



Designation: E 1295 – 01

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

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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 E 1193), 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, and on such materials as aqueous effluents (see also Guide E 1192), leachates, oils, particulate matter, sediments (see also Guide E 1383), 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) 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 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 appro-*

priate safety and health practices and determine the applicability of regulatory limitations prior to use. Specific hazard statements are given in Section 8.

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



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2. Referenced Documents

2.1 ASTM Standards:²

- D 1193** Specification for Reagent Water
- D 3978** Practice for Algal Growth Potential Testing with *Selenastrum Capricornutum*
- E 380** Practice for Use of the International System of Units (SI) (the Modernized Metric System)
- E 729** Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians
- E 943** Terminology Relating to Biological Effects and Environmental Fate
- E 1023** Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
- E 1192** Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
- E 1193** Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*
- E 1383** Guide for Collecting Sediment Toxicity Test with Fresh Water Invertebrates
- E 1706** Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates

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 A brood refers, collectively, to the young neonates released at the time of adult molt by the young/adult animal originally exposed to the control and test solutions. The number of young in each brood should increase over the period 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.

3.3 For definitions of other terms used in this standard, refer to Guide **E 729**, Terminology **E 943**, and Guide **E 1023**. For an explanation of units and symbols, refer to Practice **E 380**.

4. Summary of Guide

4.1 At the beginning of the test, at least ten *C. dubia* less than 24-h old are maintained individually in separate test chambers (or in separate compartments in two or more test chambers), exposed to control water and one (preferably 2 or more) toxicant concentrations. One or more control treatments 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. 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. 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)**³ 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 (4). 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 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.

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 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 ($\pm$ 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 or 48 hours
8)	Age of Test Organisms	< 24h old neonates, within 8–12h of same age
9)	No. Organisms/Test Chamber	1
10)	No. Replicate Test Chambers/treatment	10
11)	Feeding Regime	Feed 0.1 mL each natural food and synthetic food
12)	Test Solution Aeration	None
13)	Dilution Water	Any appropriate water as determined by purpose of test. See Section 10 for additional guidance.
14)	Test Chamber Cleaning	Brush and rinse cups between uses.
15)	Test Concentrates	Test Dependent
16)	Biological Variables	Survival and reproduction
17)	Test Acceptability	90% or greater control survival $\geq$ 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 <i>Ceriodaphnia dubia</i> . Additional criteria listed in Section 14.
	1. All <i>C. dubia</i> used in the test must be less than 24-h old and from the same broodstock.
	2. The average survival of the <i>C. dubia</i> exposed in the control sample must be $\geq$ 90%.
	3. At least 60% of the control animals must produce 3 broods in 8 days (7 days preferred), with the 3 brood average $\geq$ 15 young/female.
	4. All measured dissolved oxygen reading must 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 must be randomly assigned to a control or test treatment replicate test chamber.
	7. If required, a solvent control treatment must be included with each test.
B.	The performance based criteria for culturing <i>C. dubia</i> include:
	1. Seven day, three brood reference toxicity testing should be performed on a monthly basis. If not performed on a monthly, it might be desirable to perform a reference toxicant test concurrently with any 7 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 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.

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 E 729 and E 1192) 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, 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 E 1023), 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 μ m bacterial filter might be desirable (5). 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 (6) 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 (7). 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 (8). 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, 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 fluorocarbon plastic may be used if (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 glass, stainless steel, nylon, or fluorocarbon plastic covers or Shimatsu closures, to keep out extraneous contaminants and to reduce 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 D 1193). 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 grade⁴ 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 D 1193.

8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, 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 (9), recommended handling procedures (10), and chemical and physical properties of the test material or effluent should be studied before a test is begun. Special procedures may be necessary

⁴ *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. (USPCO, Rockville, M.D.).

with radiolabeled test materials (11) and with materials that are, or are suspected of being, carcinogenic (12).

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, the dilution water should (a) be acceptable to *C. dubia*, (b) not unnecessarily 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 (90 % or greater in the cultured animals) and reproduction (at least 15 young/surviving female animals) of *C. dubia* (see 14.1 d, e, and f).

9.1.2 The characteristics of the dilution water should be uniform 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 uniform 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 values.

9.1.4 If it is desired to study the effect of an environmental factor such as total organic carbon, (TOC), particulate matter, or dissolved oxygen on the results of a three-brood test with *C. dubia*, 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.

9.2 Source:

9.2.1 If a natural fresh water is used it should be obtained from an uncontaminated source of uniform 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- μ m mesh) to remove potential predators and competitors of *C. dubia*.

9.2.2 Widespread use of one reconstituted water will increase comparability of test results. The reconstituted fresh water described in Guide E 729 has been used successfully by several people. Addition of 5 μ g of selenium (13) and 1 μ g of crystalline vitamin B₁₂/L (14) 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 (15). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite, and both are more reliable than carbon filters, especially for removing chloramines (16). Some organic chloramines, however, react slowly with sodium bisulfite (17). In addition to residual chlorine, municipal drinking water often contains unacceptably high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. Excessive concentrations of most metals can usually be reduced with a chelating resin (18), but use of an alternative dilution water might be preferable.

9.3 Treatment:

9.3.1 Dilution water should be aerated intensively by such means as air stones, surface aerators, or column aerators, (19, 20) 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 100 % of saturation (21) 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 (22).

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 (23) 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 (5).

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*.

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 (24). 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 (25), 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.⁴ 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 two controls should be compared (see X4.6). 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.

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:

