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Standard Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests[1]

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1. Scope

1.1 This guide covers procedures for obtaining laboratory data concerning the adverse effects of a test material (added to dilution water, but not to food) on *Daphnia magna* Straus, 1820, during continuous exposure throughout a life-cycle using the renewal or flow-through techniques. These procedures also should be useful for conducting life-cycle toxicity tests with other invertebrate species, although modifications might be necessary.

1.2 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, or known mixtures. With appropriate modifications, these procedures can be used to conduct tests on temperature, dissolved oxygen, pH, and on such materials as aqueous effluents (also see Guide E1192), leachates, oils, particulate matter, sediments, and surface waters. The technique, (renewal or flow-through), will be selected based on the chemical characteristics of the test material such as high oxygen demand, volatility, susceptibility to transformation (biologically or chemically), or sorption to glass.

1.3 Modification of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of standard test procedures. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information on new concepts and procedures for conducting life-cycle toxicity tests with *D. magna*.

1.4 This guide is arranged as follows:

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

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Section

Appendix X2 Food

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *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.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

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

E943 Terminology Relating to Biological Effects and Environmental Fate

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

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

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

3. Terminology

3.1 The words "must," "should,"" may," "can," and "might" have very specific meanings in this guide.

3.2 *must*—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 used only in connection with factors that directly relate to the acceptability of the test (see 14.1).

3.3 *should*—used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors.

3.4 *may*—used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Therefore the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either" may" or "can."

3.5 For definitions of other terms used in this guide, refer to Guide E729 and Terminology E943. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 A 21-day life-cycle toxicity test for *Daphnia magna* is described. The test design allows for the test organisms to be exposed to a toxicant using either the renewal technique (with exchange of the total volume of test water and toxicant at least three times a week) or the flow-through technique (with continual water and toxicant addition, usually at least four volume additions per day). At least five concentrations of a test material, a control, and a solvent control (if applicable) replicated at least four times are recommended. Each test concentration has at least ten *Daphnia* per treatment. The technique (renewal or flow-through) which uses a minimum of ten daphnids per treatment has only one daphnid per replicate, whereas the typical technique (renewal or flow-through) utilizes four replicates with at least five daphnids per replicate (≥20 daphnids per treatment). A control consists of maintaining daphnids in dilution water to which no test material has been added to provide (*1*) a measure of the acceptability of the test by giving an indication of the quality of the test organisms and the suitability of the dilution water, food, test conditions, handling procedures, and so forth, and (*2*) the basis for interpreting data obtained from the other treatments. In each of the other treatments, the daphnids are maintained in dilution water, to which a selected concentration of test material has been intentionally added. Measurement end points obtained during the test include the concentration of the test material and final number alive, final weight, and number of progeny per daphnid. Then data are analyzed to determine the effect of the test material on survival, growth, and reproduction of *D. magna*.

5. Significance and Use

5.1 Protection of an aquatic species requires prevention of unacceptable effects on populations in natural habitats. Toxicity tests are conducted to provide data that may be used to predict what changes in numbers and weights of individuals might result from similar exposure to the test material in the natural aquatic environment. Information might also be obtained on the effects of the material on the health of the species.

5.2 Results of life-cycle tests with *D. magna* are used to predict chronic effects likely to occur on daphnids in field situations as a result of exposure under comparable conditions.

5.2.1 Life-cycle tests with *D. magna* are used to compare the chronic sensitivities of different species, the chronic toxicities of different materials, and study the effects of various environmental factors on the results of such tests.

5.2.2 Life-cycle tests with *D. magna* are used to assess the risk of materials to aquatic organisms (see Guide E1023) or derive water quality criteria for aquatic organisms (**1**).[3]

5.2.3 Life-cycle tests with *D. magna* are used to predict the results of chronic toxicity tests on the same test material with the same species in another water or with another species in the same or a different water. Most such predictions take into account the results of acute toxicity tests, and so the usefulness

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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

of the results of a life-cycle test with *D. magna* is greatly increased by also reporting the results of an acute toxicity test (see Guide E729) conducted under the same conditions. In addition to conducting an acute toxicity test with unfed *D. magna*, it may be desirable to conduct an acute test in which the daphnids are fed the same as in the life-cycle test to see if the presence of that concentration of that food affects the results of the acute test and the acute-chronic ratio (ACR) (see 10.3.1).

5.2.4 Life-cycle tests are used to evaluate the biological availability of, and structure-activity relationships between, test materials and test organisms.

5.3 Results of life-cycle tests with *D. magna* might be influenced by temperature (**2**), quality of food, composition of dilution water, condition of test organisms, and other factors.

6. Apparatus

6.1 *Facilities*—Culture and test chambers are often kept in a room maintained at about 20°C but at separate locations. Alternatively, culture and test chambers may be placed in a temperature-controlled water bath or environmental chamber or incubator. The water-supply system should provide an adequate supply of dilution water to the culture tanks and test chambers. The water-supply system should be equipped for temperature control and aeration, and strainers and air traps should be included in the water-supply system. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of air through a 0.22-µm bacterial filter might be desirable (**3**). During culturing and testing, daphnids should be shielded from disturbances to prevent unnecessary stress. The test facility should be well-ventilated and free of fumes. A timing device should be used to provide a 16-h light and 8-h dark photoperiod (**4**). A 15 to 30-min transition period when lights go on might be desirable to reduce the possibility of daphnids being stressed by instantaneous illumination; a transition period when lights go off may also be desirable.

6.1.1 When *D. magna* are fed algae, a high-light intensity might cause sufficient photosynthesis to result in an increase of pH high enough to kill daphnids (**5**). Therefore, the maximum acceptable intensity is dependent on the buffer capacity of the dilution water, species, and density of algae, and the kind of test chamber and cover. Light intensities up to 600 lx or a fluence rate of 1 w/m^2 will usually be acceptable, but higher intensities might result in an unacceptably high pH in the culture water.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which daphnids will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that can adversely affect daphnids. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, fiberglass, silicon, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Concrete and rigid (unplasticized) plastics may be used for culture tanks and in the water-supply system, but they should be soaked, preferably in flowing dilution water, for several days before use (**6**). Cast-iron pipe may be used in supply systems, but colloidal iron probably will 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, or test solutions before or during the test. Items made of neoprene rubber and other materials not previously mentioned should not be used unless it has been shown that their use will not adversely affect survival, growth, and reproduction of *D. magna* (see Section 14).

6.3 *Test Chambers:*

6.3.1 *Flow-through tests,* 500-mL to 2-L glass beakers (or equivalent) with a notch (approximately 4 by 13 cm) cut in the lip may be used to expose the *Daphnia* to the test material. The notch should be covered with 0.33-mm opening (U.S. standard sieve size No. 50) stainless steel or polyethylene screening small enough to retain first instar *Daphnia*. The screen can be attached to the beaker with silicone adhesive. The chambers should provide at least 30 mL of solution for each of the initial test daphnid(s).

6.3.2 *Renewal tests,* beaker ranging in size from 100 to 1000 mL. A notched chamber is not required for a renewal test. Each chamber should provide at least 40 mL of solution for each of the initial test daphnid(s).

