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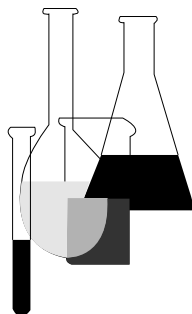# Ecological Effects Test Guidelines

OPPTS 850.1010 Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids

"Public Draft"

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 850.1010   Aquatic invertebrate acute toxicity test, freshwater daphnids.

(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 797.1300 Daphnid Acute Toxicity Test; OPP 72–2 Acute Toxicity Test for Freshwater Aquatic Invertebrates (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.

(b) **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. 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 Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present in the aquatic environment.

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

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

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 in daphnids under the carapace in response to stress conditions.

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.

Immobilization means the lack of movement by the test organisms.

Loading means the ratio of daphnid biomass (grams, wet weight) or number of daphnids 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.

1

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.

Static-renewal system means a static test system in which the test solution is renewed every 24 h.

(d) **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 min 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. In static-renewal testing the dilution water and test chamber are renewed periodically. 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 (DOC), 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 EC50 values for the test chemical at the end of 24 and 48 h.

(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 chemical (e.g. 1, 10, 100 mg/L, etc.).

(iii) A minimum of five daphnids should be exposed to each concentration of test chemical for a period of 48 h. 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.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine the concentration-response curves and the 24– and 48–h EC50 values.

(ii) A minimum of 20 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, and 64 mg/L). An equal number of daphnids should be placed in two or more

replicates. If solvents, solubilizing agents, or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test chemical. If carriers are absolutely necessary, the amount used should be the minimum necessary to achieve solution of the test substance. Triethylene glycol and dimethyl formamide are preferred, but ethanol and acetone can be used if necessary. Carrier concentrations should be kept constant at all treatment levels. The concentration of solvent should not exceed 100 mg/L. The concentration ranges should be selected to determine the concentration-response curves and EC50 values at 24 and 48 h. Concentration of test chemical in test solutions should be analyzed prior to use.

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

(iv) The DOC, temperature, and pH should be measured at the beginning and end of the test in each chamber.

(v) The test duration is 48 h. The test is unacceptable if more than 10 percent of the control organisms are immobilized during the 48–h test period. Each test chamber should be checked for immobilized daphnids at 24 and 48 h after the beginning of the test. Concentration-response curves and 24–h and 48–h EC50 values for immobilization should be determined along with their 95 percent confidence limits.

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

(vii) 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 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 should be measured in each test chamber at a minimum at the beginning and at the end of the test. In the static-renewal test, the test concentration of test chemicals should be measured in each test chamber at a minimum at the beginning and at the end of the renewal period. In the flow-through test the concentration of test chemical should be measured in each chamber at a minimum at the beginning of the test and at 48 h after the start of the test, and 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 should not vary more than ±20 percent.

(4) **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 should 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 mathematically corrected.

(ii) **Numerical.** The number of immobilized daphnids should 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–h EC50 and corresponding 95 percent interval should be calculated.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The cladocerans, *D. 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 h 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 should have originated from the same culture population.

(C) Stock daphnids may be tested periodically to determine any genetic changes in the populations which may alter the sensitivity to test chemicals.

(D) Daphnids should 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 three young per adult per day over the 7–day period prior to the test.

(*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 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

4

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 environmental conditions similar 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 should be discarded. Organisms that touch dry surfaces or are dropped or injured in handling should be discarded.

(B) Smooth glass tubes (I.D. greater than 5 mm), equipped with rubber bulbs, 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 should 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.

(*4*) An apparatus for providing a 16–h light and 8–h dark photoperiod with a 15– to 30–min transition period.

(*5*) In addition, the flow-through system should 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 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.

(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 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.

(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 specifications in the following Table 1.:

Table 1.—Water Quality Parameters

Substance	Maximum concentration
Hardness as $CaCO_3$	180 mg/L
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 parameters should 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 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 less than 0.1 mS/m is acceptable as the diluent for making reconstituted water.

6

(iv) **Cleaning.** All test equipment and test chambers should 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 should 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–h flow 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.

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

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

(ii) DOC between 60 and 105 percent saturation. Do not aerate daphnid toxicity tests. A single air bubble can get under the carapace of the daphnid and kill it, or float the daphnid to the surface where it will get trapped.

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

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

(f) **Reporting.** The sponsor must submit to the 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 40 CFR Part 792—Good Laboratory Practice Standards, 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 chemical including its source, lot number, composition (identity and concentration of major ingredients (percent active ingredient of chemical) 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, carriers and/or additives used, and their concentrations.

(4) Carriers and/or additives used and their concentrations.

(5) 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 should be reported.

(6) 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), number of test organisms per test chamber, number of replicates per treatment, lighting, method of test chemical introduction or test substance delivery system, renewal schedule (in static-renewal tests), and flow rate (in flow-through test) expressed as volume additions per 24 h.

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

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

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

(10) The 24– and 48–h EC50 values and their respective 95 percent confidence limits using the mean measured test chemical concentration, and the methods used to calculate both the EC50 values and their confidence limits.

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

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

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

(14) If it is observed that the stability or homogeneity of the test substance cannot be maintained, care should be taken in the interpretation of the results, and note made that the results may not be reproducible.