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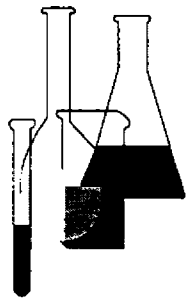
OPPTS 850.1000 Special Considerations for Conducting Aquatic Laboratory Studies



"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.

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OPPTS 850.1000 Special considerations for conducting aquatic laboratory studies.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of 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) [Reserved]

(b) **Introduction.** (1) This guideline provides additional information on how to design and conduct aquatic laboratory studies with emphasis on the importance of adequate characterization of the test material and proper understanding of how the material behaves under test conditions. This guideline also attempts to interpret those areas that need to be defined and set limits for designing and conducting laboratory studies.

(2) Agency guidance for performing aquatic testing sets forth a reasonable position and approach for testing criteria, limits, and standards. However, standards are set with the recognition that certain problems will arise and provisions must be made to accommodate unavoidable problems. This document provides for exceptions, while at the same time maintaining a high level of scientific integrity so that testing will provide information that is scientifically defensible and protective of the environment, while taking into consideration the chemistry of the test material.

(c) **General considerations.** (1) Note that for aquatic toxicity testing, the solubility and stability of the test material must be known for the conditions under which it will be tested and chemical analysis of the batch test material must be performed. Determining the solubility and stability of the test material in the mixture or test solution is an important part of these studies.

(2) The behavior of a test material should be based on experiments which are conducted under the same conditions as those occurring during the test. These include but are not limited to:

(i) Test solution characteristics (salt or freshwater).

(ii) Temperature, pH, conductivity, lighting.

(iii) With test organisms in place.

(iv) Use of the same test containers.

(v) Use of the same flow-through systems where appropriate.

(3) All chemistry methods used in preliminary trials, in range-finding tests, in establishing percent purity of batches of test material, or in measuring concentrations in test containers must be submitted with the study. The documentation must include a complete description of the method so

that a bench chemist can determine the necessary equipment and perform the analysis. It must also include the raw data, standards, and chromatograms from a representative analysis using the method. This representative analysis must be conducted with the specific media for which it will be used during the test—i.e., analysis should be performed under test conditions. The actual limit of detection (LOD) and limit of quantification (LOQ) must be identified.

(d) **Definitions.**

EEC is the effective environmental concentration.

LOD is the limit of detection below which the qualitative presence of the material is uncertain.

LOEC is the lowest-observable-effect-concentration.

LOQ is the limit of quantification below which the quantitative amount of the material is uncertain relative the amount.

Measured concentration is an analytically derived measure above the LOQ.

NOEC is the no-observed-effect-concentration.

Nominal concentration is, for aquatic tests, the nominal test level, which is the concentration that would exist if all test material added to the test solution was completely dissolved and did not dissipate in any way.

Recommended means that the procedure or test is preferred in order to avoid problems, but it is not required. If the recommended procedure or test is not performed, the study will not necessarily be rejected.

Solubility is defined as the amount of chemical retained in the supernatant of a conventionally centrifuged sample of test medium.

(e) **Stability.** (1) A test material is considered to be stable under test conditions if, under those conditions, it does not degrade, volatilize, dissipate, precipitate, sorb to test container walls, or otherwise decline to concentrations less than 70 percent of the day–0 measured concentration during the study period. If it is expected to decline to less than 70 percent of the day–0 measured concentration during the study period, either static renewal or flow-through design is needed to try to ensure that the test concentration is maintained at levels greater than or equal to 70 percent. The only exception is testing with algae and diatoms, which cannot be tested in static renewal or flow-through systems (see discussion in paragraph (m) of this guideline on testing with algae and diatoms).

(2) Static renewal is one method to ensure relatively continuous concentrations when the test material is not stable under test conditions. At

a minimum, the renewal cycle should be based on the stability of the test material under test conditions. The time to renewal (renewal cycle) should be shorter than the time it takes for the concentration of the test material to decline to < 70 percent. (The renewal cycle may be shorter than required by stability characteristics of the test material because of other factors, such as dissolved oxygen, feeding, etc.)

(f) **Sample storage.** If samples of growth medium, stock solutions, or test solutions collected for chemical analysis cannot be analyzed immediately, they should be handled and stored appropriately to minimize loss of the test material. Loss could be caused by such processes as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization. Stability determination under storage conditions, whether it refers to storing the test material before testing or storing samples awaiting analysis, is required by GLP regulation.

(g) **Preliminary trials.** (1) The Agency recommends preliminary testing for problem chemicals. The information about stability and solubility of problem chemicals should be developed under test conditions. This information can be gained while doing the currently required range-finding studies. A list of recommended preliminary tests is as follows:

(i) Stability trials should be conducted under test conditions. These trials must be documented and submitted to the Agency for review with the study to which they apply.

(ii) Solubility trials should be conducted under test conditions. These trials must be documented and submitted with the study to the Agency for review. Surfactants and charged polymers will be self-dispersing in water and should be tested at or below their dispersability limits.

(iii) If solubility is a problem (<100 ppm), trials should be conducted under test conditions using various solvents that are most likely to be effective and that are widely recognized as being nontoxic and other means to ensure that the appropriate methods are used during the laboratory tests to enhance solubility. Once a solvent is chosen based upon more simplistic, comparative evaluations, the decision should be confirmed in the preliminary trials with only that solvent.

(iv) Chemical analysis methods as detailed in paragraph (l) of this guideline.

(v) Stability of the test material in the samples to be collected for chemical analyses should be determined during the laboratory studies. This includes determining whether and how samples can be stored for future analysis.

(2) Laboratory studies must be designed taking into account this preliminary information. This means the trials described are to be conducted before the definitive laboratory studies are initiated.

(h) **Toxicity tests with poorly soluble materials.** (1) Existing OPP guidelines for aquatic toxicity tests require that chemicals be tested up to a maximum dissolved concentration of 100 ppm (milligrams per liter) for pesticides or 1,000 ppm for industrial chemicals in an effort to obtain an LC50 or EC50. This amount of test material is considered to represent a conservative measure of the most bioavailable fraction, which may include some colloidal material not removed by centrifugation in addition to the truly dissolved fraction.

(2) Applicants must demonstrate the technique used to maximize chemical dissolution in the test media under standard conditions. Consideration of the optimum technique should include use of nontoxic solvents, saturation (solubility) columns, sonication, minor adjustments to environmental conditions (i.e., temperature, pH, etc.), as appropriate. Minor adjustments should not extend outside the recommended range of conditions for the specific test organism.

(3) Current policy allows chemicals that are poorly soluble (solubility <100 ppm) or dispersible in water to be tested up to the maximum water solubility or dispersibility limit obtainable for the given test conditions employed, provided that certain prerequisites apply:

(i) Concentrations of test chemical in test media are measured at appropriate intervals and from appropriate test chambers of all test levels are determined from centrifuged supernatant or other appropriate separation (e.g., filtrate). Self-dispersing industrial chemicals (e.g., surfactants, detergents, or charged [polymers) should be sampled directly.

(ii) Testing is also performed with a more soluble formulation e.g., emulsifiable concentrate, if one exists (in addition to testing with the technical-grade material). Testing with a more soluble formulation will not, however, be required if it does not provide a twofold increase in solubility.

(4) Studies that involve radical changes in environmental test conditions outside the recommended range of values for temperature, salinity, pH, etc., will be considered on a case-by-case basis.

(i) **Methods for solubility enhancement—(1) Saturator columns.** The use of saturation columns as an aid in the dissolution of test material and in confirming maximum solubility is recommended but not required for nonvolatile test chemicals with test media solubilities of 10 ppm or less. Methods for using these columns in aquatic toxicity tests can be adapted from the methods established for their use in determining water solubility under OECD's Column Elution Method (see OPPTS 830.7840). Saturator columns may be considered to generate test solutions for static

studies or for flow-through studies. Furthermore, saturator columns for these studies need only be considered if conventional techniques for dosing the water do not result in water concentrations within twice the stated solubility of the compound.

(2) **Emulsifiers and formulation testing.** Testing with a more soluble formulation, if one exists and which may contain emulsifiers, dispersants, solubilizing agents, etc., is required for all active ingredients subject to aquatic organism testing and having a water solubility less than 100 ppm and less than an EC/LC50. A defined EC/LC50 provides a greatly improved basis for risk assessment.

(3) **Effect of temperature.** Solubility is a function of temperature and is especially sensitive at the limits of solubility. Generally, below saturation, increases of as much as 10 °C may affect the solubility up to a factor of 2. However if test solutions are close to saturation, small changes in temperature may result in supersaturated solutions. In addition, control of temperature is important because of its well-known effects on the actual toxicity of the compound.

(4) **Centrifugation.** Conventional centrifugation is required for all test media where undissolved test material, precipitate, flocculant, or colloidal suspension except for surfactants or charged polymers) are observed in the test chambers or where the solubility and (hence bioavailability) are in question. Filtration may be used instead of centrifugation if the analytical method is validated over a range of acceptable concentrations.

(j) **Measurement at initiation and termination of testing**—(1) **Initial analysis.** (i) Analysis at the 0–hour: A 30–min interval is generally required between the addition of the test substance and the introduction of the test organisms. 0-hour measurement should be made when test organisms are added. Industry will have to justify an exception from the 30–min requirement for adding test organisms if the characteristics of the test material and test system require a longer equilibration time. If preliminary trials have been performed, this delay should be predictable.

(ii) In flow-through tests, the study should be conducted with knowledge of the time it will take for the test material to reach equilibrium or steady-state in the test container. Initiation of the test and scheduling of the sampling times must be based on this information. In some cases, a flow-through system may have to be run for an extended-time pretest in an attempt to achieve equilibrium or steady-state conditions. If equilibrium or steady-state cannot be achieved, and/or it appears that the measured concentrations will be substantially below (< 70 percent) nominal, the study report should reflect that the laboratory was aware of this problem. The study report should clearly identify the problem, indicate the steps taken to mitigate it and justify the study design and dosing levels. However, if sufficient analytical methods are available and acceptable toxicity

data are produced, additional testing and evaluation with the sole objective of obtaining initial measured concentrations greater than 70 percent of nominal will not be required.

(2) **Analysis at test termination.** Where indicated, measurement at test termination is considered necessary to determine if the test organisms were exposed to the test material throughout the entire study and at what levels. A significant change in test concentration during the last part of the study may substantially alter the results. For example, if the test concentration dropped dramatically during the last few days of a study, the effects that may have been caused by such exposure may not occur. The EC50 or LC50 developed from that study would be misleading if it is called a ''96–h LC50''.

(k) **Replicates and concentration measurement.** (1) Average concentrations of replicates are used in regression analysis. When replicate test containers and measurement of test concentration are required, each replicate in each test concentration must be analyzed separately because the responses in each replicate are viewed as independent and it is necessary to know what the concentrations were so variation can be determined. Exceptions to this occur when:

(i) Replicate treatment containers under static tests or static renewal conditions are filled from a bulk preparation. In this case, only samples from the bulk supply for each test level must be analyzed.

(ii) A ''splitter'' is used in a flow-through test to feed more than one replicate. In this case, only samples from one replicate per treatment level require analysis. It is recommended that samples be collected from all replicates and be stored in case anomalous concentrations are measured in the one that is analyzed. Analyzing the other replicates may shed light on the cause and extent of the anomalous measurements.

(2) Replicates receiving flow from a splitter should be sampled and analyzed alternately. In other words, if there are two replicates (A and B), replicate A should be analyzed in the first week and replicate B in the second week, etc.

(3) To the extent possible, variability in measured concentrations should be minimized. The goal for limiting variability of measurements between replicates of the same concentration, and over time in the same concentration, is maintaining the ratio of the highest concentration to the lowest concentration at 1.5:1 or less. Generally, variability above this amount is not acceptable.

(4) An important factor in considering the limits of variability is the avoidance of overlapping mean test concentrations between test levels. High variability puts into question the reliability of the environmental chemistry method and/or the concentrations on which to base statistical

analysis and toxicological conclusions. If variability beyond the 1.5:1 ratio occurs, an exception to it should be justified.

(5) This justification should clearly state the problem, explain why it occurred, provide scientific justification, and identify all measures taken to mitigate the problem. The justification also should include the fully developed chemistry method, including the documentation necessary for a bench chemist to review and evaluate it.

(6) For cases in which variability problems are suspected, preliminary trials are strongly recommended. If it becomes clear that high variability cannot be avoided, an exception should be justified. Any justification should be provided in advance. Agency scientists will decide on the validity of the rationale for the exception, and may recommend other methods to reduce potential variability.

(l) **Use of chemical analysis to confirm exposure in aquatic testing**—(1) **Acute static tests.** Except for acute aquatic algae and diatom studies (which can only be conducted as static tests), acute static tests may be conducted only if, among other things, the test material has been shown to be stable under the test conditions, as defined in paragraph (e) of this guideline. (Other factors not addressed in this guideline may preclude conducting a static test even if the test material is stable under test conditions. These include, but are not limited to, problems in maintaining dissolved oxygen levels, feeding requirements, and concern for bacterial/microbial contaminants.) In an acute static test with a test material that is stable and readily soluble at the treatment levels, measurements of each test concentration are not absolutely required. However:

(i) For static tests, the concentration of toxicant should be measured at the beginning and end of the test in all test chambers. Further, measurement of the toxicant's degradation products is desirable, but not required.

(ii) The study may be rejected if the following occurs:

(A) The test material was not stable under test conditions.

(B) Precipitates formed.

(C) Solubility was likely to have been a problem at the levels tested.

(iii) If the recommended chemical measurements were made to verify exposure levels, the study may not be rejected. Whether the study design was modified in a scientifically defensible attempt to accommodate these chemical characteristics will also be considered.

(iv) If variability is expected to be a problem, it is recommended that measurements of test concentrations be made at each test level at 0–hour, 48–h and, for tests longer than 48 h, at test termination. Replicate

test containers should be measured separately, except as explained under paragraph (k) of this guideline.

(2) **Acute static renewal.** Refer to the general discussion of replicates under paragraph (k) of this guideline. If a static renewal test is conducted, each test chamber must be sampled for chemical analysis at the 0–hour, at the end of the first (or longest) cycle, and at test termination. It is recommended that measurements be made at the end of each renewal cycle acute flow-through.

(3) **Acute flow-through.** If a flow-through test is conducted, each test concentration must be measured at the 0–hour and at test termination. It is recommended that for 96–h tests, an intermediate measurement be made at 48–h to verify midtest exposure if variability is expected to be a problem. (All acute aquatic algae and diatom tests must be conducted as static. Flow-through and static renewal systems are not recommended for these tests, since they are conducted with microscopic organisms that cannot be protected from loss when renewing or draining water from the test containers. Static tests for *Lemna gibba* can be conducted, regardless of stability.)

(4) **Chronic static renewal.** Refer to the general discussion of replicates under paragraph (k) of this guideline. Concentrations must be measured at each test level at 0–hour, at the end of the last renewal cycle (at test termination), and at the beginning and end of an intervening cycle at least once per week. The longest cycle in a sequence should be used if variable-cycle periods are employed.

(5) **Chronic flow-through.** Refer to the general discussion of replicates under paragraph (k) of this guideline. In each concentration, measure at 0–hour, every 7 days, and at test termination. At the beginning of a study, the exact flow of the system and water output at each splitter must be documented. In addition, system flow must be metered and monitored visually or mechanically on a daily basis (every 24 h), and it is recommended that the system flow be metered and monitored twice a day (approximately every 12 h). Measurement of test concentration is required each time metering fluctuation or malfunction is detected or observed. A record of the regular inspections must be maintained and provided with the study report.

(m) **Measured concentrations versus nominal concentrations.** This section describes acceptable limits of deviation of measured from nominal concentrations.

(1) Test endpoints are used as if the organisms were exposed to the test material at the statistically developed value (LC50 or EC50) for the entire test duration. One aspect of the risk assessment is to compare concern levels based on the LC50 is to initial immediate concentrations. However, field conditions may exist in which concentrations that may be of

acute concern may last longer or occur frequently enough to be comparable to the 48–h, 96–h or 120–h test duration. Even though a pesticide may degrade rapidly under one environmental condition (in water, for example), the possibility of repeated exposure needs to be considered. Repeated exposure from reservoirs of the active ingredient, occurring in environmental compartments where persistence is greater, may occur. The Agency takes these eventualities into account in order to generate risk assessments that adequately address hazard to the aquatic ecosystem.

(2) Presumably, a safer chemical one that may degrade rapidly, has low solubility, and is used at low rates. While these characteristics may result in lower exposure levels in the field, the risk they represent can only be determined if the actual toxicity of the pesticide is known or the level below which the pesticide is not likely to result in 50 percent mortality (i.e., an LC50 > X-concentration situation.) When potentially low, realistic exposure levels are calculated and used for risk assessments, it is imperative that the actual toxicity of the pesticide at those levels be determined. If the test is conducted using nominal concentrations, the results could reflect a higher apparent effect concentration (e.g. LC50, EC50, or NOEL). As a result, potential risk may be missed because the comparison would be between a low ''realistic'' exposure and a high nominal test level that was not the true toxicity level. A risk assessment based on such a comparison and data would be faulty and could not be scientifically defended.

(3) Pesticide chemicals that are used at very low levels tend to have high biological activity. For this reason, it is imperative that the toxicity data developed for these pesticides be accurate and scientifically defensible.

(4) Measured concentrations are used when they are available because they indicate what the exposure was in the test chambers. When measured concentrations are indicated, they are considered necessary because:

(i) There are concerns that the actual concentrations to which the test organisms are exposed may differ from ''nominal.'' This variation may be due to chemical characteristics, test conditions, or mechanical apparatus.

(ii) Measured concentrations confirm that the test system was designed appropriately and is operating acceptably. Characteristics that make testing difficult (low solubility, short half-life, high binding potential, etc.) must be accounted for in the exposure estimates. They are not a reason for developing misleading toxicity values from laboratory tests.

(5) Measurement of test concentrations is not performed just to determine if the technician knows how to mix the test solution once. Among other things, it also ensures that the test solution was mixed correctly each

time. It corroborates the precision of the technician or mechanics of the test system.

(6) If test levels are not measured, the nominal values are used to calculate the LC50, EC50, NOEC and LOEC. If the test material has degraded or has become unavailable because of insolubility or sorption, the pesticide may be characterized as less toxic than it really is. For example, if based on nominal test levels, the LC50 is 5 ppm, the pesticide would be considered moderately toxic. No higher tier testing would be required and that value (5 ppm) would be the basis for developing concern levels with which to compare EECs. But if, in reality, the concentrations to which the organism was actually exposed were only between 0.1 and 1 ppm, the LC50 may well be closer 0.5 ppm. For pesticides this would result in labeling, and could trigger higher tier tests. More importantly, it would yield substantially lower concern levels with which to compare exposure levels.

(7) When a laboratory test design has been specifically modified to accommodate the instability of test material or other factors likely to cause variability in test concentrations, and the design is judged adequate based on sufficient preliminary information, the study will not be rejected solely on the grounds that measured concentrations varied by more than 30 percent of the nominal concentration. (This assumes that the preliminary stability tests were conducted under test conditions essentially identical to the actual test conditions.) An increase in measured test concentration of more than 30 percent from the nominal concentration during the test will generally not result in rejection, provided that the following conditions are met:

(i) A reasonable and scientific explanation is given, and the variability of results produced by the chemical analysis method is adequately characterized.