6.3.3 Any container made of glass, Type 316 stainless steel, or a fluorocarbon plastic may be used if (*1*) each chamber is separate with no interconnections, (*2*) each chamber contains at least 30 mL of test solution (see 12.4) per first-generation daphnid for flow-through tests and at least 40 mL for renewal tests, (*3*) there is at least 1000 mm^2 of air to water interface per daphnid, and (*4*) the test solution is at least 30 mm deep. Static test chambers should be covered with glass, stainless steel, nylon, or fluorocarbon plastic covers to keep out extraneous contaminants and to reduce evaporation of test solution. All chambers and covers in a test must be identical. Covers are not required for flow-through studies.

6.4 *Cleaning*—Test chambers and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New equipment should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 5 % concentrated nitric acid), and washed at least twice with distilled, deionized, or dilution water. Some lots of some organic solvents might leave a film that is insoluble in water. Also, stronger nitric acid, for example, 10 %, might cause deterioration of silicone adhesive; an initial rinse with 10 % concentrated hydrochloric acid might prevent such deterioration. A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid, but it might attack silicone adhesives. At the end of every test, all items that are to be used again should be immediately (*1*) emptied, (*2*) rinsed with water, (*3*) 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 (*4*) rinsed at least twice with distilled, deionized, or dilution water. Acid is useful for removing mineral deposits. Test chambers should be rinsed with dilution water just before use.

6.5 *Acceptability*—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. This test will reveal (*1*) whether *D. magna* will survive, grow, and reproduce acceptably (see Section 14) in the new facilities, (*2*) whether there are any location effects on survival, growth, or reproduction, and (*3*) the magnitude of the within-chamber and between-chamber variance.

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[4] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the test.

8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions should be minimized by wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses, and by using dip nets or tubes to remove daphnids from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**7**), recommended handling procedures (**8**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures will be necessary with radiolabeled test materials (**9**) and with materials that are, or are suspected of being, carcinogenic (**10**).

8.2 Disposal of stock solutions, test solutions, and test organisms might pose special problems in some cases; therefore, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

8.3 Cleaning equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area with no smoking allowed and no open flame, for example, pilot light, is present.

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

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

8.6 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 well-ventilated area.

9. Dilution Water

9.1 *Requirements*—The dilution water should (*1*) be acceptable to *D. magna*, (*2*) be of uniform quality, and (*3*), except as stated in 9.1.4, not unnecessarily affect results of the test.

9.1.1 The dilution water must allow satisfactory survival, growth, and reproduction of *D. magna* (see Section 14).

9.1.2 The quality of the dilution water should be uniform, allowing the brood stock to be cultured and the test conducted in water of the same quality. In particular, during culture or testing, or both, the range of hardness should be ±10 % of the average.

9.1.3 The dilution water should not unnecessarily affect results of a life-cycle test with *D. magna* because of such things as sorption or complexation of test material. Therefore, except as stated in 9.1.4, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L.

9.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of a life-cycle test with *D. magna*, 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 the laboratory's usual culture dilution water to facilitate interpretation of the results in the special water.

9.2 *Source:*

9.2.1 The use of reconstituted water might increase comparability of test results between laboratories. The hard reconstituted fresh water (160 to 180 mg/L as $CaCO_3$) described in Guide E729 has been used successfully. Addition of 2 µg of selenium(IV) and 1 µg of crystalline vitamin B_{12}/L might be desirable (**11**). Other water sources (natural or reconstituted) may be used if they have been demonstrated to provide adequate daphnid survival, growth, and reproduction.

9.2.2 Natural fresh waters have been used successfully. Natural waters should be obtained from an uncontaminated source of consistent quality. A well or spring is usually preferable to a surface water. If a surface water is used, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination and should maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron.

9.2.3 Dechlorinated water is not recommended as a dilution water for *Daphnia magna*. Dechlorinated water should be used only as a last resort because dechlorination is often incomplete and residual chlorine is quite toxic to *D. magna* (**12**). Sodium bisulfite is probably better for dechlorinating water than sodium sulfite, and both are more reliable than carbon filtration, especially for removing chloramines (**13**). Some organic chloramines, however, react slowly with sodium bisulfite (**14**). In addition to residual chlorine, municipal

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

drinking water often contains unacceptably high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. When necessary, excessive concentrations of most metals can usually be removed with a chelating resin (**15**).

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 the addition of test material. Adequate aeration will bring the pH and concentrations of dissolved oxygen and other gases into equilibrium with the air, and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % saturation to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation of dissolved gases, which might be caused by heating dilution water, should be avoided to prevent gas bubble disease (**18**,**19**).

9.3.2 Filtration through sand, sock, bag, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low (see 9.1.3).

9.3.3 Dilution water that might be contaminated with undesirable microorganisms may be passed through a properly maintained ultraviolet sterilizer (**20**) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm. Water that might be contaminated with *Aphanomyces daphniae* should be autoclaved (**3**).

9.4 *Characterization:*

9.4.1 The following items should be measured at least twice each year, and more often if, (*1*) such measurements have not been made semiannually for at least two years, or (*2*) surface water is used: hardness, alkalinity, conductivity, pH, particulate matter, TOC, organophosphorus pesticides, polychlorinated biphenyls (PCBs), chlorinated phenoxy herbicides, 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.

9.4.2 For each analytical method used (see 13.3) to measure the parameters listed in 9.4.1, quantification of the limit should be below either (*1*) the concentration in the dilution water or (*2*) the lowest concentration that has been shown to adversely affect the survival, growth, or reproduction of *D. magna* (**21**).

10. Test Material

10.1 *General*—The test material should be reagent grade[4] or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the test material:

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

10.1.2 Solubility and stability in the dilution water and solvents.

10.1.3 Measured acute toxicity to *D. magna*.

10.1.4 Measured or estimated chronic toxicity to *D. magna*.

10.1.5 Precision and bias of the analytical method at the planned concentration(s) of test material.

10.1.6 Estimate of toxicity to humans.

10.1.7 Recommended handling procedures (see 8.1).

10.2 *Stock Solutions:*

10.2.1 Stock solutions are usually prepared prior to dosing the dilution water to obtain the desired test concentrations. Water-soluble test materials can often be added directly to dilution water to prepare a stock solution (or in some cases the test solution). Test materials that are moderately soluble or insoluble in water are often dissolved in a solvent to form a stock solution that is then added to dilution water. If a stock solution is used, the concentration and stability of the test material in the stock solution should be determined before beginning the test. If the test material is subject to photolysis, the stock solution should be shielded from light. If the test material hydrolyzes or biodegrades rapidly, it might be necessary to prepare new stock solutions daily.

10.2.2 The preferred carrier for stock solutions is dilution water except possibly for tests on hydrolyzable, oxidizable, and reducible materials. Filtration or sterilization, or both, of the water might be necessary. If the hardness of the dilution water in the test system will not be affected, distilled and deionized water are also acceptable for stock solution preparation. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials (**22**). Minimum necessary amounts of strong acids and bases may be used to prepare 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 even more than the use of the minimum necessary amount of strong acid or base.

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 not affect survival, growth, or reproduction of *D. magna*. Because of their low toxicities to aquatic animals (**23**), low volatilities, and high abilities to dissolve many organic chemicals, dimethylformamide and triethylene glycol are often good organic solvents for preparing stock solutions. Other water-miscible organic solvents, such as methanol, ethanol, and acetone, may also be used as carriers, but they might stimulate undesirable growths of microorganisms, and acetone is quite volatile. If an organic solvent is used, its concentration in any test solution should not exceed 0.1 mL/L. Surfactants should not be used in the preparation of stock solutions because they might affect the form and toxicity of the test material in test solutions. (These limitations do not apply to any ingredients of a mixture, formulation, or commercial product, unless an extra amount of solvent is used in the preparation of the stock solution.)

