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Standard Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*[1]

This standard is issued under the fixed designation E1295; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining data concerning the adverse effects of an effluent or a test material (added to dilution water, but not to food) on *Ceriodaphnia dubia* Richard 1894, during continuous exposure throughout a portion of the organism's life. These procedures should also be useful for conducting life cycle toxicity tests with other Cladocera (Guide E1193), although modifications will be necessary.

1.2 These procedures are applicable to most 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, pH, dissolved ions, and on such materials as aqueous effluents (see also Guide E1192), leachates, oils, particulate matter, sediments (see also Guide E1706), and surface waters. Renewal tests might not be applicable to materials that have high oxygen demand, are highly volatile, are rapidly biologically or chemically transformed, or sorb to test chambers. If the concentration of dissolved oxygen falls below 4 mg/L or the concentration of test material decreases by more than 20 % in test solution(s) at any concentration between renewals, more frequent renewals might be necessary.

1.3 Other modifications of these procedures might be justified by special needs or circumstances. Results of tests conducted using unusual procedures are not likely to be comparable to results of many other tests. Comparisons of results obtained using modified and unmodified versions of these procedures might provide useful information on new concepts and procedures for conducting three-brood toxicity tests with *C. dubia.*

1.4 This guide is arranged as follows:

Section

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

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***A Summary of Changes section appears at the end of this standard**

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 8.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D3978 Practice for Algal Growth Potential Testing with *Pseudokirchneriella subcapitata*

D4447 Guide for Disposal of Laboratory Chemicals and Samples

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

E1193 Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

E1733 Guide for Use of Lighting in Laboratory Testing

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[3]

SI10–02 IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

3. Terminology

3.1 The words “must,” “should,” “may,” “can,” and “might” have very specific meanings in this standard. “Must” is used to express an absolute requirement, that is, to state that the test has 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 Section 14). “Should” is used to state that the specified condition is recommended and has to be met in most tests. Although a 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 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 SI10-02SI10–02 IEEE/ASTM SI 10.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *brood, n*—the young neonates released at the time of adult molt by the young/adult animal originally exposed to the control and test solutions.

3.3.1.1 *Discussion*—The number of young in each brood are typically counted over each 24 h period of the test and should increase over the duration of the test. Animals may be transferred to fresh control or test solution before completing the release of a brood, resulting in split broods. Care is needed when interpreting the results to determine the number of broods released during a test.

4. Summary of Guide

4.1 At the beginning of the test, at least ten *C. dubia* less than 24 h old, and within 8 h of age, are introduced individually in separate test chambers, and exposed to control water and a least one (preferably 2 or more) toxicant concentrations. One control treatment must be used but more may be used. Control treatments may include standard laboratory water only, or some combination of standard water(s) and uncontaminated site water, to provide a measure of organism survival and reproduction based on specific test water conditions, such as hardness, alkalinity, and so forth. Specified data on the concentration of test material and the survival and reproduction of *C. dubia* are collected and analyzed to determine the effect of the tested concentration (% effluent or ambient water) on *C. dubia*.

4.2 Table 1(**1**)[4] contains a summary of the conditions used when conducting a three-brood test with *C. dubia*. Table 2 and Section 14 list the requirements that need to be met for a test to be deemed acceptable.

5. Significance and Use

5.1 *Ceriodaphnia* was first used as a toxicity test organism by Mount and Norberg (**2**). Introduced for use in effluent and ambient water evaluations, *Ceriodaphnia* have also been a valuable addition to single chemical test procedures.

5.2 Protection of a population requires prevention of unacceptable effects on the number, weight, health, and uses of the individuals of that species, or species for which the test species serves as a surrogate. A three-brood toxicity test is conducted

[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.

TABLE 1 Test Conditions for Conducting Three-Brood Toxicity Tests with *Ceriodaphnia dubia*

	Test Criteria	Specification
1)	Test Type	Whole effluent, receiving water or reference toxicity test, or both, with static-renewal of test solution.
4)	Test Duration	6–8 days, when 60% of control animals produce 3 broods
3)	Temperature	25°C (± 1°C)
4)	Photoperiod	16 h light: 8 h dark, ambient laboratory light levels
5)	Test Chamber Size	30 mL
6)	Test Solution Volume	15 mL
7)	Renewal of Test Solution	every 24 h
8)	Age of Test Organisms	< 24 h old neonates, within 8 h of same age
9)	No. Organisms/Test Chamber	1
10)	No. Replicate Test Chambers/ treatment	10
11)	Feeding Regime	Feed 0.1 mL each YCT and *Raphidocelis subcapitata*, (formerly known as *Selenastrum capricornutum*)
12)	Test Solution Aeration	None
13)	Dilution Water	Any appropriate water as determined by purpose of test. See Section10 for additional guidance.
14)	Test Chamber Cleaning	Replace or brush and rinse cups between uses.
15)	Test Concentrations	Test Dependent
16)	Biological Variables	Survival and reproduction
17)	Test Acceptability	80 % or greater control survival ≥ 15 young/female in controls

TABLE 2 Test Acceptability Requirements for Three-Brood Toxicity Test with *Ceriodaphnia dubia*