(ii) All test containers exhibit a similar (but not necessarily identical) shift. (If concentrations in some containers go up substantially (>30 percent) and test concentrations in other containers go down substantially (>30 percent), they will not be considered to have exhibited a similar shift. The most important criterion is that test levels must not experience a shift in "order." That is, the highest test level should remain highest, the next should remain second, etc. If orders are shifted, the test may be rejected, since regression analysis would not yield statistically sound median lethal concentrations and confidence limits.)

(iii) The variability of the measured concentrations is acceptable.

(iv) A statistically valid endpoint can be derived from the measured concentrations (either an LC50, EC50, or that the LC50 or EC50 is greater than 100 ppm).

(v) The preliminary stability information is provided with complete documentation and description of methods used to derive such information.

(8) In some cases, high variability cannot be avoided because the test concentrations are approaching the limit of detection or because of unavoidable binding of the test material to the chemical analysis apparatus. When the ratio of the highest concentration to the lowest measured concentration is expected to vary by more than 1.5, the registrant is strongly advised to justify an exception to this requirement in advance of conducting the aquatic laboratory studies. This exception justification should consist of:

(i) Documentation of the preliminary trials indicating this problem.

(ii) The specific steps that will be taken to reduce the variation.

(iii) The fully developed chemical analysis method.

(iv) The raw data, standards, and chromatogram from a representative analysis using the method. For each chemistry method, the actual minimum detection level and level of quantification must be identified.

(9) The Agency will decide on each exception justification on a case-by-case basis. However, if a series of aquatic tests are to be conducted with one chemical and it is anticipated that these limits will be exceeded, one exception justification may cover more than one study. The Agency will then exercise judgment in evaluating studies with test materials that are difficult to measure.

(10) Conducting flow-through or static renewal tests with aquatic algae is not feasible with the current state of the practice. Therefore, the following is recommended for a test material that, based on preliminary stability testing, is expected to degrade to less than 70 percent of the nominal concentration. The study should be conducted normally, with concentrations measured at 0-hour and at test termination. Although it is undesirable to allow the concentrations to decline throughout the study, the problem may be unavoidable. In this case, the LC50 regression analysis is based on the mean measured concentration. If the concentration is expected to decline to less than the minimum detection level before the end of the study, then it is recommended that interim chemical measurements be made to determine the decline rate.

(11) For purposes of consistency, the aquatic test with a vascular plant (*Lemna gibba*) need not be done using a flow-through or static renewal system with the sole purpose of maintaining test concentrations. There may be other reasons for conducting a static renewal study.

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Prevention, Pesticides
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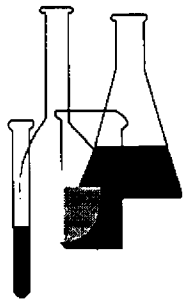
Ecological Effects Test Guidelines

OPPTS 850.1010 Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids



"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.*).

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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.

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

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.

(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.

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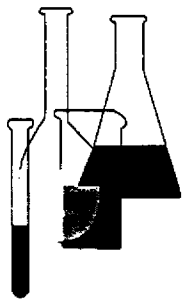
OPPTS 850.1020 Gammarid Acute Toxicity Test



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OPPTS 850.1020 Gammarid acute 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 is 40 CFR 795.120 Gammarid Acute Toxicity Test.

(a) **Purpose**. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures subject to environmental effects test regulations. This guideline describes a test to develop data on the acute toxicity of chemicals to gammarids. The data from this test will be used in assessing the hazard of a chemical to aquatic organisms.

(b) **Definitions**. The definitions in section 3 of TSCA and in Part 792, Good Laboratory Practice Standards, apply to this test guideline. The following definitions also apply to this guideline:

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

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.

LC50 means the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of the test batch of organisms within a particular period of exposure (which shall be stated).

Loading means the ratio of the biomass of gammarids (grams, wet weight) to the volume (liters) of test solution in either a test chamber or passing through it in a 24–hour period.

Solvent means a substance (e.g., acetone) which is combined with the test substance to facilitate introduction of the test substance into the dilution water.

Static system means a test chamber in which the test solution is not renewed during the period of the test.

(c) **Test procedures**—(1) **Summary of the test.** In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. In a static test, the test substance is introduced into each test chamber. In a flow-through test, the rate in 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 gammarids, which have been acclimated to the test conditions, into the test chambers. Gammarids in the test chambers are observed periodically during the test; the dead gammarids are removed and the findings recorded. Dissolved oxygen concentration, pH, temperature, and the concentration of test substance in test chambers are measured at specified intervals. Data collected during the test are used to develop concentration—response curves and LC50 values for the test substance.

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

(ii) The gammarids shall be exposed to a wide-range of concentrations of the test substance (e.g. 1, 10, 100 mg/L, etc.), usually under static conditions.

(iii) A minimum of five gammarids should be exposed to each concentration of test substance for a period of 96 hours. The exposure period may be shortened if data suitable for determining concentrations in the definitive test can be obtained in less time. Nominal concentrations of the test substance may be acceptable.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine the 24, 48, 72, and 96—hour LC50 values and the concentration-response curves.

(ii) A minimum of 20 gammarids per concentration shall be exposed to five or more concentrations of the test substance 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). The range and number of concentrations to which the organisms are exposed shall be such that in 96 hours there is at least one concentration resulting in mortality greater than 50 and less than 100 percent, and one concentration causing greater than zero and less than 50 percent mortality. An equal number of gammarids may be placed in two or more replicate test chambers. Solvents should be avoided, if possible. If solvents have to be used, a solvent control, as well as a dilution control, shall be tested at the highest solvent concentration employed in the treatments. The solvent should not be toxic or have an effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/L.

(iii) Every test shall include a concurrent control using gammarids from the same population or culture container. The control group shall be exposed to the same dilution water, conditions and procedures, except that none of the test substance shall be is added to the chamber.

(iv) The dissolved oxygen concentration, temperature and pH of the test solution shall be measured at the beginning of the test and at 24, 48, 72 and 96 hours in at least one replicate each of the control, and the highest, lowest and middle test concentrations.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die during the test.

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

(vii) Gammarids shall be randomly assigned to the test chambers. Test chambers shall be positioned within the testing area in a random manner or in a way in which appropriate statistical analyses can be used to determine whether there is any variation due to placement.

(viii) Gammarids shall be introduced into the test chambers after the test substance has been added.

(ix) Observations on compound solubility shall be recorded. The investigator should record the appearance of surface slicks, precipitates, or material adhering to the sides of the test chambers.

(4) **Analytical measurements**—(i) **Water quality analysis.** The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water shall be measured at the beginning of each definitive test.

(ii) **Collection of samples for measurement of test substance.** Each sample to be analyzed for the test substance concentrations shall be taken at a location midway between the top, bottom, and sides of the test chamber. Samples should not include any surface scum or material dislodged from the bottom or sides. Samples shall be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photogradation, chemical reaction, volatilization, or sorption.

(iii) **Measurement of test substance.** (A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) shall be measured in each test chamber at least at the beginning (0–hour, before gammarids are added) and at the end of the test. During flow-through tests, the concentration of dissolved test substance shall be measured in each test chamber at least at 0 and 96–hours and in at least one chamber whenever a malfunction of the test substance delivery system is observed.

(B) The analytical methods used to measure the amount of test substance in a sample shall be validated before beginning the test. This involves adding a known amount of the test substance to each of three water samples taken from a chamber containing dilution water and the same number of gammarids as are placed in each test chamber. The nominal concentrations of the test substance in these samples should span the concentration range to be used in the test. Validation of the analytical method

should be performed on at least two separate days prior to starting the test.

(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(D) Among replicate test chambers, the measured concentrations shall not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test shall not vary more than plus or minus 30 percent from the measured concentration in that chamber at zero time.

(E) The mean measured concentration of dissolved test substance shall be used to calculate all LC50's and to plot all concentration-response curves.

(d) **Test conditions for definitive test**—(1) **Test species**—(i) **Selection.** (A) The amphipods, *Gammarus fasciatus, G. pseudolimnaeus,* and *G. lacustris* are specified for this test.

(B) Gammarids can be cultured in the laboratory or collected from natural sources. If collected, they must be held in the laboratory for at least 14 days prior to testing.

(C) Gammarids used in a particular test shall be of similar age and/or size and from the same source or culture population.

(ii) **Acclimation.** If the holding water is from the same source as the dilution water, acclimation to the dilution water shall be done gradually over a 48–hour period. The gammarids then shall be held at least 7 days in the dilution water prior to testing. Any changes in water temperature should not exceed 2 °C per day. Gammarids should be held for a minimum of 7 days at the test temperature prior to testing.

(iii) **Care and handling.** Gammarids shall be cultured in dilution water under similar environmental conditions to those used in the test. Organisms shall 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, gammarids shall be observed carefully for signs of stress and mortality. Dead and abnormal individuals shall be discarded.

(iv) **Feeding.** The organisms shall not be fed during testing. During culturing, holding, and acclimation, a sufficient quantity of deciduous leaves, such as maple, aspen, or birch, should be placed in the culture and holding containers to cover the bottom with several layers. These leaves should be aged for at least 30 days in a flow-through system before

putting them in aquaria. As these leaves are eaten, more aged leaves should be added. Pelleted fish food may also be added.

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

(*1*) Containers for culturing, acclimating and testing gammarids;

(*2*) Containers for aging leaves under flow-through conditions;

(*3*) A mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods;

(*4*) Apparatus for straining particulate matter, removing gas bubbles, or aerating the dilution water, as necessary; and

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

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

(C) Test chambers shall be covered loosely 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.** Construction materials and equipment that may contact the stock solution, test solution or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test substances. Glass, stainless steel, and perfluorocarbon plastic should be used wherever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be aged prior to use. Rubber, coopper, brass, galvanized metal, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) **Test substance delivery system.** In flow-through tests, diluters, metering pump systems or other suitable devices shall be used to deliver the test substance to the test chambers. The system used shall be calibrated before each test. The general operation of the test substance delivery system shall be checked twice daily during a test. The 24–h flow shall be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from one test chamber to another.

(iv) **Test chambers.** Test chambers shall contain at least one liter of test solution. Test chambers made of stainless steel should be welded,

not soldered. Test chambers made of glass should be glued using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber. A substrate, such as a bent piece of stainless steel screen, should be placed on the bottom of each test chamber to provide cover for the gammarids.

(v) **Cleaning of test system.** Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed sequentially with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) **Dilution water.** (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if gammarids will survive in it for the duration of the culturing, acclimating, and testing periods without showing signs of strees. The quality of the dilution water should be constant enough that the month-to-month variation in hardness, acidity, alkalinity, conductivity, TOC or COD, and particulate matter is not more than 10 percent. The pH should be constant within 0.4 unit. In addition, the dilution water should meet the following specifications measured at least twice a year:

Substance	Maximum concentration
Particulate matter	20 mg/L
Total organic carbon (TOC) or	2 mg/L
chemical oxygen demand (COD)	5 mg/L
Boron, fluoride	100 µg/L
Un-ionized ammonia	1 µg/L
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc	1 µg/L
Residual chlorine	3 µg/L
Cadmium, mercury, silver	100 ng/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus:	
polychlorinated biphenyls (PCBs) or	50 ng/L
organic chlorine	25 ng/L

(B) If the dilution water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass-distilled or carbon-filtered deionized water with a conductivity less than 1 µmho/cm is acceptable as the diluent for making reconstituted water.

(C) The concentration of dissolved oxygen in the dilution water shall be between 90 and 100 percent saturation. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use.

(3) **Test parameters.** Environmental parameters during the test shall be maintained as specified below:

(i) Water temperature of 18 ± 1°C.

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

(iii) The number of gammarids placed in a test chamber shall not be so great as to affect the results of the test. Ten gammarids per liter is the recommended level of loading for the static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

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

(e) **Reporting.** The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition, the test report shall include, but not necessarily be limited to, the following information:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and completed.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing the data.

(4) The test substance identified by name, Chemical Abstracts (CAS) number or code number, source, lot or batch number, strength, purity, and composition, or other appropriate characteristics.

(5) Stability of the test substance under the conditions of the test.

(6) A description of the methods used, including:

(i) The source of the dilution water, its chemical characteristics (e.g., hardness, pH, etc.) and a description of any pretreatment.

(ii) A description of the test substance delivery system, test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., test substance addition), the loading, the lighting, and the flow rate.

(iii) Frequency and methods of measurements and observations.

(7) The scientific name, weight, length, source, and history of the organisms used, and the acclimation procedures and food used.

(8) The concentrations tested, the number of gammarids and replicates per test concentration. The reported results should include:

(i) The results of dissolved oxygen, pH and temperature measurements.

(ii) If solvents are used, the name and source of the solvent, the nominal concentration of the test substance in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determination in water and solvents.

(iii) The measured concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(iv) In each test chamber at each observation period, the number of dead and live test organisms, the percentage of organisms that died, and the number of test organisms that showed any abnormal effects in each test chamber at each observation period.

(v) The 48, 72 and 96–h LC50's and their 95 percent confidence limits. When sufficient data have been generated, the 24–h LC50 value also. These calculations should be made using the mean measured test substance concentrations.

(vi) The observed no-effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects), if any.

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

(9) A description of all circumstances that may have affected the quality or integrity of the data.

(10) The names of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(11) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the calculated LC50 value, 95 percent confidence limits, slope of the transformed concentration-response line, and the results of a goodness-of-fit test (e.g., X^2 test).

(12) The signed and dated reports prepared by any individual scientist or other professional involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(13) The locations where all specimens, raw data, and the final report are stored.

(14) The statement prepared and signed by the quality assurance unit.

United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

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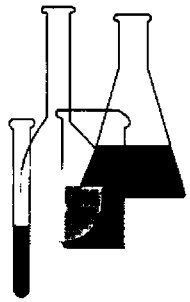
Ecological Effects Test Guidelines

OPPTS 850.1025 Oyster Acute Topxicity Test (Shell Deposition)



"Public Draft"

INTRODUCTION

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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 850.1025 Oyster acute toxicity test (shell deposition).

(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 797.1800 Oyster Acute Toxicity Test and OPP 72–3 Acute Toxicity Test for Estuarine and Marine Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **Purpose.** This guideline prescribes tests to be used to develop data on the acute toxicity of chemical substances and mixtures (''chemicals'') to Eastern oysters, *Crassostrea virginica* (Gmelin). The Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **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.

Acute toxicity is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. For aquatic animals this usually refers to continuous exposure to the chemical in water for a period of up to 4 days. The effects (lethal or sublethal) occurring may usually be observed within the period of exposure with aquatic organisms. In this test guideline, shell deposition is used as the measure of toxicity.

EC50 is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular exposure period which should be stated.

Shell deposition is the measured length of shell growth that occurs between the time the shell is ground at test initiation and test termination 96 h later.

Umbo means the narrow end (apex) of the oyster shell.

Valve height means the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(d) **Test procedures**—(1) **Summary of the test.** (i) The water solubility and the vapor pressure of the test chemical should be known. Prior to testing, the structural formula of the test chemical, its purity, stability

in water and light, *n*-octanol/water partition coefficient, and pK_a values should be known prior to testing. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water should also be known.

(ii) For chemicals with limited solubility under the test conditions, it may not be possible to determine an EC50. If it is observed that the stability or homogeneity of the test chemical cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(iii) Test chambers are filled with appropriate volumes of dilution water. The flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each test chamber and the flow-rate adjusted to establish and maintain the desired concentration in each test chamber. Test oysters, which have been acclimated and prepared by grinding away a portion of the shell periphery, are randomly introduced into the test and control chambers. Oysters in the test and control chambers are observed daily during the test for evidence of feeding or unusual conditions, such as shell gaping, excessive mucus production or formation of fungal growths in the test chambers. The observations are recorded and dead oysters removed. At the end of 96 h the increments of new shell growth are measured in all oysters. The concentration-response curve and EC50 value for the test chemical are developed from these data.

(2) **Range-finding test.** A range-finding test should be conducted to establish test chemical concentrations for the definitive test. The test is conducted in the same way as the definitive test except a widely spaced chemical concentration series (i.e. log-interval) is used.

(3) **Definitive test.** (i) Oysters which meet condition criteria (age, size, reproductive status, health) and which have been acclimated to test conditions should have approximately 3 to 5 mm of the shell periphery, at the rounded (ventral) end, ground away with a small electric disc grinder or other appropriate device, taking care to remove the shell rim uniformly to produce a smooth, rounded, blunt profile. The oyster's valves should be held together tightly during grinding to avoid vibrating the shell and injuring the adductor muscle. Oysters from which so much of the shell rim has been removed that an opening into the shell cavity is visible should not be used.

(ii) It is desirable to have shell growth values for the low and high concentrations relatively close to, but different from, 0 and 100 percent. Therefore, the range of concentrations to which the oysters are exposed should be such that in 96 h relative to the controls, very little shell growth occurs in oysters exposed to the highest concentration and shell growth is slightly less than controls at the lowest concentration. Oysters in the

remaining concentrations should have increments of shell growth such that the concentration producing 50 percent shell growth relative to the growth is bracketed with at least one concentration above and one below it.

(iii) The test should be carried out without adjustment of pH unless there is evidence of marked change in the pH of the solution. In this case, it is advised that the test be repeated with pH adjustment to that of the dilution water and the results reported.

(iv) The test begins when at least 20 prepared oysters are placed in each of the test chambers containing the appropriate concentrations of test substance and controls. The steady-state flows and test chemical concentrations should be documented. At least five test chemical concentrations should be used. The dilution factor between concentrations should not exceed 1.8.

(v) Test oysters should be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. The oysters should be spread out equidistantly from one another so that the entire test chamber is used. The oysters should also be placed with the left (cupped) valve down and the open, unhinged ends all oriented in the same direction facing the incoming flow of test solution.

(vi) The oysters are inspected at least after 24, 48, 72, and 96 h. Oysters are considered dead if touching of the gaping shell produces no reaction. Dead oysters are removed when observed and mortalities are recorded. Observations at 3 h and 6 h are also desirable.

(vii) Shell growth is the primary criterion used in this test guideline to evaluate the toxicity of the test chemical. Shell growth increments in all oysters should be measured after 96–h exposure. Record the length of the longest ''finger'' of new shell growth to the nearest 0.1 mm. Oysters should be handled very gently at this stage to prevent damage to the new shell growth.

(viii) Records should be kept of visible abnormalities such as loss of feeding activity (failure to deposit feces), excessive mucus production (stringy material floating suspended from oysters), spawning, or appearance of shell (closure or gaping).

(ix) The criteria for a valid definitive test are:

(A) The mortality in the controls should not exceed 10 percent at the end of the test.

(B) The dissolved oxygen concentration should be at least 60 percent of air saturation throughout the test.

(C) If evidence of spawning is observed, the test should be repeated.

(D) There should be evidence that the concentration of the substance being tested has been satisfactorily maintained over the test period. The concentration of the test substance should be measured:

(*1*) In each chamber at time 0-h.

(*2*) In each chamber at 96–h; and

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

(E) Dissolved oxygen, temperature, salinity, and pH measurements should be made at the beginning and end of the test in each chamber.

(F) A minimum of 2 mm of new shell growth should be observed in control oysters (solvent and dilution water).

(4) **Test results.** (i) At the end of the test, appropriate statistical analysis should be conducted on the oyster shell deposition test data. The probit transformation should then be applied to the response variable and then regressed, using least squares regression, on dose or log-dose. An F Test for linearity should be conducted to determine whether the chosen regression technique adequately describes the experimental data.

(ii) Calculate the ratio of the mean shell growth for each group of test oysters (exposed to each of the test chemical concentrations) to the mean shell growth of the group of control oysters. From these data the concentration-response curve is drawn and an EC50 along with the 95 percent confidence limits on the value are determined from the curves. The mean measured concentration of test chemical should be used to calculate the EC50 and to plot the concentration-response curve.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The Eastern oyster, *Crassostrea virginica,* should be used as the test organism.

