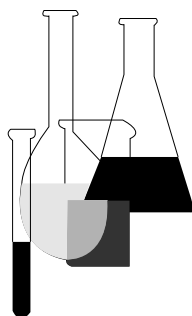




Ecological Effects Test Guidelines

OPPTS 850.1350

Mysid 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 850.1350 Mysid 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 797.1950 Mysid Shrimp 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.

(b) **Purpose.** This guideline prescribes tests using mysids as test organisms to develop data on the chronic toxicity of chemicals. The Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

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

Chronic toxicity test is a method used to determine the concentration of a substance that produces an adverse effect from prolonged exposure of an organism to that substance. In this test, mortality, number of young per female, and growth are used as measures of chronic toxicity.

Death is the lack of reaction of a test organism to gentle prodding.

Flow-through is 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.

G1 (Generation 1) are those mysids which are used to begin the test, also referred to as adults; *G2 (Generation 2)* are the young produced by *G1*.

LC50 is the experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of time.

Loading is the ratio of test organism biomass (gram, wet weight) to the volume (liters) of test solution in a test chamber.

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

Retention chamber is a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating washout from test chambers.

(d) **Test procedures**—(1) **Summary of the test.** (i) In preparation for the test, the flow of test solution through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. The rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into retention chambers within the test and the control chambers. Mysids in the test and control chambers are observed periodically during the test, the dead mysids removed, and the findings reported.

(ii) Dissolved oxygen concentration (DOC), pH, temperature, salinity, the concentration of test substance, and other water quality characteristics are measured and recorded at specified intervals in selected test chambers.

(iii) Data collected during the test are used to develop an MATC and to quantify effects on specific chronic parameters.

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

(ii) The mysids 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 10 mysids 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 test substance are acceptable.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine concentration-response curves, LC50 values, and effects of a chemical on growth and reproduction during chronic exposure.

(ii) A minimum of 40 mysids per concentration should be exposed to five or more concentrations of the test 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). Mysids should be physically separated into replicate groups of no more than eight individuals when most of the mysids reach sexual maturity (usually 10–14 days after the beginning of the test). 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 substance. The concentration of solvent should not exceed 0.1 mL/L. The concentration ranges should be selected to determine the concentration response curves, LC50 values, and MATC. Con-

centration of test substance in test solutions should be determined prior to use.

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

(iv) The DOC, temperature, salinity, and pH should be measured weekly in each chamber.

(v) The test duration is 28 days. The test is unacceptable if more than 25 percent of first generation females in the control groups fail to produce young or if the average number of young produced per female in the controls is less than three per day. The number of dead mysids in each chamber should be recorded on days 7, 14, 21, and 28 of the test. The number of male and female mysids in each test chamber should be recorded at the time when sexual characteristics become discernible. This generally occurs after 10–12 days in the control, but may be delayed in those mysids exposed to the test substance. Females are identified by the presence of a ventral brood pouch. Body length (as measured by total midline body length, from the anterior tip of the carapace to the posterior margin of the uropod) should be recorded for males and females at the time when sex can be determined simultaneously for all mysids in control and treatment groups. This time cannot be specified because of possible delays in sexual maturation of mysids exposed to test substances. A second observation of male and female body lengths should be conducted on day 28 of the test. To reduce stress on the mysids, body lengths can be recorded by photography through a stereomicroscope with appropriate scaling information. As offspring are produced by the G1 mysids (approximately 13 to 16 days in controls), the young should be counted and separated into retention chambers at the same test substance concentration as the chambers where they originated. If available prior to termination of the test, observations on the mortality, number of males and females and male and female body length should be recorded for the G2 mysids. Concentration-response curves, LC50 values and associated 95 percent confidence limits for the number of dead mysids (G1) should be determined for days 7, 14, 21, and 28. An MATC should be determined for the most sensitive test criteria measured (cumulative mortality of adult mysids, number of young per female, and body lengths of adult males and females).

(vi) In addition to death, 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 substance in the chambers should be measured as often as is feasible during the test. The measured concentration of the test substance should not vary more than 20 percent among replicate test chambers of a treatment concentration. The concentration of test substance should be measured:

(A) At each test concentration at the beginning of the test and on days 7, 14, 21, and 28.

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

(4) **Analytical measurements**—(i) **Test chemical.** Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be employed 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.** (A) The number of dead mysids, cumulative young per female, and body lengths of male and female mysids should be recorded during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the day-7, -14, -21, and -28 adult (G1) death concentration-response curves.

(B) A 7-, 14-, 21- and 28-day LC50, based on adult (G1) death, and corresponding 95 percent confidence intervals should 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 mortality of adults, cumulative number of young per female, and body lengths of adult male and females) on designated days. An MATC should be calculated using these chronic tests criteria.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The mysid *Mysidopsis bahia*, is the organism specified for these tests. Juvenile mysids, ≤24-h old, are to be used to start the test. It has recently been proposed, under paragraph (g)(2) of this guideline, to place this species in a new genus, *Americamysis*.

(B) Mysids to be used in chronic toxicity tests should originate from laboratory cultures in order to ensure the individuals are of similar age and experimental history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural

areas. Because of similarities with other mysid species, taxonomic verification should be obtained from the commercial supplier, by experienced laboratory personnel, or by an outside expert.

(C) Mysids used in a particular test should be of similar age and be of normal size and appearance for their age.

