred to incubation cups using small plastic spoons. Several flow-through incubation cup designs have been used successfully (**90**, **92**). The embryos must be periodically suspended by gentle water currents during incubation for a complete hatch. Most embryos hatch within 48 h after incubation at 18°C. The embryo test should probably be reinitiated if control survival is less than 70 %, 48 h after initiation of the test. Information on the remainder of the test is covered in X11.3.2.

X11.3.2 *Prolarvae*—Prolarvae are best transferred from holding tanks to test compartments with a small cup, moving a few individuals at a time. Density of prolarvae in the compartments should not exceed 25 per L (**90**) and should be thinned to 60 prolarvae per treatment at 9 days posthatch. A compartment (2 per treatment) can be a 3.8-L round glass jar 200 mm deep containing 2.5 L of test solution with a center standpipe covered with 500-µm mesh polypropylene screen and painted black (**90**). Polycarbonate compartments (4 per treatment) with nylon screen covered windows (**92**) and 1.9 L glass jars with a 3.8-cm diameter screen-covered hole drilled in the side to allow the retention of 1 L of test solution (**91**) have also proven satisfactory. The larvae are susceptible to impingement on screens used for water discharge. The compartments are suspended inside glass aquaria. The larvae can be released from the compartments into aquaria at 22 days posthatch; density of larvae in the aquaria should not exceed 4 per L (**90**).

X11.3.2.1 Dead fish should be recorded and removed daily. Live fish should be counted weekly using multiple counts from at least two people. Fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic and grossly abnormal in either swimming behavior or physical appearance should also be counted.

X11.3.2.2 Larvae should be fed a minimum of 3 times daily. The following feeding regime is based on the number of days posthatch (**86**):

1 to 5	No feeding required. Some researchers recommend introducing brine shrimp nauplii at 2 days posthatch for imprinting of larvae on prey.
6 to 10	Brine shrimp nauplii (see Practice E1203) at a minimum density of 10/mL in compartments.
11 to 15	Brine shrimp nauplii at a minimum density of 10/mL in compartment and 1 mg of commercial small mesh food (for example, Biotrainer #1[A]) per fish.
16 to 20	Brine shrimp nauplii at a minimum density of 10/mL in compartment and 2 mg of commercial small mesh food (for example, Biotrainer #1 and Biodiet #2[A]) per fish.
21 to 25	Brine shrimp nauplii at a minimum density of 5/mL and 4 mg of commercial small mesh food (for example, Biodiet #2 and Salmon Starter[B]) per fish.
26 to 45	Commercial small mesh food (for example, Salmon Starter) at 6 to 9 % of expected body weight per fish.

[A]Bioproducts Incorporated, P.O. Box 429, Warrenton, OR 97146.
[B]Nelson-Murray Elevators, 118 W. 4800 South, Murray, UT 84107.

Food density must be maintained to discourage cannibalism. The compartments and aquaria should be cleaned daily by brushing and siphoning to prevent the buildup of nitrogenous wastes from the high feeding density. The diet may be supplemented with additional brine shrimp nauplii if larvae are having difficulty in switching over to dry, commercial food.

X11.3.2.3 Fish should not be fed for the last 24 h prior to termination on day 45. At termination, determine weight of each surviving fish. If the fish exposed to the test material appear to be edematous compared to control fish, both dry weight (dried at 60°C for 24-h) and wet weight of all fish should be determined. Swim bladder inflation may affect the survival and growth of larvae and should be reported (**93**). One method of determining swim bladder inflation is anesthesia with MS-222; fish without swim bladders lie on their sides on the bottom and waves of muscle contractions sweep from head to tail (**94**, **95**, **96**). Another method of determination is microscopic examination.

X11.4 *Test Acceptability*—Striped bass embryos and larvae are difficult to work with. Further, an inherent variability is probably associated with the quality of the gametes which will limit the success of a specific test. An early life-stage test with striped bass is unacceptable if: (*a*) the average overall survival of controls from embryos to 48 h posthatch is less than 70 % with a minimum in any control chamber of 60 %, (*b*) the average overall survival of controls from 48 h (prolarval stage) to 9 days posthatch is less than 60 % with a minimum in any control chamber of 50 %, and (*c*) the average overall survival of controls from 9 days to 45 days posthatch (metamorphosis and test termination) is less than 80 % with a minimum in any control chamber of 70 %.

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