(B) Oysters used in the same test should be 30 to 50 mm in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height should be less than 20 percent of the mean.

(C) Oysters used in the same test should be from the same source and from the same holding and acclimation tanks.

(D) Oysters should be in a prespawn condition of gonadal development prior to and during the test as determined by direct or histological observation of the gonadal tissue for the presence of gametes.

(ii) **Acquisition.** Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(iii) **Acclimation.** (A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters should be held for at least 12 to 15 days before testing. All oysters should be maintained in dilution water at the test temperature for at least 2 days before they are used.

(B) During holding, the oysters should not be crowded, and the dissolved oxygen concentration should be above 60 percent saturation. The temperature of the holding water should be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth and such that test results are not affected as confirmed by previous testing.

(C) Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(D) A batch of oysters is acceptable for testing if the percentage mortality over the 7-day period prior to testing is less than 5 percent. If the mortality is between 5 and 10 percent, acclimation should continue for 7 additional days. If the mortality is greater than 10 percent, the entire batch of oysters should be rejected. Oysters which appear diseased or otherwise stressed or which have cracked, chipped, bored, or gaping shells should not be used. Oysters infested with mudworms (*Polydora* sp.) or boring sponges (*Cilona cellata*) should not be used.

(2) **Test facilities**—(i) **Apparatus.** (A) In addition to normal laboratory equipment, an oxygen meter, equipment for delivering the test chemical, adequate apparatus for temperature control, and test tanks made of chemically inert material are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the oysters should be avoided.

(ii) **Dilution water.** A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation, and testing periods. Natural seawater is recommended, although artificial seawater with food added may be used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and should be delivered at a flowrate of at least 1 and preferably 5 L/h per oyster. The flowrate should be ±10 percent of the nominal flow. A dilution water is acceptable if oysters will survive

and grow normally for 14 days without exhibiting signs of stress; i.e. excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water should have a salinity in excess of 12 ppt, and should be similar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 ppt and a monthly range in pH of less than 0.8 unit. Artificial seawater salinity should not vary more than 2 ppt nor more than 0.5 pH unit. Oysters should be tested in dilution water from the same origin.

(3) **Test parameters**—(i) **Carriers.** Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used the control oysters should be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 mL/L.

(ii) **Dissolved oxygen.** The dissolved oxygen concentrations should be at least 60 percent of the saturation value and should be recorded daily.

(iii) **Loading.** The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) **Temperature.** The test temperature should be 20 °C. Temporary fluctuations (less than 8 h) within ±5 °C are permissible. Temperature should be recorded continuously.

(v) **pH.** The pH should be measured at the beginning and end of the test in each test chamber.

(f) **Reporting.** In addition to the reporting requirements as specified under EPA Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the following specific information should be reported:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature, and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g. the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including the age and/or size (i.e. height), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, the loading rate, and the flowrate.

(5) The methods of preparation of stock and test solutions, and the test chemical concentrations used.

(6) The number of dead and live test organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(7) The 96–h shell growth measurements of each oyster; the mean, standard deviation and range of the measured shell growth at 96 h of oysters in each concentration of test substance and control.

(8) The calculated 96–h EC50 and its 95 percent confidence limits and the statistical methods used to calculate these values.

(9) When observed, the 96–h observed no-effect concentration (the highest concentration tested at which there were no mortalities, abnormal behavioral or physiological effects and at which shell growth did not differ from controls).

(10) A graph of the concentration-response curve based on the 96–h chemical concentration and shell growth measurements upon which the EC50 was calculated.

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

(12) Any incidents in the course of the test which might have influenced the results.

(13) A statement that the test was carried out in agreement with the prescriptions of the test guideline given above (otherwise a description of any deviations occurring).

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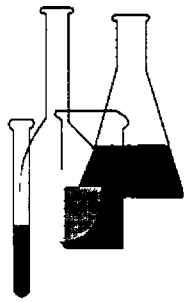
OPPTS 850.1035 Mysid Acute Toxicity Test



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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.

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OPPTS 850.1035 Mysid acute 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 797.1930 Mysid Shrimp Acute Toxicity Test and OPP 72–3 Acute Toxicity Test for Estuarine and Marine Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **Purpose.** This guideline prescribes a test using mysids as test organisms to develop data on the acute 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 test guideline.

Concentration-response curve is the curve produced from toxicity tests when percent response (e.g. mortality) values are plotted against concentration of test substance for a given length of exposure.

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

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.

LC50 means 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 means the ratio of test organisms biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

No observed effect concentration (NOEC) is the highest tested concentration in an acceptable toxicity test which did not cause the occurrence of any specified adverse effect (statistically different from the control at 95 percent level), and below which no tested concentration caused such an occurrence.

Retention chamber means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms, and eliminating loss of organisms in outflow water.

Static system means a test chamber in which the test solution is not renewed during the period of the test.

(d) **Test procedures**—(1) **Summary of the test.** In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. In a flow-through test, 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 the test chambers. Mysids in the test chambers are observed periodically during the test, dead mysids are removed, and the findings recorded. Dissolved oxygen concentration, pH, temperature, salinity, the concentration of test substance, and other water quality characteristics are measured at specified intervals in test chambers. Data collected during the test are used to develop concentration-response curves and LC50 values for the test substance.

(2) **Range-finding test.** (i) A range-finding test should be conducted to determine:

(A) Which life stage (juvenile or young adult) is to be utilized in the definitive test.

(B) The test solution concentrations for the definitive test.

(ii) The mysids should be exposed to a series of widely spaced concentrations of test substance (e.g. 1, 10, 100 mg/L, etc.), usually under static conditions.

(iii) This test should be conducted with both newly hatched juvenile (<24 h old) and young adult (5 to 6 days old) mysids. For each age class (juvenile or young adult), a minimum of 10 mysids should be exposed to each concentration of test substance for up to 96 h. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. The age class which is most sensitive to the test substance in the range-finding test should be utilized in the definitive test. When no apparent difference in sensitivity of the two life stages is found, juveniles should be utilized in the definitive test. No replicates are required, and nominal concentrations of the test chemical are acceptable.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine the concentration-response curves and the 48– and 96–h LC50 values with the minimum amount of testing beyond the range-finding test.

(ii) The definitive test should be conducted on the mysid life stage (juveniles or young adults) which is most sensitive to the test substance being evaluated.

(iii) A minimum of 20 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). An equal number of mysids are introduced into the test and control chambers by stratified random assignment and 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 substance. Preferred carriers are dimethyl formamide, triethylene glycol, acetone, or ethanol. Use of carriers should be avoided, if possible, as they may serve as a carbon source for bacteria. The concentration of solvent should not exceed 0.1 mL/L. The concentration ranges should be selected to determine the concentration-response curves and LC50 values at 48 and 96 h.

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

(v) The dissolved oxygen concentration, temperature, salinity, and pH should be measured at the beginning and end of the test in each chamber.

(vi) The test duration is 96 h. The test is unacceptable if more than 10 percent of the control organisms die or exhibit abnormal behavior during the 96–h test period. Each test chamber should be checked for dead mysids at 24, 48, 72, and 96 h after the beginning of the test. Concentration-response curves and 24–, 48–, 72– and 96–h LC50 values should be determined along with their 95 percent confidence limits.

(vii) In addition to death, 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 or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(ix) The concentration of the test substance in the chambers should be measured as often as is feasible during the test. During static tests, the concentration of test substance should be measured at a minimum at the beginning and at the end of the tests. During the flow-through test, the concentration of test substance should be measured at the beginning and end of the test and in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(4) **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 by appropriate laboratory practices before beginning the test. 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 mathematically corrected.

(ii) **Numerical.** The number of dead mysids should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48– and 96–h LC50 and corresponding 95 percent interval should be calculated. An NOEC and the slope of the dose-response curve should also be determined.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The mysid, *Mysidopsis bahia*, is the organism specified for these tests. Either juvenile (<24 h old) or young adult (5 to 6 days old) mysids 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 acute 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. 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 dilution 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 under paragraph (g)(l) of this guideline can be used during holding, culturing, and testing periods.

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

(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, for flow-through tests, flow-through chambers and a test substance delivery system are required. Furthermore, it is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss of test organisms through outflow water. For static tests, suitable chambers for exposing test mysids to the test substance are required. Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(B) 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 test 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 dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(B) For use in the flow-through test, 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 μm prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than 1 μohm/cm at 12 °C is acceptable 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.** In flow-through tests, proportional diluters, metering pumps, or other suitable systems should be used to deliver test substance to the test chambers. The system to be used 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 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 among test 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 °C. Excursions from the test temperature should be not greater than ±2 °C.

(ii) 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.

(iii) The number of mysids placed in a test solution should not be so great as to affect results of the test. Loading should not exceed 30 mysids per liter for a static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

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

(v) Salinity of 20±3 ppt.

(f) **Reporting.** The sponsor should submit to the EPA all data developed during the test that are suggestive or predictive of acute toxicity and

all concomitant toxicologic manifestations. In addition to the reporting requirements as specified under Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the following specific information should be reported:

(1) The nature of the test, laboratory, name of the investigator, test substance, and dates of test should be supplied.

(2) A detailed description of the test substances should be provided. This information should include the source, lot number, composition, physical and chemical properties, shelf life and storage conditions, and any carrier or additives used.

(3) Detailed information about the shrimp should be provided: Common and scientific names, source of supply, age, history, weight, acclimation procedure, and feeding history should be reported.

(4) A description of the experimental design including the number of test solution concentrations, number of replicates, and number of shrimp per replicate should be provided.

(5) The source of the dilution water, its chemical characteristics (e.g. salinity), and a description of any pretreatment.

(6) A description of the test chambers, the depth and volume of solution in the chamber, the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system and flow rate expressed as volume additions per 24 h.

(7) The concentration of the test substance in each test chamber before the start of the test and at the end.

(8) The number of dead shrimp and measurements of water temperature, salinity, and dissolved oxygen concentration in each test chamber should be recorded at the protocol-designated times.

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

(10) Recorded data for the holding and acclimation period (temperature, salinity, etc.).

(11) Concentration-response curves should be fitted to mortality data collected at 24, 48, 72, and 96 h. A statistical test of goodness-of-fit should be performed.

(12) For each set of mortality data, the 48– and 96–h LC50 and 95 percent confidence limits should be calculated on the basis of the average measured concentration of the test substance. When data permits, LC50 values with 95 percent confidence limits should be computed for 24– and

72–h observations. The NOEC and slope of the dose-response curves should also be calculated.

(13) The methods used in calculating the concentration-response curves and the LC50 values should be fully described.

(g) **References.** The following references should be consulted for additional background material 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, E.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* (Pericarda: Mysidacea: Mysidae), from the Gulf of Mexico. *Proceedings of the Biological Society of Washington* 107:680-698 (1994).

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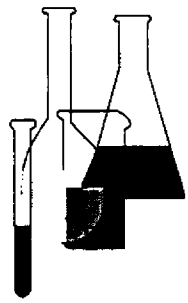
Ecological Effects Test Guidelines

OPPTS 850.1045 Penaeid Acute 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.*).

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OPPTS 850.1045 Penaeid acute 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 797.1970 Penaid Shrimp Acute Toxicity Test and OPP 72–3 Acute Toxicity Test for Estuarine and Marine Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **Purpose.** This guideline prescribes tests using penaeid shrimp as test organisms to develop data on the acute 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 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this guideline:

Concentration-response curve is the curve produced from toxicity test data when percent response (e.g. mortality) values are plotted against concentration of test substance for a given length of exposure.

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

Flow-through is a continuous passage of test solution or dilution water through a test chamber, holding, or acclimation tank with no recycling.

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

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

No-observed-effect-concentration (NOEC) is the highest tested concentration in an acceptable toxicity test which did not cause the occurrence of any specified adverse effect (statistically different from the control at the 95 percent level), and below which no tested concentration caused such an occurrence.

ppt is parts per thousand (salinity units).

(d) **Test procedures**—(1) **Summary of the test.** Prior to testing, the bottoms of the test chambers are covered with 2 to 3 cm of sand and

then filled with appropriate volumes of dilution water. The flow is adjusted to the rate desired to achieve loading requirements. Penaeids are introduced into the test chambers according to the experimental design. The shrimp are acclimated by maintaining them in the test chambers for a period of 3 to 7 days prior to the beginning of the test. The test begins when the test substance is introduced into the test chambers. The rate of flow is adjusted to maintain the desired test substance concentration in each chamber. The shrimp are observed during the test; dead shrimp are counted, removed, and the findings recorded. Dissolved oxygen concentration (DOC), pH, temperature, salinity, test substance concentration, and other water quality characteristics are measured at specified intervals in selected test chambers. The concentration of test substances with low water solubility may have to be determined with more frequency. Data collected during the test are used to develop concentration-response curves and LC50 values for the test substance.

(2) **Range-finding test.** (i) A range-finding test should be conducted to determine the test substance concentrations to be used for the definitive test. Substances which have low solubility and/or unusual adsorbance characteristics may require special handling procedures (physical procedures or the use of carrier substances) and attention to the type of materials used in the testing chambers to enhance solubility or decrease adsorption.

(ii) The shrimp should be exposed to a series of widely spaced concentrations of test substance (e.g. 1, 10, 100 mg/L, etc.).

(iii) A minimum of five penaeid shrimp should be exposed to each concentration of test substance for up to 96 h. 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 48– and 96–h LC50 values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 shrimp 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 shrimp are introduced into the test and control chambers by stratified random assignment and 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 substance. Preferred carriers are dimethyl formamide, triethylene glycol, acetone, or ethanol. Use of carriers should be avoided, if possible, as they may serve as a carbon source for bacteria. The concentration of solvent should not exceed 0.1 mL/L. The concentration ranges should be selected to determine the requested concentration-response curves and LC50 values. The concentration of test substance in test solutions should be determined

prior to use and at designated times. Abnormal or unexpected observations should trigger chemical analysis of the test water. If a specific test chamber seems to be affected, its water should be analyzed.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures, and shrimp from the same population or culture container, except that none of the chemical is added. If carriers are used, a separate carrier control should also be included.

(iv) The DOC, temperature, salinity, and pH should be measured at the beginning of the test and at 24, 48, 72, and 96 h in each test chamber.

(v) The test duration is 96 h. The test is unacceptable if more than 10 percent of the control organisms die or appear to be stressed or diseased during the 96–h test period. Each test chamber should be checked for dead shrimp at 3, 6, 12, 24, 48, 72, and 96 h after the beginning of the test. Concentration-response curves and 48– and 96–h LC50 values should be determined along with their 95 percent confidence limits.

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

(vii) Distribution of shrimp among test chambers should be randomized. 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 dissolved test substance (that which passes through a 0.45 μm filter) in the test chambers should be measured as often as is feasible during the test. The concentration of test substance should be measured:

(A) In each chamber at the beginning of the test and at 48 and 96 h after the start of the test.

(B) In at least one chamber containing the next to the lowest test substance concentration at least once every 24 h during the test.

(C) 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 substance should not vary more than 20 percent.

(ix) Observations on compound solubility should be recorded. The investigator should report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chambers.

(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 dead shrimp should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48– and 96–h LC50 and corresponding 95 percent intervals should be calculated. An NOEC and the slope of the dose response curve should also be determined.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** This test should be conducted using one of three species of penaeid: *Penaeus aztecus* (brown shrimp), *Penaeus duorarum* (pink shrimp), or *Penaeus setiferus* (white shrimp). Post-larval juvenile shrimp should be utilized. Shrimp may be reared from eggs in the laboratory or obtained directly as juveniles or adults. Shrimp used in a particular test should have been obtained from the same source, be of similar age, and be of normal size and appearance. Shrimp 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 control group.

(ii) **Acclimation.** During acclimation, shrimp should be maintained in facilities with background colors and light intensities similar to those of the testing areas. In addition, any change in the temperature and chemistry of the dilution water used for holding and acclimating 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 2 percent.

(iii) **Care and handling.** Upon arrival at the test facility, the shrimp should be transferred to water closely matching the temperature and salinity of the transporting medium. Shrimp should be held in glass tanks of 30 L capacity or larger. No more than 22 to 24 shrimp should be placed in a 30 L tank unless the flow-through apparatus can maintain dissolved oxygen levels above 60 percent of saturation. With species of the genus *Penaeus*, a minimum flow rate of 7.5 L/g body weight day should be provided. Larger flows, up to 22 L/g body weight day, may be desirable to ensure dissolve oxygen concentrations above 60 percent of saturation and the removal of metabolic products. The period of acclimation to ambient laboratory conditions should be at least 4 to 7 days.

(iv) **Feeding.** Penaeid shrimp should not be fed during testing. Every 2 or 3 days during the acclimation period, shrimp should be fed fish pieces approximately 1 cm^2. Uneaten food should be removed daily.

(2) **Facilities**—(i) **Apparatus.** (A) Facilities which may be needed to perform this test include: Flow-through tanks for holding and acclimating penaeid shrimp; a mechanism for controlling and maintaining the water temperature and salinity during the holding period; apparatus for straining particulate matter, removing air bubbles, or aerating water when necessitated by water quality requirements; and an apparatus providing a 12–h light and 12–h dark photoperiod with a 15– to 30–min transition period. Facilities should be well ventilated, free of fumes, and free of all other disturbances that may affect test organisms.

(B) Acid-washed sand, free of excess organic matter, should be placed in the bottom of test chambers to a depth of 2–3 cm.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation, minimize entry of dust and other particles, and prevent escape of the shrimp.

(ii) **Cleaning.** Test substance delivery systems and test chambers should be cleaned before each test following standard laboratory practices.

(iii) **Construction materials.** Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(iv) **Dilution water.** (A) Natural or artificial seawater is acceptable as dilution water if shrimp will survive in it without signs of stress, such as unusual behavior or discoloration. Shrimp should be acclimated and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a 5 μm filter with a pore size <20 μm prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than 0.1 mS/m at 12 °C is acceptable 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 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 94–h flow through a test chamber should be equal

to a least 5× the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent among test chambers or across time.

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

(i) Temperature of 23 ± 1 °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 substance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of shrimp placed in a test solution should not be so great as to affect results of the test. Loading requirements 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 12–h light and 12–h darkness, with a 15– to 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 acute toxicity and all other toxicological 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 nature of the test, laboratory, name of the investigator, test substance, and dates of the test should be supplied.

(2) A detailed description of the test substances should be provided. This information should include the source, lot number, composition, physical and chemical properties, shelf life, storage conditions, and any carrier or additives used.

(3) Detailed information about the shrimp should be provided: Common and scientific names, source of supply, age, history, weight, acclimation procedure, and feeding history should be reported.

(4) A description of the experimental design including the number of test solution concentrations, number of replicates, and number of shrimp per replicate should be provided.

(5) The source of the dilution water, its chemical characteristics (e.g. salinity), and a description of any pretreatment.

(6) A description of the test chambers, the depth and volume of solution in the chamber, the number of organisms per treatment, the number

of replicates, the loading, the lighting, the test substance delivery system, and flow rate expressed as volume additions per 24 h.

(7) The concentration of the test substance in each test chamber before the start of the test and at the end.

(8) The number of dead shrimp and measurements of water temperature, salinity, and DOC in each test chamber should be recorded at the protocol-designated times.

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

(10) Recorded data for the holding and acclimation period (temperature, salinity, etc.).

(11) Concentration-response curves should be fitted to mortality data collected at 24, 48, 72, and 96 h. A statistical test of goodness-of-fit should be performed.