10.2.4 If a solvent other than water is used as a carrier, at least one solvent control, using solvent from the same batch used to make the stock solution, in addition to the dilution-water control, must be included in the test.

10.2.4.1 If the test contains both a dilution-water control and a solvent control, the survival, growth, and reproduction of *D. magna* in the two controls should be compared (see X1.4). If a statistically significant difference in either survival, growth,

or reproduction is detected between the two controls, the solvent control is normally used for meeting the requirements specified in Section 14 and as the basis for the calculation of results. Judgment might be required in the choice of which control data to use to compare with treatments, especially when the solvent concentration is not constant in the treatments. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements specified in Section 14 and as the basis for calculating the results.

10.2.5 If a solvent other than water is used as a carrier, 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 *Test Concentration(s):*

10.3.1 If the test is intended to provide a good estimate of the highest concentration that will not unacceptably affect the survival, growth, or reproduction of *D. magna*, the test concentrations (see 12.1.1.1) should bracket the best prediction of that concentration. Such a prediction is usually based on the results of an acute toxicity test (see Guide E729) with the test material using the same dilution water and *D. magna* neonates (for example, individuals less than 24-h old). Because the food used in the life-cycle test sometimes affects the results of the acute test (**24**,**25**), acute tests should be conducted with and without the food added to the dilution water prior to conducting the chronic study. If an acute-chronic ratio has been determined for the test material with a species of comparable sensitivity, the result of the acute test with *D. magna* can be divided by the acute-chronic ratio. Except for a few materials (**26**), acute-chronic ratios determined with daphnids are typically less than ten. Thus, the highest concentration of test material in a life-cycle test with *D. magna* is typically selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test.

10.3.2 In some situations (usually regulatory), it is only necessary to determine whether one specific concentration of test material unacceptably affects survival, growth, or reproduction. These situations usually arise when the concentration resulting from the direct application of a material to a body of water is known, or when the material is thought to be nontoxic at its solubility limit in water. When there is only interest in one specific concentration, it is often only necessary to test that specific concentration (see 12.1.2).

11. Test Organisms

11.1 *Species—D. magna* has been extensively used for acute and life-cycle toxicity tests because it is one of the largest cladoceran species, is easy to identify, and is available from many laboratories and commercial sources. These procedures might also be suitable for other daphnid species, although modifications might be necessary. The identities of daphnids obtained from laboratories and commercial sources should be verified, regardless of any information that comes with the organisms. *D. magna* should be verified using the scheme of Brooks (**27**). The identification of other daphnids may vary with the taxonomic reference used (**28**,**29**).

11.2 *Age*—Life-cycle tests with *D. magna* should begin with organisms less than 24-h old.

11.3 *Source*—All daphnids used in a test should be from the same brood stock. This brood stock must have been cultured for at least two generations using the same food, water, and temperature as will be used in the life-cycle test. This will not only acclimate the daphnids, but will also demonstrate the acceptability of the food, water, and so forth, before the test.

11.4 *Brood Stock:*

11.4.1 Brood stock can be obtained from another laboratory or a commercial source. When daphnids are brought into the laboratory, they should be acclimated to the dilution water by gradually changing the water in the culture chamber from the water in which they were transported to 100 % dilution water over a period of two or more days. Daphnids should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 3°C within 12 h until the desired temperature is reached. Generally, acclimation to pH should not exceed more than 1.5 pH units per day.

11.4.2 *D. magna* has been cultured in a variety of systems, such as in large groups in aquaria, in groups of one to five in 100 to 250-mL beakers, or in specially designed chambers (**30**).

11.4.3 To maintain *D. magna* in good condition, the brood stock should be cultured so as to avoid unnecessary stress due to crowding, rapid changes in temperature, and water quality. Daphnids should not be subjected to more than a 3°C change in water temperature in any 12-h period and preferably not more than a 3°C change in any 72-h period. 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 daphnids is kept low, for example, no more than 1 daphnid/30 mL, the surface water/air interface should provide adequate dissolved oxygen. Organisms used for testing must produce at least 60 young per adult during a 21-day test.

11.5 *Food*—Various combinations (see Appendix X2) of trout chow, yeast, alfalfa, and algae, such as *Ankistrodesmus convolutus*, *Ankistrodesmus falcatus*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) (**31**), have been successfully used for culturing and testing *D. magna*. The concentration of test material (number of cells for algae) in the batch of food used should be determined. The experience gained over the past decade has shown that it is very important to incorporate algae into the diet to maintain consistently healthy daphnids (**32-34**).

11.6 *Handling—D. magna* should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that the daphnids are not unnecessarily stressed. Daphnids should be introduced into solutions beneath the air-water interface. Daphnids that touch dry surfaces or are dropped or injured during handling should be discarded. Smooth glass tubes with an inside diameter of at least 5 mm should be used for transferring adult *D. magna*, and

the amount of solution carryover should be minimized. Equipment used to handle daphnids should be sterilized between use by autoclaving or by treatment with an iodophor **(35)** or with 200 mg of hypochlorite/L for at least 1 h (see 6.4).

11.7 *Harvesting Young*—Young less than 24-h old can be obtained using specially designed chambers **(27)** or by transferring to chambers containing dilution water and food, allowing an overnight period for brood release.

11.8 *Quality*—To decrease the chances of a test being unacceptable (see 14.1), the test should not begin with young that were in the first brood **(32)**, nor with young from a daphnid that (*1*) is sick **(3,36)** or incompletely developed **(11)**, (*2*) is more than 50 days old, (*3*) did not produce young before Day 10, (*4*) did not produce at least nine young in the previous brood, or (*5*) is from a culture in which ephippia were produced or in which substantial mortality (>10 %) occurred during the week prior to the test. These factors are most easily monitored if an appropriate number of daphnids from brood stock are individually isolated for the seven days prior to the test, and young produced by these daphnids are used to start the test.

12. Procedure

12.1 *Experimental Design*—It is recommended that at least four chambers per treatment containing at least ten daphnids per treatment be used for renewal and flow-through tests. As a minimum for flow-through and renewal tests, ten daphnids per treatment could be used when each chamber contains only one daphnid and ten chambers per treatment are used. A comparison of the experimental design for renewal and flow-through tests is presented in Table 1.

12.1.1 Decisions concerning the various aspects of experimental design, such as the number of treatments, dilution factor, and numbers of test chambers and daphnids per treatment, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 15).

12.1.1.1 A life-cycle test intended to allow calculation of an end point (see X1.2) usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the controls, daphnids are exposed to dilution water to which neither test material nor solvent has been added. One or more solvent controls might also be necessary (see 10.2.3). Except for the control(s) and the high 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 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 uncertain (see 10.3.1), six or seven concentrations might be desirable.

12.1.1.2 If the purpose of the test is to determine whether a specified concentration causes adverse effects (see 10.3.2), only that concentration and appropriate 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.

12.1.2 The primary focus of the physical and experimental test design and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned **(37)**. Therefore, the test chamber is the experimental unit. All chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature.

