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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 E 1295; 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 E 1193), although modifications will be necessary. Tests of effluents present particular challenges relative to dilution water, acclimation, etc.

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, 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 40 % of saturation 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 appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 8.

1.5 This guide is arranged as follows:

[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01on Aquatic Toxicology.

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Culture Techniques	X2
Test Chambers	X3
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2. Referenced Documents

2.1 *ASTM Standards:*

D 1193 Specification for Reagent Water[2]

D 3978 Practice for Algal Growth Potential Testing with *Selenastrum Capricornutum*[3]

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[4]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[3]

E 943 Terminology Relating to Biological Effects and Environmental Fate[3]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[3]

E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*[3]

3. Terminology

3.1 The words "must," "should,"" may," "can," and "might" have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought 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 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 In each of two or more treatments, at least ten organisms less than 12-h old at the beginning of the test, *C. dubia* are maintained individually in separate test chambers (or in separate compartments in two or more test chambers). One or more additional groups of ten less than 12-h old *C. dubia* serve as control treatments, that 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, etc., 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*.

5. Significance and Use

5.1 *Ceriodaphnia* was first used as a toxicity test organism by Mount and Norberg **(1)**.[5] Introduced for use in effluent and ambient water evaluations, the organism with adequate methods development should be 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 what changes in the number of neonates produced, survival, and perhaps length or weight, or both, of individuals of the test species result from exposure to the test material.

5.3 Results of three-brood toxicity tests with *C. dubia* might be used to predict 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 used to compare 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 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 (see 9.3). However, in the three-brood ambient water and effluent tests it is not unusual to observe acute toxicity.

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

[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 11.05.

[4] *Annual Book of ASTM Standards*, Vol 14.02; excerpts in gray pages of Vol 11.04.

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

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 µm bacterial filter might be desirable **(2)**. 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. A15-to 30-min transition period **(3)** 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 **(4)**. Thus the maximum acceptable intensity is dependent on the buffer capacity of the dilution water, species and density of algae, and kind of test chamber and cover. Intensities up to 600 lx 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 **(5)**. 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 *c*, *d*, and *e*).

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, etc., 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, reproduction, and when measured length or weight, or both, 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 salad-dressing 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 (11.4.3, 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 grade[6] 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 such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, glasses, and by using tubes to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans **(6)**, recommended handling procedures **(7)**, 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 **(8)** and with materials that are, or are suspected of being, carcinogenic **(9)**.

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, it should be understood that when this is done one might not only remove the confounding results of upstream toxicity, but also other factors (suspended solids, humic acids, etc.,) that might otherwise act to reduce or increase the toxicity of the effluent.

9.1.1 The dilution water must allow satisfactory survival, and reproduction of *C. dubia* (see 14.1 *d*, *e*, and *f*). The dilution water should not unnecessarily affect length or weight, or both, of *C. dubia* if these characteristics are to be measured.

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 threebrood 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 (80-µm 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 hard reconstituted fresh water described in Guide E 729 has been used successfully by several people (but see X1.1). Addition of 5 µg of selenium **(10)** and 1 µg of crystalline vitamin B_{12}/L **(11)** might be desirable. *C. dubia* has also been cultured and tested in reconstituted soft water. Acclimation in one reconstituted water

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

and testing in another of different hardness or alkalinity should be avoided.

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 **(12)**. 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 **(13)**. Some organic chloramines, however, react slowly with sodium bisulfite **(14)**. 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 **(15)**, 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, **(16, 17)** 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 **(18)** 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 **(19)**.

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 **(20)** equipped with an intensity meter and flow controls, or passed through a membrane filter with a pore size of 0.20 µm. Water that might be contaminated with *Aphanomyces daphniae* should be autoclaved **(2)**.

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, organophosphorus pesticides, organic chlorine, (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, 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 **(21)**. 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 **(22)**, 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 as solvents, 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.[6] 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, (if length or weight, or both, of *C. dubia* is to be measured they too should be independent of the concentration of solvent), or (*b*) such a solvent test must have already been conducted using the same dilution water and *C. dubia*. If either survival, reproduction, or length or weight, or both, is found to be related to the concentration of solvent, a threebrood toxicity test with *C. dubia* in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival, reproduction, or length or weight, or both, 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, (and length or weight, or both, if these characteristics are measured), of *C. dubia* in the two controls should be compared (see X4.6). If a statistically significant difference in either survival, reproduction, (and length or weight, or both) 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*:

10.3.1 *Sampling Point*—The effluent sampling point should be the same as that specified in the National Polutant Discharge Elimination System, (NPDES) monitoring permit. 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, reproduction, and length or weight, or both, of *C. dubia*, the test concentrations (see 12.10.2.2) 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 E 729) on the test material using the same dilution water and *C. dubia* less than 12-h old. Because the food used in a threebrood toxicity test sometimes affects the results of the acute test **(23)**, 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.

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, reproduction, (or length or weight, or both). 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 section 12.1.1.2).

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 retention time of the effluent. 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 Renewal toxicity tests should generally 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 24 h, a grab sample should be collected daily, diluted appropriately, and used for daily renewals.

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 **(24)**.

10.6 *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.

10.7 *Treatment*—The sample of effluent must not be altered except that it may be filtered through an 80-µm sieve or screen to remove potential predators. Undissolved materials should be uniformly dispersed by gentle agitation immediately before any sample of effluent is distributed to test chambers.

11. Test Organisms

11.1 *Species*—The genus *Ceriodaphnia* is undergoing a revision. Berner **(25)** 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 12-h old. Using neonates born within a narrow age range, for example, less than 12-h old and born within 4-h of each other is desirable.