(12) For each set of mortality data, the 48– and 96–h LC50 and 95 percent confidence limits should be calculated on the basis of the average measured concentration of the test substance. When data permits, LC50 values with 95 percent confidence limits should be computed for 24– and 72–h observations. The NOEC and slope of the dose-response curves should also be calculated.

(13) The methods used in calculating the concentration-response curves and the LC50 values should be fully described.

(g) **References.** The following references should be consulted for additional background material 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, FL 1978).

(2) [Reserved]

United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

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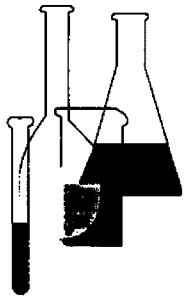
Ecological Effects Test Guidelines

OPPTS 850.1055 Bivalve Acute Toxicity Test (Embryo-Larval)



"Public Draft"

INTRODUCTION

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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.*).

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OPPTS 850.1055 Bivalve acute toxicity test (embryo-larval).

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

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is OPP 72–3 Acute Toxicity Test for Estuarine and Marine Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **Purpose.** This guideline prescribes tests to be used to develop data on the acute toxicity of chemical substances and mixtures (''chemicals'') to Eastern oysters (*Crassostrea virginica*), Pacific oysters (*Crassostrea gigas*), quahogs (*Mercenaria mercenaria*), or bay mussels (*Mytilus edulis*). The Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **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 guideline. The following definitions also apply to this test guideline.

Acute toxicity is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. The effects (lethal or sublethal) occurring may usually be observed within the period of exposure with aquatic organisms. In this test guideline, abnormal development or death is used as the measure of toxicity.

48–h EC50 (Effective Median Concentration) is that experimentally derived concentration of a chemical in water in which 50 percent of the larvae exposed to test material are dead or abnormally developed compared to larvae in the controls (not exposed to test material) after a 48–h exposure.

Embryo is the stage between the fertilization of the egg and the trochophore (2 to 8 cell stage).

Larva includes the trochophore and the straight hinge stage.

LOEC is the lowest observed effect concentration.

NOEC is the no observed effect concentration.

Veliger is the larval stage in which the ciliated velum (swimming organ) is present.

(d) **Test procedures**—(1) **Summary of the test.** (i) The water solubility and the vapor pressure of the test chemical should be known. Prior to testing, the structural formula of the test chemical, its purity, stability

in water and light, *n*-octanol/water partition coefficient, and pK values should be known. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water is also desirable.

(ii) It may be possible to determine an EC50 for a chemical with limited solubility under the test conditions. If the stability or homogeneity of the test chemical cannot be maintained, care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(iii) This study consists of a static 48–h exposure that is used to evaluate the proportion of living and normal D-shaped veligers exposed to the test material compared to the proportion of the same in controls not exposed to test material. The concentration-response curve and EC50 value for the test chemical are developed from these data.

(2) **Range-finding test.** A range-finding test should be conducted to establish test chemical concentrations for the definitive test. The test is conducted in the same way as the definitive test except a widely spaced chemical concentration series (i.e. log-interval) is used.

(3) **Definitive test.** (i) The test is started about 4 h after fertilization while the embryos are in the 2– to 4–cell stage (determined microscopically). At this stage embryos (15–30 embryos/mL/replicate) are added to the test solution. The endpoint for this test is the determination of a 48–h EC50. This will be based on the proportion of normal larvae (those that are alive with completely developed shells containing meat) exposed to test solution as compared to normal larvae in controls. An LOEC and an NOEC are also to be calculated. Constant conditions should be maintained in the test facilities as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters, and all operations and tests should be carried out in an environmental free from harmful concentrations of dust, vapors, and gases and in such a way to avoid cross-contamination. Any disturbances that may change the behavior of the test organisms should be avoided.

(ii) The test chemical concentrations are to be documented in all tests. At least five test concentrations are to be used with a dose separation factor not to exceed 1.8 between concentrations.

(iii) Test organisms are to be impartially distributed among test chambers in such a manner that the test results show no significant bias from the distributions.

(iv) Test organisms are inspected at regular intervals. Dead bivalves are removed when observed.

(v) The criteria for a valid definitive test are:

(A) Mortality or aberrant development in the controls are not exceed 30 percent percent for oysters or 40 percent for clams at the end of each test.

(B) The dissolved oxygen concentration should be at least 60 percent of air saturation throughout all tests.

(C) Embryos were not more than 4–h old from fertilization at the beginning of the test.

(D) The difference between the time-weighted-average (TWA) measured temperatures for any two test chambers from the beginning to the end of the test should not be greater than 1 °C. No single measured temperature in any test chamber should be more than 3 °C different from the mean of the TWA measured temperatures for the individual test chambers. The difference between the measured temperatures in any two test chambers should not be more than 2 °C at any one time.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) Eastern oysters (*C. virginica*) are the preferred test species, but Pacific oysters (*C. gigas*), quahogs (*M. mercenaria*), or bay mussels (*M. edulis*) may also be used.

(B) The test must begin with embryos within 4-h of fertilization when embryos are in the 2– to 4–, and 8–cell stages.

(C) Embryos used to start a test should be obtained from females and males that have been maintained for at least 2 weeks in the dilution water in the laboratory before they are stimulated to spawn.

(D) The spawning of bivalve test organisms is induced by rapidly elevating the temperature 5–10 °C above the conditioning temperature. An added stimulus of heat-killed bivalve sperm may be used. To fertilize the eggs, sufficient sperm suspension should be added to the egg suspension to yield 10^5 to 10^7 sperm/mL in the final mixture. Additional guidance may be found in paragraph (g)(1) of this guideline.

(ii) **Acquisition.** Bivalves may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(2) **Test facilities**—(i) **Apparatus.** (A) Test vessels, equipment and facilities that contact stock solutions, test solutions, or any water into which any brood stock or test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect test organisms.

(B) Test chambers are defined as the smallest physical units between which there are no water connections. Tests are usually conducted in glass chambers that are 1– to 2–L in capacity.

(ii) **Dilution water.** A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation, and testing periods. The dilution water should be acceptable to adult bivalve molluscs and their embryos and larvae. For oysters, at least 70 percent of the embryos resulting from eggs and sperm of appropriately conditioned adults result in normal larvae while being maintained in the dilution water for 48 h. For clams, this should be 60 percent of the embryos resulting in normal larvae. Also, a dilution water is acceptable if adult oysters or clams will survive and grow normally for 14 days without exhibiting signs of stress, i.e., excessive mucus production (stringy material floating suspended from oysters) lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. Natural seawater is recommended, although artificial seawater with food added may be used. The dilution water is to have a salinity in excess of 12 ppt. A natural seawater should have a weekly range in salinity of less than 10 ppt and a monthly range in pH of less than 0.8 unit. Artificial seawater salinity should not vary more than 2 ppt nor more than 0.5 pH unit. Oysters are to be tested in dilution water from the same origin.

(3) **Test parameters**—(i) **Carriers**. Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersant of low toxicity to oysters. When such carriers are used the control oysters are to be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 mL/L.

(ii) **Dissolved oxygen**. The dissolved oxygen concentrations are to be at least 60 percent of the saturation value and should be recorded daily.

(iii) **Loading.** The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) **Temperature.** Tests with *C. gigas* should be conducted at 20 °C, with *C. virginica* and *M. mercenaria* at 25 °C, and with *M. edulis* at 16 °C. The temperature for *C. gigas*, *C. virginica*, and *M. mercenaria* should never exceed 32 °C, nor 20 °C for *M. edulis* (even during spawning induction). Temperature should be recorded continuously.

(v) **pH.** The pH is to be measured at the beginning and end of the test in each test chamber.

(f) **Reporting.** In addition to the reporting requirements prescribed in 40 CFR Part 792—Good Laboratory Practice Standards, the report is to contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature, and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g., the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including the age and/or size (i.e., height), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, the loading rate, and the flowrate.

(5) The methods of preparation of stock and test solutions, and the test chemical concentrations used.

(6) The number of dead and live test organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(7) The calculated 48–h EC50 and its 95 percent confidence limits and the statistical methods used to calculate these values.

(8) The calculated LOEC and a NOEC must also be developed.

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

(10) Any incidents in the course of the test which might have influenced the results.

(11) A statement that the test was carried out in agreement with the prescriptions of the test guideline given above (otherwise a description of any deviations occurring).

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

(1) ASTM. Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Apecies of Saltwater Bivalve Molluscs. E 724-89. American Society for Testing and Materials, Philadelphia, PA. 18 pp (1989).

(2) [Reserved]

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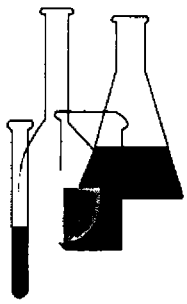
Ecological Effects Test Guidelines

OPPTS 850.1075
Fish Acute Toxicity Test, Freshwater and Marine



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OPPTS 850.1075 Fish acute toxicity test, freshwater and marine.

(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 797.1400 Fish Acute Toxicity Test; OPP 72–1 Acute Toxicity Test for Freshwater Fish and 72–3 Acute Toxicity Test for Estuarine and Marine Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982; and OECD 203 Fish Acute Toxicity Test.

(b) **Purpose.** The purpose of the acute toxicity test with fish species is to help in the assessment of possible risk to similar species in natural environments, as an aid in determination of possible water quality criteria for regulatory purposes, and for use in correlation with acute testing of other species for comparative purposes. Data on a cold and warm freshwater species are generally required. The rainbow trout, *Oncorhynchus mykiss*, and bluegill sunfish, *Lepomis macrochirus*, are preferred species to meet this requirement since they are sensitive indicator species and a large data base which characterizes the response to environmental contaminants is available. Other species as identified in paragraph (e)(4)(i)(A) of this guideline may be used. However, under certain circumstances, when potential environmental exposures may lead to significant risks, data on the preferred species may be required for risk assessment purposes so that the Agency can conduct comparative analyses with alternative chemical substances. Historically, it appears that many chemical classes are subject to comparative analyses. Development of a good data base could ultimately result in the use of other species in comparative analyses. In any case, the results of such a study should not be construed to represent behavior of the test material in the natural environment where other factors may come into play, but rather as a indicator of effects which might occur under comparable conditions as those utilized in the study.

(c) **Principle of the test**—(1) **Definitive test.** The goal of the definitive test is to determine concentration-response curves for fish mortality, the LC50's, and the 95 percent confidence intervals for each species tested at 24, 48, 72, and 96 h in a static, static-renewal, or flow-through system.

(2) **Range-finding or limit testing.** Definitive testing may be waived if limit testing with at least 30 organisms shows LC50 levels to be greater than 1,000 mg/L based on 100 percent active ingredients (AI), or the limits of water solubility or dispersibility. For pesticides, a lower level of 100 mg AI/L may be tested when estimated environmental concentrations are not expected to exceed 100 mg/L (ppm) as might occur with pesticide use. Prior to selection of definitive test concentrations it may be advisable

to conduct a range-finding test. Results of any range-finding and limit tests should be reported with results of the definitive test.

(3) **Information on the test substance.** The material to be tested should be technical grade unless the test is designed to test a specific formulation, mixture, or effluent. The degree of purity must be recorded for technical ingredients and mixtures. The percentage of each impurity should be reported and percentages should total 100 percent. A complete description of physicochemical characteristics (i.e. solubility, vapor pressure, hydrolysis in pH 5, 7, and 9) should be included with description of the AI used in specific chemical testing. A reliable analytical method for quantification of test substance concentrations must be available.

(d) **Validity of the test.** (1) Maximum-allowable control or solvent control mortality is 10 percent (or 1 mortality if 7 to 10 control fish are used) for a 96–h period of testing. If the test is continued past 96 h, the maximum-allowable additional mortality is 10 percent.

(2) Constant conditions must be maintained throughout the test period. Flow-through procedures are preferred over static-renewal or semistatic procedures and static-renewal procedures are preferred over a static test procedure.

(3) In static tests, the dissolved oxygen (DO) in each replicate should at all times be greater than 60 percent saturation. In flow-through tests, the DO should be maintained above 75 percent saturation.

(4) Measured concentrations are required if the test chemical is unstable or a flow-through diluter system is employed. Exception may be made in cases where hydrolysis studies indicate chemical to be stable (< 5 percent degradation) in 96 h at a pH comparable to test dilution water. In any case there must be evidence that test concentrations remained at least 80 percent of the nominal concentrations throughout the test or that mean measured concentrations are an accurate representation of exposure levels maintained throughout the test period.

(e) **Description of the method**—(1) **Apparatus.** Normal laboratory equipment and especially the following is necessary:

(i) Equipment for determination of water hardness, etc.

(ii) Adequate apparatus for temperature control.

(iii) Tanks constructed of chemically inert material and of suitable capacity to allow recommended loading levels.

(2) **Water.** (i) Clean surface or ground water, seawater (for estuarine or marine species), and reconstituted water are acceptable as dilution water. Dechlorinated water should not be used because some forms of chlorination are difficult to remove adequately. If dechlorinated tap water

is used, then daily chlorine analysis should be performed. Reconstituted or natural water is preferred.

(ii) Chemical analysis of water used in testing should include the following elements and limitations on maximum concentrations based on at least biannual testing:

Substance	Maximum concentration
Particulate matter	20.0 mg/L
Chemical oxygen demand (COD)	5.0 mg/L
Total organic carbon (TOC)	2.0 mg/L
Boron and fluoride	<100.0 mg/L
Residual chlorine	0.003 mg/L
Un-ionized ammonia	0.020 mg/L
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, and zinc.	0.001 mg/L
Cadmium, mercury, and silver	<0.100 µg/L
Total organophosphorus pesticides	0.050 µg/L
Total organochlorine pesticides + PCBs or organic chlorine	0.050 µg/L
Specific conductivity	<1.0 µohms

(iii) Salinity should be 20 ± 5 ppt for estuarine species.

(iv) Hardness should range between 40 and 180 mg/L as $CaCO_3$ for freshwater species.

(v) Water hardness or salinity, as appropriate, should be measured at the beginning of each test.

(vi) In marine flow-through tests, salinity should be recorded at the beginning of the test, on day 4, and if extended, on days 7 and 14.

(3) **Solutions of test water.** (i) Distilled water should be used in making stock solutions of the test substance. If the stock volume is more than 10 percent of the test solution volume, dilution water should be used. If a carrier, i.e. a solvent and/or dispersant, is absolutely necessary to dissolve the test substance, the amount used should not exceed the minimum volume necessary to dissolve or suspend the test substance in the dilution water. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

(ii) Solvent concentration may not exceed 0.5 mL/L in static-renewal or static testing, and 0.1 mL/L in flow-through testing.

(iii) Preferred solvents are dimethyl formamide, triethylene glycol, methanol, acetone, or ethanol. Solvent use should be avoided if possible.

(iv) Solvent concentrations selected should be kept constant in the solvent control and all test solutions. The concentration of solvent in highest treatment level should be used in the solvent control.

(v) The use of a solubility (saturation) column is permitted in the preparation of stock solutions. This may help to ensure the aqueous solubility limit is attained for poorly soluble test materials.

(vi) The pH should not be adjusted after the addition of the test chemical or stock solution into dilution water.

(vii) The pH should be measured in each replicate at the beginning of the test and every 24 h thereafter.

(viii) The pH must be monitored in low, medium, and high test concentrations and must remain > 6.0 and < 8.0 for freshwater testing and > 7.5 and < 8.5 for marine testing.

(ix) The pH may be adjusted in stock solutions to match the pH of dilution water if pH change does not affect stability of compound in water. HCl and NaOH may be used for this adjustment if warranted.

(4) **Selection of test species**—(i) **Test species.** One or more of the following species may be used:

(A) Freshwater species—Atlantic salmon, *Salmo salar*; bluegill sunfish, *Lepomis macrochirus*; brook trout, *Salvelinus fontinalis*; channel catfish, *Ictalurus punctatus*; coho salmon, *Oncorhynchus kisutch*; common carp, *Cyprinus carpio*; fathead minnnow, *Pimephales promelas*; guppy, *Poecilia reticulata*; rainbow trout, *Oncorhynchus mykiss*; red killifish, *Oryzias latipes*; threespine stickleback, *Gasterosteus aculeatus*; and zebrafish, *Brachydanio rerio*.

(B) Saltwater species—Atlantic silverside, *Menidia menidia*; sheepshead minnow, *Cyprinodon variegatus*; and tidewater silverside, *Menidia penisulae*.

(C) Data on both a warm and a cold freshwater species are generally required. The preferred warm water species is the bluegill sunfish. The rainbow trout is the preferred cold water species. When data on a marine or estuarine species is desired, the Atlantic silversides is preferred.

(ii) **Acclimation.** (A) A minimum 12–day acclimation period is required with 14 days recommended. A minimum of 7 days of the acclimation period must be performed in test dilution water.

(B) Holding water should come from the same source as the test dilution water, if not, acclimation to the dilution water should be done gradually over a 48–h period.

(C) No disease treatments may be administered within 48 h of test initiation or during testing.

(D) No feeding is permitted within 48 h of test initiation.

(E) Pretest mortality must be < 5 percent during acclimation. If pretest mortality is > 10 percent, then the entire batch must be rejected and a new batch begun in acclimation.

(F) Any changes in water temperature should not exceed 3 °C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing.

(G) During the final 48 h of acclimation fish should be maintained in facilities with background colors and light intensities similar to those of testing area.

(iii) **Age and size of test fish.** (A) Juvenile fish must be tested. Juvenile fish < 3.0 g should be used and the longest should not be more than twice the length of the shortest. The fish should be of normal size and appearance for their age. All fish must be of the same age.

(B) Wild caught fish may be used to satisfy testing guidelines if size, age, and source requirements are satisfied. Wild caught fish should be quarantined 7 days before acclimation procedures begin.

(C) Fish must originate from the same source and population. Records should be kept regarding the source of the initial stock and/or culturing techniques.

(D) Fish should not be used for a test if they appear stressed, or if more than 5 percent die during the 48 h immediately prior to the test, or if they were used in previous tests for treatments or controls.

(iv) **Temperature.** The recommended test temperatures are:

Species	Temperature, °C
Atlantic salmon	12±2.0
Atlantic silverside	22±2.0
Bluegill sunfish	22±2.0
Brook trout	12±2.0
Channel catfish	22±2.0
Coho salmon	12±2.0
Common carp	22±2.0
Fathead minnow	23±2.0
Guppy	23±2.0
Rainbow trout	12±2.0
Red killifish	23±2.0
Sheepshead minnow	22±2.0
Threespine stickleback	10±2.0
Tidewater silverside	22±2.0
Zebra-fish	23±2.0

(v) **Feeding.** Feeding of test fish daily until 48 h prior to test initiation is suggested.

(f) **Performance of the test**—(1) **Test design**—(i) **Test duration.** Acute testing must be performed for a minimum of 96 h.

(ii) **Controls.** Every test should include controls consisting of the same dilution water, conditions, procedures, and test population, except that no test substance is added. Solvent (carrier) controls are also required if a solvent was used.

(iii) **Introduction of fish.** Fish should be added to test chambers within 30 min of addition of the test material to dilution water. Fish may be added prior to addition of test material. Fish should be introduced randomly to individual replicates.

(iv) **Number of test organisms.** A minimum of seven fish per replicate is required. The use of 10 fish per replicate is preferred to obtain a more statistically accurate representation of the dose-response curve, to allow for mortality which may occur, yet be unrelated to chemical effect, and to avoid unnecessary repetitions of the test due to excessive control mortality.

(v) **Replicates.** (A) Two replicates per test concentration are preferred to avoid test repetition due to system failures, and to provide a stronger statistical baseline.