12.1.3 A renewal test system should consist of at least five test concentrations plus a control and solvent control (if necessary). At least four chambers should be used for each treatment and control, with at least five daphnids per chamber. A design that is frequently used is five treatment levels with ten chambers each with one daphnid per chamber.

12.1.4 The flow-through test can be any of several designs and should be capable of (*1*) delivering at least five test concentrations plus a control and solvent control; (*2*) delivering test material concentrations that vary less than ±30 % of the mean measured amount over a 21-day period, and (*3*) supplying four to six volume exchanges of each test solution per day. At least four chambers must be used for each treatment and control, with at least ten daphnids per test concentration. A design that is frequently used is five treatments plus controls with four chambers per treatment and with ten daphnids per chamber.

12.1.5 *Test Material Measurement*—A general guide is that the highest values for a given treatment level divided by the lowest measured value for the same treatment level should not vary by more than a factor of 1.5. This varies for chemicals for which the method of analysis is not precise or for chemicals which are measured at extremely low levels. In these cases, every effort should be made to make the measurements as accurate and precise as possible.

TABLE 1 Experimental Design

Design Parameter	Renewal Test	Flow-Through Test
Number of test concentrations	≥5	≥5
Control	Yes	Yes
Solvent control	If appropriate	If appropriate
Number of chambers	At least 4	At least 4
Minimum number of daphnids/treatment	10 (individual daphnid/chamber) 20 (multiple daphnids/chamber)	10 (individual daphnid/chamber) 20 (multiple daphnids/chamber)
Number of daphnids/test chamber	At least 1	At least 5
Feeding	Once daily	2 to 3 times daily (or continuous)
Renewal of test solution	At least 3 times/week	At least 1 volume replacement/day
Temperature	20°C	20°C
Water chemistry	New solutions at each renewal, old solutions after longest time hour interval	Initially and at least weekly thereafter
Analytical confirmation of test material	Initially and at least weekly thereafter, old solutions at least once during the study	Initially and at least weekly thereafter

12.1.6 Assignment of *Daphnia* to the chambers within the treatments as well as assignment of treatment chambers within the test system must be randomized. The following format is suggested: (*1*) random assignment of treatment chambers to the test system, (*2*) random selection of the sequence of chambers to be followed when placing the *Daphnia* into the system, and (*3*) random assignment of the *Daphnia* to the beakers in a given sequence.

12.2 *Dissolved Oxygen*—The dissolved oxygen (DO) concentration in each test chamber should be at least 3.0 mg/L for both the renewal and flow-through tests. Because results are based on measured rather than calculated concentrations of test material, some loss of test material by aeration is not necessarily detrimental and test solutions may be aerated gently when needed to maintain dissolved oxygen levels. Vigorous aeration, however, should be avoided because it can stress daphnids, resuspend fecal matter, and greatly increase volatilization and evaporative losses. Because gaseous exchange occurs at the water/air interface and during diluter cycling, additional aeration is usually unnecessary. Renewal tests might require aeration since dissolved oxygen levels typically drop with time. Also, the use of carrier solvents might reduce the concentration of dissolved oxygen. Aeration, when used, should be the same in all test chambers, including the controls, at all times during the test.

12.3 *Temperature:*

12.3.1 Life-cycle tests with *D. magna* should be conducted at 20 ± 2°C. Other temperatures may be used to study the effect of temperature on the reproduction of *D. magna* or to study the effect of temperature on the chronic toxicity of the material to *D. magna*.

12.3.2 For each test chamber in which temperature is measured, the time-weighted average temperature measured at the end of the test should be within 2°C of the selected test temperature. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Each individual measured temperature must be within 3°C of the mean of the time-weighted averages. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C.

12.4 *Loading*—There should be at least 30 mL of test solution per each first-generation daphnid in flow-through tests and 40 mL per each daphnid in renewal tests.

12.5 *Selection of Test System:*

12.5.1 A renewal test can be used for test materials that are stable in the dilution water and testing conditions. Also, when testing at or near the test material's water solubility, the renewal allows for more time to adequately stir test solutions to approach expected water solubility.

12.5.2 A flow-through test system can be used for most test materials, but should be selected for test materials that have a tendency to dissipate rapidly by hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization. Several diluter systems are currently in use. Mount and Brungs diluters (**38**) have been successfully modified for *Daphnia* testing and other diluter systems have also been useful (**39-45**).

12.6 *Beginning the Test:*

12.6.1 *Selecting the Test System and Preparing Test Solutions:*

12.6.1.1 For a renewal test, fresh test solutions containing appropriate amounts of test material and food should be prepared less than 4 h before each renewal. The fresh test solutions should be placed in each chamber. The test organisms should be added after the food has been added. Analytical confirmation of the test material concentrations prior to the initiation of the test is recommended. Test solutions should be renewed at least three times a week. The test concentrations should vary less than ±30 % of the mean measured amounts over a 21-day period. If test material concentrations decline by more than 30 % over the longest interval between renewals, the beakers might be preexposed (for example, preconditioned) to the test material to help maintain constant test concentrations. The test chambers can be preconditioned by allowing the appropriate test solutions to sit in the test chambers for at least 1 h at which time these solutions would be discarded. The test chambers would then be refilled with the appropriate test solutions.

12.6.1.2 For a flow-through test, the diluter system should be turned on before a test is begun to verify that it is functioning properly: (*1*) the total volume of water being delivered to each treatment and control is within 10 % of predicted, (*2*) each flow splitter divides the volume of water delivered into approximately four equal flows (±10 %), (*3*) the number of times the diluter cycles per hour (intermittent diluters) is correct or the total volume of flow per test concentration per hour (continuous-flow diluters) is correct, and (*4*) the chemical delivery system is functioning properly. Analytical confirmation of the test concentrations are required before the test may begin. A careful check of the diluter system can save time, effort, and the need for repeating test material analyses. The diluter system typically needs to operate for at least two days prior to starting the test to check the reliability of the system and provide time for the toxicant to reach the desired concentration in each test chamber.

12.6.1.3 Mean measured concentrations of the test material should vary less than ±30 % from the intended nominal concentration for a test.

12.6.2 The test begins when test organisms are first placed in the test solutions. Daphnids less than 24-h old should be impartially distributed to the test chambers by placing one daphnid in each test chamber from each treatment, and then a second daphnid in each test chamber from each treatment, and continuing the process until each test chamber contains the appropriate number of daphnids. Alternatively, the daphnids may be assigned by total randomization (see 12.1.6).

12.7 *Care and Maintenance*—The test chambers should be brushed and rinsed with dilution water at least three times a week. A common way of doing this is to remove the *Daphnia* by pipet and place it in 100 mL of test solution. Pour the remaining test solution through a fine-mesh screen into a clean test chamber. The test solution is returned to the cleaned test chamber and the *Daphnia* are then returned to the test solution. More frequent cleaning might be necessary if bacterial growth

appears or if the DO content drops below 4.0 mg/L. The test chamber screens (flow-through tests) should be brushed clean daily.

12.7.1 In renewal tests, new solutions will be placed in clean test chambers before the first-generation daphnids are returned after removal from old solutions. A duplicate set of test chambers can be used to facilitate the renewal procedure and allow for preconditioning of the test chambers, if needed.