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, water, and temperature as will be used in the threebrood test. This will not only acclimate the organisms, but will also help demonstrate the acceptability of the food, water, etc., 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.

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.

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. If culture chambers are properly cleaned and the density of organisms is no more than 1 to 2 organisms/15 mL, surface aeration should provide adequate dissolved oxygen.

11.5 *Food*—Various combinations (see Appendix X1) of trout chow, yeast, rye grass powder,[7] and algae **(26)** such as *Ankistrodesmus convolutus, A. falcatus, Chlamydomonas reinhardtii*, and *Selenastrum capricornutum*, have been successfully used for culturing and testing *C. dubia*. The food should be analyzed for the test material, if it might be present.

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 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 uses by autoclaving or by treatment with an iodophor **(27)**.

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* nor with young from *C. dubia* that (*a*) is sick **(2, 28)** or incompletely developed, (*b*) did not produce at least 6 to 8 young in the previous brood, or (*c*) is 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 *Demonstration of Feasibility*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain dilution water with no added test material or effluent, to determine before the first toxicity test (*a*) whether *C. dubia* will survive, and reproduce acceptably (see 14.1 *d*, *e*, and *f*) in the new facilities, (*b*) whether the food, water, handling procedures, etc., are acceptable, (*c*) whether there are any location effects on survival, and reproduction, and length or weight, or both, if these are to be determined, and (*d*) to evaluate the magnitude of the within and between chamber variances.

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 X4.2) usually consists of one or

[7] Rye grass powder available as Cerophyl®, from Wards Biological Supply Co., has been found suitable for this purpose. See Chemical Abstract Service Number, CAS 1000842-92-0.

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, reproduction, (and length or weight, or both) 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. If the estimate of chronic toxicity is particularly nebulous (see 10.4.1), six or seven concentrations might be desirable.

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.

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 **(29)**. 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.

12.2.3 The effect of the test material or effluent on survival, reproduction, (and length or weight, or both, if evaluated), cannot be determined accurately 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 **(30)**. If such calculations are not made, at least two test chambers and ten physically separated individual organisms must be in each treatment (test concentration and control). Replicate test chambers (that is, experimental units) are necessary in order to allow estimation of experimental error **(28)**. 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*—The concentration of dissolved oxygen in each test chamber must be between 40 and 100 % of saturation **(18)** 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 50 and 100 % of saturation. If the concentration of dissolved oxygen falls below 40 % of saturation 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, some loss of test material 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. Three broods can be obtained in 7 days 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.

12.5 *Preparing Test Solutions*:

12.5.1 Except possibly for effluents, 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 7-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 Except possibly for effluents, the concentration of test material in a test solution should be no more than 30 % higher or lower than the nominal concentration. 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.

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 12-h old is placed in each test chamber, or compartment, that already contains test solution. The organisms must be either (*a*) impartially assigned to the test chambers (or compartments in the test chambers) by placing one *C. dubia* in one test chamber or in one compartment in each treatment, and then a *C. dubia* in a second chamber or compartment in each treatment, and continuing the process until each chamber or compartment contains one *C. dubia*, or (*b*) assigned either by random assignment of one *C. dubia* to each treatment, random assignment of a second *C. dubia* to each treatment, etc., or by total randomization, or (*c*) 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 usually acceptable. The minimum acceptable renewal frequency is after the majority of control organisms have produced a brood. When this occurs all organisms are transferred to new solutions. 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 she was removed. The live and dead offspring from each first-generation *C. dubia* should be separately counted, recorded, and discarded. 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 D 1193) or dilution water. If the test chambers are to be conditioned, dilution water containing test material, but not food, should be placed in the chamber and then discarded just before the next renewal.

12.9 *Duration of Test*—A test begins when less than 12-h old neonates are first placed in test solutions. At 25°C control organisms should produce three broods in 7 days. Periodically controls might not produce three broods in 7 days. When this occurs 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 15°C, time to third brood production will be increased.

12.10 *Biological Data*:

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

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 It might be desirable to determine the length or weight, or both, of each first generation *C. dubia* that is alive at the end of the test. Determining dry weight requires a sensitive balance; length can be determined using a calibrated microscope equipped with an ocular micrometer. There is not a consensus amongst the toxicological community regarding the value of length or weight measurements, or both, of Cladocera in evaluating potential impact **(26, 31-35)**. However, including length or weight measurements, or both, when conditions warrant might provide insight not achieved with data on survival and reproduction. Dry weight (dried at 60°C **(36)** to constant weight) might be preferable to length (distance from apex of helmet to base of spine). Wet weight is not acceptable. It might be desirable to determine the size of each

first-generation organism that dies before the end of the test. However, whether or not this can be accomplished is dependent, in part, on the age (size) of the organism at the time of death (it is very difficult, if not impossible, to determine the dry weight of a <5-day old neonate, or males).

12.10.4 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, 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. 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. Measurements on old test solutions might require a composite from replicate test chambers of the same test concentration. Dissolved oxygen concentrations must 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) is desirable. Temperature should be monitored throughout the test. 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.

12.11.2 *Test Material*:

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

12.11.2.2 The concentration of test material in each treatment must be measured at least at the beginning and end of a test. It is preferable to measure the concentrations at the beginning and end of each renewal period. Samples from old test solutions should be obtained by pooling the test solutions for each treatment and removing duplicate samples. Analysis of additional samples after filtration or centrifugation is desirable to determine the percentage of test material that is not dissolved or is associated with particulate matter.

12.11.2.3 Within each treatment the highest of all the measured concentrations obtained during the test in fresh test solutions divided by the lowest must be less than two. The variability of the sampling and analytical procedures should be determined before the beginning of the test to determine how many samples should be taken and analyses performed at each sampling point to ensure that this requirement is not violated just because of sampling or analytical variability.