(B) Each test chamber should contain an equal volume of test solution and equal numbers of test fish. Replicate test chambers should be physically separated.

(vi) **Loading.** (A) The number of fish placed in each replicate should not be so great as to affect the test results.

(B) In static or static-renewal tests, loading should not exceed 0.8 g (fresh weight) of fish per liter of test solution in a replicate at any one time.

(C) In flow-through tests, loading should not exceed 0.5 g fresh weight of fish (FWF) per liter of test solution passing through a replicate within 24 h.

(vii) **Test chambers and support equipment.** (A) Construction materials and equipment that contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, no. 316 stainless steel, nylon screen, and perfluorocarbon plastic (e.g. Teflon) are acceptable materials and should be used whenever possible. Concrete, fiberglass, or plastic (e.g. PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be thoroughly conditioned before use. Rubber, copper, brass, galvanized metal, epoxy glues, lead, and flexible tubing

should not come in contact with the dilution water, stock solution, or test solution.

(B) Test chambers should be loosely covered to reduce evaporation and to minimize the entry of dust or other particulates into solutions and to prevent loss of test fish.

(C) Size. Many different sizes of test chambers have been used successfully. The size, shape, and depth of the test chamber is acceptable if the specified flow rate and loading requirements can be achieved. Test vessels must be of adequate size to maintain a load rate of FWF > 0.8 g FWF/L for static or static-renewal tests, or FWF > 0.5 g FWF/L for flow-through tests.

(D) Test substance delivery system. (*1*) In flow-through tests, proportional diluters, metering pump systems, or other suitable systems should be used to deliver the test chemical to the test chambers. The choice of a specific delivery system depends on the specific properties and requirements of the test substance.

(*2*) The system should be calibrated before and after each test. Calibration includes determining the flow rate and test concentration in each replicate. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to each replicate.

(*3*) A closed flow-through system may be used to test volatile compounds when more than 20 percent of the test substance would be lost through volatility or the test substance would cause oxygen levels may fall below 60 percent of the saturation level. A design description of this type of system should be included in the study report.

(E) Aeration. Gentle aeration of test vessels used in static systems during the exposure period is permitted only in cases where oxygen levels are in danger of dropping below 60 percent saturation due to chemical characteristics of the test material. Test concentrations must be measured during the test if aeration is used. No aeration of actual test vessels may be utilized in flow-through tests.

(viii) **Light.** (A) The photoperiod with 15 to 30 min transition periods is suggested. Photoperiods may range from 12D/12N to 16D/8N, where D = day, and N = night.

(B) Light intensity should range from 30 to 100 lm at the water surface; the intensity selected should be duplicated as closely as possible in all replicates.

(ix) **Temperature.** (A) Temperatures must be recorded in all replicates at the beginning of the test and every 24 h thereafter. The temperature should be recorded at least hourly in one replicate throughout the

test. Temperature should vary no more then 1.0 °C in any given 24–h period.

(B) The test system should be equipped with an automatic alarm system to alert staff of temperature changes in excess of 2.0 °C.

(C) If the water is heated, precautions should be taken to ensure that supersaturation of dissolved gases is avoided.

(x) **Dissolved oxygen.** DO concentrations should be measured in each replicate at the beginning of the test and every 24 h thereafter.

(xi) **Feeding.** Fish may not be fed during the treatment period.

(xii) **Disturbances.** Any disturbance which might change the behavior of the test fish should be avoided.

(2) **Test concentrations.** (i) A minimum of five test concentrations must be employed.

(ii) Five or more concentrations in a geometric series should be tested. Test concentrations must be at least 50 percent greater than the next lowest test concentration (not to exceed 120 percent). Range-finding studies prior to testing may allow more accurate selection of test concentrations.

(iii) No more than 25 percent variation is allowed between test concentrations within the same treatment during the test.

(iv) Concentration selection. (A) Test concentrations should be selected to produce a no-observable-effect concentration (NOEC) and, preferably, at least two partial mortalities, i.e. one greater than and the other less than 50 percent, after 96 h. The highest test concentration should not exceed the chemical's aqueous solubility limit if the chemical is not a surfactant or the chemical's self-dispersibility limit if the chemical is a surfactant or a charged polymer.

(B) Exceptions may be required in testing certain pesticide AIs as products. Product formulations may increase the solubility of the AI beyond its aqueous solubility limit.

(v) Concentration analysis. (A) Concentration analysis must be performed at initiation and every 48 h of the study thereafter.

(B) In static tests, the test substance concentration should be measured in each replicate minimally at the beginning (0–hour, before test organisms are added), at 48 h, and at the end of the test.

(C) In static-renewal tests, the test substance should be measured in each replicate at the beginning and end of test and just before and after each renewal.

(D) In flow-through tests, the test substance should be measured as follows:

(*1*) In each replicate at 0, 48, and 96 h, and every 96 h thereafter, as long as the test is continued.

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

(3) **Collection of samples for measurement.** (i) Water samples must be removed from a central point within the test vessel, not from inflow or outflow points.

(ii) These samples should not contain any surface particulates or material dislodged from the bottom or sides. Samples should be analyzed immediately, or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(iii) The test solution volume should not be reduced during the test by more than 10 percent as a result of sampling.

(iv) Samples from each test concentration replicate should not be pooled for analyses.

(v) Diluter systems must be monitored for proper adjustment, and operation every 24 h, and should be monitored during the first hour of operation.

(vi) Surface films and precipitates must be reported should they occur.

(vii) The flow rate to each replicate should be measured at the beginning and end of each test.

(viii) During a test, the flow rates should not vary more than a factor of 10 from any one replicate to another.

(ix) Minimum number of test vessel replacements should be 6 to 10 per 24–h period for flow-through testing.

(4) **Observations.** (i) Mortality observations should be recorded at 6, 24, 48, 72, and 96 h.

(ii) If the test is continued past 96 h, additional observations should be made every 24 h until termination.

(iii) In addition to mortality, any abnormal behavior should be recorded, such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, and changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging.

(g) **Data and reporting—(1) Treatment of results.** The cumulative percentage mortality for each exposure period is plotted against concentration on logarithmic paper. Normal statistical procedures are then employed to calculate the LC50 for the appropriate exposure period. Confidence limits (CI) with p = 0.95 for the calculated LC50 values are to be included.

(2) **Test report.** (i) The test report must include the following:

(ii) Test facilities, test dates, and personnel must be reported.

(iii) Identification of the test substance and purity.

(iv) Water quality characteristics as reported in the laboratory records for the study. These must include 24-h records of DO, pH, and temperature.

(v) Methods of stock solution preparation and the concentrations used in definitive testing.

(vi) All test concentrations measured during the test and at termination.

(vii) The number of test organisms in each replicate and/or test concentration.

(viii) The LC50 concentration-response curves, LC50 values, and associated 95 percent CI should be determined for 24, 48, 72 and 96 h, whenever sufficient data exist.

(ix) A graph of the concentration-mortality curve at test termination. Any control mortality observed during the acclimation or study period.

(x) An NOEL for the 96–h test should also be reported.

(xi) If no LC50 value is determined, but it can be demonstrated that the concentrations tested were the highest possible due to the test chemical's aqueous solubility limit, self-dispersibility limit, or other physicochemical limitations, then the data will be considered for acceptance. Explanation should include details of the solvents which were tried prior to initiation of the final study.

(xii) Any abnormal behavior displayed by the test fish.

(xiii) Any protocol deviations or occurrences which may have influenced the final results of the test.

(xiv) A quality control methods and quality assurance statement should accompany all final study reports.

(xv) Raw data must be available to support study author's conclusions and should be presented with the study report.

(xvi) Methods of statistical analysis should be reported.

(xvii) Methods used in analysis of test concentrations of the test chemical should be described. The accuracy of the method (i.e. detection limit and quantification limit) should be given.

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians, E 729–88a. American Society Testing Materials, Philadelphia, PA. Approved Nov. 21, 1988.

(2) Organization of Economic Cooperation and Development, Guidelines for Testing of Chemicals, Guideline 203 "Fish Acute Toxicity Test." Adopted July 17, 1992.

(3) Test Guideline EG–9, Fish Acute Toxicity Test, Office of Pollution Prevention and Toxics, Office of Prevention, Pesticides and Toxic Substances, U.S. Environmental Protection Agency, Washington DC.

(4) Standard Evaluation Procedure Acute Toxicity Test for Freshwater Fish, EPA–540/9–85–006, Office of Pesticide Programs, Office of Prevention Pesticides and Toxic Substances, U.S. Environmental Protection Agency, Washington DC. Revised June 1985.

(5) Acute Toxicity Test for Estuarine and Marine Organisms (Estuarine Fish 96–Hour Acute Toxicity Test), EPA 540/9–85–009, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, U.S. Environmental Protection Agency, Washington DC. Revised June 1985.

(6) Federal Insecticide, Fungicide, Rodenticide Act, Subdivision E, Hazard Evaluation, Wildlife and Aquatic Organisms, U.S. Environmental Protection Agency. October 1982.

(7) Finney, D.J., Probit Analysis. 3rd Edition. Cambridge University Press: London and New York (1971).

(8) Stephen, C.E., "Methods for Calculating an LC50" Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, American Society of Testing and Materials, Philadelphia, PA (1977).

(9) Canada, Environment Canada. Biological test method: acute lethality test using threespine stickleback (*Gasterosteus aculeatus*). Environmental Protection, Conservation and Protection, Environment Canada, Report EPS 1/RM/10 (1990).

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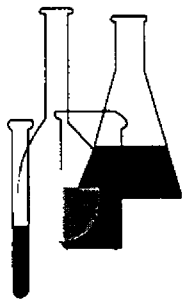
Ecological Effects Test Guidelines

OPPTS 850.1085
Fish Acute Toxicity
Mitigated by Humic Acid



"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 850.1085 Fish acute toxicity mitigated by humic acid.

(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 is 40 CFR 797.1460 Fish Acute Toxicity Mitigated by Humic Acid.

(b) **Purpose.** This guideline may be used to develop data on the acute toxicity of chemical substances and mixtures under static or static renewal conditions, subject to environmental effects testing. This guideline prescribes procedures to be used to develop data on the acute toxicity of chemicals to fish with and without the presence of naturally occurring dissolved organic substances (.e.g., humic acids and their salts). EPA will use data from these tests in assessing the hazard of a chemical to the environment. For additional background information on this test guideline see OPPTS 850.1075.

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

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

Acute toxicity test means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g. 96 h). In this guideline, death is used as the measure of toxicity under static or static renewal conditions only.

Carrier means a solvent used to dissolve a test substance prior to delivery to the test chamber.

Death means the lack of opercular movement by a test fish.

Dissolved organic carbon (DOC) means various organic molecules occurring in lotic and lentic ecosystems, which in this test are restricted to a heterogeneous group of humic substances.

Total organic carbon (TOC) means the sum of all organic carbon molecules, which are dissolved, particulate, and suspended, occurring in test dilution waters.

Humic substances means humic acids (HAs), fulvic acids, and humin fractions, and their various salts, resulting from chemical fractionation of this heterogeneous naturally-occurring organic substance. For purposes of

this test, HA, sodium salt (e.g. Aldrich Catalog No. H1,675–2; mention of a commercial company or product does not constitute approval or endorsement by the Agency) may be used as the source of DOC.

LC50 means that the test substance concentration calculated from experimentally-derived mortality data is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

Loading means the ratio of fish biomass (in grams, wet weight) to the volume (in liters) of test solution in a test chamber or passing through it in a 24–h period.

Static means the test solution is not renewed during the period of the test.

Test solution means the dilution water containing the dissolved test substance to which test organisms are exposed.

(d) **Test procedures**—(1) **Summary of the test.** (i) This test is designed to determine the acute effects of the test substance on one of three species of fish with HA. Test chambers are filled with appropriate volumes of dilution water.

(ii) The test substance is introduced into each test chamber. Some test chambers contain only dilution water; other contain a concentration of spiked HA.

(iii) Test fish which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Fish in the test and control chambers are observed periodically during the test; dead fish are removed at least twice each day and the findings are recorded.

(v) The dissolved oxygen (DO) concentration, pH, and temperature are measured at intervals in selected test chambers.

(vi) A concentration-response curve and LC50 value for the test substance in dilution water spiked with a known amount of HA are developed from the mortality data collected during the test.

(2) **Range-finding test.** (i) If the toxicity of the test substance in HA is not already known, a range-finding test should be performed to determine the range of concentrations to be used in the definitive test. The highest concentration of test substance for use in the range-finding test should not exceed its solubility in water or the permissible amount of carrier used.

(ii) Initially, two fish test is performed at 20 mg/L of HA. In some cases, the 20 mg HA/L concentration may be so high that no toxicity will

be present due to the formation of viscous, colloidal complexes. If this occurs, the 20 mg HA/L concentration should be decreased to 15 mg/L, or an appropriately lower concentration.

(3) **Definitive test.** (i) A minimum of 20 fish should be exposed to each of five or more test substance concentrations in dilution water spiked with a known amount of HA. The range of test substance concentrations to which the fish are exposed should be such that in 96 h there are at least two partial mortality exposures bracketing 50 percent survival.

(ii) For exposure to each concentration of a test substance in dilution water spiked with a known amount of HA, an equal number of test fish should be placed in two or more replicate test chambers. Test fish should be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions.

(iii) Every test should include a control consisting of the same dilution water, conditions, procedures, and fish from the same group used in the test, except that none of the test substance is added. Every test should also include negative controls consisting of dilution water with HA alone.

(iv) Mortality data collected during the test are used to calculate a 96–h LC50 value. The 24–, 48–, and 72–h values should be calculated whenever there is sufficient mortality data to determine such values.

(v) Test fish should not be fed while they are being exposed to the test substance under static conditions.

(4) **Test results**. (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substances on the presence of a known amount of HA.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance of physiology, such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging should be recorded.

(iii) Observations on compound solubility and/or dispersibility should be recorded. The investigator should report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chamber.

(iv) Each test and control chamber should be checked for dead fish and observations recorded at 24, 48, 72, and 96 h after the beginning of the test or within 1 h of the designated times. If the test is continued past 96 h, additional observations should be made every 24 h until termination.

(v) The mortality data are used to calculate LC50 values and their 95 percent confidence limits, and to plot concentration-response curves

for each time interval whenever sufficient data exists. The methods recommended for use in calculating LC50 values include probit, logit, binomial, and moving average angle.

(vi) A test is be unacceptable if more than 10 percent of the control fish die or exhibit abnormal behavior during a 96–h test.

(5) **Analytical measurements**—(i) **Water quality analysis.** (A) The hardness, acidity, alkalinity, pH, conductivity, TOC, or chemical oxygen demand (COD), and total suspended solids (TSS) of the dilution water should be measured at the beginning of each static test. The month-to-month variation of the above values should be less than 10 percent and the pH should vary less than 0.4 units.

(B) During static tests, the DO concentration, temperature, and pH should be measured in each test chamber at the beginning and end of the test. The test solution volume should not be reduced by more than 10 percent as a result of these measurements.

(ii) **Dissolved organic carbon.** The naturally-occurring DOC selected for this test should be HA, which is available from the Aldrich catalog, (No. H1,675–2).

(iii) **Collection of samples for measurement of TOC.** Samples to be analyzed for TOC should be taken from the control chambers midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides.

(iv) **Measurement of TOC.** (A) For static tests, DOC should be measured (as TOC) at a minimum in each test chamber at the beginning (time 0, before fish are added) of the test. Three TOC measurements should be made and the average reported.

(B) The analytical methods used to measure the TOC in a sample should be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions. This involves adding a known amount of the dissolved organic carbon source to three water samples taken from a chamber containing dilution water to be used in the test. The normal concentration of dissolved organic carbon in those samples should span the TOC concentration range to be used in the test.

(C) The nominal concentration of test substance based on 100 percent active ingredient (AI) should be used to calculate all LC50 values and to plot all concentration-response curves.

(e) **Test conditions**—(1) **Test species**— (i) **Selection**. The test species for this test are the rainbow trout (*Oncorhynchus mykiss = Salmo*

gairdneri), bluegill (*Lepomis macrochirus*), and fathead minnow (*Pimephales promelas*).

(ii) **Age and condition of fish.** Juvenile fish should be used. Fish used in a particular test should be the same age and be of normal size and appearance for their age. The longest fish should not be more than twice the length of the shortest. All newly acquired fish should be quarantine and observed for at least 14 days prior to use in a test. Fish should not be used for a test if they appear stressed or if more than 5 percent die during the 48 h immediately prior to the test.

(iii) **Acclimation of test fish**. If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48–h period. The fish should be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3 °C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing. During the final 48–h of acclimation, fish should be maintained in facilities with background colors and light intensities similar to those of the testing area and should not be fed.

(2) **Facilities**—(i) **General**. Facilities needed to perform this test include:

(A) Flow-through tanks for holding and acclimating fish.

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation, and test periods.

(C) Apparatus for straining particulate matter, removing gas bubbles, or insufficient dissolved oxygen, respectively.

(D) Apparatus for providing a 16–h light and 8–h dark photoperiod with a 15– to 30–min transition period.

(E) Chambers for exposing test fish to the test substance.

(ii) **Construction materials**. Construction materials and commercially purchased equipment that may contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g. PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be thoroughly conditioned before use. If cast iron pipe is used in freshwater supply systems, colloidal iron may leach into the dilution water and strainers or filters should be used to remove rust particles. Rubber, copper, brass, galvanized

metal, epoxy glues, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) **Test chambers**. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(iv) **Cleaning of test system**. Test chambers should be cleaned before each test. They should be washed with detergent and rinsed in sequence with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(v) **Dilution water.** (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if the the test fish will survive in it for the duration of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration, hemorrhaging, disorientation, or other unusual behavior. The quality of the clean dilution water (without spiked HA) should be constant and should meet the specifications in the following Table 1., measured at least twice a year:

Table 1.—Specifications for Dilution Water

Substance	Maximum Concentration
Total suspended solids	20 mg/L
Total organic carbon (TOC), or chemical oxygen demand (COD).	2 mg/L, or 5 mg/L, respectively
Un-ionized ammonia	20 µg/L
Residual chlorine	1 µg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs),or organic chlorine.	50 ng/L, or 25 ng/L, respectively
Hardness (as $CaCO_3$ during testing)	180 mg/L

The quality of the dilution water after spiking with HA should meet all the previous specifications except for TOC or COD.

(B) The DO concentration in the dilution water should be between 90 and 100 percent saturation; 9.8 to 10.9 mg/L for tests with trout, and 8.0 to 8.9 mg/L for tests with bluegill or fathead minnow at sea level. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use. Buffered soft water should be aerated before but not after the addition of buffers.

(C) Diseased organisms present in the dilution water in sufficient number to cause infection of the fish should be killed or removed by suitable equipment.

(D) Glass-distilled or carbon-filtered deionized water with a conductivity less than 1 μS/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity and TOC should be measured on each batch.

(vi) **Carriers.** Only distilled water should be used in making stock solutions of the test substance. However, if the stock volume is more than 10 percent of the test solution volume, dilution water should be used. Carbon-based carriers cannot be used in this test. If necessary, stock solution pH should be adjusted to pH 7.

(3) **Test parameters**—(i) **Loading.** The number of fish placed in a test chamber should not be so great as to affect the results of the test. The loading should not be so great that the test substance concentrations are decreased by more than 20 percent due to uptake by the fish. Loading should not exceed 0.5 g of fish/L of solution in the test chamber at any one time. These loading rates should be sufficient to maintain the DO concentration above the recommended levels and the ammonia concentration below 20 μg/L.

