

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 number and percentage of organisms that died or showed any other adverse effects in the control and in each treatment at each observation period.

(6) Concentration-response curves shall be fitted to mortality data collected at 24, 48, 72, and 96 hours. A statistical test of goodness-of-fit shall be performed and the results reported.

(7) The 96-hour LC<sub>50</sub> and when sufficient data have been generated, the 24-, 48-, and 72-hour LC<sub>50</sub>'s and the corresponding 95-percent confidence limits and the methods used to calculate the values. These calculations shall be made using the average measured concentration of the test substance.

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

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

(f) *References.* For additional background information on this test guideline the following references should be consulted:

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

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19068, May 20, 1987; 52 FR 26150, July 13, 1987]

#### § 797.1950 Mysid shrimp 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 tests using mysids as test organisms to develop data on the chronic toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

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

(1) "Chronic toxicity test" means 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.

(2) "Death" means the lack of reaction of a test organism to gentle prodding.

(3) "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.

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

(5) "LC<sub>50</sub>" means that 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.

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

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

(8) "Retention chamber" means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating washout from test chambers.

(c) *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, pH, temperature, salinity, the concentration of test substance and other water quality characteristics are measured at specified intervals in selected test chambers.

(iii) Data collected during the test are used to develop a MATC (Maximum Acceptable Toxicant Concentration) and quantify effects on specific chronic parameters.

(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 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 chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine concentration-response curves, LC<sub>50</sub> values, and effects of a chemical on growth and reproduction during chronic exposure.

(ii) A minimum of 40 mysids per concentration shall be exposed to four 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 mysids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be

used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the concentration response curves, LC<sub>50</sub> values and MATC. Concentration of test substance in test solutions should be analyzed 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 chemical is added.

(iv) The dissolved oxygen concentration, temperature, salinity, and pH shall be measured weekly in each chamber.

(v) The test duration is 28 days. The test is unacceptable if more than 20 percent of the control organisms die, appear stressed or are diseased during the test. The number of dead mysids in each chamber shall be recorded on days 7, 14, 21, and 28 of the test. At the time when sexual characteristics are discernible in the mysids (approximately 10 to 12 days in controls; possible delays may occur in mysids exposed to test substances), the number of males and females (identified by ventral brood pouch) in each chamber shall be recorded. Body length (as measured by total midline body length, from the anterior tip of the carapace to the posterior margin of the uropod) shall 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 shall 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 shall 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 shall be recorded for the G2 mysids. Concentration-response curves, LC<sub>50</sub> values and associated 95 percent confidence limits for the number of dead mysids (G1) shall be determined for days 7, 14, 21, and 28. An MATC shall 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 shall also be reported.

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

(viii) The concentration of the test substance in the chambers should be measured as often as is feasible during the test. The concentration of test substance shall be measured:

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

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

Equal aliquots of test solutions may be removed from each test 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.

(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 employed 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*. (A) The number of dead mysids, cumulative young per female, and body lengths of male and female mysids shall be recorded during each definitive test. Appropriate statistical analyses shall 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 LC<sub>50</sub>, based on adult (G1) death, and corresponding 95 percent confidence intervals shall 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 shall be calculated using these chronic tests criteria.

(d) *Test conditions*—(1) *Test species*—(i) *Selection*. (A) The mysid shrimp, *Mysidopsis bahia*, is the organism specified for these tests. Juvenile mysids, ≤24 hours old, are to be used to start the test.

(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 shall be of similar age and be of normal size and appearance for their age.

(D) Mysids shall 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-hour 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 (f)(1) of this section 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 hours 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; and (4) an apparatus for providing a 14-hour light and 10-hour dark photoperiod with a 15- to 30-minute 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 shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Cleaning.* Test substance delivery systems and test chambers shall 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 shall be cultured and tested in dilution water from the same origin.

(B) Natural seawater shall be filtered through a filter with a pore size of >20 microns 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 1 µohm/cm at 12 °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) shall 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 shall 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-hour flow rate through a chamber shall 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 shall be maintained as specified below:

(i) The test temperature shall be 25 °C. Excursions from the test temperature shall be no greater than ±2 °C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation.

Aeration, if needed to achieve this level, shall be done before the addition of the test substance. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of mysids placed in a test solution shall 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 shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 14 hours light and 10 hours darkness, with a 15-30 minute transition period.

(v) Salinity of 20 parts per thousand  $\pm 3$  percent.

(e) *Reporting.* The sponsor shall 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 in part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data shall 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 shall be reported.

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

(10) An  $LC_{50}$  value based on the number of dead adults with corresponding 95 percent confidence intervals for days 7, 14, 21 and 28. These calculations shall 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.

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

(f) *References.* For additional background information on this test guideline the following references should be consulted:

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

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19069, May 20, 1987]

### PART 798—HEALTH EFFECTS TESTING GUIDELINES

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Sec.

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798.5195 Mouse biochemical specific locus test.  
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798.6050 Functional observational battery.  
798.6200 Motor activity.  
798.6400 Neuropathology.  
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798.6560 Subchronic delayed neurotoxicity of organophosphorus substances.

AUTHORITY: 15 U.S.C. 2603.

SOURCE: 50 FR 39397, Sept. 27, 1985, unless otherwise noted.

#### Subparts A-B [Reserved]

#### Subpart C—Subchronic Exposure

##### § 798.2250 Dermal toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the dermal route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.* (1) Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by dermal application for part (approximately 10 percent) of a life span.

(2) Dose in a dermal test is the amount of test substance applied to the skin (applied daily in subchronic tests).