the test chemical. If a carrier other than nutrient medium is absolutely necessary to dissolve the chemical, the volume used shall not exceed the minimum volume necessary to dissolve or suspend the chemical in the test solution.

(3) *Test parameters.* (i) The test temperature shall be 24 °C for *Selenastrum* and 20 °C for *Skeletonema.* Excursions from the test temperature shall be no greater than ±2 °C. Temperature should be recorded hourly during the test.

(ii) Test chambers containing *Selenastrum* shall be illuminated continuously and those containing *Skeletonema* shall be provided a 14-hour light and 10-hour dark photoperiod with a 30 minute transition period under fluorescent lamps providing 300 ±25 uEin/m^2 sec (approximately 400 ft-c) measured adjacent to the test chambers at the level of test solution.

(iii) Stock algal cultures should be shaken twice daily by hand. Test containers shall be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/minute for *Selenastrum* and at approximately 60 cycles/minute for *Skeletonema* during the test. The rate of oscillation should be determined at least once daily during testing.

(iv) The pH of nutrient medium in which algae are subcultured shall be 7.5 (±0.1) for *Selenastrum* and 8.1 (±0.1) for *Skeletonema,* and is not adjusted after the addition of the algae. The pH of all test solutions shall be measured at the beginning and end of the test.

(v) Light intensity shall be monitored at least daily during the test at the level of the test solution.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of acute phytotoxicity. In addition to the general reporting requirements prescribed in part 792—*Good Laboratory Practice Standards of this Chapter,* the following shall be reported:

(1) Detailed information about the test organisms, including the scientific name, method of verification, and source.

(2) A description of the test chambers and containers, the volumes of solution in the containers, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus.

(3) The concentration of the test chemical in the control and in each treatment at the end of the test and the pH of the solutions.

(4) The number of algal cells per milliliter in each treatment and control and the method used to derive these values at the beginning, 24, 48, and 72 hours, and end of the test; the percentage of inhibition or stimulation of growth relative to controls; and other adverse effect in the control and in each treatment.

(5) The 96-hour EC_{10}, EC_{50}, and EC_{90} values, and when sufficient data have been generated, the 24, 48, and 72 hour LC_{50}'s and 95 percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination.

(6) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(7) The results of any optional analyses such as: Microscopic appearance of algae, size or color changes, percent mortality of cells and the fate of subcultured cells, the concentration of test substance associated with algae and test solution supernate or filtrate.

(8) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l or saturation concentration) had no effect on the algae, report the results and concentration and a statement that the chemical is of minimum phytotoxic concern.

(9) If the range-finding test showed greater than a 50 percent inhibition of algal growth at a test concentration below the analytical detection limit, report the results, concentration, and a statement that the chemical is phytotoxic below the analytical detection limit.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987]

§ 797.1300 Daphnid acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on

the acute toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes an acute toxicity test in which daphnids (*Daphnia magna* or *D. pulex*) are exposed to a chemical in static and flow-through systems. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present in the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) *Brood stock* means the animals which are cultured to produce test organisms through reproduction.

(2) EC_{50} means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(3) *Ephippium* means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(4) *Flow-through* means a continuous or an intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(5) *Immobilization* means the lack of movement by the test organisms except for minor activity of the appendages.

(6) *Loading* means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time, or passing through the test chamber during a specific interval.

(7) *Static system* means a test system in which the test solution and test organisms are placed in the test chamber and kept there for the duration of the test without renewal of the test solution.

(c) *Test procedures*—(1) *Summary of the test.* (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test, the flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each treatment chamber. The addition of test chemical in the flow-through system is conducted at a rate which is sufficient to establish and maintain the desired concentration in the test chamber. The test is started within 30 minutes after the test chemical has been added and uniformly distributed in static test chambers or after the concentration of test chemical in each flow-through test chamber reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured and acclimated in accordance with the test design are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, the immobile daphnids removed, and the findings recorded.