(ii) **Dissolved oxygen concentration.** During static tests with rainbow trout, the DO should be maintained above 5.5 mg/L in each test chamber. In tests with bluegill and fathead minnow, the DO should be greater than 4.5 mg/L in each test chamber.

(iii) **Temperature.** The test temperature should be 22 °C for bluegill and fathead minnow, and 12 °C for rainbow trout. Deviations from the test temperature should be no greater than ±2 °C. The temperature should be measured at least hourly in one test chamber.

(iv) **Light.** A 16–h light and 8–h dark photoperiod should be maintained.

(f) **Reporting**. The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition to the reporting requirements prescribed in 40 CFR Part 792—Good Laboratory Practice Standards, the reported test data should include the following:

(1) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC, COD, and total suspended solids.

(2) The source of the HA (e.g., batch number), as well as a complete description and chemical characterization.

(3) A description of the test chambers, the depth and volume of solution in the chamber, and the specific way the test was begun (e.g., conditioning and test substance additions).

(4) Detailed information about the test fish, including the scientific name and method of verification, average weight (grams, wet weight), standard length, age, source, history, observed diseases, treatments and mortalities, acclimation procedures, and food use.

(5) The number of replicates used, the number of organisms per replicate, and the loading rate.

(6) The measured DO, pH, and temperature and the lighting regime.

(7) A description of preparation of the stock solution. If the pH of the stock solution was adjusted, describe the adjustment.

(8) The concentrations of the dissolved organic carbon as TOC from the HA control just before the start of the test, all triplicate measurements, and average TOC values.

(9) Results from any range-finding tests performed at 20 mg/L of HA.

(10) The number of dead and live tests organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(11) The 96–h LC50, and when sufficient data have been generated, the 24–, 48–, 72–h LC50 values, their 95 percent confidence limits, and the methods used to calculate the LC50 values and their confidence limits.

(12) When observed, the no-observed-effect-concentration (the highest concentration tested at which there were no mortalities, abnormal behavioral, or physiological effects) in treatments.

(13) The concentration-response curve at each observation period for which LC50 values are calculated.

(14) Methods and data records of all chemical analyses of water quality parameters, TOC, including method validations and reagent blanks.

United States
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Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–120
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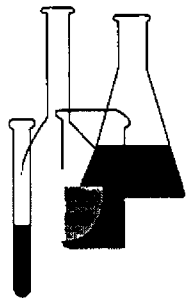
EPA

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.*).

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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.

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Prevention, Pesticides
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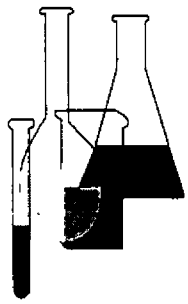
Ecological Effects Test Guidelines

OPPTS 850.1350
Mysid Chronic Toxicity Test



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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 determined 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 μm 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 °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× 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 °C. Excursions from the test temperature should be no greater than ±2 °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).

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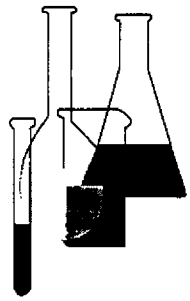
Ecological Effects Test Guidelines

OPPTS 850.1400
Fish Early-Life Stage 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 850.1400 Fish early-life stage 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 797.1600 Fish Early Life Stage 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 210 Fish Early-Life Stage Toxicity Test.

(b) **Introduction.** (1) Tests with the early-life stages of fish are intended to define the lethal and sublethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sublethal effects of the substance on other fish species.

(2) This guideline is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.

(c) **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.

LOEC (Lowest-observed-effect-concentration) is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

NOEC (No-observed-effect-concentration) is the test concentration immediately below the LOEC.

(d) **Principle of the test.** The early-life stages of fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions, or where appropriate, semistatic conditions. The test is begun by placing fertilized eggs in the test chambers and is continued at least until all the control fish are free-feeding. Lethal and sublethal effects are assessed and compared with control values to determine the LOEC and the NOEC.

(e) **Information on the test substance.** (1) Results of an acute toxicity test (see OPPTS 850.1075), preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapor pressure of the test substance are known and a reliable

analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

(2) Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow}, and results of a test for ready biodegradability. (See OPPTS 835.3110 through 835.3160.)

(f) **Validity of the test.** For a test to be valid the following conditions apply:

(1) The dissolved oxygen concentration must be between 60 and 100 percent of the air saturation value throughout the test.

(2) The water temperature must not differ by more than ± 1.5 °C between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (Tables 4. and 5. under paragraphs (h)(1)(ii) and (h)(1)(iii) of this guideline).

(3) Evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ± 20 percent of the mean measured values.

(4) Overall survival of fertilized eggs in the controls and, where relevant, in the solvent-only controls must be greater than or equal to the limits defined in Tables 4. and 5. under paragraphs (h)(1)(ii) and (h)(1)(iii) of this guideline.

(5) When a solubilizing agent is used it must have no significant effect on survival nor produce any other adverse effects on the early-life stages as revealed by a solvent-only control.

(g) **Description of the method**—(1) **Test chambers.** Any glass, stainless steel, or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomized block design with each treatment being present in each block is preferable to a completely randomized design. The test chambers should be shielded from unwanted disturbance.

(2) **Selection of species.** (i) Recommended fish species are:

(A) Freshwater—rainbow trout, *Oncorhynchus mykiss*; fathead minnow, *Pimephales promelas*; zebra fish, *Danio rerio*; ricefish, *Oryzias latipes*.

(B) Salt water—sheepshead minnow, *Cyprinodon variegatus*.

(ii) The Office of Prevention, Pesticides and Toxic Substances prefers rainbow trout (*O. mykiss*) or fathead minnow (*P. promelas*) as test species for freshwater fish and sheepshead minnow (*C. variegatus*) or silverside

(*Menidia menidia*, *M. beryllina*, or *M. peninsulae*) as test spcies for estuarine or marine fish. This does not preclude the use of other species. Examples of other well-documented species which have also been used are:

(A) Freshwater—coho salmon, *Oncorhynchus kisutch*; chinook salmon, *Oncorhynchus tschawytscha*; brown trout, *Salmo trutta*; Atlantic salmon, *Salmo salar*; brook trout, *Salvelinus fontinalis*, lake trout, *Salvelinus namaycush*; northern pike, *Esox lucius*; white sucker, *Catostomus commersoni*; bluegill, *Lepomis macrochirus*; channel catfish, *Ictalurus punctatus*; flagfish, *Jordanella floridae*; three-spined stickleback, *Gasterosteus aculeatus*; common carp, *Cyprinus carpio*.

(B) Salt water—Atlantic silverside, *M. menidia*; Tidewater silverside, *M. peninsulae*.

(iii) Feeding and handling requirements of brood and test animals, test conditions, duration, and survival criteria for these species can be found in Tables 1. and 2. under paragraph (g)(3) of this guideline, and Tables 4. and 5. under paragraphs (h)(1)(i) and (h)(1)(ii) of this guideline.

(iv) The test procedure might have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

(3) **Holding of the brood fish.** (i) Details on holding the brood stock under satisfactory conditions may be found in the references cited under paragraphs (j)(1), (j)(2), and (j)(3) of this guideline.

(ii) Conditions for recommended species are provided in the following Table 1. Abbreviations in the table have the following meanings: BW = body weight; FBS = frozen brine shrimp—adults *Artemia* sp.; BSN = brine shrimp nauplii—newly hatched; BSN48 = brine shrimp nauplii—48 h old.

Table 1.—Feeding and Handling Requirements of Brood and Test Animals of Recommended Species

Species	Food					Post-Hatch Transfer Time[1]	Time to First Feeding
	Brood Fish	Newly-Hatched Larvae	Juveniles				
			Type	Amount	Frequency		
Freshwater							
Oncorhynchus mykiss, rainbow trout	trout food	none [2]	trout starter	4% BW per day	2–4 feeds/ day	14–16 days post-hatch or at swim-up[3]	19 days post-hatch or at swim-up
Pimephales promelas, fathead minnow	FBS	BSN	BSN48		ad libitum	once hatching is 90%	within 2 days of hatching
Danio rerio, Zebra fish	BSN48, flake food	protozoa [4] protein [5]	BSN48			not necessary	6–7 days after spawning
Oryzias latipes, ricefish	flake food	BSN, flake food (or protozoa or rotifers)	BSN48, flake food (or rotifers)		BSN once daily; flake food twice daily *or* flake food and rotifers once daily	from hatch to swim-up	within 24 h of hatch/swim-up
Saltwater							
Cyprinodon variegatus, Sheepshead minnow	FBS or flake food	BSN	BSN48		2–3 feeds per day	not applicable	within 1 day first hatch

[1] if applicable
[2] yolk-sac larvae require no food
[3] not essential
[4] filtered from mixed culture
[5] granules from fermentation process

(iii) Conditions for other species are provided in the following Table 2.:Abbreviations in the table have the following meanings: BSN = brine shrimp nauplii, newly hatched; BSN48 = brine shrimp nauplii, 48 hours old; FBS = frozen brine shrimp; adult *Artemia* sp.

Table 2.—Feeding and Handling Requirements of Brood and Test Animals of Other Well-Documented Species

Species	Food					Post-Hatch Transfer Time (if applicable)	Time To First Feeding
	Brood fish	Newly-hatched larvae	Juveniles				
			Type	Amount	Frequency		
Freshwater							
Oncorhynchus kisutch, Coho salmon	trout food	none [1]	trout starter	4% BW per day	2–4 feeds/ day	26–36 days post-hatch or at swim-up	after swim-up at transfer
Oncorhynchus tschawytscha, Chinook salmon	trout food	none	trout starter	4% BW per day	2–4 feeds/ day	26–36 days post-hatch or at swim-up	26 days post-hatch at swim-up
Salmo trutta, brown trout	trout food	none	trout starter	4% BW per day	5 feeds/day	21 days post-hatch or at swim-up	at swim-up
Salmo salar Atlantic salmon	trout food	none	trout starter	4% BW per day	5 feeds/day	21 days post-hatch or at swim-up	at swim-up
Salvelinus fontinalis, brook trout	trout food	none	trout starter	4% BW per day	5 feeds/day	21 days post-hatch or at swim-up	at swim-up

Table 2.—Feeding and Handling Requirements of Brood and Test Animals of Other Well-Documented Species—Continued

Species	Food					Post-Hatch Transfer Time (if applicable)	Time To First Feeding
	Brood fish	Newly-hatched larvae	Juveniles				
			Type	Amount	Frequency		
Salvelinus namaycush, lake trout	trout food	none	trout starter	4% BW per day	5 feeds/day	21 days post-hatch or at swim-up	at swim-up
Esox lucius, Northern pike	live minnows	BSN48	larval fish			transfer hatched fish daily	1 week post-hatch or swimming yolk-sac stage
Catostomus commersoni, white sucker	FBS	none	BSN48		3 feeds/day	once all embryos have hatched	7–8 days post-hatch or at swim-up
Lepomis macrochirus, bluegill	FBS, trout food	BSN	BSN48		3 feeds per day		at swim-up
Ictalurus punctatus, channel catfish	catfish food	modified Oregon	modified Oregon		at least 3 feeds per day	6–7 days at 26 °C [2]	within 48 h of swim-up
Jordanella floridae, flagfish	FBS, flake food, BSN	BSN48, flake food, or protozoa/rotifers[3]	BSN48, flake food		*Artemia nauplii* once daily; flake food twice daily *or* flake food and protozoa & rotifers once daily	from hatch to swim-up	within 24 h of hatch
Gasterosteus aculeatus, three-spined stickleback	Tetramin FBS	*Brachionus rubens* (rotifer)	BSN48, Tetramin		BSN48, 2–3 feeds per day; Tetramin once daily	several hours after hatch[2]	within 24 hours of hatch
Cyprinus carpio, common carp	Proprietary carp food; freeze-dried tubifex or trout food	BSN	BSN48, ground; trout starter, or flake food		3–4 feeds per day	once hatching complete	36–48 h post-hatch
Saltwater							
Menidia menidia, Atlantic silverside	BSN48, flake food	days 1–8	rotifers[3]		3 feeds per day	not applicable	within 24 h of first hatch
		days 9–11	BSN48 and rotifers[3]		2 feeds per day	not applicable	within 24 h of first hatch
		days 11–end	BSN48			not applicable	within 24 h of first hatch
Menidia peninsulae, Tidewater silverside	BSN48, flake food	days 1–8	rotifers[3]		3 feeds per day	not applicable	within 24 h of first hatch
		days 9–11	BSN48 and rotifers[3]		2 feeds per day	not applicable	within 24 h of first hatch
		days 11–end	BSN48			not applicable	within 24 h of first hatch

[1] yolk-sac larvae require no food
[2] fish may be handled with a 6 mm internal diameter glass siphon tube
[3] rotifers—*Brachionus plicatilis*

(4) **Handling of embryos and larvae.** (i) Initially, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Nonturbulent flow through these small vessels may

be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged. Fertilized eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching.

(ii) Where egg containers, grids, or mesh have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch, according to the advice in Table 1. except that mesh should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air, and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.

(5) **Water.** Any water in which the test species shows control survival, at least as good as that described in Table 4. under paragraph (h)(1)(ii) of this guideline, and Table 5. under paragraph (h)(1)(iii) of this guideline, is suitable as a test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of test substance) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, sulfate), pesticides, total organic carbon, and suspended solids should be made, for example, every 3 months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in the following Table 3:

Table 3.—Some Chemical Characteristics of an Acceptable Dilution Water

Substance	Maximum Concentration
Particulate matter	< 20 mg/L
Total organic carbon	< 2 mg/L
Un-ionized ammonia	< 1 µg/L
Residual chlorine	< 10 µg/L
Total organophosphorus pesticides	< 50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/L
Total organic chlorine	< 25 ng/L

(6) **Test solutions.** (i) For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10 percent throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 h has been found suitable (see paragraph (j)(1) of this guideline).

(ii) The use of solvents or dispersants (solubilizing agents) may be required in some cases in order to produce a suitably concentrated stock solution.

(iii) For the semistatic technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels while a proportion (at least two-thirds) of the test water is changed.

(h) **Procedure.** Useful information on the performance of fish early-life stage tests is available in the literature, some examples of which are included under paragraphs (j)(1), and (j)(4) through (j)(8) of this guideline.

(1) **Conditions of exposure**—(i) **Duration.** The test should start as soon as possible after the eggs have been fertilized, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test should continue at least until all the control fish have been free-feeding. Test duration will depend upon the species used.

(A) Data for recommended species are provided in the following Table 4.:

Table 4.—Test Conditions, Duration, and Survival Criteria for Recommended Species

Species	Test Conditions		Recommended Duration of Test	Survival of Controls (minimum percent)	
	Temperature (°C)	Photoperiod (hours)		Hatching Success	Post-Hatch Success
Freshwater					
Oncorhynchus mykiss, Rainbow trout.	10±2 [1] 12±2 [2 3]	14 [4]	2 weeks after controls are free-feeding (or 60 days post-hatch)	>66	70
Pimephales promelas, Fathead minnow.	25±2	16	32 days from start of test (or 28 days post-hatch)	>66	70
Danio rerio, Zebra fish	25±2	12–16 [6]	30 days post-hatch		70
Oryzias latipes, ricefish	24±1 (a) 23±2 [2 5]	12–16 [6]	30 days post-hatch		80
Saltwater					
Cyprinodon variegatus, Sheepshead minnow [7].	25±2	12–16 [6]	32 days from start of test (or 28 days post-hatch)	>75	80

[1]for embryos
[2]for larvae and juvenile fish
[3]the particular strain of rainbow trout tested may necessitate the use of other temperatures; brood stock must be held at the same temperature as that to be used for the eggs
[4]darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12–16 h photoperiod (6))
[5]this supersedes the requirement for temperature control given earlier on in the test
[6]for any given test conditions, light regime should be constant
[7]salinity shall be at 15–30; for any given test this shall be performed to ±2 percent.

(B) Data for other species are provided in the following Table 5:

Table 5.—Test Conditions, Duration and Survival Criteria for Other Well-Documented Species

Species	Test Conditions		Recommended Duration of Test	Survival of Controls (minimum percent)	
	Temperature (°C)	Photoperiod (hours)		Hatching Success	Post-Hatch Success
Freshwater					
Oncorhynchus kisutch, Coho salmon.	10[1], 12[2]	12–16[3]	60 days post-hatch	>66	70
Oncorhynchus tschawytscha, Chinook salmon.	10[1], 12[2]	12–16[3]	60 days post-hatch	>66	70
Salmon trutta, brown trout	10	12–16[3]	60 days post-hatch	>66	70
Salmo salar, Atlantic salmon	10	12–16[3]	60 days post-hatch	>66	70
Salvelinus fontinalis, brook trout .	10	14[3]	60 days post-hatch	>66	70
Salvelinus namaycush, Lake trout	12–18	16	60 days post-hatch	>66	70
Esox lucius, Northern pike	7	12–16[3]	32 days from start of test	>66	70
Catostomus commersoni, White sucker.	15	16	32 days from start of test	>66	80
Lepomis macrochirus, Bluegill	28	16	32 days from start of test		75
Ictalurus punctatus, Channel catfish.	26	16	32 days from start of test		65 (overall)
Jordanella floridae, Flagfish	24–26	16			
Gasterosteus aculeatus, Three-spined stickleback.	18–20	12–16	28 days	80	80
Cyprinus carpio, common carp ...	21–25	12–16	28 days post-hatch	>80	75
Saltwater					
Menidia menidia, Atlantic silverside[4].	22–25	13	28 days	>80	60
Menidia peninsulae, Tidewater silverside[4].	22–25	13	28 days	>80	60

[1]for embryos
[2]for larvae and juvenile fish
[3]darkness for larvae until 1 week after hatching except when they are being inspected, then subdued lighting throughout test (12–16 h photoperiod unless otherwise specified, but constant regime for a given test)
[4]salinity 20

(ii) **Loading.** The number of fertilized eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 60 eggs, divided equally between at least two replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60 percent of the air saturation value (ASV) can be maintained without aeration. For flow-through tests, a loading rate not exceeding 0.5 g/L/24 h and not exceeding 5 g/L of solution at any time has been recommended (see paragraph (j)(1) of this guideline).

(iii) **Light and temperature.** The photoperiod and water temperature should be appropriate for the test species as given in Table 4. under paragraph (h)(1)(ii) of this guideline.

(iv) **Feeding.** Food and feeding are critical, and it is essential that the correct food for each stage should be supplied at an appropriate time and at a level sufficient to support normal growth. Feeding should be ad libitum while minimizing the surplus. Surplus food and feces should be removed as necessary to avoid accumulation of waste. Detailed feeding regimes are given in Table 1. under paragraph (g)(3)(ii) of this guideline,

but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimize growth. Effort should therefore be made to confirm the proposed regime with acknowledged experts.

(v) **Test concentrations.** (A) Normally five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC50 to period of exposure in the acute study should be considered when selecting the range of test concentrations. The use of fewer than five concentrations, for example in limit tests, and a narrower concentration interval may be appropriate in some circumstances. Justification should be provided if fewer than five concentrations are used. Concentrations of the substance higher than the 96–h LC50 or 10 mg/L, whichever is the lower, need not be tested.

(B) Where a solubilizing agent is used, its concentration should not be greater than 0.1 mL/L and should be the same in all test vessels. However, every effort should be made to avoid the use of such materials.

(vi) **Controls.** One dilution-water control and also, if relevant, one control containing the solubilizing agent should be run in addition to the test series.