12.8 *Feeding*—Sufficient food should be provided to ensure an acceptable level of reproduction. Each test chamber should receive the same concentration. The use of algae, vitamins, alfalfa, or other materials in various combinations have been used successfully.

12.8.1 *Flow-Through Tests*—A recommended regime is at least two feedings per day (preferably three feedings per day) where each feeding results in at least 1 mg/L trout chow suspension (optional) or 1.0×10^8 algae cells/L, or both, in the test solutions. Continuous feeding methods have also been used successfully. A peristaltic pump is usually used to pump the food to the mixing cells of the diluter.

12.8.2 *Renewal Tests*—Daily feeding is recommended. This is accomplished by adding food to the test solutions each time the test solutions are renewed and once a day on days when the test solutions are not renewed. Sufficient food should be provided to result in at least 1-mg/L trout chow suspension (optional) or at least 1.0×10^8 algae cells/L, or both, in the test solutions.

12.8.3 The previously recommended amounts of food are suggested because they have been demonstrated to work. Other levels of food can be used as long as the number of young produced in the control treatments meets the minimum criteria for acceptance, that is, 60 young per adult in 21 days.

12.9 *Duration*—The test ends on Day 21, at which time the first generation (parent) daphnids are counted, growth measurements are taken, and the number of young, since last cleaning or renewal, both alive and dead, in each beaker are recorded.

12.10 *Biological Data:*

12.10.1 The death of all first generation daphnids must be recorded daily. The criteria for death are absence of heartbeat, white or opaque coloration, lack of movement of appendages, and lack of response to gentle prodding. The daphnids in each chamber will be observed daily. Mean control survival must be ≥70 % for the test to be acceptable.

12.10.2 Reproductive counts should be made at least three times weekly after Day 7; for example, every Monday, Wednesday, and Friday (that is, Days 9, 12, 14, 16, 19, and 21 if the test was started on a Wednesday). A convenient way to count the young (noting living or dead) after the adults have been removed is to pour the old test solution through a small screen, rinse the young into a watchglass, and count over a piece of black plastic by removing the young with a Pasteur pipet. After the young *Daphnia* have been counted, they can be discarded. A data recording system must be used that records survival and reproduction for each test vessel.

12.10.3 The size of first-generation daphnids (adults) that are alive at the end of the test must be determined using dry weight (normally, a mean dry weight is determined for pooled adults from each chamber) or length. Dry weight (wet weight is not acceptable) is determined by drying daphnids to a constant weight; at 60°C for 72 h or at 100°C for 24 h (**46,47**). Dry weight is often preferred to length measurements because it provides an indication of the effects of the test substance on the biomass production and hence energy transfer from one trophic level to the next. Length is measured as the distance from apex of the helmet to the base of the spine or may be extrapolated from a standard curve of dry weight to body length.

12.10.4 The day when first reproduction of the first-generation daphnids are observed for each chamber will be recorded (that is, time to first brood).

12.10.5 Both first- and second-generation daphnids should be carefully and regularly observed during the test for abnormal development and aberrant behavior, such as inability to maintain position in the water column, uncoordinated swimming, and cessation of feeding. Although developmental and behavioral effects are often difficult to quantify and might not provide suitable end points, they might be useful for interpreting effects on survival and growth and for deciding whether the test should be extended beyond the minimum duration (see 12.8).

12.10.6 Morphological examination of first-generation daphnids alive at the end of the test in each treatment, before they are dried, might be desirable. Biological and histological examination and measurement of test material in exposed daphnids will probably not be possible unless additional daphnids are exposed specifically for such purposes.

12.10.7 It might be desirable to obtain data on the effect of the test material on survival, development, and behavior of a few second-generation daphnids for four or more days.

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

12.11 *Other Measurements:*

12.11.1 *Water Quality (Flow-Through and Renewal)*—Hardness, alkalinity, conductivity, dissolved oxygen, and pH should be measured at least weekly for the dilution water (not test water) used in the test. Dissolved oxygen and pH should be measured at the beginning and end of the test, and at least weekly during the test on the control(s) and each treatment. Hardness, alkalinity, and conductivity should also be measured in at least the highest test concentration at least once during the test to determine whether they are affected by the test material. For renewal tests, hardness, alkalinity, conductivity, dissolved oxygen, and pH should be measured in old solutions at least weekly. Measurements of calcium, magnesium, sodium, potassium, chloride, sulfate, particulate matter, and TOC, or chemical oxygen demand (COD) may be desirable for both flow-through and renewal tests.

12.11.2 *Temperature*—Throughout the test duration, temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily in at least one test chamber. Near the beginning, middle, and end of the test, temperature must be concurrently measured in all test chambers. If the test chambers are in a water bath, the temperature of the water bath may be measured as a substitute

for measurements in the test vessels. In this case, temperature must be measured or monitored at least hourly in the water bath or the maximum and minimum temperatures must be measured daily. If the test chambers are in a constant-temperature room or incubator, measuring or monitoring the air temperature at least hourly or measuring of the maximum and minimum air temperature daily may be made instead of normal measurements in the test chambers, provided that measurements are made weekly to show that the test solutions are at the same test temperature as the air.

12.11.3 *Test Material:*

12.11.3.1 The concentration of the test material in each treatment should be frequently measured 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 these test materials will probably have to be monitored by such indirect means as radioanalysis, turbidity, TOC, or by measurement of one or more chemical specific components.

12.11.3.2 The concentration of the test material in each treatment should be measured at least weekly, including the control(s). For renewal tests, the old solutions must be measured at least twice during the study (preferably on the old solutions from longest renewal interval). Analysis of additional samples after filtration or centrifugation may be desirable for both flow-through and renewal tests to determine the percentage of test material that is not dissolved or is associated with particulate matter. When test concentrations are measured, at least two samples from two or more chambers should be measured.

12.11.3.3 In each treatment, the highest of all the measured concentrations obtained during the test divided by the lowest must be less than two.

12.11.3.4 If the daphnids are possibly being exposed to substantial concentrations of one or more impurities or degradation or reaction products, measurement of the impurities and products is desirable.

13. Analytical Methodology

13.1 The methods used to analyze water samples for test material may determine the usefulness of the test results because all results are based on measured concentrations. For example, if the analytical method measures any reaction or biodegradation 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 no interfering products are present. Separate measurement of major products is usually desirable.

13.2 If samples cannot be analyzed immediately, they should be handled and stored appropriately **(48)** 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 where ASTM standards do not exist or are not sufficiently sensitive enough, methods should be obtained from other reliable sources **(49)**. The concentration of nonionized ammonia can be calculated from pH, temperature, and concentration of total ammonia **(50)**.

13.4 Methods used to analyze food (see 11.5) or daphnids (see 11.8) should be obtained from appropriate sources **(51)**.

13.5 The precision and bias of each analytical method used should be determined in an appropriate matrix, that is, in water samples from a brood-stock tank or control test chamber, in food, and in daphnids. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed. The limit of detection of the method and the limit of quantification of the analytical instrument should be determined.

14. Acceptability of Test

14.1 A life-cycle test with *D. magna* should be considered unacceptable if one or more of the following occurred.

14.1.1 Daphnids were not randomly assigned to test chambers and there were less than four chambers per treatment or ten daphnids per treatment.