(ii) Dissolved oxygen concentration, pH, temperature, the concentration of test chemical and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to develop concentration-response curves and determine EC_{50} values for the test chemical.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The daphnids should be exposed to a series of widely spaced concentrations of the test chemical (e.g., 1, 10, 100 mg/1, etc.), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test chemical for a period of 48 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration-response curves and the 24- and 48-hour EC_{50} values with the

minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/l). An equal number of daphnids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test chemical. The concentration of solvent should not exceed 0.1 mg/l. The concentration ranges shall be selected to determine the concentration-response curves and EC_{50} values at 24 and 48 hours. Concentration of test chemical in test solutions should be analyzed prior to use.

(iii) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning and end of the test in each chamber.

(v) The test duration is 48 hours. The test is unacceptable if more than 10 percent of the control organisms are immobilized during the 48-hour test period. Each test chamber shall be checked for immobilized daphnids at 24 and 48 hours after the beginning of the test. Concentration-response curves and 24-hour and 48-hour EC_{50} values for immobilization shall be determined along with their 95 percent confidence limits.

(vi) In addition to immobility, any abnormal behavior or appearance shall also be reported.

(vii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(viii) The concentration of the test chemical in the chambers should be measured as often as is feasible during the test. In the static test the concentration of test chemical shall be measured, at a minimum, at the beginning of the test and at the end of the test in each test chamber. In the flow-through test the concentration of test chemical shall be measured at a minimum:

(A) In each chamber at the beginning of the test and at 48 hours after the start of the test;

(B) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

Among replicate test chambers of a treatment concentration, the measured concentration of the test chemical shall not vary more than ±20 percent.

(5) [Reserved]

(6) *Analytical measurements.* (i) *Test chemical.* Deionized water should be used in making stock solutions of the test chemical. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test chemical in a sample shall be validated before beginning the test by appropriate laboratory practices. Any analytical method is not acceptable if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* The number of immobilized daphnids shall be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 24- and 48-hour EC_{50} and corresponding 95 percent interval shall be calculated.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The cladocerans, *Daphnia magna* or *D. pulex*, are the test species to be used in this test. Either species may be used for testing of a particular chemical. The species identity of the test organisms should be verified using appropriate systematic keys. First instar daphnids, ≤24 hours old, are to be used to start the test.

(B) Daphnids to be used in acute toxicity tests should be cultured at the

test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test shall have originated from the same culture population.

(C) Daphnids shall not be used for a test (*1*) if cultures contain ephippia; (*2*) if adults in the cultures do not produce young before day 12; (*3*) if more than 20 percent of the culture stock die during the 2 days preceding the test; (*4*) if adults in the culture do not produce an average of at least 3 young per adult per day over the 7-day period prior to the test and (*5*) if daphnids have been used in any portion of a previous test, either in a treatment or in a control.

(ii) *Acclimation.* (A) Brood daphnids shall be maintained in 100-percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in the dilution water at the test temperature. During production of neonates, daphnids should not be fed.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(iii) *Care and handling.* (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully, and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage and mortality. Dead and abnormal individuals shall be discarded. Organisms that touch dry surfaces or are dropped or injured in handling shall be discarded.

(B) Smooth glass tubes (I.D. greater than 5 mm) equipped with rubber bulb should be used for transferring daphnids with minimal culture media carry-over. Care should be exercised to introduce the daphnids below the surface of any solution to avoid trapping air under the carapace.

(iv) *Feeding.* A variety of foods (e.g., unicellular green algae) have been demonstrated to be adequate for daphnid culture. Daphnids shall not be fed during testing.

(2) *Facilities*—(i) *Apparatus.* (A) Facilities needed to perform this test include: (*1*) Containers for culturing and acclimating daphnids; (*2*) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation, and test periods; (*3*) apparatus for straining particulate matter, removing gas bubbles, or aerating the water as necessary; and (*4*) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15 to 30 minute transition period. In addition, the flow-through system shall contain appropriate test chambers in which to expose daphnids to the test chemical and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Construction materials.* (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results.

(B) For static tests, daphnids can be conveniently exposed to the test chemical in 250 ml beakers or other suitable containers.