(2) **Frequency of analytical determinations and measurements.** (i) During the test, the concentrations of the test substance are determined at regular intervals to check compliance with the validity criteria. A minimum of five determinations is necessary. In studies lasting more than 1 month, determinations should be made at least once a week. Samples may need to be filtered (e.g. using a 0.45 μm pore size) or centrifuged to ensure that the determinations are made on the substance in true solution.

(ii) During the test, dissolved oxygen, pH, total hardness and salinity (if relevant), and temperature should be measured in all test vessels. As a minimum, dissolved oxygen, salinity (if relevant), and temperature should be measured weekly, and pH and hardness should be measured at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel.

(3) **Observations**—(i) **Stage of embryonic development**. The embryonic stage at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared.

(ii) **Hatching and survival.** Observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae, and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals

not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

(A) For eggs: Particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance.

(B) For embryos: Absence of body movement and/or absence of heart-beat.

(C) For larvae and juvenile fish: Immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

(iii) **Abnormal appearance.** The number of larvae or fish showing abnormality of body form should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several percent in the controls in some species. Abnormal animals should only be removed from the test vessels on death.

(iv) **Abnormal behavior.** Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence, and atypical feeding behavior should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data and influence a decision to extend the exposure period beyond the recommended duration.

(v) **Weight.** At the end of the test all surviving fish must be weighed. Individual weights are preferred but, if the fish are especially small, they may be weighed in groups by test vessel. Dry weights (24 h at 60 °C) are preferable to wet weights (blotted dry).

(vi) **Length**. At the end of the test, measurement of individual lengths is recommended: Standard, fork, or total length may be used. If however, caudal fin rot or fin erosion occurs, standard lengths should be used.

(vii) **Data for statistical analysis.** These observations will result in some or all of the following data being available for statistical analysis:

(A) Cumulative mortality.

(B) Numbers of healthy fish at end of test.

(C) Time to start of hatching and end of hatching.

(D) Numbers of larvae hatching each day.

(E) Length and weight of surviving animals.

(F) Numbers of deformed larvae.

(G) Numbers of fish exhibiting abnormal behavior.

(i) **Data and reporting**—(1) **Treatment of results.** (i) It is recommended that a statistician be involved in both the design and analysis of the test results since this test guideline allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilized eggs, and number of parameters measured.

(ii) In view of the options available in test design, specific guidance on statistical procedures is not given here. However, it will be necessary for variations to be analyzed within each set of replicates using analysis of variance or contingency table procedures. To make a multiple comparison between the results at the individual concentrations and those for the controls, Dunnett's method might be found useful (see paragraphs (j)(9) and (j)(10) of this guideline). However, care must be taken where applying such a method to ensure that chamber-to-chamber variability is estimated and is acceptably low. Other useful methods are also available (see paragraphs (j)(1), (j)(6), and (j)(11) of this guideline).

(2) **Interpretation of results.** The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.

(3) **Test report.** The test report must include the following information:

(i) **Test substance.** (A) Physical nature and, where relevant, physicochemical properties.

(B) Chemical identification data.

(ii) **Test species.** Scientific name, strain, source and method of collection of the fertilized eggs, and subsequent handling.

(iii) **Test conditions.** (A) Test procedure used (e.g. semistatic or flow-through design).

(B) Photoperiods.

(C) Test design (e.g. number of test chambers and replicates, number of embryos per replicate).

(D) Method of preparation of stock solutions and frequency of renewal (the solubilizing agent and its concentration must be given, when used).

(E) Nominal test concentrations, means of the measured values, their standard deviations in the test vessels, and the method by which these

were attained, and evidence that measurements refer to concentrations of the test substance in true solution.

(F) Dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured), and any other measurements made.

(G) Water quality within test vessels: pH, hardness, temperature, and dissolved oxygen concentration.

(H) Detailed information on feeding (e.g. type of feed, source, amount given, and frequency).

(iv) **Results.** (A) Evidence that controls met the overall survival acceptability standard of the test species (Tables 4. and 5.).

(B) Data on mortality/survival at embryo, larval, and juvenile stages and overall mortality/survival.

(C) Days to hatch and numbers hatched.

(D) Data for length and weight.

(E) Incidence and description of morphological abnormalities, if any.

(F) Incidence and description of behavioral effects, if any.

(G) Statistical analysis and treatment of data.

(H) NOEC for each response assessed.

(I) LOEC (at $p = 0.05$) for each response assessed.

(J) Any concentration-response data and curves available.

(v) **Discussion of the results.** [Reserved]

(j) **References.** The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials (ASTM). Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. ASTM E 1241–92, p. 180–207, Philadelphia, PA (1992).

(2) Brauhn, J.L. and Schoettger, R.A., Acquisition and Culture of Research Fish: Rainbow trout, Fathead minnows, Channel catfish and Bluegills. p. 54, Ecological Research Series, EPA–660/3–75–011, Duluth, MN (1975).

(3) Brungs, W.A. and Jones, B.R., Temperature Criteria for Freshwater Fish: Protocol and Procedures. p. 128, Ecological Research Series EPA–600/3–77–061, Duluth, MN (1977).

(4) Hansen, D.J. and Parrish, P.R., Suitability of sheepshead minnows (*Cyprinodon variegatus*) for life-cycle toxicity tests. In *Aquatic Toxicology and Hazard Evaluation* (edited by F.L. Mayer and J.L. Hamelink), pp. 117–126, ASTM STP 634 (1977).

(5) McKim, J.M. et al., Metal toxicity to embryos and larvae of eight species of freshwater fish-II: Copper. *Bulletin of Environmental and Contamination Toxicology* 19:608–616 (1978).

(6) Rand, G.M. and Petrocelli, S.R., Fundamentals of Aquatic Toxicology. Hemisphere Publication Corporation, NY (1985).

(7) USEPA, Recommended Bioassay Procedure for Fathead Minnows, *Pimephales promelas* (Rafinesque), Chronic Tests. p. 13, National Water Quality Laboratory, Duluth, MN (1972).

(8) USEPA, Recommended Bioassay Procedure for Bluegill. *Lepomis macrochirus* (Rafinesque), Partial Chronic Tests. p. 11, National Water Quality Laboratory, Duluth, MN (1972).

(9) Dunnett, C.W., A multiple comparisons procedure for comparing several treatments with a control. *Journal of the American Statistical Association* 50: 1096–1121 (1955).

(10) Dunnett, C.W., New tables for multiple comparisons with a control. *Biometrics* 20:482–491 (1964).

(11) McClave, J.T. et al., Statistical Analysis of Fish Chronic Toxicity Test Data. Proceedings of 4th Aquatic Toxicology Symposium, ASTM, Philadelphia, PA (1980).

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Prevention, Pesticides
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(7101)

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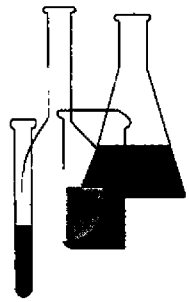
Ecological Effects Test Guidelines

OPPTS 850.1500
Fish life cycle toxicity



"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.*).

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OPPTS 850.1500 Fish life cycle toxicity.

(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 is OPP 72–5 Life-Cycle Tests of Fish (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **When required.** (1) Data obtained from a life-cycle test of fish are required by 40 CFR 158.145 to support the registration of an end-use product intended to be applied directly to water or expected to transport to water from the intended use site, and when any of the following conditions apply:

(i) If the estimated environmental concentration is equal to or greater than one-tenth of the no-effect level in the fish early life-stage or invertebrate life-cycle test; or

(ii) If studies of other organisms indicate the reproductive physiology of fish may be affected.

(2) See 40 CFR 158.50, ''Formulators' exemption'', to determine whether these data must be submitted. Section II-A of this Subdivision provides an additional discussion on this subject.

(c) **Test standards.** Data sufficient to satisfy the requirements in 40 CFR 158.145 should be derived from tests which comply with the general test standards in § 70-3 and the following test standards:

(1) **Test substance.** Data shall be derived from testing conducted with the technical grade of each active ingredient in the product.

(2) **Duration of tests.** Fish should be cultured in the presence of the test substance from one stage of the life cycle to at least the same stage of the next generation (e.g. egg to egg).

(3) **Species.** Testing should be performed on a freshwater fish (e.g. fathead minnow). An estuarine species (e.g. sheepshead minnow) may be used if the pesticide is expected to enter the estuarine environment.

(4) **Concentration analysis.** The concentration of the test substance in the water should be determined at the start of the study and periodically throughout the study to verify concentrations.

(d) **Reporting and evaluation of data.** In addition to the basic information provided in § 70-4, the test report should contain the following information (where appropriate):

(1) Reproductive effects;

(2) Detailed records of spawning, egg numbers, fertility, and fecundity;

(3) No-effect level, and mortality data;

(4) Statistical evaluation of effects;

(5) Locomotion, behavioral, physiological, and pathological effects;

(6) Definition of the criteria used to determine effects;

(7) Summary of general observation of signs of intoxication or other effects;

(8) Stage of life cycle in which organisms were tested;

(9) Duration of the test; and

(10) Concentration analysis.

(e) **Acceptable protocol**—(1) **Freshwater fish life-cycle test.** An example of an acceptable protocol is found in the following reference:

National Water Quality Laboratory Committee on Aquatic Bioassays. 1971. Recommended bioassay procedure for fathead minnow *Pimephales promelas* (Rafinesque) chronic tests. (Revised January, 1972). Pp. 15-24 in Biological Field and Laboratory Methods. U. S. Environmental Protection Agency, Office of Res. and Dev. EPA-670/4-73-001.

(2) **Estuarine fish life-cycle test.** Examples of acceptable protocols are found in the following references:

(i) Schimmel, S.C., and D.J. Hansen. 1974. Sheepshead minnow *Cyprinodon variegatus*: an estuarine fish suitable for chronic (entire lifecycle) bioassays. *Proc. 28th Ann. Cong. S.E. Assoc. Game-Fish Comm.* Pp. 392-398.

(ii) Hansen, D.J., P.R. Parrish, S.C. Schimmel, and L.R. Goodman. 1978. Life-cycle toxicity test using sheepshead minnows (*Cyprinodon variegatus*). Pp. 109-116 in Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Research and Development. EPA-600/9-78-010.

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Ecological Effects Test Guidelines

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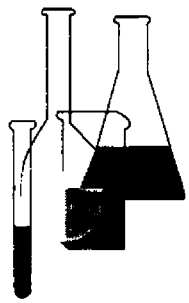
Oyster BCF



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OPPTS 850.1710 Oyster BCF.

(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 materials used in developing this harmonized OPPTS test guideline are 40 CFR 797.1830 Oyster Bioconcentration Test and OPP 72–6 Aquatic Organism Accumulation Tests (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982.

(b) **Purpose.** This guideline is to be used for assessing the propensity of chemical substances to bioconcentrate in tissues of estuarine and marine molluscs. This guideline describes a bioconcentration test procedure for the continuous exposure of Eastern oysters (*Crassostrea virginica*) to a test substance in a flow-through system. EPA will use data from this test in assessing the hazard a chemical or pesticide may present to the 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 are applicable to this test guideline. The following definitions also apply:

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

(2) *Bioconcentration* is the net accumulation of a chemical directly from water into and onto aquatic organisms.

(3) *Bioconcentration factor (BCF)* is the quotient of the concentration of a test chemical in tissues of aquatic organisms at or over a discrete time period of exposure divided by the concentration of test chemical in the test water at or during the same time period.

(4) *Depuration* is the elimination of a test chemical from a test organism.

(5) *Depuration phase* is the portion of a bioconcentration test after the uptake phase during which the organisms are in flowing water to which no test chemical is added.

(6) *EC50* is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular period of exposure (which should be stated).

(7) *Loading* is the ratio of the number of oysters to the volume (liters) of test solution passing through the test chamber per hour.

(8) *Organic chlorine* is the chlorine associated with all chlorine-containing compounds that elute just before lindane to just after mirex during gas chromatographic analysis using a halogen detector.

(9) *Organochlorine pesticides* are those pesticides which contain carbon and chlorine, such as aldrin, DDD, DDE, DDT, dieldrin, endrin, and heptachlor.

(10) *Steady-state* is the time period during which the amounts of test chemical being taken up and depurated by the test oysters are equal, i.e. equilibrium.

(11) *Steady-state bioconcentration factor* is the mean concentration of the test chemical in test organisms during steady-state divided by the mean concentration of the test chemical in the test solution during the same period.

(12) *Stock solution* is the concentrated solution of the test substance which is dissolved and introduced into the dilution water.

(13) *Test chamber* is the container in which the test oysters are maintained during the test period.

(14) *Test solution* is dilution water containing the dissolved test substance to which test organisms are exposed.

(15) *Umbo* is the narrow end (apex) of the oyster shell.

(16) *Uptake* is the sorption of a test chemical into and onto aquatic organisms during exposure.

(17) *Uptake phase* is the initial portion of a bioconcentration test during which the organisms are exposed to the test solution.

(18) *Valve height* is the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(d) **Test procedures**—(1) **Summary of the test.** Oysters are continuously exposed to a minimum of one constant, sublethal concentration of a test chemical under flow-through conditions for a maximum of 28 days. During this time, test solution and oysters are periodically sampled and analyzed using appropriate methods to quantify the test chemical concentration. If, prior to day 28, the tissue concentrations of the chemical sampled over three consecutive sampling periods have been shown to be statistically similar (i.e. steady-state has been reached), the uptake phase of the test is terminated, and the remaining oysters are transferred to untreated flowing water until 95 percent of the accumulated chemical residues have been eliminated, or for a maximum depuration period of 14 days. The mean test chemical concentration in the oysters at steady-state

is divided by the mean test solution concentration at the same time to determine the bioconcentration factor (BCF). If steady-state is not reached during 28 days of uptake, the steady-state BCF should be calculated using non-linear parameter estimation methods.

(2) [Reserved]

(3) **Range-finding test**. The oyster acute toxicity test is used to determine the concentration levels to be used in the oyster bioconcentration test.

(4) **Definitive test**. (i) The following data on the test chemical should be known prior to testing:

(A) Solubility in water.

(B) Stability in water.

(C) Octanol-water partition coefficient.

(D) Acute toxicity (e.g. propensity to inhibit shell deposition) to oysters.

(E) The validity, accuracy, minimum detection, and minimum quantification limits of selected analytical methods.

(ii) At least two concentrations should be tested to assess the propensity of the compound to bioconcentrate. The concentrations selected should not stress or adversely affect the oysters and should be less than one-tenth the EC50 or <EC10 determined in either the rangefinding or 96-h definitive test under OPPTS 850.1025 of these guidelines. The test concentration should be less than the solubility limit of the test substance in water and should be close to the potential or expected environmental concentration. The limiting factor of how low one can test is based on the detection and quantification limits of the analytical methods. The concentration of the test material in the test solution should be at least 10 times greater than the detection limit in water. The mean measured concentration of the test material should be 80 percent of the nominal concentration. However, this may be difficult to achieve for chemicals with high octanol-water partition coefficients.

(iii) It should be documented that the potential to bioconcentrate is independent of the test chemical concentration, and at least two concentrations should be tested which are at least a factor of 10 apart.

(iv) To determine the duration of this test, an estimation of the uptake phase should be made prior to testing based upon either previous experience with the same chemical in a different species, a test with a similar material, the results of a preliminary range-finding test, or, from the water solubility or octanol-water partition coefficient of the test chemical. This

estimate should also be used to designate a sampling schedule. The uptake phase should continue until steady-state has been reached. The uptake phase should continue for at least 4 days, but need not be longer than 28 days.

(A) The time to steady-state (S in hours) can be estimated from the water solubility of the octanol-water partition coefficient for chemicals whose uptake and depuration follow a two-compartment, two-parameter model (ASTM, 1986, under paragraph (g)(1) of this guideline). The following equations were developed from data on fish but are considered useful in this test as well:

$$S = 3.0/\text{antilog}(0.431 \log W - 2.11)$$

or

$$S = 3.0/\text{antilog}(-0.414 \log P + 0.122)$$

where

W = water solubility (mg/L)

P = octanol-water partition coefffient

For example, S for a chemical of log P 4.0 would be estimated as 3.0/antilog(-0.414(4.0) + 0.122) = 3.0/0.029 = 103.4 h.

Bioconcentration kinetic studies have also been performed specifically for molluscs, e.g. as investigated by Hawker and Connell (under paragraph (g)(2) of this guideline) and these may also be consulted.

(B) The depuration phase should continue until at least 95 percent of the accumulated test substance and metabolites have been eliminated, but no longer than 14 days.

(C) Based on the estimate of the time to steady-state, one of the following sampling schemes may be used to generate the appropriate data.

Table—Time to Steady-State in Days

Test Period	S<4	S>4<14	S>15<21	S>21
	Sampling days	Sampling days	Sampling days	Sampling days
Exposure[2]	1[1]	4[1]	1	1
.....	6[1]	1	3	3
.....	1	3	7	7
.....	2	7	10	10
.....	3	10	14	14
.....	4	12	18	21
.....		14	22	28

Table—Time to Steady-State in Days—Continued

Test Period	S<4	S>4<14	S>15<21	S>21
	Sampling days	Sampling days	Sampling days	Sampling days
Depuration[2]	11	1	1	1
.....	61	2	3	3
.....	121	4	7	7
.....	1	6	10	10
.....				14

[1] Hours
[2] Additional sampling times may be needed to confirm that steady-state has been attained

(v) The following criteria should be met in order for the test to be valid:

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

(B) The mortality in the controls should not exceed 10 percent at the end of the test.

(C) The dissolved oxygen concentration should be >60 percent of saturation throughout the test.

(D) There should be evidence (using measured test chemical concentrations) that the concentration of the chemical being tested has been satisfactorily maintained over the test period.

(E) If evidence of spawning is observed, the test should be discontinued and later repeated.

(F) Temperature variations from 20 °C should be held to a minimum, preferably ±2 °C.

(vi) The following methodology should be followed:

(A) The test should not be started until the test chemical delivery system has been observed to be functioning properly and the test chemical concentrations have equilibrated (i.e. the concentration does not vary more than 20 percent). Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium. At initiation (time 0), test solution samples should be collected immediately prior to the addition of oysters to the test chambers.

(B) The appropriate number of oysters (see paragraph (d)(4)(vii)(A) of this guideline) should be brushed clean and should be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. The number of oysters used in ths test will depend on the length of the test, number of replicate test chambers used, and if, in addition to a nonsolvent control, a solvent-control is used. Also important are the size of each oyster and the size of the test chamber. For example, in a 28–day test, a minimum of 28 oysters in the uptake (exposure) phase and an additional 20 oysters in the depuration phase per test chemical concentration would be needed. These oysters could be distributed among two or more replicates at each concentration. A minimum of 48 oysters would be required for each control. The oysters should be spread out equidistant from one another and placed with the left (cupped) valve down and the unhinged ends (opposite from umbo) all oriented in the same direction facing the incoming flow.

(C) Oysters should be exposed to the test chemical during the uptake phase until steady state has been reached or for a maximum of 28 days. The uptake phase should be a minimum of at least 4 days. Then the remaining oysters should be transferred to untreated flowing water and sampled periodically to determine if depuration of the test chemical occurs. Every test should include a control consisting of the same dilution water, conditions, procedures, and oysters from the same group used in the test, except that none of the test chemical is added. If a carrier is present in the test chamber, a separate carrier control is required.

(D) Oysters should be observed (and data recorded) at least daily for feeding activity (deposition of feces) or any unusual conditions such as excessive mucus production (stringy material floating suspended from oysters), spawning, or appearance of shell (closure or gaping). If gaping is noted, the oyster(s) should be prodded. Oysters which fail to make any shell movements when prodded are to be considered dead, and should be removed promptly with as little disturbance as possible to the test chamber(s) and remaining live oysters.