12.11.2.4 If the organisms are probably being exposed to substantial concentrations of one or more impurities or degradation or reaction products (see 12.5.1), measurement of the impurities and products is desirable.

13. Analytical Methodology

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

13.2 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(37)** to minimize loss of test material by hydrolysis, microbial degradation, oxidation, photolysis, reduction, sorption, and volatilization.

13.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources **(38)**. The concentration of unionized ammonia may be calculated from pH, temperature, and concentration of total ammonia **(39)**.

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

14. Acceptability of Test

14.1 A three-brood toxicity test with *C. dubia* should usually be considered unacceptable if one or more of the following occurred: except that if, for example, temperature was measured numerous times, a deviation of more than 3°C (see section 14.1.1) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature had been measured more often.

(*a*) Treatments were not randomly assigned to test chamber locations.

(*b*) The test was begun with organisms more than 12-h old.

(*c*) A required dilution water control or solvent control was not included in the test or if the concentration of solvent was not the same in all treatments that contained test material, the concentration of solvent affected survival, productivity, and length or weight, or both, of *C. dubia* if determined.

(*d*) More than 20 % of the first-generation organisms died in any required control treatment.

(*e*) Organisms which lived to produce 3 broods in the control(s) did not produce, on the average, at least 15 young, in a pattern of increasing brood sizes and the young were not produced in 8 days.

(*f*) Ephippia was produced in the control(s).

(*g*) Temperature, dissolved oxygen, and concentration of test material were not measured as specified in 12.10.

(*h*) Any measured concentration of dissolved oxygen was not between 40 and 100 % of saturation or the time-weighted average-measured dissolved-oxygen concentration from the beginning to the end of the test for any test chamber was not between 50 and 100 % of saturation.

(*i*) The difference between the time-weighted averagemeasured temperatures for any two test chambers was greater than 1°C.

(*j*) Any individual measured temperature in any test chamber was more than 3°C different from the mean of the time-weighted average-measured temperatures for the individual test chambers.

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

(*l*) The highest measured concentration of test material in fresh test solution was more than twice the lowest in the same treatment.

(*m*) Test solutions were not renewed at a frequency consistent with the objectives of the test as influenced by the material being tested.

(*n*) All test chambers (and compartments) and covers were not identical.

14.2 An assessment should be made of the significance of the concentrations of test material in the controls, treatments, food (see 11.5), and brood stock (see 11.4).

15. Calculation

15.1 The primary data to be analyzed from a 7-day test with *C. dubia* are those on (*a*), the number of live young produced by each first-generation *C. dubia*, (*b*) survival, (*c*) the dry weight or length of adult organisms, or both, surviving the 7-day test when these measurements are made, and (*d*) the concentration of test material or percent effluent in the test solutions in each treatment.

15.2 The variety of procedures that can be used to calculate the results of life-cycle toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (*a*) the advantages and disadvantages of various alternative procedures, and (*b*) appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of the results should be appropriate to the experimental design (see 12.2). The major alternative procedures and points to be considered when selecting and using the procedures for calculating results of life-cycle toxicity tests with *C. dubia* are discussed in Appendix X4.

16. Report

16.1 The record of the results of an acceptable life-cycle toxicity test with *C. dubia* should include the following information either directly or by reference to available documents.

16.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of test.

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

16.1.3 Source of dilution water, its chemical characteristics, and a description of any pretreatment.

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

16.1.5 Description of the experimental design and test chambers (and compartments) and covers, the depth and volume of solution in the chambers, number of first-generation organisms, and test chambers (and compartments) per treatment, conditioning, lighting, and renewal schedule.

16.1.6 Procedure used to prepare food, concentration of test material and other contaminants in the food, feeding, method, frequency, and ration.

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

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

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

16.1.10 Methods used for, and results (with sample size and standard deviations, or confidence limits) of, chemical analyses of water quality and concentration(s) of test material (in fresh and old test solutions) impurities, and reaction and degradation products, including validation studies and reagent blanks.

16.1.11 A table of data on survival, and reproduction in each test chamber (and compartment) in each treatment, including the control(s), in sufficient detail to allow independent statistical analysis. A table of data on length and weight measurements of surviving first-generation organisms should be included if these data were collected.

16.1.12 Methods used for, and results of, statistical analyses of the data.

16.1.13 Summary of general observations on other effects or symptoms.

16.1.14 Results of all associated acute toxicity tests.

16.1.15 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

16.2 Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

APPENDIXES

(Nonmandatory Information)

X1. FOOD

X1.1 *Introduction*—A wide variety of foods have been used in the culture and testing of *C. dubia*. The foods termed "synthetic" are usually made from one or more ingredients such as a trout chow, yeast, etc. The foods termed "natural" consist of one or more species of algae. "Combination" foods contain both "synthetic" and "natural" ingredients.

X1.1.1 Each food type given has been shown to be advantageous by the investigators using that particular type. However, not all food types have been successfully used by all investigators. What works in one laboratory sometimes works poorly in another laboratory. What works under field conditions involving the testing of effluents or contiguous source samples on site might not be particularly advantageous when testing in reconstituted waters. No test should be started until a food has been demonstrated adequate for the conditions under which the test is to be conducted.

X1.2 *Natural Food:*

X1.2.1 Various natural foods have been used with different amounts of success. Natural foods are probably of greatest advantage when used with reconstituted waters, or natural waters with low bacterial counts. Natural foods also tend to be less effective chelators than synthetic foods. Although it requires more effort to prepare a natural food, than a synthetic food, use of a natural food might be cost effective if it decreases the number of unacceptable tests.