A.	The following performance criteria must be met when conducting a three-brood test with*Ceriodaphnia dubia*. Additional criteria listed in Section 14. 1. All *C. dubia* used in the test must be less than 24 h old (hatched within 8 h window) and from the same brood stock. 2. The average survival of the *C. dubia* exposed in the control sample must be ≥ 80 %. Control treatments may include standard laboratory water only, or some combination of standard water(s) and uncontaminated site water, to provide a measure of organism survival and reproduction based on specific test water conditions, such as hardness, alkalinity, and so forth. A control treatment consists of maintaining organisms in 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 test organisms 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 other treatments the ten organisms are maintained in water to which a selected concentration of test material (percentage of effluent or river, or lake water) has been added. 3. At least 60 % of the control animals must produce 3 broods in 8 days (6–8 days preferred), with the 3 brood average in the control treatment of ≥ 15 young/surviving female. 4. All measured dissolved oxygen measurements be between 4.0 and 8.4 mg/L. 5. All test containers must be the same and must be randomly assigned to the control or test treatment. 6. Test animals should be stocked in test chambers via random block loading design, for example, all A replicates come from the same brood cup, all B replicates come from the same brood cup and so on and so forth. 7. If required, a solvent control treatment must be included with each test.
B.	The performance based criteria for culturing *C. dubia* include: 1. Seven day, three brood reference toxicity testing (12.1) should be performed on a monthly basis for labs routinely conducting three brood tests. For laboratories conducting tests only occasionally, reference toxicant tests should be conducted at least once in association with each period of testing. For laboratories testing only occasionally, it may be desirable for a reference toxicant test to be conducted prior to initiating other testing, as a means to ensure that the testing procedures, facilities, and staff can successfully support conduct of the procedure. If not performed on a monthly, it might be desirable to perform a reference toxicant test concurrently with any 6 day to 8 day 3 brood test with chemicals or environmental samples. If tested concurrently, both tests must use the same lot of test animals and same control/dilution water. 2. Survival and reproduction of the culture animals and food stocks used in culture should be tracked and recorded. Culture restarts should be tracked in this same logbook. 3. Characteristics such as pH, hardness, alkalinity, conductivity, dissolved oxygen and temperature should be recorded for each batch of culture water. 4. Water and food should be routinely analyzed for background contamination. This can include chemical analysis, as well as side-by-side testing of new and old lots of food and water to determine the suitability of the new food and water for use in culture or testing, or both.

to help determine changes in survival and the number of neonates produced that result from exposure to the test material.

5.3 Results of three-brood toxicity tests with *C. dubia* might be used to predict chronic or partial chronic effects on species in field situations as a result of exposure under comparable conditions.

5.4 Results of three-brood toxicity tests with *C. dubia* might be compared with the chronic sensitivities of different species and the chronic toxicities of different materials, and to study the effects of various environmental factors on results of such tests.

5.5 Results of three-brood toxicity tests with *C. dubia* 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 are based on the results of acute toxicity tests, and so the usefulness of the results of a three-brood toxicity test with *C. dubia* might be greatly increased by also reporting the results of an acute toxicity test (see Guides E729 and E1192) conducted under the same conditions. In addition to conducting an acute test with unfed *C. dubia*, it might also be desirable to conduct an acute test in which the organisms are fed the same as in the three-brood test, to see if the presence of that concentration of that food affects the results of the acute test and the acute chronic ratio (see 10.4.1).

5.5.1 A 48 or 96-h EC50 or LC50 can sometimes be obtained from a three-brood toxicity test with a known test material, but often all the concentrations in the test will be below the EC50 or LC50. In addition, it is usually desirable to know the EC50 or LC50 before beginning the three-brood test, as a means to determine the concentrations for use in the chronic test (see 10.4.1). It should be noted that results from an acute test may not necessarily correspond to those of a chronic test, due to the addition of food to the chronic test.

5.6 Three-brood toxicity tests with *C. dubia* might be useful for studying biological availability of, and structure activity relationships between, test materials.

5.7 Results of three-brood toxicity tests with *C. dubia* can vary with temperature, quality and quantity of food, dissolved ion concentrations, quality of the dilution water, condition of the test organisms, and other factors.

5.8 Results of three-brood toxicity tests with *C. dubia* 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.

6. Apparatus

6.1 *Facilities*—Culture and test chambers should be maintained in a constant temperature room, incubator, or recirculating water bath. If dilution water is not prepared batchwise, it is usually piped directly from the source of an elevated headbox so it can be gravity-fed into culture tanks and containers used to prepare test solutions. Strainers and air traps should be included in the water supply system. The head-box should be equipped for temperature control and aeration. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of air through a 0.22 µm bacterial filter might be desirable (**3**). The facility should be well ventilated and free of fumes. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, the culture tanks should not be in a room in which toxicity tests are conducted, stock or test solutions are prepared, effluent or test material is stored, or equipment is cleaned. During culture and testing, organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress. A timing device should be used to provide a 16-h light and 8-h dark photoperiod. A 15- to 30-min transition period (**4**) when lights go on might be desirable to reduce the possibility of organisms being stressed by instantaneous illumination; a transition period when lights go off might also be desirable.

6.1.1 When *C. dubia* are fed algae, a high light intensity might cause sufficient photosynthesis to result in a pH high enough to kill Cladocera (**5**). Thus the maximum acceptable intensity is dependent on the buffer capacity of the dilution water, species and density of algae, and the kind of test chamber and cover. Ambient laboratory light levels will usually be acceptable, but higher intensities might be better or worse, depending on other conditions.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, effluents, test solutions, or any water into which organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that can adversely affect organisms. In addition, equipment and facilities that contact stock solutions, test solutions, or effluents should be chosen to minimize sorption of test materials and components of effluents from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Concrete and rigid plastics may be used for culture tanks and in the water supply system, but they should be soaked, preferably in flowing dilution water, for several days before use (**6**). Cast iron pipe may be used in supply systems, but colloidal iron might be added to the dilution water and strainers will be needed to remove rust particles. Copper, brass, lead, galvanized metal, and natural rubber should not contact dilution water, stock solutions, effluents, or test solutions before or during the test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect either survival, reproduction, or when measured length or weight, or both, of *C. dubia* (see 14.1).

6.3 *Test Chambers:*

6.3.1 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, tubes, cups, and so forth, may be used to create two or more compartments within each chamber if (*a*) first instar *C. dubia* cannot move from one compartment to another, and (*b*) it has been shown that survival and reproduction are the same when only some of the compartments in a chamber contain first-generation organisms (organisms used to initiate a test) as when all the compartments in a chamber contain first-generation organisms. Thus, test solution can flow (chambers are not considered replicates in static tests), 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, will be more similar between compartments in the same test chamber than between compartments in different test chambers in the same treatment.

6.3.2 Many seven-day toxicity tests with *C. dubia* have been conducted with each test organism in a separate 30 mL beaker containing 15 mL of test solution or disposable plastic food quality cups. Any container made of glass, Type 316 stainless steel, or a polystyrene may be used if as long as the composition of the cup does not interact unacceptably with the test material (*a*) each first generation *C. dubia* is in a separate chamber or compartment, and (*b*) each chamber contains sufficient test solution to provide adequate surface area to maintain dissolved oxygen concentrations acceptable to the test organisms (12.2). All chambers (and compartments) in a test must be identical. Chambers should be covered with clean glass, stainless steel, nylon, or fluorocarbon plastic covers or Shimatsu closures, to keep out extraneous contaminants and to limit evaporation of test solution.

6.4 *Cleaning*—Test chambers and equipment used to prepare and store dilution water, stock solutions, effluent, and test solution, should be cleaned before use. The methods used to clean the test containers might depend in part on the material from which they are made. New glass and stainless steel items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid), and at least twice with water that meets the specifications of ASTM Type II (see Specification D1193). Some lots of some organic solvents might leave a film that is insoluble in water. At the end of a 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

ASTM Type II water. Test chambers should be rinsed with dilution water just before use. (**Warning—**Cleaning procedures which use dichromate-sulfuric acid or hypochlorite are discouraged because they are hazardous and might leave residues which might contaminate test solutions.)

7. Reagents and Materials

7.1 *General*—The test material should be reagent grade[5] or better, unless a test on an effluent, a formulation, commercial product, or technical-grade or use-grade material is specifically needed.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D1193.

8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, guidelines for the safe handling and disposal of hazardous materials should be strictly followed (Guide D4447). Skin contact with all test materials, effluents, and solutions of them should be minimized, by wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, glasses, and by using pipets 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 (**7**), recommended handling procedures (**8**), and chemical and physical properties of the test material or effluent should be studied before a test is begun. Special procedures may be necessary with radiolabeled test materials (**9**) and with materials that are, or are suspected of being, carcinogenic (**10**).

8.2 Although disposal of stock solutions, test solutions, effluents, 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 materials or effluents might be desirable before disposal of solutions.

8.3 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.

8.4 Acidic solutions and hypochlorite solutions should not be mixed because hazardous fumes might be produced.

8.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and mixing concentrated acid with water should be performed only in a fume hood.

8.6 Because dilution water and test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help prevent electrical shocks.

9. Dilution Water

9.1 *Requirements*—Besides being available in adequate supply, dilution water should (*a*) be acceptable to *C. dubia*, (*b*) not affect results of the test, and (*c*) be of uniform characteristics. In effluent testing, upstream dilution water might be toxic. If the objective of the effluent test is to determine the toxicity of the effluent independent of the upstream water, a reconstituted water of similar hardness, alkalinity and pH may be used as the dilution water. However, use of a reconstituted water will not only remove the confounding results of upstream toxicity, but also other factors (suspended solids, humic acids, and so forth) that might otherwise act to reduce or increase the toxicity of the effluent.

9.1.1 The dilution water must allow satisfactory survival (80 % or greater in the cultured animals) and reproduction (at least 15 live young/surviving female animals) of *C. dubia* (see 14.1 *d, e*, and *f*). However, acceptable dilution water typically will produce >90 % survival and >30 live young/surviving female during a 6 day-8 day test using healthy cultures.

9.1.2 The characteristics of the dilution water should be consistent over time so that brood stock is cultured, and the test conducted, in water of the same characteristics. In tests to evaluate the toxicity of ambient waters, additional controls should be considered using acceptable quality dilution water (see 9.1.1) with similar chemical characteristics (for example, pH, hardness, and alkalinity).

9.1.3 The characteristics of the dilution water should be consistent during the test. The range of hardness during the test should be less than 5 mg/L or 10 % of the average, whichever is higher. In effluent testing where upstream water is used as dilution water the variance associated with hardness might naturally exceed these ranges.

9.1.4 If it is desired to study the effect of an environmental factor such as total organic carbon (TOC), particulate matter, dissolved ion concentrations, or dissolved oxygen on the results of a three-brood test with *C. dubia*, it will be necessary to use a water that is naturally or artificially high or low in that environmental factor. 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.

9.2 *Source:*

9.2.1 If a natural fresh water is used it should be obtained from an uncontaminated source of consistent characteristics. A well or spring that has been shown to be of acceptable characteristics is usually preferable to a surface water. If a surface water is used, the intake should be positioned to minimize fluctuations in characteristics and the possibility of contamination, and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. Surface waters should be filtered (60-µm mesh) to remove potential predators and competitors of *C. dubia*.