(D) Mysids should not be used for a test if they exhibit abnormal behavior, or if they have been used in a previous test, either in a treatment or in a control group.

(ii) **Acclimation.** (A) Any change in the temperature and chemistry of the water used for holding or culturing the test organisms to those of the test should be gradual. Within a 24-h period, changes in water temperature should not exceed 1 °C, while salinity changes should not exceed 5 percent.

(B) During acclimation mysids should be maintained in facilities with background colors and light intensities similar to those of the testing areas.

(iii) **Care and handling.** Methods for the care and handling of mysids such as those described in paragraph (g)(1) of this guideline can be used during holding, culturing, and testing periods.

(iv) **Feeding.** Mysids should be fed during testing. Any food utilized should support survival, growth, and reproduction of the mysids. A recommended food is live *Artemia* spp. nauplii (approximately 48-h old).

(2) **Facilities—(i) Apparatus.** (A) Facilities which may be needed to perform this test include:

(1) Flow-through or recirculating tanks for holding and acclimating mysids.

(2) A mechanism for controlling and maintaining the water temperature during the holding, 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 14-h light and 10-h dark photoperiod with a 15- to 30-min transition period. In addition, flow-through chambers and a test substance delivery system are required. It is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss through outflow water.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect 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) **Cleaning.** Test substance delivery systems and test chambers should be cleaned before each use following standard laboratory practices.

(iii) **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) Retention chambers utilized for confinement of test organisms can be constructed with netting material of appropriate mesh size.

(iv) **Dilution water.** (A) Natural or artificial seawater is acceptable as dilution water if mysids will survive and successfully reproduce in it for the duration of the holding, acclimating, and testing periods without showing signs of stress, such as reduced growth and fecundity. Mysids should be cultured and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a filter with a pore size of $> 20 \mu\text{m}$ prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized or glass-distilled water. Deionized water with a conductivity less than 0.1 mS/m at $12 \text{ }^\circ\text{C}$ is acceptable as the diluent for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) should be measured on each batch.

(v) **Test substance delivery system.** Proportional diluters, metering pumps, or other suitable systems should be used to deliver test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate 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 chamber should be equal to at least $5\times$ the volume of the chamber. The flow rates should not vary more than 10 percent among chambers or across time.

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

(i) The test temperature should be $25 \text{ }^\circ\text{C}$. Excursions from the test temperature should be no greater than $\pm 2 \text{ }^\circ\text{C}$.

(ii) DOC between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test sub-

stance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of mysids placed in a test solution should not be so great as to affect results of the test. Loading requirements for the test will vary depending on the flow rate of dilution water. The loading should not cause the DOC to fall below the recommended levels.

(iv) Photoperiod of 14 h light and 10 h darkness, with a 15–30 min transition period.

(v) Salinity of 20 ± 3 ppt.

(f) **Reporting.** The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of chronic toxicity and all concomitant toxicologic manifestations. In addition to the general 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 source of the dilution water, its chemical characteristics (e.g. salinity, pH, etc.) and a description of any pretreatment.

(2) Detailed information about the test organisms, including the scientific name and method of verification, average length, age, source, history, observed diseases, treatments, acclimation procedures, and food used.

(3) A description of the test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g. conditioning, test substance additions, etc.), the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system, and the flow rate expressed as volume additions per 24 hours.

(4) The measured concentration of test substance in test chambers at the times designated.

(5) The first time (day) that sexual characteristics can be observed in controls and in each test substance concentration.

(6) The length of time for the appearance of the first brood for each concentration.

(7) The means (average of replicates) and respective 95 percent confidence intervals for:

(i) Body length of males and females at the first observation day (depending on time of sexual maturation) and on day 28.

(ii) Cumulative number of young produced per female on day 28.

(iii) Cumulative number of dead adults on day 7, 14, 21, and 28.

(iv) If available prior to test termination (day 28), effects on G2 mysids (number of males and females, body length of males and females, and cumulative mortality).

(8) The MATC is calculated as the geometric mean between the lowest measured test substance concentration that had a significant ($p < 0.05$) effect and the highest measured test substance concentration that had no significant ($p < 0.05$) effect in the chronic test. The most sensitive of the test criteria for adult (G1) mysids (cumulative number of dead mysids, body lengths of males and females, or the number of 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 ($p < 0.05$)) 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 chemical effects. The statistical tests employed and the results of these tests should be reported.

(9) Concentration-response curves should be fitted to the cumulative number of adult dead for days 7, 14, 21, and 28. A statistical test of goodness-of-fit should be performed and the results reported.

(10) An LC50 value based on the number of dead adults with corresponding 95 percent confidence intervals for days 7, 14, 21, and 28. These calculations should be made using the average measured concentration of the test substance.

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

(12) The data records of the holding, acclimation and test temperature and salinity.

(g) **References.** The following references should be consulted for additional background information on this test guideline:

(1) Environmental Protection Agency, *Bioassay Procedures for the Ocean Disposal Permit Program*, EPA Report No. 600/9-78-010 (Gulf Breeze, Florida, 1978).

(2) Price, W.W. et al. Observations on the genus *Mysidopsis* Sars, 1864 with the designation of a new genus, *Americamysis*, and the descriptions of *Americamysis alleni* and *A. stucki* (Peracarida: Mysidacea: Mysidae), from the Gulf of Mexico. *Proceedings of the Biological Society of Washington* 107:680-698 (1994).