X1.2.2 The four species of algae which have been commonly used to culture and test *C. dubia* are *Ankistrodesmus convolutus*, *A. falcatus*, *Chlamydomonas reinhardtii*, and *Selenastrum capricornutum*. Cultures of these species can be purchased from several sources.[8] Generally, the cultures are supplied on agar slants that can be kept for several months in a dark refrigerator at 4°C. The algae are transferred to a liquid nutrient medium to grow large amounts for feeding organisms. Algae are grown using aseptic techniques although controlled bacterial contamination does not appear to greatly influence results.

X1.2.3 *Selenastrum capricornutum* may be cultured as food for *C. dubia* following procedures based on the algal assay bottle test (see Practice D 3978 or **(40)**). This medium may be prepared in large quantities by adding 1 mL of each of the following concentrates to each 1000 mL of ASTM Type II water plus magnetic stirring bar in 2-L Erlenmeyer flasks.

Concentrate	Number of g/500 mL, store in dark
No. 1	
$NaNO_3$	12.75
$MgCl_2 \cdot 6H_2O$	6.082
$CaCl_2 \cdot 2H_2O$	2.205
No. 2	
$MgSO_4 \cdot 7H_2O$	7.35
No. 3	
K_2HPO_4	0.522
No. 4	
$NaHCO_3$	7.5

After the addition of all four concentrates, autoclave the foam-plugged flasks for 30 min, at 121°C.

Concentrate No. 5	Number of g/500 mL, sterile filter, store in refrigerator[A]
H_3BO_3	92.76
$MnCl_2 \cdot 4H_2O$	207.69
$ZnCl_2$	1.635
$FeCl_3 \cdot 6H_2O$	79.88
$CoCl_2 \cdot 6H_2O$	0.714
$Na_2MoO_4 \cdot 2H_2O$	3.63
$CuCl_2 \cdot 2H_2O$	0.006
$Na_2EDTA \cdot 2H_2O$	150.0
Na_2SeO_4	1.196[B]

[A] Add 1 mL aseptically to cool, sterilized medium.

[B] Not in medium from algal assay bottle procedure, but might be beneficial to Cladocera.

X1.2.3.1 Inoculate, aseptically, each L of media with 30 mL of 7-day old *S. capricornutum* culture with optical density (O.D.) = 0.10 to 0.15 as measured at 750 nm with 16.8 mm light path. Cultures with optical density readings of between 0.10 to 0.15 as just determined will have approximately 2.5 to 2.8×10^6 cells/mL of medium.

X1.2.3.2 Incubate the algal cultures on magnetic stirrers at 24 ± 2°C, and an average of 2500 lx of illumination at the media surface during a 16-h light photoperiod. Magnetic stirrers generate heat. In order to maintain 24 ± 1°C in the culture medium, an air temperature of 21 ± 1°C might be required.

X1.2.3.3 On Day 2 after inoculating the fresh media, 10 mL of the following vitamin solution should be added/L of medium (modified from Murphy **(41)**.

Vitamin	mg[A]
D-pantothenic (Hemi calcium salt)	140.0
Vitamin B_{12} (Cyanocobalamine)	0.006
Thiamine HCl (Vitamin B_1)	12.0
Riboflavin (Vitamin B_2)	8.0
Niacinamide (Nicotinamide)	26.0
D-Biotin (Vitamin H)	6.0
Putrescine (Dihydrochloride)	6.0
Myoinositol	220.0
Choline chloride	100.0

[A] Prepare in 500 mL quantities, store in the dark under refrigeration.

X1.2.3.4 On Day 3 remove one half of the *S. capricornutum*

[8] Algal species available from the Starr Collection at the University of Texas in Austin, TX, or the American Type Culture Collection in Rockville, MD, have been found suitable for this purpose.

cultures and store in the refrigerator.

X1.2.3.5 On Day 7 remove the remaining *S. capricornutum* cultures. Mix equal volumes of 3-day old and 7-day old cultures. Centrifuge the combined algae at 10 000 r/min for 10 min. Pour off the algal medium and resuspend the algal pellet (the pellet from 1 L of 3-day + 7-day mixture resuspends and stores conveniently in 60 mL of reconstituted water in a 100-mL plastic bottle) in appropriate dilution or reconstituted water depending on anticipated needs. The algae can be stored in a refrigerator for as long as 1 year and still retain its quality as a food.

X1.2.3.6 When the alga is to be used as a food, remove it from the refrigerator, allow it to warm up and adjust its O.D. to 0.4 at 750 nm and 16.8 mm light path. Food prepared in this manner generally contains between 10 and 14 $\times$ 10^6 cells/mL.

X1.2.3.7 The 0.4 O.D. food can be used to culture and test *D. magna, D. pulex,* and *C. dubia* although the amounts used to culture and test each species varies. The addition of rye grass powder to the algal feeding suspension appears to facilitate productivity of *C. dubia* (cereal leaves, and wheat grass powder may substitute for rye grass powder). Rye grass powder should be prepared by adding it to ASTM Type II water at a ratio of 7.5 gm/L and blending at high speed for 5 min. The blended solution should be refrigerated overnight at 4°C. After refrigeration the solution should be filtered through a 40-µm mesh sieve and stored in the refrigerator. Rye grass powder prepared in this manner appears to lose its beneficial qualities as a food supplement after about 1 week. To prepare the algal-rye-grass-powder feeding suspension, remove the algae and rye grass powder from the refrigerator and allow them to warm to room temperature. Count the number of cells in the algal concentrate and multiply by the volume of concentrate to obtain the total number of cells in the concentrate. Divide the total number of cells in the concentrate by the number of cells per mL desired in the final feeding suspension. Do not dilute to the final volume at this time. Divide the *calculated* final volume of the algal feeding suspension by the number of mL to be added to a test chamber. Multiply this number by 0.120 to get the volume of rye grass powder to add to the algal concentrate before bringing the algal-rye-grass-powder mixture to the final desired volume. Prepare only enough of this mixture at a time for one 7-day test.

