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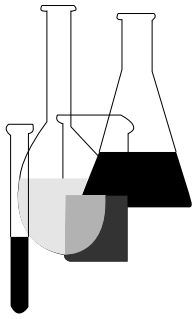
Ecological Effects Test Guidelines

OPPTS 850.1300 Daphnid Chronic Toxicity Test



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

OPPTS 850.1300 Daphnid chronic toxicity test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 797.1330 Daphnid Chronic Toxicity Test; OPP 72–4 Fish Early Life-Stage and Aquatic Invertebrate Life-Cycle Studies (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982; and OECD 202, *Daphnia* sp. Acute Immobilisation Test and Reproduction Test.

(a) **Purpose.** This guideline prescribes a chronic toxicity test in which daphnids are exposed to a chemical either in a static-renewal or a flow-through system. The Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present to the aquatic environment. No preference is given in this guideline on the type of test system to be used, either static-renewal or flow-through. However, the former works well if individual daphnids need to be monitored during the test. The latter works well with chemicals that are volatile, have low water solubilities, and an oxygen demand, and for those that degrade, hydrolyze, or photolyze easily. Flow-through systems allow maintenance of near constant chemical concentrations throughout the test.

(b) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

Brood stock means the animals which are cultured to produce test organisms through reproduction.

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.

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.

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

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

Immobilization means the lack of movement by daphnids except for minor activity of the appendages.

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.

LOEC (lowest observed effect concentration) means the lowest concentration of a material used in this test that has an adverse effect on the test organisms and is the test concentration immediately above the NOEC.

MATC (maximum acceptable toxicant concentration) means the maximum concentration at which a chemical can be present and not be toxic to the test organism.

NOEC (no observed effect concentration) means the highest concentration of a material used in this test that does not have an adverse effect on the test organisms and is the test concentration immediately below the LOEC.

Static-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 min after the test substance has been added and uniformly distributed in the test chambers in the static-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. At the end of the test, the growth of surviving adults is measured as the total body length or dry weight or both.

(2) **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.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine concentration-response curves, EC50 values, and effects of a chemical on immobilization and reproduction during chronic exposure.

(ii) A minimum of 10 daphnids per concentration should 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). In flow-through testing, an equal number of daphnids (minimum of 20 per concentration) should be placed in two or more replicates or test chambers, e.g. four replicates each with five daphnids, for each concentration. In static-renewal tests, 10 or more replicates of one daphnid each, for each concentration, should be used. The concentration ranges should be selected to determine the concentration-response curves, EC50 values, and MATC. Solutions should be analyzed for chemical concentration at designated times during the test.

(iii) Every test should 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 invalid and unacceptable if any of the following occur:

(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 should be recorded on day 21 of the test. After offspring are produced, they should be counted and removed from the test chambers every 2 or 3 days. Concentration-response curves, EC50 values, and associated 95 percent con-

fidence limits for adult immobilization should be determined for day 21. An MATC should 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) Growth of daphnids is determined by measuring total body length or dry weight, or both, of each surviving adult. It is preferred that both measures be taken.

(vii) In addition, any abnormal behavior or appearance should also be reported.

(viii) Test organisms should 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 should 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.

(4) **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 should 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 should 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 EC50 based on adult immobilization and corresponding 95 percent confidence intervals should 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 should 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 h 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 should have originated from the same culture population.

(B) Daphnids should 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 three 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 should be fed the same diet and with the same frequency as that used for culturing and acclimation. All treatments and controls should 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 of dilution water or test solution. For manual once-a-day feeding, a suggested rate is 15 mg food (dry weight) per liter of dilution water or test solution.

(iv) **Loading.** The number of test organisms placed in a test chamber should not affect test results. Loading should not exceed 40 daphnids per liter in the static-renewal system. In the flow-through test, loading limits will vary depending on the flow rate of the dilution water. Loading should 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 has 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 should be discarded. Organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

(C) Smooth glass tubes (I.D. > 5 mm) 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 in order not to trap air under the carapace.

(vi) **Acclimation.** (A) Brood daphnids should be maintained in 100 percent dilution water at the test temperature for at least 48 h prior to the start of the test. This is easily accomplished by culturing them in dilution water at the test temperature. During acclimation, daphnids should 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–h light and 8–h dark photoperiod.

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

(*6*) In addition, the flow-through test should 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 static-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 should 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 cm 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 should 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 should 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–h flow rate through a test chamber should be equal to at least 5× 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. For the static-renewal test, test substance dilution water should 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 specifications in the following Table 1.:

Table 1.—Specifications for Dilution Water

	Substance
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:	50 ng/L
Organic chlorine	25 ng/L

(B) The water quality characteristics listed above should 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 should 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) should 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 μohm/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 at a concentration ≤0.1mL/L. Triethylene glycol and dimethyl formamide are preferred solvents, but ethanol or acetone can be used if necessary.

(v) **Cleaning of test system.** All test equipment and test chambers should 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 should be 20 °C. Excursions from the test temperature should be no greater than ±1 °C.

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

(C) Photoperiod of 16–h light and 8–h darkness.

(ii) Additional measurements include:

(A) The concentration of the test substance in the chambers should 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.

(C) The dissolved oxygen concentration, temperature, and pH should 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 should submit to the EPA 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 under Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the reporting of test data should 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, percent active ingredient, 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 should 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 static-renewal process and schedule for the static-renewal chronic test, the test substance delivery system and flow rate expressed as volume additions per 24 h 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 static-renewal and flow-through tests.

(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, the number of offspring per adult in the control replicates and in

each treatment replicate, and the growth of surviving adults measured as total length or dry weight or both.

(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 (LOEC) and the highest measured test substance concentration that had no significant ($p \leq 0.05$) effect (NOEC) on day 21 of the test. The most sensitive of the test criteria (number of adult animals immobilized, the number of young per surviving female, the number of immobilized young per female, and the growth of surviving females) 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) should be used to test for significant test substance effects. The statistical tests employed and the results of these tests should be reported.

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

(14) An EC50 value based on adult immobilization with corresponding 95 percent confidence limits when sufficient data are present for day 21. These calculations should be made using the average measured concentration of the test substance.