[5] *Reagent Chemicals, American Chemical Society Specifications*, American. Chemical Society., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals* , BDH Ltd., Poole, Dorset,U.K. and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC0, Rockville, M.D.

9.2.2 Widespread use of one reconstituted water will increase comparability of test results. The reconstituted fresh water described in Guide E729 has been used successfully by several people. Addition of 5 µg of selenium **(11)** and 1 µg of crystalline vitamin B_{12}/L **(12)** might be desirable, (but see X1.1). *C. dubia* has also been cultured and tested in reconstituted soft water. Acclimation in one reconstituted water and testing in another of different hardness or alkalinity should be avoided to minimize stress due to routine water quality changes.

9.2.3 Chlorinated water should not be used as, or in the preparation of, dilution water because residual chlorine is quite toxic to Cladocera **(13)**. 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 **(14)**. Some organic chloramines, however, react slowly with sodium bisulfite **(15)**. 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 reduced with a chelating resin **(16)**, but use of an alternative dilution water might be preferable.

9.3 *Treatment:*

9.3.1 Dilution water should be aerated gently by such means as air stones, surface aerators, or column aerators, **(17, 18)** prior to addition of test material. Adequate aeration will bring the pH and concentrations of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 % and 110 % of saturation **(19)** to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation by dissolved gases that can be caused by heating the dilution water should be avoided **(20)**.

9.3.2 Filtration through sand, rock, bag, or depth type cartridge filters may be used to keep the concentration of particulate matter acceptably low (see 9.2.1), and as a pretreatment before ultraviolet sterilization or filtration through a finer filter.

9.3.3 Dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer **(21)** equipped with an intensity meter and flow controls, passed through a membrane filter with a pore size of 0.20 µm, or autoclaved. Water that might be contaminated with *Aphanomyces daphniae* should be autoclaved **(3)**.

9.4 *Characterization*—The following items should be measured in the dilution water 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: hardness, alkalinity, conductivity, pH, particulate matter, total dissolved solids, total suspended solids, TOC, selected pesticides (such as those found in USGS Schedules 2001/2010), organic chlorine, PCBs, phthalate esters, ammonia, cyanide, sulfide, chloride, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, sodium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc. For each method used (see 13.3), the detection limit should be below either (*a*) the concentration in the dilution water, or (*b*) the lowest concentration that has been shown to affect adversely the survival, and reproduction of *C. dubia*.

9.5 *Control water*—Dilution water can be analogous to the laboratory control water treatment to which no test material has been added. The control provides *(a)* a measure of the acceptability of the test by giving an indication of the quality of the test organisms and the suitability of the dilution water, food, test conditions, handling procedures, and so forth, and *(b)* the basis for interpreting data obtained from the other treatments.

9.5.1 Additional controls, also referred to as secondary controls, can be useful for distinguishing effects due to a test material from the water in which samples are tested. Surface water samples or tested aqueous samples can contain ion concentrations or water quality characteristics that are above or below the tolerance range of *C. dubia* **(22, 23, 24)**. Including a secondary control that reflects the sample water quality and ion concentrations can identify if effects to a *C. dubia* endpoint are due to the test material or toxicant alone or if effects are also associated with the background water quality of the sample.

10. Test Material

10.1 Before a test is begun with material other than effluents, the following should be known about the test material:

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

10.1.2 Solubility and stability in the dilution water.

10.1.3 An estimate of the lowest concentration of test material that is acutely toxic to *C. dubia*.

10.1.4 Accuracy and precision of the analytical method at planned test concentration(s).

10.1.5 Estimate of toxicity to humans and recommended handling procedures (see 8.1).

10.2 *Stock Solution:*

10.2.1 In some cases the test material can be added directly to dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

10.2.2 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 of the dilution water will not be affected, distilled and deionized water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(25)**. Minimum amounts of strong acids or bases may be used in the preparation of aqueous stock solutions, but such reagents might affect the pH of test solutions appreciably. Use of a more

soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more or less than use of the minimum amounts of strong acids and bases.

10.2.3 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 survival or reproduction of *C. dubia* (and length or weight, or both, if these characteristics are to be measured). Because of its low toxicity to aquatic animals **(26)**, 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 growth of microorganisms and besides, acetone is quite volatile. If an organic solvent is used, it should be reagent grade or better.[5] 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 test solutions.

10.2.4 If a solvent other than water is used, (*a*) at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test and (*b*) a dilution water control, must be included in the test. If no solvent other than water is used, a dilution water control must be included in the test.

10.2.4.1 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.

10.2.4.2 If the concentration of solvent is not the same in all test solutions that contain test material, either (*a*) a solvent test must be conducted to determine whether the survival, or reproduction of *C. dubia* is related to the concentration of the solvent over the range used in the toxicity test, or (*b*) such a solvent test must have already been conducted using the same type of dilution water and the same source of *C. dubia*. If either survival or reproduction is found to be related to the concentration of solvent, a three-brood toxicity test with *C. dubia* in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival or reproduction is found to be related to the concentration of solvent, a three-brood toxicity test with *C. dubia* in that water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

10.2.4.3 If the test contains both a dilution water control and a solvent control, the survival, and reproduction of *C. dubia* in the controls should be compared (see X4.7 and Guide E1847). If a statistically significant difference in either survival or reproduction, is detected between the two controls, only the solvent control may be used for meeting the requirements of 14.1 *c, d*, and *e* as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of 14.1 *c, d*, and *e* as the basis for calculation of results. Likewise, a secondary control to account for background water quality (for example, high or low TOC or dissolved ions) may be appropriate and would function as does the solvent control described here.