X1.2.4 *Ankistrodesmus convolutus, A. falcatus, Chlamydomonas reinhardtii,* and *Selenastrum capricornutum* can be cultured using the following procedures.

X1.2.4.1 Nutrient medium (Table 1 and Table 2) is prepared by adding specified amounts of stock solutions to ASTM Type II water. The quality of the water must be exceptionally good to obtain consistent growth and food value of the algae. Nutrient medium is sterilized prior to the addition of the algae, either by filtration through a 0.22-µm membrane filter or by autoclaving.

X1.2.4.2 A vitamin solution for addition to the nutrient medium should contain the following **(45)**:

TABLE 1 Modified Bold Basal pH 6.6 (Modified Bristol Solution) (42)

NOTE 1—The specified volumes of eleven stock solutions (six macronutrient solutions, three minor constituent solutions, and two micronutrient solutions) are added to 900-mL high-quality distilled or deionized water and diluted to 1 L.

Macronutrients (use 10 mL of each):	
$NaNo_3$	25 g/L
$CaCl_2 \cdot 7H_2O$	2.5 g/L
$MgSO_4 \cdot 7H_2O$	7.5 g/L
K_2HPO_4	7.5 g/L
KH_2PO_4	17.5 g/L
NaCl	2.5 g/L
EDTA (use 1 mL):	
EDTA	50 g/L
KOH	31 g/L
Iron (use 1 mL):	
$FeSO_4 \cdot 7H_2O$	4.98 g/L
H_2SO_4	1.0 ml/L
Boron (use 1 mL):	
H_3BO_3	11.42 g/L
Micronutrients (use 1 mL):	
$ZnSO_4 \cdot 7H_2O$	8.82 g/L
$MnCl_2 \cdot H_2O$	1.44 g/L
MoO_3	0.71 g/L
$CuSO_4 \cdot 5H_2O$	1.57 g/L
$Co(NO_3)_2 \cdot 6H_2O$	0.49 g/L
Micronutrients (use 1 mL):	
$NiCl_2$	0.0015 g/L
Na_2SeO_4	0.002 g/L
$SnCl_4$	0.001 g/L
KI	0.003 g/L
$VOSO_4 \cdot 2H_2O$	0.002 g/L

TABLE 2 Algal Media

NOTE 1—For either medium, prepare one stock solution for each macronutrient and use 1 mL of each stock solution per L of medium.

	Woods Hole MBL **(43)** *gm/L in stock solution*	ASM-1 **(44)** *gm/L in stock solution*
	Macronutrients [A]	
$CaCl_2 \cdot 2H_2O$	36.76	29.41
$MgSO_4 \cdot 7H_2O$	36.97	49.3
$MgCl_2 \cdot 6H_2O$	...	40.57
$NaHCO_3$	12.60	...
K_2HOP_4	8.71	17.41
$NaNO_3$	85.01	170.00
$Na_2SiO_3 \cdot 9H_2O$	28.42	...
	Micronutrients [B]	
Na_2EDTA	4.36	7.44
$FeCl_3 \cdot 6H_2O$	3.15	1.081
$CuSO_4 \cdot 5H_2O$	0.01	0.000186
$CoSO_4 \cdot 6H_2O$	0.01	0.019
$ZnSO_4 \cdot 7H_2O$	0.022	0.920
$MnCl_2 \cdot 4H_2O$	0.18	1.385
$Na_2MoO_4 \cdot 2H_2O$	0.006	0.010
H_3BO_3	1.0	2.47

[A] Macronutrients should be prepared as separate solutions.
[B] Micronutrients can be mixed as single stock solution.

	mg/L
Biotin	5
Thiamine	100
Pyridoxine	100
Pyridoxamine	3
Calcium pantothenate	250
B_{12}	1
Nicotinic Acid	50
Nicotinamide	50
Folic Acid	20
Riboflavin	30
Inositol	90

After filtration through a 0.22-µm membrane filter, this solution can be stored in a dark sterile bottle in a refrigerator for at least 3 years or portions can be frozen. One mL of this vitamin solution should be added to each liter of nutrient medium before the medium is sterilized.

X1.2.5 The general principles of sterile technique should be observed to prevent contamination of the algal culture with fungi, bacteria, or other algal species. Glassware should be washed and sterilized as recommended for *C. dubia*. Although the algae will grow acceptably at 20 ± 2°C with 1000 to 4500 lx for 14 to 16 h/day, they will grow faster at 24 ± 2°C with continuous light at 4300 to 4500 lx. The light should be from a broad spectrum fluorescent bulb. If the algal culture is aerated by bubbling air through it, the air should be filtered through a 0.22-µm bacterial filter.

X1.2.6 When sterile nutrient medium with vitamins is first inoculated with algae, there is usually a lag phase of 1 to 2 days before growth becomes visible. This is followed by a log phase of rapid growth that gradually levels off as the maximum cell density is approached. When the maximum crop is reached, the cell concentration will remain fairly constant but the individual cells will continue to grow and age. Algae for feeding *C. dubia* should be harvested during the log growth phase to ensure that the algae are in a healthy growth condition. The time it takes to go from inoculation to harvest depends on the nutrient medium, vessel size, light intensity, photoperiod, degree of aeration, temperature, and amount and condition of the inoculum. As a general rule, a culture with adequate light and aeration is about one week from maximum cell density when the medium turns visibly green.