14.1.2 The test was begun with daphnids more than 24-h old or with daphnids from a culture that had not been maintained for at least two generations with acceptable reproduction.

14.1.3 Appropriate dilution-water controls (and solvent controls if necessary) were not included in the test.

14.1.4 The test lasted less than 21 days.

14.1.5 More than 30 % of the first-generation daphnids died in the control treatment(s) within 21 days.

14.1.6 Daphnids that lived for 21 days in the control treatment(s) did not produce, on average, at least 60 young in the 21 days.

14.1.7 Ephippia were produced in the control treatment(s).

14.1.8 Temperature, dissolved oxygen, and concentration of test material were not measured as specified in 12.11.2.

14.1.9 The mean measured dissolved oxygen concentration in any treatment was <3.0 mg/L or any measured dissolved oxygen concentration was <1.5 mg/L.

14.1.10 The mean measured temperature in any treatment was not between 18 and 22°C or any measured temperature was below 17 or above 23°C. Except, for example, if temperature was measured numerous times, a deviation of more than 3°C in any one measurement might be inconsequential. However, if temperature was only measured a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if the temperature had been measured more often.

14.1.11 The highest measured concentration of test material in a treatment was more than twice the lowest in the same treatment.

15. Calculation of Results

15.1 The primary data obtained from a life-cycle test with *Daphnia magna* are (*1*) the number of adults alive at the end of the test, (*2*) the number of live young produced per adult reproduction day or the total number of live young produced per chamber (*3*) time to first brood, (*4*) the dry weight (or length) of the first-generation daphnids (individuals from each chamber can be pooled) alive at the end of the test, and (*5*) the concentration of test material in the test solutions in each

treatment. Other assessment end points may be obtained and evaluated (for example, time to appearance of the primiparous instar in the brood chamber, mean number of reproduction days, mean brood size, total number of broods produced per treatment, and mean number of broods produced per female.

15.1.1 Reproductive data usually consist of three parameters indicative of reproductive success: time to first brood, total number of young, and young per adult reproduction day (YAD). Time to first brood is calculated as the number of days after test initiation until the instar are first observed for each chamber. The total number of young is the cumulative number of young produced per chamber during the test. The YAD is determined from the total number of young produced and the number of adult reproduction days during the test. The adult reproduction days are based on the number of days daphnids are reproducing and the number of adult daphnids alive on each day. The number of reproduction days (normally 13 to 15) is counted from the day first neonate production is observed (first reproduction day) to the last day of the test. If reproduction is first observed on Day 7 of a test in a chamber containing ten adult daphnids and no mortality occurs for the duration of the tests, then the chamber would have a total of 150 adult reproduction days (10 adults × 15 reproduction days). The value for adult reproduction days for each chamber is calculated by summing the number of adult daphnids alive in each chamber for each reproduction day. These data are available from the daily survival data. An adult daphnid is considered dead for the whole 24 h preceding observed death. For example, if an adult daphnid is observed dead on Day 21, then that chamber would have one less adult reproduction day.

Example: Test Level 1 Chamber B
Day of first brood = Day 7 (6 days without reproduction)
Number of reproduction days = 15 (that is, 21 – 6 = 15)
Number of surviving adults from Day 7 to Day 10 = 10
10 adults × 4 reproduction days = 40 adult reproduction days
Number of surviving adults from Day 11 to Day 21 = 9
9 adults × 11 reproduction days = 99 adult reproduction days
Total adult reproduction days = 139
Total number of young = 1737

$$\frac{\text{1737 young}}{\text{139 adult reproduction days}}$$

= 12.50 young/ adult reproduction days

15.2 The statistical procedures that can be used to analyze 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 the advantages and disadvantages of various alternative procedures and appropriate preliminary tests, such as those for outliers and for heterogeneity. The statistical procedure(s) and interpretation of the results should be appropriate to the experimental design (see 12.1). For example, if results are calculated from daphnids that were all exposed in the same laboratory, the results only apply directly to that batch of daphnids in that laboratory and do not necessarily apply to other batches or other laboratories. The major alternative procedures and points to be considered when selecting and using statistical procedures for analyzing results of life-cycle toxicity tests with *D. magna* are discussed in Appendix X1.

16. Report

16.1 The record of the results of an acceptable life-cycle toxicity test with *D. magna* 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 the dilution water, its chemical characteristics, and a description of any pretreatment.

16.1.4 Source of the 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 daphnids at the beginning of the test.

16.1.5 Description of the experimental design, test chambers, compartments and covers, the depth and volume of solution in the chambers, number of daphnids per chamber, test chambers 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, and feeding method, frequency, and ration.

16.1.7 Range and time-weighted average measured concentration of dissolved oxygen (as percent of saturation) for each treatment and description of any aeration performed on test solutions before or during the 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 the methods used to obtain, prepare, and store the samples.

16.1.10 Methods used for, the results (with 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 in sufficient detail to allow independent statistical analyses on survival, growth, and reproduction of daphnids in each test chamber and in each treatment, including the control(s).

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

16.1.13 Summary of general observations on other effects.

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 procedures used and the quality of the results.

17. Keywords

17.1 chronic; *Daphnia magna*; flow-through; invertebrate; life-cycle; renewal; toxicity

APPENDIXES

(Nonmandatory Information)

X1. STATISTICAL GUIDANCE

X1.1 *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: (*1*) preliminary and diagnostic graphical displays, (*2*) pair-wise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (*3*) analysis of variance (ANOVA) and corresponding contingency table tests, (*4*) multiple comparison techniques for simultaneous pair-wise comparison of other treatment groups with control groups, (*5*) concentration-effect curve analyses, and (*6*) multiple regression. If used correctly, each of these techniques can provide useful information concerning the results of an acceptable life-cycle test with *D. magna*.

X1.1.1 The three kinds of data that can be obtained from toxicity tests are dichotomous or categorical (for example, mortality), count or enumeration (for example, number of young), and continuous (for example, length and 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 arising from different application areas, different terminologies and computing tools were developed for analyzing the three kinds of data.

X1.2 *End Point*—The end point of life-cycle toxicity tests with *D. magna* generally has been defined in terms of whether differences from control daphnids are statistically significant at the 5 % level. One of the main conceptual problems with the definition of end point is that biological importance and statistical significance are logically distinct. Effects of considerable biological importance might not be statistically significant if sample sizes are small or if effects are extremely variable, or both. Conversely, biologically trivial effects might be highly statistically significant if sample sizes are large or if effects are very reproducible. An end point based solely on statistical significance might depend as much or more on sample sizes as on the magnitudes of the effects.

X1.2.1 An alternative approach is to define the end point in terms of a specified absolute or relative amount of difference in a biological attribute from the control treatment(s). A regression-type model would be fitted to the data, and the concentration associated with a specified amount of difference from the control treatment(s) would be estimated using the model. For example, the concentration resulting in a specified percent decrease in number of live young might be estimated along with confidence limits on the estimated concentration. The result of a life-cycle test would then be reported as a point estimate, preferably with confidence limits, of the concentration expected to cause an amount of effect that has been preselected as being biologically unacceptable.

X1.2.2 In general, an end point defined in terms of a statistically significant difference is calculated using ANOVA, 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 presented in reports to permit calculation of end points other than those chosen by the authors and to allow other uses of the data, such as modeling.

