

(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.

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§ 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) *EC₅₀* 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) *Ehippium* 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.

(9) *Renewal system* means the technique in which test organisms are periodically transferred to fresh test solution of the same composition.

(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 then adjusted to the rate desired. The test substance is introduced into each test chamber. The addition of test substance in the flow-through system is done at a rate which is sufficient to establish and maintain the desired concentration of test substance in the test chamber.

(ii) The test is started within 30 minutes after the test substance has been added and uniformly distributed in the test chambers in the renewal test or after the concentration of test substance in each test chamber of the flow-through test system reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured or 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, immobile adults and offspring produced are counted and removed, and the findings are recorded. Dissolved oxygen concentration, pH, temperature, the concentration of test substance, and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to determine any significant differences ($p \leq 0.05$) in immobilization and reproduction as compared to the control.

(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 substance (e.g., 1, 10, 100 mg/l), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate chronic test concentrations. 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 concentration-response curves, EC_{50} values and effects of a chemical on immobilization and reproduction during chronic exposure.

(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, 64 mg/l). An equal number of daphnids shall be placed in two or more replicates. The concentration ranges shall be selected to determine the concentration-response curves, EC_{50} values and MATC. Solutions shall be analyzed for chemical concentration at designated times during the test.

(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 test duration is 21 days. The test is unacceptable if:

(A) More than 20 percent of the control organisms appear to be immobilized, stressed or diseased during the test.

(B) Each control daphnid living the full 21 days produces an average of less than 60 young.

(C) Any ephippia are produced by control animals.

(v) The number of immobilized daphnids in each chamber shall be recorded on day 21 of the test. After offspring are produced, they shall be counted and removed from the test

chambers every 2 or 3 days. Concentration-response curves, EC_{50} values and associated 95 percent confidence limits for adult immobilization shall be determined for day 21. An MATC shall be determined for the most sensitive test criteria measured (number of adult animals immobilized, number of young per adult, and number of immobilized young per adult).

(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 as in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(5) [Reserved]

(6) *Analytical measurements.* (i) *Test chemical.* Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, 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 adults, total offspring per adult, and immobilized offspring per adult shall be counted during each test. Appropriate statistical analyses should provide a goodness-of-fit determination for the adult immobilization concentration-response curves calculated on day 21. A 21-day EC_{50} based on adult immobilization and corresponding 95 percent confidence intervals shall also be calculated. Appropriate statistical tests (e.g., analysis of variance, mean separation test) should be used to test for significant chemical effects on chronic test criteria (cumulative number of immobilized adults, cumulative

number of offspring per adult and cumulative number of immobilized offspring per adult) on day 21. An MATC shall be calculated using these chronic test criteria.

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

(B) First instar daphnids, ≤ 24 hours old, are to be used to start the test.

(ii) *Acquisition*. (A) Daphnids to be used in chronic 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.

(B) Daphnids shall not be used for a test if:

(1) Cultures contain ephippia.

(2) Adults in the cultures do not produce young before day 12.

(3) More than 20 percent of the culture stock die in the 2 days preceding the test.

(4) 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.

(5) Daphnids have been used in any portion of a previous test either in a treatment or in a control.

(iii) *Feeding*. (A) During the test the daphnids shall be fed the same diet and with the same frequency as that used for culturing and acclimation. All treatments and control(s) shall receive, as near as reasonably possible, the same ration of food on a per-animal basis.

(B) The food concentration depends on the type used. Food concentrations should be sufficient to support normal growth and development and to allow for asexual (parthenogenic) reproduction. For automatic feeding devices, a suggested rate is 5 to 7 mg food (either solids or algal cells, dry weight) per liter dilution water or test solution. For manual once-a-day feeding, a suggested rate is 15 mg food (dry weight) per liter dilution water or test solution.

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

(v) *Care and handling of test organisms*. (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. A variety of foods have been demonstrated to be adequate for daphnid culture. They include algae, yeasts and a variety of mixtures.

(B) 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 during handling shall be discarded.

(C) Smooth glass tubes (I.D. greater than 5mm) equipped with a rubber bulb can be used for transferring daphnids with minimal culture media carry-over.

(D) Care should be exercised to introduce the daphnids below the surface of any solution so as not to trap air under the carapace.

(vi) *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 dilution water at the test temperature. During acclimation, daphnids shall be fed the same food as will be used for the definitive test.

(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.

(2) *Facilities*—(i) *General*. (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 when water supplies contain particulate matter, gas bubbles, or insufficient dissolved oxygen, respectively.

(4) An apparatus for providing a 16-hour light and 8-hour dark photoperiod.

(5) An apparatus to introduce food if continuous or intermittent feeding is used.

(6) In addition, the flow-through test shall contain appropriate test chambers in which to expose daphnids to the test substance and an appropriate test substance delivery system.

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

(ii) *Test chambers.* (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 test results.

(B) For renewal tests, daphnids can be conveniently exposed to the test solution 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. Such containers shall be suspended in the test chamber in such a manner to ensure 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.

(D) 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.

(iii) *Test substance delivery system.* (A) In the flow-through test, proportional diluters, metering pump systems or other suitable systems should be used to deliver the test substance to the test chambers.

(B) The test substance delivery system shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber shall be equal to at least five times the volume of the test chamber. During a test, the flow rates shall not vary more than 10 percent from any one test chamber to another. For the renewal test, test substance dilution water shall be completely replaced at least once every 3 days.

(iv) *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/l.
Total organic carbon or	2 mg/l.
Chemical oxygen demand	5 mg/l.
Un-ionized ammonia	20 µg/l.
Residual chlorine	<3 µg/l.
Total organophosphorus pesticides	50 ng/l.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) or organic chlorine	50 ng/l.
	25 ng/l.

(B) The water quality characteristics listed above shall be measured at least twice a year or when 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 of less than 1 microhm/cm is acceptable as the diluent for making reconstituted water.

(D) If the test substance is not soluble in water an appropriate carrier should be used.

(v) *Cleaning of test system.* All test equipment and test chambers shall be cleaned before each use following standard laboratory procedures. Cleaning of test chambers may be necessary during the testing period.

(3) *Test parameters.* (i) Environmental conditions of the water contained in test chambers should be maintained as specified in this paragraph:

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

(B) 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 substance. All treatment and control chambers shall be given the same aeration treatment.

(C) Photoperiod of 16-hours light and 8-hours darkness.

(ii) Additional measurements include:

(A) The concentration of the test substance in the chambers shall be measured during the test.

(B) At a minimum, the concentration of test substance should be measured as follows:

(1) In each chamber before the test.

(2) In each chamber on days 7, 14, and 21 of the test.

(3) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(4) An apparatus for providing a 16-hour light and 8-hour dark photoperiod.

(C) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning of the test and on days 7, 14, and 21 in at least two chambers of the high, middle, low, and control test concentrations.

(e) *Reporting.* The sponsor shall submit to the U.S. Environmental Protection Agency all data developed by the test that are suggestive or predictive of chronic toxicity and all associated toxicologic manifestations. In addition to the reporting requirements prescribed in the 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 substance including its source, lot number, composition (identity and concentration of 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), 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 methods. 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 substance additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the renewal process and schedule for the renewal chronic test, the test substance delivery system and flow rate expressed as volume additions per 24 hours for the flow-through chronic test, and the method of feeding (manual or continuous) and type of food.

(6) The concentration of the test substance in test chambers at times designated for renewal and flow-through tests.

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(7) The number and percentage of organisms that show any adverse effect in each test chamber at each observation period.

(8) The cumulative adult and offspring immobilization values and the progeny produced at designated observation times, the time (days) to first brood and the number of offspring per adult in the control replicates and in each treatment replicate.

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

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

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

(12) The MATC to be reported is calculated as the geometric mean between the lowest measured test substance concentration that had a significant ($p \leq 0.05$) effect and the highest measured test substance concentration that had no significant ($p \leq 0.05$) effect on day 21 of the test. The most sensitive of the test criteria (number of adult animals immobilized, the number of young per female and the number of immobilized young per female) is used to calculate the MATC. The criterion selected for MATC computation is the one which exhibits an effect (a statistically significant difference between treatment and control groups; $p \leq 0.05$) at the lowest test substance concentration for the shortest period of exposure. Appropriate statistical tests (analysis of variance, mean separation test) shall be used to test for significant test substance effects. The statistical tests employed and the results of these tests shall be reported.

(13) Concentration-response curves utilizing the average measured test substance concentration shall be fitted to cumulative adult immobilization data at 21 days. A statistical test of goodness-of-fit shall be performed and the results reported.

(14) An EC_{50} value based on adult immobilization with corresponding 95 percent confidence limits when sufficient data are present for day 21. These calculations shall be made using the aver-

age measured concentration of the test substance.

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§ 797.1400 Fish acute toxicity test.

(a) *Purpose.* This guideline may be used to develop 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 tests to be used to develop data on the acute toxicity of chemicals to fish. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the 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. The following definitions also apply to this guideline:

(1) *Acclimation* means the physiological compensation by test organisms to new environmental conditions (e.g., temperature, hardness, pH).

(2) *Acute toxicity test* means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). In this guideline, death is used as the measure of toxicity.

(3) *Carrier* means a solvent used to dissolve a test substance prior to delivery to the test chamber.

(4) *Conditioning* means the exposure of construction materials, test chambers, and testing apparatus to dilution water or to test solutions prior to the start of a test in order to minimize the sorption of the test substance onto the test facilities or the leaching of substances from the test facilities into the dilution water or test solution.

(5) *Death* means the lack of opercular movement by a test fish.

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

(7) *Incipient LC_{50}* means that test substance concentration, calculated from