X1.2.7 Algae are usually cultured in static or semicontinuous systems.

X1.2.7.1 Static cultures are usually maintained in Erlenmeyer flasks stoppered with loose cotton, plastic foam plugs, Shimatsu closures, or covered with beakers. If the flasks are kept on a shaker table or well-mixed by bubbling air, the nutrient medium can be filled to 50 % of the total volume of the flask. If mixing is done once or twice a day by hand, the flask should be filled to only 40 % of its volume. Small static cultures can be maintained in 250 to 500-mL flasks, but 2 to 4-L Erlenmeyer flasks can be used to grow large amounts of algae for food. The entire contents should be harvested just prior to maximum cell density. New cultures should be inoculated often enough that at least one culture is available for harvesting during the log growth phase every time food is needed.

X1.2.7.2 Semicontinuous cultures allow for continuous production of large amounts of algae while maintaining the algae in log growth phase by periodic removal of a portion of the algal culture and replacement with fresh medium. Convenient culture vessels for this system are large aspirator bottles set on magnetic stirrers and provided with an air line and a tube connected to a reservoir of sterile medium. With this system, algae can be drawn off several times a week and fresh medium gravity fed into the culture vessel. Semicontinuous cultures are more likely to become contaminated by bacteria than are static cultures.

X1.2.7.3 Algae can be separated from the nutrient medium by centrifugation or by letting the algae settle in a refrigerator. The period of time required for the algae to settle is in part species dependent. It is not necessary to remove all the medium but only to concentrate the algae so that addition of medium to *C. dubia* cultures and test solutions is minimal.

X1.3 *Synthetic Food:*

X1.3.1 Numerous synthetic foods have been used to culture and test *C. dubia. Ceriodaphnia* have been maintained using only yeast or only rye grass powder. Yeast or rye grass powder should not be used alone with reconstituted water or other water with low bacteria counts. A recipe for preparing a synthetic food combining trout chow, rye grass powder, and yeast, is given in the following paragraphs. This food or variations of this recipe are often referred to as YTC, or TCY **(46)**.

X1.3.2 Digested trout chow is prepared by adding 5 gm of trout chow to 1 L ASTM Type II water. Mix well and aerate continuously (digest) for one week at ambient laboratory temperature. At the end of one week, replace any water lost during digestion and filter 900 mL of the supernatant through a 100-µm mesh filter. Discard the remainder.

X1.3.3 Rye grass powder is prepared by adding 10.0 gm of the powder to 1 L of distilled water. Store the mixture overnight.

X1.3.4 Yeast is prepared by adding 5 gm of dry yeast[9] to 1 L of ASTM Type II water. Stir with a magnetic stirrer until well dispersed or use a blender at low speed for 5 min. Place in a refrigerator overnight. Prior to use resuspend the yeast mixture.

X1.3.5 Combined trout chow rye-grass-powder-yeast food is prepared by mixing equal volumes of the three solutions. A suspended solids analysis should be conducted on each new batch of food to provide data on the consistency of the preparation procedures and on the uniformity of each batch. Place aliquots of the final mixture in small screw cap bottles. Fresh or thawed food is stored in the refrigerator between feedings, and is used for a maximum of 1 week.

[9] Yeast such as Fleischman's or St. Regis has been found suitable for this purpose.

X2. CULTURE TECHNIQUES

X2.1 Two techniques that have been used to culture *C. dubia* are:

X2.1.1 Cultures initiated by adding 20 >12-h old neonates to 360 mL of reconstituted hard or soft water contained in 500-mL jars. Feed these cultures at a rate of 12 mL of 0.4 O.D. algae plus rye grass powder (see X1.2.3) daily. Transfer organisms to fresh reconstituted water on Day 3 prior to the addition of algae rye grass powder. After the 2nd brood has been produced (generally Day 5 or 6 at 25°C) isolate the original females into 100-mL beakers containing 60 mL of the appropriate reconstituted water (50-mL beakers containing 30 mL of medium will suffice). To these beakers add 1 mL of 0.4 O.D. algae/30 mL of media. Discard the mass culture and 1st and 2nd brood young contained therein.

X2.1.1.1 Use the 3rd brood young to start new cultures and experiments. The isolated females generally produce between 10 and 16 third brood neonates for use in starting cultures and experiments.

X2.1.1.2 This *technique* is particularly useful when the cohort experimental design (blocking on females) is used. One mass culture of this type is generally sufficient to produce at least 10 females whose young are within 12 h of each other. Cultures may be started on consecutive days to ensure that sufficient organisms are present on any given day.

X2.1.2 Use 1-L glass beakers as culture vessels. Maintain cultures in several separate vessels to provide back-up in case one is lost due to accident or other problems such as low dissolved oxygen (D.O.), or lack of food. Fill the 1-L culture vessels with 900 mL of media. A new culture is started each week, and the oldest culture is discarded. Using this schedule, 1-L cultures will provide 500 to 1000 neonates per week. Feeding the proper amount of food is extremely important in *C. dubia* culturing. The trout chow-rye-grass-powder yeast suspension (see X1.3.1) will provide adequate nutrition if fed daily at a rate of 1 to 4 mL/L of medium containing 200 to 1000 organisms.

X2.1.3 The culture medium in each vessel should be replaced with fresh medium weekly by pouring one half of the contents of a culture vessel (450 mL of the 900 mL) into a shallow container. Dispose of the remainder of the media and animals unless needed in a test. Clean the vessel and add about 100 mL of fresh medium in the clean culture vessel. Remove approximately 100 *C. dubia* from the shallow container and place in the fresh media along with a small amount of the old medium to provide seed bacteria for the new culture, and carefully add sufficient additional fresh medium to bring the total volume to 900 mL **(46)**.

X3. TEST CHAMBERS

X3.1 Test chambers for natural and synthetic foods are in part objective dependent but also are somewhat food type dependent. In experiments in which effluents are being tested using synthetic foods, *C. dubia* may be tested in 30-mL beakers containing 15 mL of media, fed at a rate of 0.1 mL TCY per day. Researchers have also found that disposable plastic salad dressing cups can be used in *C. dubia* testing. The cups are convenient because they are disposable and because they fit in the viewing field of most stereomicroscopes.

X3.2 Similar tests using natural foods might require test chambers that provide a somewhat greater depth of solution. The depth of solution (15 mL in 30-mL beaker) appears inadequate to maintain sufficient algae in suspension for 24 h.

X3.3 In either case, larger beakers may be utilized by adjusting the food concentration. One reason for using 30-mL beakers is that they fit conveniently under the objective of a stereomicroscope, aiding in counting neonates. Viewing larger beakers on a black background from the top with a light source from the back permits reasonably easy capture of neonates. Slowly rotating the beaker causes the neonates to swim away from the light source. At times during ambient water toxicity tests or effluent tests, suspended solids settle out imparting a light background to the bottom of the test chambers. When this occurs it is often easier to view the test chambers from the front with the light source behind and slightly above the test chamber.

X4. STATISTICAL GUIDANCE

X4.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies, in data. The data display and statistical techniques most commonly used to achieve these goals are: (*a*) preliminary and diagnostic graphical displays, (*b*) pairwise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (*c*) analysis of variance (ANOVA) and corresponding contingency table tests, (*d*) multiple comparison techniques for simultaneous pairwise comparison of treatment groups with control groups, (*e*) regression analysis, and (*f*) concentration effect curve analyses. If used correctly, each of these techniques can provide useful information about the results of an acceptable *C. dubia* 7-day test.

X4.1.1 The three kinds of data obtained from toxicity tests

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

X4.2 *End point*—The primary end point of a life-cycle toxicity test with *C. dubia* is based on the reduction in numbers of live neonates produced by first-generation *C. dubia* during the test. The end point generally has been defined in terms of whether differences from control organisms were statistically significant at the 5 % level. One of the main conceptual problems associated with such a definition of the end points is that the notions of biological importance and statistical significance are logically distinct. Effects of considerable biological importance might not be statistically significant if sample sizes are small or effects are extremely variable or both. Conversely, biologically trivial effects might be highly statistically significant if sample sizes are large or effects are very reproducible. An end point based solely on statistical significance might depend as much or more on sample sizes as on the magnitude of the effect.

X4.2.1 An alternative is to define the end point in terms of a specified absolute or relative amount of difference from control organisms. A regression model would be fitted to the data and a concentration associated with a specified amount of difference from the control(s) would be estimated using the model. For example, the concentration resulting in a specified percent decrease in number of live offspring might be estimated along with confidence limits on the estimated concentration. Results of 7-day tests would then be reported as point estimates, with confidence limits, of the concentration expected, to cause an amount of effect that was preselected as being unacceptable. However, no consensus currently exists concerning what constitutes significant preselected biological effects.

X4.2.2 In general, an end point defined in terms of a statistically significant difference is calculated using analysis of variance, contingency tables, or other hypothesis testing procedures. An end point defined in terms of a specified amount of effect is calculated using regression analysis, concentration effect curve analysis, or other point estimation procedures. Regardless of the procedure used, sufficient data should be present in reports (see 16.1) to permit calculation of end points other than those chosen by the authors, and to allow other uses of the data, such as modelling.

X4.3 *Graphical Displays*—These should be an integral part of every data analysis **(47)**. Preliminary scatter plots are desirable because they might provide insights into the structure of the data and reveal the presence of unanticipated relations or anomalies. Histograms are useful for examining the distribution of data before hypothesis testing. The advent of modern computers and statistical computing packages,[10] has made the inspection of data patterns both easy and inexpensive. Feder and Collins **(48)** illustrate the use of various types of preliminary and diagnostic graphical displays in analysis of data from chronic toxicity tests.

X4.4 *Outlier Detection Procedures*—Data that do not appear to be in conformance with the substantial majority are often referred to as outliers, and might be due to random variation or to clerical or experimental errors. Statistical outlier detection procedures are screening procedures that indicate whether a datum is extreme enough to be considered, not due just to random variation. Barnett and Lewis **(49)** describe many outlier detection procedures, and Feder and Collins **(48)** illustrate the use of several outlier detection procedures with aquatic toxicological data. If outliers can be shown to be due to clerical or experimental error, they should be either corrected or deleted from the data prior to analysis. If outliers are not known to be erroneous values, the question of how to deal with them is a matter of judgment. It is often desirable to analyze the data both with and without questionable values in order to assess their importance, because one or a few extreme outliers can sometimes greatly affect the outcome of an analysis.

X4.5 *Data Transformations*—Many standard statistical procedures such as regression analysis and ANOVA, are based on the assumption that experimental variability is homogeneous across treatments. This assumption typically does not hold for certain kinds of data. If data displays or tests of heterogeneity demonstrate that variability is not homogeneous across treatments, variance stabilizing transformations of the data might be necessary. The arc sine, square root, and logarithmic transformations are often used on dichotomous, count, and continuous data, respectively **(50)**. The question of whether to transform raw data should be decided on a case-by-case basis after studying data displays, tests of heterogeneity, and similar data from previous tests. In reality, ANOVA and regression are not very sensitive to departures from normality and small deviations from this assumption are not prohibitive. Nonparametric procedures might be important aids in analyzing heterogeneous data.