X1.3 *Preliminary Data Analysis:*

X1.3.1 *Graphical Displays*—These should be performed every time data for any biological attribute are analyzed using either regression analysis or hypothesis testing **(52)**. Preliminary scatterplots are desirable because they might provide insights into the structure of the data and reveal the presence of unanticipated relations or anomalies. Every time a regression-type model is fitted to data, a graph of predicted and observed values should be examined to assess the goodness of fit of the model; a graph of residuals from the fit should be examined to assess departures from the model. Histograms are useful for examining the distribution of the data before hypothesis testing. The advent of modern computers and statistical computing packages, for example, Minitab, SAS, BMDP, and SPSS **(53)**, has made the preparation of graphs both easy and inexpensive. Feder and Collins **(54)** illustrate the use of various types of preliminary and diagnostic graphical displays in the analysis of data from chronic toxicity tests.

X1.3.2 *Outlier Detection Procedures*—Data points 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 outside the range of a random variation. Barnett and Lewis **(55)** describe many outlier detection procedures, and Feder and Collins **(54)** 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 either be corrected or deleted from the data set prior to analysis. If outliers are not known to be erroneous values, the question of how to deal with them is a matter of judgment. Data analysis should be performed 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.

X1.3.3 *Data Transformations*—Many standard statistical procedures such as regression analysis and ANOVA are based on the assumption that experimental variability is homogeneous across treatments. This assumption typically does not hold for certain kinds of data. If data displays or tests of

heterogeneity demonstrate that variability is not homogeneous 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 (**56**). 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.

X1.4 *Comparison of Solvent Control and Dilution Water Control*—If both solvent and dilution water controls are included in the test, the results should be compared using a *t*-test for count and continuous data and Fisher's exact test or a 2 by 2 contingency table test for categorical data (**57**). Adjustments for chamber-to-chamber heterogeneity might be necessary. The use of a large alpha level (for example, 0.25) will make it more difficult to accept the null hypothesis when it should not be accepted. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

X1.5 *Analysis of Variance and Contingency Table Analyses*—The 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. Feder and Collins (**54**) illustrate transformation of data before use of a contingency table test, if necessary.

X1.5.1 Both contingency table tests and ANOVA followed by *t*-tests make no assumption about the particular form for the relationship between effects and concentrations. Therefore, they are not designed to be particularly sensitive to the one-sided, monotonic trends characteristically observed in toxicity tests. Specialized tests have been designed to be more sensitive to relations of this type. Some such tests are the one-sided measure of association test, the Cochran-Armitage test for categorical data, and tests based on linear or polynomial regression models for continuous data (**58**). The ANOVA tests are based on normal distribution theory and assume that the data within treatments are a random sample from an approximately normal distribution and that error variance is constant between treatments.

X1.5.2 As a part of the ANOVA, residuals should be plotted against concentration to determine whether there are any obvious violations of the assumptions of normality and homoscedasticity, that is, constant error variance. When results of an ANOVA are reported, the ANOVA model and table, the *F* -statistic and its significance level, the minimum detectable difference, and the power of the test should be presented.

X1.6 *Multiple Comparison Procedures*—The usual approach to analyzing data from life-cycle tests is to compare data for each concentration of the test material to data for the control(s). In Fisher's Protected Test, which should be used only if the ANOVA *F*-test is significant (**59**), each concentration of test material is compared to the control(s) using the *t*-test. If the investigator desires to set an experiment-wise α, rather than a comparison-wise α, Dunnett's procedure (**59**,**60**) can be used without the ANOVA *F*-test. Williams' procedure (**59**,**61**) 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 (**62**) No Statistical Significance of Trend (NOSTASOT) test can be used with the same assumptions as Williams' procedure. Shirley (**63**) has developed a nonparametric equivalent for Williams' test, and Williams (**64**) has modified and corrected Shirley's procedure to increase its power to detect the alternative hypothesis.

X1.6.1 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 detectable difference, and the power of the test.

X1.7 *Regression Analysis and Concentration-Effect Curve Estimation* —An alternative to tests for statistically significant differences is to fit concentration-effect models or multiple regression models to the data and estimate the concentration that corresponds to a specified amount of difference from the control treatment(s) (**65**). Regression models are commonly used to fit concentration-effect data so that estimates may be made of the concentration that corresponds to a specified amount of difference from the control treatment(s). The probit and logit models are commonly used to describe trends in dichotomous data, such as survival. Nonlinear or linearized models, or both, are used for continuous data, such as length, weight, or young per adult reproductive day. Toxicity tests should be designed to avoid the need for extrapolation, because it can introduce biases into the estimates.

X1.7.1 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 (**2**) discusses the probit model in considerable detail, and Draper and Smith (**66**) and Neter, Wasserman, and Kutner (**25**) discuss most practical aspects of multiple regression analysis. Feder and Collins (**54**) discuss use of these techniques in aquatic toxicology.

X1.7.2 When a regression model or concentration-effect curve model is fitted, data for each experimental unit are plotted against concentration. If necessary, transformation of the effect data or concentration data, or both, should be performed to stabilize the variance 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 there is no further structure in the residuals to be explained. Presentation of results of regression or concentration-effect curve analysis should include the intercept or other point estimate and the slope and their 95 % confidence limits.

X2. FOOD

X2.1 *Introduction*—A wide variety of foods have been used for culturing and testing *D. magna* (**67**). The foods termed *synthetic* are usually made from one or more ingredients such as a trout chow or yeast. The foods termed *natural* consist of one or more green algae and diatoms. Combination foods contain both synthetic and natural ingredients. A number of studies have compared the abilities of various foods to support survival, growth, and reproduction of *D. magna* (**68**). Although the results of such comparisons have shown that there are substantial differences between foods, definitive general conclusions are not yet possible because (*1*) a food that works well in one laboratory sometimes works very poorly in another laboratory, (*2*) substantial differences in composition and nutritional value appear to occur within and between brands and formulations of trout chow, (*3*) some ingredients of synthetic foods are occasionally contaminated by pesticides and metals, (*4*) the daphnids might be feeding on secondary food, such as bacteria, that contaminate the food or grow in the test chamber, and (*5*) if a food does not contain an essential trace metal, daphnids might be able to obtain the metal from some dilution waters, but not from others. Therefore the following information is intended to be helpful, but should not be considered definitive. No test should be started until a food has been demonstrated adequate under the conditions for which the test is to be conducted (see 6.5 and 11.3).

X2.2 *Synthetic Foods:*

X2.2.1 Numerous synthetic foods have been used with varying degrees of success (**69**). The following formula has been used successfully in several laboratories as synthetic food, but the quality of the food will obviously depend mostly on the quality of the trout chow used. Other ingredients, such as a vitamin mix (see X2.3.1), may be added if desired.

X2.2.2 Place 12 g of trout chow, 3 g of active dry yeast, and 400 mL of deionized, distilled, or dilution water in a blender and blend for 5 min at high speed. Pour into a 1-L graduated cylinder and bring to volume. Mix well and let settle for 10 min. Siphon the top 800 mL into a container and cover. While the 800 mL of food mixture is being stirred vigorously, remove three 10-mL samples from the central portion of the container by means of a serological pipet with a tip opening of about 2.5 mm. Place each sample in a tared aluminum weighing pan. Slowly evaporate the liquid portion to avoid spattering and dry for 24 h at 60°C. Cool in a desiccator and weigh. Calculate the milligrams of dry solids per millilitre in each sample, *Z*, as follows:

$$Z = \frac{S}{10\ \text{mL}} \qquad \text{(X2.1)}$$

where:

S = solids in weighing pan, mg.