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

10.3 *Effluent:*

10.3.1 *Sampling Point*—The effluent sampling point should be based on the purpose of the test. The collection point for the National Pollutant Discharge Elimination System (NPDES) permit testing purposes is often strictly defined. In some cases, a sampling point between last treatment and the discharge point might provide much better access. If the waste is chlorinated, it might be desirable to have sampling points both upstream and downstream of the chlorine contact point to determine the toxicity of both chlorinated and unchlorinated effluent. The schedule of effluent sampling should be based on an understanding of the short- and long-term operations and schedules of the discharger. Although it is usually desirable to evaluate an effluent sample that most closely represents the normal or typical discharge, conducting tests on atypical samples might also be informative.

10.4 *Test Concentration(s):*

10.4.1 If the test is intended to provide a good estimate of the highest concentration of test material or effluent that will not unacceptably affect the survival, and reproduction of *C. dubia*, the test concentrations (see 12.12.2) should bracket the best prediction of that concentration. Such a prediction is usually based on the results of a 48-h static-acute toxicity test (see Guide E729) on the test material using the same dilution water and *C. dubia* less than 24-h old. Because the food used in a three-brood toxicity test sometimes affects the results of the acute test **(27)**, the acute test should be conducted with and without the food added to the dilution water. If an acute chronic ratio has been determined for the test material with a species of comparable sensitivity, the results of the acute test with *C. dubia* can be divided by the acute-chronic ratio to predict an appropriate range of concentrations for the chronic test.

10.4.2 In some (usually regulatory) situations, it is only necessary to determine whether one specific concentration of test material or effluent unacceptably affects survival or reproduction. For example, the specific concentration might be the concentration occurring in a receiving water, the concentration resulting from the direct application of a material to a body of water, or the solubility limit of a material in water. When there is interest only in a specific concentration, it is often necessary to test only that specific concentration (see 12.2.1.3). However, use of multiple concentrations will provide data useful for determining toxicity thresholds and evaluating the concentration response relationship **(28)**.

10.5 *Collection:*

10.5.1 Several different methods may be used to collect effluent samples for toxicity tests. Selection of a method should be based on the type of test that is to be conducted, the characteristics of the effluent, any treatment technologies

employed, the rate and manner by which the effluent is discharged into the receiving water, and the average wastewater retention time. Industrial or municipal facilities occasionally discharge directly, with no provision for effluent retention. In the more typical situation, however, holding and treatment ponds provide some duration of effluent retention. The retention time should be measured because channeling sometimes causes the average retention time to be substantially less than the calculated or design retention time.

10.5.2 It is recommended that renewal toxicity tests be conducted on effluent obtained by the following methods:

10.5.2.1 If the average retention time of the effluent is less than 24 h, a 24-h composite sample should be collected daily, diluted appropriately, and used for daily renewals (see 10.5.2.3).

10.5.2.2 If the average retention time is greater than 14 days, a grab sample should be collected daily, diluted appropriately, and used for daily renewals. If the average retention time is greater than 24h and less than 14 days, either composite or grab sampling can be used to collect effluent samples.

10.5.2.3 If an effluent is known, or suspected, of being highly variable in terms of constituents and retention time is less than 24 h, grab samples might be more representative of toxicity potential. In addition, more frequent renewal intervals might be desirable.

10.5.2.4 In most cases composite or grab sampling as described will be suitable. It is recommended that provisions be made for cooling samples to 4°C during the collection of composite samples. In some cases, flow-proportional sampling might be desirable. Such situations will be governed by the effect of flow variation on the retention time of the effluent, and in turn, the effect of altered retention time on loss of components of the effluent. Generally, losses will occur either (*a*) in a treatment basin, or (*b*) due to hydrolysis or other naturally occurring phenomenon. Flow-proportional sampling, therefore, is recommended only when the variation in flow has a substantial effect relative to these factors. Other sampling techniques are described in detail by Shelly **(29)**.

10.6 *Sample Containers*—Samples should be collected and stored in containers appropriate for the effluent or toxicant sample of concern. Samples containing dissolved metals should be collected and stored in plastic containers, due to the potential of absorption of the dissolved metals to glass. **(30)**

10.7 *Preservation*—If samples are not used within 2 h of collection, they should be preserved by storing them in the dark at about 4°C. Storage time is in part dependent on effluent type but should not exceed 72 h. A sample storage time of ≤36h should be used if logistically feasible.

10.8 *Treatment*—The sample of effluent must not be altered except that it may be filtered through an 80-µm sieve or screen (60-µm preferred) to remove potential predators. Undissolved materials should be uniformly dispersed by gentle agitation immediately before any sub-sample of effluent is drawn for solution preparation and before test solutions are distributed to test chambers. For hydrophobic chemicals, studies have shown that vigorous agitation of at least one minute will desorb them.

11. Test Organisms

11.1 *Species*—The genus *Ceriodaphnia* went through a revision. Berner **(31)** investigated the taxonomy of *Ceriodaphnia* in U.S. EPA cultures and based on this study the early published reference in toxicological literature to *C. dubia/affinis* was most likely *C. dubia*. Identification of the species employed in testing is the responsibility of the reporting investigator.

11.2 *Age*—Three-brood toxicity tests with *C. dubia* should be started with organisms less than 24 h old. Using neonates born within a narrow age range, for example, less than 24 h old and born within 8 h of each other is required.