X4.6 *Comparison of Solvent Control and Dilution-Water Control*—If both solvent and dilution water controls are included in the test, they should be compared (for example, using a *t*-test for count and continuous data, Fisher's Exact Test, or a 2 by 2 contingency table test for categorical data **(51)**). Adjustments for chamber-to-chamber heterogeneity might be necessary. The use of a large alpha level (for example, 0.25) will make it more difficult to accept the null hypothesis when it should not be accepted. The test statistic, its significance level, and the power of the test should be reported.

X4.7 *Analysis of Variance and Contingency Table*

[10] Statistical computer packages such as *BMDP Biomedical Computer Programs, P-series*, available from UCLA, Los Angeles, CA, and *SAS User's Guide, Statistics*, available from SAS Institute, Cary, NC, and *Statistical Package for the Social Sciences*, published by McGraw-Hill, New York, NY, 1970; and *Minitab*, published by Duxbury Press, North Scituate, MA, all have been found suitable for this purpose.

Analyses—ANOVA tests are often appropriate for untransformed continuous data, and for transformed categorical and count data. Contingency table tests are usually appropriate for untransformed categorical data. If evidence of chamber-to-chamber heterogeneity is found, standard contingency table analyses might be inappropriate for categorical data. In this case it might be appropriate to apply an arc-sine variance stabilizing transformation to the proportion dead within each experimental unit, and perform an ANOVA on the transformed proportions. Feder and Collins **(48)** illustrate transformation of data before use of a contingency table test.

X4.7.1 Both contingency table tests and ANOVA *F*-tests are overall tests that do not assume any particular form for the relation between effects and concentrations. They are thus not designed to be particularly sensitive to one-sided, monotone trends characteristically observed in toxicity tests. Specialized tests have been designed to be more sensitive to relations of this type. Some such tests are the One-Sided Measure of Association Tests, the Cochran-Armitage Test for categorical data, and tests based on linear or polynomial regression models for continuous data **(29, 52)**.

X4.7.2 ANOVA tests are based on normal distribution theory and assume (*a*) that the data within treatments are a random sample from an approximately normal distribution, and (*b*) that error variance is constant between treatments. As a part of the ANOVA, statistical tests for the assumptions of normality and homoscedasticity should be performed to determine whether there are any obvious violations of these assumptions. When results of an ANOVA are reported, the ANOVA model and table, the *F* statistic and its significance level, and the power of the test should be presented.

X4.8 *Multiple Comparison Procedures*—The usual approach to analyzing data from sublethal tests is to compare data for each concentration of the test material to data for the controls. In Fisher's Protected Test, which is only used if the ANOVA *F*-test is significant **(53)**, each concentration of test material is compared to the control(s) using the *t*-test. If the investigator desires to set the experiment wise alpha, rather than a comparison wise alpha, Dunnett's procedure **(53, 54)** can be used without the ANOVA *F*-test. Williams' procedure **(53, 55)** also tests the control(s) versus each concentration, but makes the additional assumption that the true mean follows a monotonic relation with increasing concentration. The latter procedure is more powerful if the assumption is correct. Alternatively, Tukey's **(56)** No Statistical Significance of Trend, (NOSTASOT) test can be used with the same assumptions as Williams' procedure. Shirley **(57)** has developed a nonparametric equivalent for Williams' test and Williams **(58)** has modified and corrected Shirley's procedure to increase its power to detect the alternative hypothesis. Care must be taken when using any of these procedures that an appropriate estimate of variability is used, incorporating any chamber-to-chamber variation that is present. Presentation of results of each comparison should include the test statistic, its significance level, the minimum significant difference, and the power of the test.

X4.9 *Regression Analysis and Concentration-Effect Curve Estimation*—An alternative to tests for statistically significant differences is to fit concentration effect models or regression models to the data and estimate the concentration that corresponds to a specified amount of difference from the control treatment **(59)**. Concentration effect curve models, such as probit and logit, are commonly used to describe trends in dichotomous data on survival. Linear and quadratic polynomial regression models are commonly used to describe trends in quantitative data on growth and reproduction. Toxicity tests should be designed to avoid the need for extrapolation, which can introduce biases into the estimates.

X4.9.1 Point estimates, such as the EC10, EC25, and EC50, are examples of end points calculated using regression analysis. Whenever a point estimate is calculated, its 95 % confidence interval should also be calculated. Finney **(60)** discusses the probit model in considerable detail, and Draper and Smith **(61)** and Neter, Wasserman, and Kutner **(62)** discuss most practical aspects of regression analysis. Feder and Collins **(48)** discuss use of these techniques in aquatic toxicology.

X4.9.2 When a regression model or concentration effect curve model is fitted, data for each experimental unit are plotted against concentration. If necessary, transformation of the effect data or concentration data, or both, should be performed to stabilize variability across treatments and to produce a smooth trend. For example, if effects or concentrations cover a range of one or more orders of magnitude, a logarithmic transformation of either concentration or effect, or both, might be appropriate. On the basis of preliminary graphs, a regression model should be postulated and fitted to the data using a linear or nonlinear regression fitting technique. Residuals from the model should be calculated and plotted against appropriate variables. Any systematic structure in the residuals indicates lack of fit of the model and the model should be modified and the procedure repeated. This cycling should continue until the pattern associated with the residuals is minimized. Presentation of results of regression or concentration effect curve analysis should include the entire regression equation in its final form, along with the standard error of the residuals.

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