Dilute the food mixture to approximately 5 mg of dry solids per millilitre by adding Y mL of water to the mixture, where:

$$Y = \frac{(Z\ \text{mg/mL})\ (770\ \text{mL})}{5\ \text{mg/mL}} - 770\ \text{mL} \qquad \text{(X2.2)}$$

With the resulting mixture stirring vigorously, remove three 10-mL samples, weigh as described in X2.2.2, and calculate the mean and standard deviation. If the standard deviation is more than 5 % of the mean, the sampling should be repeated. The mean measured solids concentration is the value used to calculate the millilitre of food mixture required for addition to the dilution water (see 12.5). Cover the mixture and store in a refrigerator for up to 14 days.

X2.2.3 This food has often been used at 30 mg of dry solids per litre in test solutions because at lower concentrations, small increases in the concentration of food resulted in substantial increases in reproduction. At about 30 mg/L and above, higher concentrations of food resulted in only slight increases in reproduction. Although 30 mg of solids/L might be suspected of causing trouble to filter feeders and substantially reducing the dissolved oxygen concentration, survival and reproduction of *D. magna* do not appear to be adversely affected by up to 60 mg/L. With some trout chows, it might be possible to use much less than 30 mg/L.

X2.3 *Natural Foods:*

X2.3.1 Various natural foods have been used with different degrees of success, depending on the species of green algae and diatoms used, the medium in which the algae and diatoms are grown, and the dilution water in which the daphnids are cultured. Although it requires more effort to prepare a natural food than a synthetic food, use of natural foods is strongly recommended because diets that contain natural foods have been shown to produce daphnids with high lipid content, large brood sizes, and acceptable survival rates (**32-34**).

X2.3.2 The four species of green algae most commonly used are *Ankistrodesmus convolutus, A. falcatus, Chlamydomonas reinhardtii*, and *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*). The diatom, *Nitschia frustulum*, might be a desirable dietary supplement. Cultures of these species can be purchased from several sources. Generally, the cultures are supplied on agar slants, which can be kept for several months in a dark refrigerator at 4°C. The green algae and diatoms are transferred to a liquid nutrient medium to grow large amounts for feeding daphnids.

X2.3.3 Nutrient media are prepared by adding specified amounts of stock solutions to deionized or distilled water. To

obtain consistent growth and food value of the green algae and diatoms, the quality of the water must be exceptionally good. Nutrient medium should be sterilized prior to the addition of algae and diatoms, either by filtration through a 0.22-µg membrane filter or by autoclaving. Examples of nutrient media that are known to produce high-quality algae are presented in Table X2.1 and Table X2.2. Other media may be used if data are available to show that daphnid-fed algae grown on this media consistently meet the criteria for acceptable reproduction over several generations.

X2.3.4 The vitamin solution should contain the following (**54**):

	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, the vitamin solution can be stored in a dark sterile bottle in a refrigerator for at least 3 years or portions can be frozen. One millilitre of this vitamin solution should be added to each litre of nutrient medium after the medium is sterilized.

X2.3.5 The general principles of sterile technique should be observed to prevent contamination of the cultures of green algae and diatoms with fungi, bacteria, or other species of algae. Glassware should be washed and sterilized as recommended for daphnids. Although the green algae and diatoms grow acceptably at 20 ± 2°C with 3800 to 4500 lx for 14 to 16 h/day, they will grow faster at 24 ± 2°C and with continuous light at 4300 to 4500 lx. The light should be from a broad-spectrum fluorescent bulb. If cultures are aerated by bubbling air through them, the air should be filtered through a 0.22-µm bacterial filter.

X2.3.6 When a sterile nutrient medium with vitamins is first inoculated with green algae or diatoms, there is usually a lag phase of one to two days before growth becomes visible. This is followed by a log phase of rapid growth that gradually levels off as the maximum cell density (standing crop) is approached. When the maximum cell density is reached, the density will remain fairly constant, but the individual cells will continue to grow and age. Green algae and diatoms for feeding daphnids should be harvested during the log growth phase to ensure that the algae and diatoms 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. Cultures with adequate light and aeration are usually about one week from maximum cell density when the medium turns visibly green.

X2.3.7 Algae are usually cultured using either the static or the partial replacement technique.

X2.3.7.1 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

TABLE X2.1 Modified Bold Basal pH 6.6 (Modified Bristol Solution) (70)

NOTE 1—The specified volumes of eleven stock solution (six macronutrient solution, 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 (concentrated)	1.0 mL/L
Boron (use 1 mL):	
H_3BO_4	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 X2.2 Algal Nutrient Media

NOTE 1—For either medium, prepare two stock solutions and use 1 mL of each stock solution per litre of medium. The above media are examples of media that are known to provide adequate algal growth. Other media may be substituted if they are shown to be suitable.

	Woods Hole MBL (71), g/L in stock solution	ASM-1 (72), g/L in stock solution
Macronutrients:		
$CaCl_2 \cdot 2H_2O$	36.76	29.41
$MgSO_4 \cdot 7H_2O$	36.97	49.3
$MgCl_2 \cdot 6H_2O$	...	40.67
$NaHCO_3$	12.6	...
K_2HPO_4	8.71	17.41
$NaNO_3$	85.01	170.0
$Na_2SiO_3 \cdot 9H_2O$	28.42	...
Micronutrients:		
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	1.384
$Na_2MoO_4 \cdot 2H_2O$	0.006	0.010
H_3BO_3	1.0	2.47

kept on a shaker table or are 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 flasks can be used to grow large amounts of green algae and diatoms. The entire contents should be harvested just prior to maximum cell density. New cultures should be inoculated often enough to provide at least one culture for harvesting during the log growth phase every time food is needed.

X2.3.7.2 The partial replacement technique allows for a continuous production of large amounts of green algae and diatoms while maintaining them in the log growth phase by periodic removal of a portion of the culture solution and replacement with fresh nutrient medium. Convenient culture vessels for this technique 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 technique, green algae and diatoms can be drawn off several times a week and fresh medium can be gravity fed into the culture vessel. However, partial replacement cultures are more likely to become contaminated than are static cultures.

X2.3.8 Harvesting of the green algae and diatoms can be accomplished by centrifugation, filtration, or by settling overnight in a refrigerator. It is not necessary to remove all the medium, but only to concentrate the green algae and diatoms so that the addition of medium to daphnid cultures and test solutions is minimal. Either dry weight or actual cell counts, or both, will be used to identify the concentration of the harvested food. Actual cell counts can be made by using a microscope and counting cells or by using electronic counters. Dry weight would be calculated in the same manner described in X2.2.2.

X2.3.9 Daphnids have been maintained in cultures and in life-cycle test on (*1*) 1.0×10^8 algae cells/L of dilution water/day, (*2*) 0.2 mg of algae/daphnid/day, or (*3*) 1.25 mg (dry weight) of algae/L of dilution water/day, if the solution is renewed on a strict every-other-day schedule or 2.5 mg (dry weight)/L/day, if the solution is renewed three times a week. Daphnids do better being fed a multispecies algae diet with or without the addition of YTC (**34**).

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