11.3 *Source*—All organisms used in a test must be from the same brood stock. The two (and preferably five) prior generations must have been raised from birth using the same food, type of water, and temperature as will be used in the three-brood test. This will not only acclimate the organisms, but will also help demonstrate the acceptability of the food, water, and so forth, before the test. Acclimation of organisms for effluent tests in which natural dilution waters are used might be difficult to achieve. In some cases available (upstream) dilution water might be toxic and an alternative dilution water will have to be employed in those cases in which effluent toxicity independent of ambient water toxicity is the testing objective. When a lab cannot raise organisms in the dilution water, a concurrent performance control should be included (see 9.5).

11.4 *Brood Stock:*

11.4.1 *C. dubia* is generally available from government, academic, and private laboratories, engaged in toxicity testing. Brood stock can be obtained from another laboratory or a commercial source. When organisms are brought into the laboratory, the water in which they were transported should be gradually replaced with new dilution water over a period of two or more days. The water temperature should be changed at a rate not to exceed 3°C within 12 h until the desired temperature is reached.

11.4.2 *C. dubia* has been cultured in a variety of systems, such as in large groups of aquaria, in smaller mass cultures, and individually in a variety of smaller chambers. Use of individual cultures allows the survival and reproduction of specific animals to be tracked. This provides a means to measure the health of individual animals and to determine the suitability of the animals for use in chronic tests. All culture productivity should be tracked and the results recorded in a culture laboratory notebook.

11.4.3 Brood stock should be cultured so they are not unnecessarily stressed. To maintain *C. dubia* in good condition and avoid unnecessary stress, crowding and rapid changes in temperature or water quality should be avoided. In general, organisms should not be subjected to more than a 3°C change in water temperature in any 12-h period, and preferably not more than 3°C in 72 h. Cultures should be regularly fed enough food to support adequate reproduction. Culture chambers should be cleaned periodically to remove feces, debris, and uneaten food, or replaced. If culture chambers are properly cleaned and the density of organisms is no more than 1 to 2

brood/adult organisms/15 mL, surface aeration should provide adequate dissolved oxygen.

11.5 *Food*—Various combinations (see Appendix X1) of trout chow, flake food, yeast, rye grass powder, cereal leaves, alfalfa, and algae **(32)** such as *Ankistrodesmus convolutus, A. falcatus, Chlamydomonas reinhardtii*, and *Selenastrum capricornutum* (also known as *Raphidocelis subcapitata* **(33)**, have been successfully used for culturing and testing *C. dubia*. The food should be analyzed for the test material, if it might be present, as well as for possible contaminants as described in 9.4 for dilution water.

11.6 *Handling—C. dubia* should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the organisms are not unnecessarily stressed. Organisms should be introduced into solutions beneath the air water interface. Organisms that touch dry surfaces or are dropped or injured during handling should be discarded. Smooth glass pipettes or tubes with an inside diameter of at least 3 mm should be used for transferring adult *C. dubia*, and the amount of solution carry over should be minimized. Equipment used to handle organisms should be sterilized between exposures by autoclaving or by treatment with an iodophor **(34)**.

11.7 *Quality*—To increase the chances of a test being acceptable (see 14.1), the test should not be begun with young that were in the first or second brood from *C. dubia* brood stock nor with young from *C. dubia* that (*a*) appear diseased or stressed **(3, 35)** or incompletely developed, (*b*) did not produce at least 8 young in the previous brood, or (*c*) are from a culture in which ephippia was produced or in which substantial mortality occurred during the week prior to the test. Organisms used to initiate a test should be able to survive, without food, for a minimum of 48 h in the appropriate dilution water. If the dilution water might contain food it might be desirable to filter it through a 0.22-µm filter to ensure removal of potential food.

12. Procedure

12.1 *Reference Toxicant Testing*—Reference toxicant tests should be conducted monthly in laboratories conducting tests routinely; for laboratories conducting tests only occasionally, reference toxicant tests should be conducted at least once in association with each period of testing. For laboratories testing only occasionally, it may be desirable for a reference toxicant test to be conducted prior to initiating other testing, as a means to insure that the testing procedures facilities, and staff can successfully support conduct of the procedure. Reference toxicity testing will help to determine (*a*) whether *C. dubia* will survive, and reproduce acceptably (see 14.1 *d, e*, and *f*) in the facilities, (*b*) whether the food, water, handling procedures, and so forth, are acceptable, (*c*) whether there are any location effects on survival, and reproduction, and (*d*) to evaluate the magnitude of the within and between chamber variances. (See Table 1.) A failed reference toxicant test would result from young per female or survival criteria not being met in the control (See Table 2). An endpoint in the reference toxicity test (for example, the EC25 or LC50) well outside of the normal expected range may indicate a problem with test organisms causing them to be more sensitive or less sensitive than is typical. Organism health should be evaluated by a review of culture records and neonate production and corrective actions may be needed if more than 1 in 20 reference toxicity test results is more than 2 or 3 standard deviations from the long-term running average from the past 20 tests.

12.2 *Experimental Design:*

12.2.1 Decisions concerning experimental design, such as number of treatments, dilution factor, and numbers of test chambers and organisms per treatment, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see 15.1). One of the following two types of experimental designs will probably be appropriate in most cases.

12.2.1.1 A three-brood toxicity test intended to allow calculation of an end point (see X4.3) usually consists of one or more control treatments and a geometric series of at least five concentrations of test material or effluent. In the dilution water or solvent control(s), or both, organisms are exposed to dilution water to which no test material has been added. In tests on effluents, a performance control (one group of 10 replicates in dilution water normally used to culture the organisms in the laboratory) is included in the experimental design. Results from these replicates help ensure, especially in those cases in which the organisms have been transported to a testing site, that the organisms' survival and reproduction are comparable to results routinely obtained in the laboratory.

12.2.1.2 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.

12.2.1.3 If it is necessary only to determine whether a specified concentration causes adverse effects (see 10.4.2), only that concentration and the control(s) are necessary. Two additional concentrations at about one-half and two times the specified concentration might be desirable to increase confidence in the results. It can be helpful to evaluate the concentration-response relationship when a serial dilution or multiple concentrations of a substance are tested. A serial dilution indicating a nonmonotonic relationship may indicate a problem with the test that can compromise the interpretation of some statistics and/or indicate the presence of pathogens affecting the test results **(28)**.

12.2.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, that is defined as the smallest physical entity to which treatments can be independently assigned **(36)**. Because test solution can flow from one compartment to another, but not from one test chamber to another (see 6.3.1), 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, generally the width of the confidence interval on a point estimate decreases and the power of a hypothesis test increases. With respect to

factors that might affect results within test chambers and, therefore, the results of the test, all test chambers in a test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations. A randomized block design (with each treatment being present in each block, which may be a row or rectangle) is preferable to a completely randomized design. When using volatile toxicants, a randomized block (or completely randomized) design creates some potential for cross-contamination; however, because of the importance of randomization to avoid unintended bias, it is generally better to control cross-contamination potential through other means (such as increased airflow) than to fail to have randomization.

12.2.3 The effect of the test material or effluent on survival, growth, and reproduction cannot be determined if any factors that affect them are too dissimilar between experimental units. Because survival, growth, and reproduction might be affected by the number of first- and second-generation organisms in the chamber or compartment, or the concentration or amount of available food, the best experimental design is to physically separate each first-generation daphnid (that is, place each first-generation daphnid in a separate test chamber or in a separate compartment within a test chamber), remove young daily, and feed each first-generation daphnid daily. Although increasing the number of test chambers per treatment and increasing the number of separated organisms per treatment both improve the experimental design, statistically the best use of any specific number of test organisms is to place each one in a separate chamber.

12.2.4 The minimum desirable number of test chambers and individual organisms per treatment should be calculated from (*a*) the expected variance within test chambers (*b*) the expected variance between test chambers within a treatment and, (*c*) either the minimum difference that is desired to be detectable using hypothesis testing, or the maximum acceptable confidence interval on a point estimate **(37)**. If such calculations are not made, at least two test chambers and ten physically separated individual organisms should be in each treatment (test concentration and control). Testing fewer replicates should only be pursued with consideration of the potential impact on statistical power (see Guide E1847). Replicate test chambers (that is, experimental units) are necessary in order to allow estimation of experimental error **(35)**. If (*a*), more than five concentrations of test material or effluent are tested, and (*b*), each test concentration is more than 50 % of the next higher one and (*c*), the data are to be analyzed using regression analysis, fewer 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 organisms for each of the control treatment(s) than for each of the other treatments.

12.3 *Dissolved Oxygen and Test Material Concentration*—The concentration of dissolved oxygen in each test chamber must be between 4.0 mg/L and 8.4 mg/L (100 % of saturation at 25°C) **(19)** at all times during the test and the time-weighted average measured concentration for each test chamber from the beginning to the end of the test must be between 4.2 mg/L and 8.4 mg/L. If the concentration of dissolved oxygen falls below 4.0 mg/L or the concentration of test material decreases by more than 20 % in test solution(s) between renewals, more frequent renewals might be necessary. Under some circumstances the concentration of dissolved oxygen in natural waters can be greater than 100 % of saturation. The tests should be run under the conditions that exist unless such conditions interfere with the objectives of the test. Because results are generally based (effluents are obvious exceptions) on measured rather than calculated concentrations of test material, the loss of a minimal amount of test material (less than 10 %) by aeration is not necessarily detrimental and test solutions may be aerated gently during the test. Turbulence, however, should be avoided because it might stress organisms, resuspend fecal matter, and greatly increase volatilization. Because aeration readily occurs at the surface, efficient aeration can be achieved with minimum turbulence by using an air lift to transfer solution from the bottom to the surface. Aeration should be the same in all test chambers, including the control(s), throughout the test.

12.4 *Temperature:*

12.4.1 Reproduction in *C. dubia* is in large part a function of temperature, quality of dilution water, and quantity and quality of food. Typically, three-broods can be obtained in 7 days or less if the test is conducted at 25 °C.

12.4.2 In lieu of measuring temperature in individual test chambers at a frequency that might jeopardize the health of the test organism, the relationship between test chamber temperature and constant temperature bath, incubator, or room may be established. Temperature in the constant temperature bath, incubator, or room should ensure that the temperature of the test solutions are within ±1°C of the selected temperature. The use of small diameter temperature probes makes it possible to safely measure the temperature of the initial and final test solutions in randomly selected test cups.

12.5 *Preparing Test Solutions:*

12.5.1 To ensure that all treatments receive the same dilution water, the batch of dilution water should be large enough to fill all the test chambers and the control(s) during the 6 day-8 day test and to perform chemical analysis. To ensure that all treatments and control(s) receive the same food, enough food should be prepared for the entire test period.

