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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.