(C) For flow-through tests, daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. The containers should be suspended in the test chamber in such a manner to insure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least 5 centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(iii) *Dilution water.* (A) Surface or ground water, reconstituted water or

dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

Substance	Maximum concentration
Particulate matter	20 mg/liter.
Total organic carbon or	2 mg/liter.
Chemical oxygen demand	5 mg/liter.
Un-ionized ammonia	1 μg/liter.
Residual chlorine	<3 μg/liter.
Total organophosphorus pesticides	50 ng/liter.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) or.	50 ng/liter.
Organic chlorine	25 ng/liter.

(B) The above water quality parameters under paragraph (d)(2)(iii)(A) of this section shall be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis shall be performed.

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon-filtered deionized water with a conductivity less than 1 μohm/cm is acceptable as the diluent for making reconstituted water.

(iv) *Cleaning.* All test equipment and test chambers shall be cleaned before each use using standard laboratory procedures.

(v) *Test substance delivery system.* In flow-through tests, proportional diluters, metering pump systems, or other suitable devices should be used to deliver test chemical to the test chambers. The system shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test chemical in each chamber. The general operation of the test substance delivery system should be checked twice during a test. The 24-hour flow through a test chamber shall be equal to at least 5 times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from any one test chamber to another.

(3) *Test parameters.* Environmental parameters of the water contained in test chambers shall be maintained as specified below:

(i) The test temperature shall be 20 °C. Excursions from the test temperature shall be no greater than ±2 °C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test chemical. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of daphnids placed in a test chamber shall not affect test results. Loading shall not exceed 40 daphnids per liter test solution in the static system. In the flow-through test, loading limits will vary depending on the flow rate of dilution water. Loading shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness.

(e) *Reporting.* The sponsor shall submit to the U.S. EPA all data developed by the test that are suggestive or predictive of acute toxicity and all concomitant gross toxicological manifestations. In addition to the reporting requirements prescribed in part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data shall include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator, and dates of testing.

(2) A detailed description of the test chemical including its source, lot number, composition (identity and concentration or major ingredients and major impurities), known physical and chemical properties and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical characteristics (e.g., conductivity, hardness, pH, etc.) and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of

verification, age, source, treatments, feeding history, acclimation procedures, and culture method. The age of the daphnids used in the test shall be reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g., conditioning, test chemical additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the method of test chemical introduction or the test substance delivery system and the flow rate (in flow-through test) expressed as volume additions per 24 hours.

(6) The concentration of the test chemical in each test chamber at times designated for static and flow-through tests.

(7) The number and percentage of organisms that were immobilized or showed any adverse effects in each test chamber at each observation period.

(8) Utilizing the average measured test chemical concentration, concentration-response curves should be fitted to immobilization data at 24 and 48 hours. A statistical test of goodness-of-fit should be performed and the results reported.

(9) The 24- and 48-hour EC_{50} values and their respective 95 percent confidence limits using the mean measured test chemical concentration and the methods used to calculate both the EC_{50} values and their confidence limits.

(10) All chemical analyses of water quality and test chemical concentrations, including methods, method validations and reagent blanks.

(11) The data records of the culture, acclimation and test temperatures.

(12) Any deviation from this test guideline and anything unusual about the test, e.g., diluter failure, temperature fluctuations, etc.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19059, May 20, 1987]

§ 797.1330 Daphnid chronic toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the chronic toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes a chronic toxicity test in which daphnids are exposed to a chemical in a renewal or a flow-through system. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in part 792 *Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) *Brood stock* means the animals which are cultured to produce test organisms through reproduction.

(2) *Chronic toxicity test* means a method used to determine the concentration of a substance in water that produces an adverse effect on a test organism over an extended period of time. In this test guideline, mortality and reproduction (and optionally, growth) are the criteria of toxicity.

(3) EC_{50} means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(4) *Ephippium* means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(5) *Flow-through* means a continuous or intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(6) *Immobilization* means the lack of movement by daphnids except for minor activity of the appendages.

(7) *Loading* means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time or passing through the test chamber during a specific interval.

(8) *MATC (Maximum Acceptable Toxicant Concentration)* means the maximum concentration at which a chemical can be present and not be toxic to the test organism.