12.5.2 The measured concentrations of test material in a test solution should not differ by greater than 30 % from the nominal test concentrations. If the difference is more than 30 %, the cause should be identified. If the concentration in the test chamber is too high, the stock solution or test solution might have been prepared incorrectly or evaporation of the test solution might have occurred. If the concentration is too low, additional possible causes are microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization. If it is likely that the organisms are being exposed to substantial concentrations of one or more reaction or degradation products, measurement of the product(s) is desirable. It might also be desirable to renew the test solutions more often.

12.6 *Conditioning Test Chambers*—Test chambers should be conditioned if the concentration of test material in a

chamber decreases by more than 20 % between renewals and the decrease can be attributed to sorption onto the test chamber. To condition the chambers, dilution water containing test material, but not food, should be placed in each test chamber 24 to 72 h before the test is to begin and before each renewal. This may help to alleviate the sorption of some materials, but not all.

12.7 *Beginning a Test:*

12.7.1 The test chambers should be conditioned if necessary.

12.7.2 Fresh test solutions containing appropriate amounts of test material and food should be prepared less than 4 h before the test is to begin.

12.7.3 Fresh test solution should be placed in each chamber.

12.7.4 The test begins when one *C. dubia* less than 24-h old, and within 8-h of age is placed in each test chamber, or compartment, that already contains test solution. The organisms must be either assigned and identified as cohorts. In the cohort procedure one neonate from a female is assigned to one test chamber or compartment of each treatment and the cohort of each first-generation organism is tracked throughout the test. The cohort procedure might be especially useful with *C. dubia* because some cohorts may produce no young in any treatment. Deletion of data for all individuals in such cohorts from all treatments is a valid way of analyzing the data. This allows the investigator to track the performance of young from each female used.

12.8 *Renewing Test Solutions*—The frequency with which test solutions should be renewed is dependent on several factors (see 10.5.2). The most significant factor is related to the rate of change of the test solutions and how this change might influence results. Solutions that change rapidly might not be effectively tested using renewal techniques. Renewing test solutions at 24-h intervals is acceptable. The minimum acceptable renewal frequency is every other day. At each renewal each first-generation *C. dubia* should be recorded as alive or dead and each live one should be transferred to a chamber containing the same concentration of test material or effluent as that from which it was removed. The live and dead offspring from each first-generation *C. dubia* should be separately counted, recorded, and discarded. If being resued, the chambers from which the first-generation *C. dubia* were removed and the young counted, should be emptied, brushed or washed to loosen debris, and rinsed with ASTM Type II water (see Specification D1193) or dilution water. It is also acceptable to have two sets of chambers to be alternated back and forth with each renewal.

12.9 *Duration of Test*—A test begins when less than 24-h old neonates (all preferably within an 8 h age window) are first placed in test solutions. At 25 °C, control organisms should produce three broods in 6 days–8 days. When less than 60 % of the control animals produce less than three broods in 7 days, the test should be continued for an additional day unless some obvious factors (presence of males, or nonreproducing females) suggest that doing so will not increase the quality of the data collected. At temperatures less than 25 °C, time to third brood production will be increased. A test is considered unacceptable when less than 60 % of the control animals fail to produce three broods over the 6 day–8 day test duration.

12.10 *Biological Data:*

12.10.1 The date of immobility or death of each first-generation *C. dubia* must be recorded. The criteria for immobility are lack of movement and lack of response to gentle prodding.

12.10.2 At each renewal the number of neonates produced by each first-generation *C. dubia* in each brood must be recorded.

12.10.3 Both first- and second-generation organisms should be carefully observed during the test for abnormal development or behavior, such as uncoordinated swimming. Although developmental and behavioral effects are often difficult to quantify and might not provide suitable end points, they might be useful for interpreting effects on survival, growth, and reproduction. Morphological examination of first-generation organisms alive at the end of the test, in each treatment, might be desirable.

12.11 *Other Measurements:*

12.11.1 *Water Quality*—Hardness, alkalinity, pH, ammonia, and conductivity should, at a minimum, be measured at the beginning and end of the test. Measurements using electrodes should not be made in chambers containing organisms; instead, such measurements should be made on old test solutions after the adult has been transferred and the offspring counted. Measurements on old test solutions might require a composite from replicate test chambers of the same test concentration. Alkalinity and pH should also be measured in the highest test concentration at least once in new and old test solutions to determine whether these are affected by the test material. Measurements on new test solutions may be performed on the solution prior to its distribution into the test chambers. Dissolved oxygen concentrations should be measured in old test solutions from the control(s) and low, medium, and high concentrations of test material near the beginning, middle, and end of the test. Dissolved oxygen can be measured on pooled samples, although it is preferable to make individual measurements. For effluents that might have high oxygen demands, dissolved oxygen should be measured at the beginning and end of each renewal period. Measurement of calcium, magnesium, sodium, potassium, chloride, sulfate, particulate matter, and TOC or chemical oxygen demand, (COD) may be desirable.

12.11.2 Temperature should be monitored throughout the test and reported with the test results; a once a day measurement of the test temperature is not sufficient and a 24 h cycle of temperature must be reported. If the test chambers are in a water bath, a constant temperature room, or incubator, measurement or monitoring the temperature at least hourly, or daily measurement of the maximum and minimum temperature, may be made. However, measuring temperature in this manner does not preclude the necessity of documenting the relationship of temperature in the test chambers and that of the constant temperature bath, incubator, or room.

12.11.3 Ammonia should be measured in any test of an effluent or biologically active sample. The concentration of unionized ammonia may be calculated from pH, temperature, and concentration of total ammonia (**38**).