(E) For oysters sampled, careful examination of all the tissues should be made at the time of shucking for any unusual conditions, such as a watery appearance or differences in color from the controls.

(F) Observations on compound solubility should also be recorded. These include the appearance of surface slicks, precipitates, or material adsorbing to the test chamber.

(vii) **Sampling.** (A) At each of the designated sampling times, triplicate water samples and enough oysters should be collected from the test chamber(s) to allow for tissue analyses of at least four oysters. The concentration of test chemical should be determined in a minimum of four oysters analyzed individually at each sampling period. If individual analy-

sis is not possible, due to limitations of the sensitivity of the analytical methods, then pairs, triplicates or more oysters may be pooled to constitute a sample for measurement. A similar number of control oysters should also be collected at each sample point, but only those collected at the first sampling period and weekly thereafter, should be analyzed. Triplicate control water samples should be collected at the time of test initiation and weekly thereafter. Test solution samples should be removed from the approximate center of the water column.

(B) At each sampling period the appropriate numbers of oysters are removed and treated as follows:

(*1*) The valve height of each oyster should be measured.

(*2*) Oysters should be shucked as soon as practical after removal and should never be refrigerated or frozen in the shell. The shell should be opened at the hinge, the adductor muscle severed and the top valve removed. The remaining adductor muscle should be severed where it attaches to the lower valve and the entire oyster removed.

(*3*) The shucked oysters should then be drained 3 min, blotted dry, weighed and analyzed immediately for the test chemical. If analyses are delayed, the shucked oysters should be wrapped individually in aluminum foil (for organic analysis) or placed in plastic or glass containers (for metal analysis) and frozen.

(C) If a radiolabeled test compound is used, a sufficient number of oysters should also be sampled at termination to permit identification and quantitation of any major (greater than 10 percent of parent) metabolites present. It is crucial to determine how much of the activity present in the oyster is directly attributable to the parent compound, and to correct the bioconcentration factor appropriately.

(5) **Test results** (i) Steady-state has been reached when the mean concentrations of test chemical in whole oyster tissue for three consecutive sampling periods are statistically similar (F test, $P = 0.05$). A BCF is then calculated by dividing the mean tissue residue concentration during steady-state by the mean test solution concentration during the same period. A 95 percent confidence interval should also be derived from the BCF. This should be done by calculating the mean oyster tissue concentration at steady-state (X_0) and its 97.5 percent confidence interval $X_0 \pm t$ (S.E.) where t is the t statistic at $P = 0.025$ and S.E. is the one standard error of the mean. This calculation would yield lower and upper confidence limits (L_0 and U_0). The same procedure should be used to calculate the mean and 97.5 percent confidence interval for the test solution concentrations at steady-state, $XS \pm t$ (S.E.), and the resulting upper and lower confidence limits (L_s and U_s). The 95 percent confidence interval of the BCF would then be between L_0/U_s and U_0/L_s. If steady-state was not reached during the maximum 28-day uptake period, the maximum BCF should be cal-

culated using the mean tissue concentration from that and all the previous sampling days. An uptake rate constant should then be calculated using appropriate techniques. This rate constant is used to estimate the steady-state BCF and the time to steady-state.

(ii) If 95 percent elimination has not been observed after 14 days depuration then a depuration rate constant should also be calculated. This rate constant should be based on the elimination of the parent compound.

(iii) Oysters used in the same test should be 30 to 50 mm in valve height and should be as similar in age/size as possible to reduce variability. The standard deviation of the height should be less than 20 percent of the mean (N = 30).

(6) **Analytical measurements.** (i) All samples should be analyzed using EPA methods and guidelines whenever feasible. The specific methodology used should be validated before the test is initiated. The accuracy of the method should be measured by the method of known additions. This involves adding a known amount of the test chemical to three water samples taken from an aquarium containing dilution water and a number of oysters equal to that to be used in the test. The nominal concentration of these samples should be the same as the concentration to be used in the test. Samples taken on two separate days should be analyzed. The accuracy and precision of the analytical method should be checked using reference or split samples or suitable corroborative methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

(ii) An analytical method should not be used if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. Atomic absorption spectrophotometric methods for metal and gas chromatographic methods for organic compounds are preferable to colorimetric methods. Spectrophotometry is also acceptable provided Beer's law is followed and an acceptable extinction coefficient can be determined.

(iii) In addition to analyzing samples of test solution at least one reagent blank should also be analyzed when a reagent is used in the analysis.

(iv) When radiolabelled test compounds are used, total radioactivity should be measured in all samples. At the end of the uptake phase, water and tissue samples should be analyzed using appropriate methodology to identify and estimate the amount of any major (at least 10 percent of the parent compound) degradation products or metabolites that may be present.

(e) **Test conditions**—(1) **Test species.** (i) The Eastern oyster, *Crassostrea virginica,* should be used as the test organism.

(ii) Oysters used in the same test should be 30 to 50 mm in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height should be less than 20 percent of the mean.

(iii) Oysters used in the same test should be from the same source and from the same holding and acclimation tank(s).

(iv) Oysters should be in a prespawn condition of gonadal development prior to and during the test as determined by direct or histological observation of the gonadal tissue for the presence of gametes.

(v) Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(vi) The holding and acclimation of the oysters should be as follows:

(A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms, and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters should be held for at least 12 days before testing. All oysters should be maintained in dilution water at the test temperature for at least 2 days before they are used.

(B) During holding, the oysters should not be crowded, and the dissolved oxygen concentration should be above 60–percent saturation. The temperature of the holding waters should be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth, such that test results are not affected, as confirmed by previous testing. Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(C) A batch of oysters is acceptable for testing if the percentage mortality over the 7–day period prior to testing is less than 5 percent. If the mortality is between 5 and 10 percent, acclimation should continue for 7 additional days. If the mortality is greater than 10 percent, the entire batch of oysters should be rejected. Oysters which appear diseased or otherwise stressed or which have cracked, chipped, bared, or gaping shells should not be used. Oysters infested with mudworms (*Polydora* sp.) or boring sponges (*Cilona cellata*) should not be used.

(2) **Facilities**—(i) **Apparatus.** (A) An oxygen meter, dosing equipment for delivering the test chemical, adequate apparatus for temperature control, test tanks made of chemically inert material and other normal laboratory equipment are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the oysters should be avoided.

(C) The test chambers should be made from materials that will not absorb the test substance. Delivery systems and test chambers should be cleaned before and after each use. If absorption of the test substance occurs, those applicable parts of the delivery system should be discarded.

(D) The test substance delivery system used should accommodate the physical and chemical properties of the test substance and the selected exposure concentrations. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution (sea) water to the test chambers. The introduction of the test substance should be done in such a way as to maximize the homogeneous distribution of the test substance throughout the test chamber.

(ii) **Dilution water.** A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation, and testing periods. Natural seawater is recommended, although artificial seawater with food (algae) added may be used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and should be delivered at a flow rate of at least one, and preferably 5 L/h per oyster. The flowrate should be ± 10 percent of the nominal flow. A dilution water is acceptable if oysters will survive and grow normally over the period in which the test is conducted without exhibiting signs of stress, i.e. excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water should have a salinity in excess of 12 ppt, and should be similiar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 ppt and a monthly range in pH of less than 0.8 units. Artificial seawater should not vary more than 2 ppt nor more than 0.5 pH units. Oysters should be tested in dilution water from the same origin. If natural sea water is used, it should meet the following specifications, measured at least twice a year.

Substance	Concentration
Suspended solids	<20 mg/L
Un-ionized ammonia	<20 mg/L

Substance	Concentration
Residual chlorine	<3 µg/L
Total organophosphorus pesticides	<50 µg/L
Total organophosphorus pesticides plus PCB's	<50 µg/L

(3) **Test parameters**—(i) **Carriers.** Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used, the control oysters should be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 mL/L (100 mg/L).

(ii) **Dissolved oxygen.** This dissolved oxygen concentration should be at least 60 percent of the air saturation value and should be measured daily in each chamber.

(iii) **Loading.** The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) **Temperature.** The test temperature should be 20 °C. Temporary excursions (less than 8 h) within ± 5 °C are permissible. Temperature should be recorded continually.

(v) **pH.** The pH should be measured daily in each test chamber.

(vi) The amount of total organic carbon (TOC) in the dilution water can affect the bioavailability of some chemicals. Thus, TOC should be measured daily.

(f) **Reporting.** In addition to the reporting requirements prescribed in 40 CFR Part 792—Good Laboratory Practice Standards, the report should contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, TOC, temperature, and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g. the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including age, size (i.e. height), weight (blotted dry), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, sampling schedule, loading rate and flowrate.

(5) The methods of preparation of stock and test solutions and the test chemical concentrations used.

(6) The number of dead and live organisms, the percentage of oysters that died and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

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

(8) Description of sampling, sample storage (if required) and analytical methods of water and tissue analyses for the test chemical.

(9) The mean, standard deviation and range of the concentration of test chemical in the test solution and oyster tissue at each sampling period.

(10) The time to steady-state.

(11) The steady-state or maximum BCF and the 95 percent confidence limits.

(12) The time to 95 percent elimination of accumulated residues of the test chemical from test oysters.

(13) Any incidents in the course of the test which might have influenced the results.

(14) If the test was not done in accordance with the prescribed conditions and procedures, all deviations should be described in full.

(g) **References.**

(1) American Society for Testing and Materials. ASTM E 1022–84. Standard practice for conducting bioconcentration tests with fishes and saltwater bivalve molluscs. In 1986 Annual Book of ASTM Standards, vol. 11.04: Pesticides; resource recovery; hazardous substances and oil spill response; waste disposal; biological effects, pp. 702–725 (1986).

(2) Hawker, D.W. and D.W. Connell, Bioconcentration of lipophilic compounds by some aquatic organisms, *Ecotoxicology and Environmental Safety* 11:184–197 (1986).

(3) Schimmel, S.C. and R.L. Garnas, Interlaboratory comparison of the ASTM bioconcentration test method using the eastern oyster, pp. 277–287. In R.C. Bahner and R.T. Hansen (eds.), Aquatic Toxicology and Hazard Assessment: Eighth Symposium, ASTM STP 891, American Society for Testing and Materials, Philadelphia, PA (1985).

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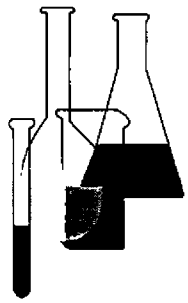
Ecological Effects Test Guidelines

OPPTS 850.1730
Fish BCF



"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 850.1730 Fish BCF

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

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.1520 Fish Bioconcentration Test; OPP guideline 72–6 Aquatic Organism Bioavailability/ Biomagnification/Toxicity Tests (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09–82–024, 1982 and OPP 165–4 Laboratory Studies of Pesticide Accumulation in Fish (Pesticide Assessment Guidelines, Subdivision N—Environmental Fate) EPA report 540/09–82–031, 1982; and OECD 305E Bioaccumulation: Flow-Through Fish Test.

(b) **Introduction**—(1) **Purpose.** The purpose of the study is to determine uptake and depuration rate constants and bioconcentration factors (BCFs) for fish exposed to a test chemical in aqueous solution. Another purpose is to identify and quantify major degradates at steady state. BCF values for the test chemical should always be based on concentrations of the chemical in fish tissue and exposure water, and not on total radiolabeled residues. BCFs may be used to help assess risks to the fish and to nontarget organisms (including humans) above them in the food chain.

(2) **Criteria for performing test.** The test is most commonly required for chemicals that are relatively persistent (stable) in water and have a relatively high potential for bioaccumulation as indicated by log P_{ow} (log of the octanol/water partition coefficient) values less than or equal to 1.0.

(3) **Criteria for degradate characterization.** BCFs based on total radiolabeled residues in fish tissue and exposure water can be used to help determine whether major degradates should be identified and quantified. If the BCF in terms of total radiolabeled residues is greater than or equal to 1,000, OPP requires that an attempt be made to identify and quantify pesticide degradates representing greater than or equal to 10 percent of total residues in fish tissues at steady state. If degradates representing greater than or equal to 10 percent of total radiolabeled residues in the fish tissue are identified and quantified, then degradates in the test water should also be identified and quantified.

(4) **Desired information on the test chemical.** To determine whether a BCF test is warranted (see paragraph (b)(2) of this guideline), it is necessary to know aqueous fate characteristics of the test chemical that determine its persistence in water and its octanol/water partition coefficient. Aqueous fate characteristics include rates of abiotic hydrolysis, biodegradation, direct photolysis in natural sunlight, and volatilization from water. Henry's law constant (approximated by the ratio of the chemi-

cal's vapor pressure to its solubility in water) is a good indicator of volatilization potential. It is necessary to know the test chemical's solubility in water to ensure that exposure concentrations do not exceed it. It is also necessary to know the toxicity of the chemical to test fish to ensure exposure concentrations do not adversely effect them (see paragraph (g)(2) of this guideline). The purity of the test chemical should be known as well as its radiopurity if radiolabeled. The structure and radiolabeled positions should be known. An appropriate analytical method, of known accuracy, precision, and sensitivity, for the quantification of the substance in the test solutions and in biological material must be available, together with details of sample preparation and storage. Analytical detection limit of test substance in both water and fish tissues should also be known.

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

Bioconcentration/bioaccumulation is the increase in concentration of the test substance in or on an organism (specified tissues thereof) relative to the concentration of test substance in the surrounding medium.

The bioconcentration factor (BCF or KB) at any time during the uptake phase of this accumulation test is the concentration of test substance (expressed in milligrams per gram or parts per million) in/on the fish or specified tissues thereof, divided by the concentration of the chemical in the surrounding medium ($BCF = C_f/C_w$).

The *depuration (loss)* rate constant (k_2) is the numerical value defining the rate of reduction in the concentration of the test substance in the test fish (or specified tissues thereof) following the transfer of the test fish from a medium containing the test substance to a medium free of that substance (k_2 is expressed in day^{-1}).

The *exposure or uptake phase* is the time during which fish are exposed to the test chemical.

Kinetic concentration factors (BCFK) are bioconcentration factors calculated directly from kinetic rate constants (k_1/k_2).

The *octanol-water partition coefficient* (P_{ow}) is the ratio of the solubility of a chemical in *n*-octanol and water at equilibrium and can also be expressed as K_{ow}. Log P_{ow} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

A *plateau or steady-state* is reached when the the plot of yhe concentration of test substance in fish (C_f) against time becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least 2 days are within ± 20 percent of each other, and there are no significant differences among the three sampling periods. At

least four successive analyses are required when pooled samples are analyzed. For test substances which are taken up slowly, the intervals would more appropriately be 7 days.

The *postexposure* or *depuration (loss)* phase is the time, following the transfer of the test fish from a medium containing test substance to a medium free of that substance, during which the depuration (or the net loss) of the substance from the test fish (or specified tissue thereof) is studied.

The *steady state bioconcentration factor* is found when the BCF does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period of time.

The *uptake rate constant* (k_1) is the numerical value defining the rate of increase in the concentration of test substance in/on test fish (or specified tissues thereof) when the fish are exposed to that chemical (k_1 is expressed in day^{-1}).

(d) **Principle of test**—(1) **Uptake and depuration phase.** The test consists of two phases—the exposure (uptake) and postexposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance until steady state is achieved or to a maximum of 28–60 days (see paragraph (g)(3) of this guideline). They are then transferred to a medium free of the test substance for a depuration phase of adequate duration (see paragraph (g)(4) of this guideline). The concentration of the test substance in/on the fish (or specified tissue thereof) and in water is followed through both phases of the test.

(2) **Determination of rate constants and BCFs.** (i) Concentrations of the test chemical in fish tissue and water as a function of time throughout the uptake and depuration phases are used to determine the uptake (k_1) and depuration (k_2) rate constants (see paragraph (i)(1) of this guideline.

(ii) Both the steady state and kinetic bioconcentration factors should be calculated (see paragraph (i)(2) of this guideline). The steady state bioconcentration factor (BCFs) is calculated as the ratio of the concentration in the fish (C_f) and to that in the water (C_w) at apparent steady-state. The kinetic bioconcentration factor (BCFK) is calculated as the ratio of the uptake rate constant (k_1) to the depuration rate constant (k_2) assuming first-order kinetics

(iii) At a minimum, BCFs should be computed for the whole fish. Whenever possible, they should also be calculated for edible and nonedible tissue. BCFs should be related to both the weight and lipid content of

the fish. If first-order kinetics are obviously not obeyed, more complex models should be employed under paragraph (d)(2)(iv) of this guideline.

(iv) **Model discrimination.** Most bioconcentration data have been assumed to be reasonably well described by a simple two-compartment/two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in fish, during the depuration phase, when these are plotted on semilog paper. (Where these points cannot be described by a rectilinear curve then more complex models should be employed, see paragraph (k)(20) of this guideline.)

(A) Graphical method for determination of depuration (loss) rate constant k_2.

Plot the concentration of the test substance found in each sample of fish against sampling time on semilog paper. The slope of that line is k_2.

$$k_2 = \ln (C_{f1}/C_{f2})/(t_2 - t_1)$$

Note that deviations from a straight line may indicate a more complex depuration pattern than first order kinetics. A graphical method may be applied for resolving types of depuration deviating from first order kinetics.

(B) Graphical method for determination of uptake rate constant k_1.

Given k_2, calculate k_1 as follows:

Equation 1

$$k_1 = C_f k_2/C_w \; T1\times (1 - e^{-k2t})$$

The value of C_f is read from the midpoint of the smooth uptake curve produced by the data when log concentration is plotted versus time (on an arithmetical scale).

(C) Computer method for calculation of uptake and depuration (loss) rate constants.

The preferred means for obtaining the bioconcentration factor and k_1 and k_2 rate constants is to use nonlinear parameter estimation methods on a computer. These programs find values for k_1 and k_2 given a set of sequential time concentration data and the model:

Equation 2

$$C_f = C_w \times k_1/k_2 \times (1 - e^{-k2t}) \qquad 0 < t < t_c$$

Equation 3

$$C_f = C_w \times k_1/k_2 \times (e^{-k2(t - tc)} - e^{k-2t} \qquad t > t_c$$

where t_c = time at the end of the uptake phase.

This approach provides standard deviation estimates of k_1 and k_2.

(D) As k_2 in most cases can be estimated from the depuration curve with relatively high precision, and because a strong correlation exists between the two parameters k_1 and k_2 if estimated simultaneously, it may be advisable first to calculate k_2 from the depuration data only, and subsequently calculate k_1 from the uptake data using nonlinear regression.

(e) **Materials—(1) Exposure tanks and tubes.** Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on the fish. Standard rectangular or cylindrical tanks, made of chemically inert material and of a suitable capacity in compliance with loading rate (see paragraph (e)(7) of this guideline), can be used. The use of soft plastic tubing should be minimized. Use Teflon, stainless steel and/or glass tubing. Experience has shown that for substances with high adsorption coefficients, such as the synthetic pyrethroids, silanized glass may be required. In these situations the equipment will have to be discarded after use.

(2) **Diluter.** For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. Preferably allow at least five volume replacements through each test chamber per day. The flow rates of stock solutions and dilution water should be checked both 48 hours before and then at least daily during the test. Include in this check the determination of the flow-rate through each test chamber and ensure that it does not vary by more than 20 percent either within or between chambers. The flow-through mode is to be preferred, but where this is not possible (e.g. when the test organisms are adversely affected) a semi-static technique may be used provided that the validity criteria are satisfied (see paragraph (g)(11) of this guideline).

(3) **Dilution water.** (i) Natural water is generally used in the test and should be obtained from uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behavior. Ideally, it should be demonstrated that the test species can survive, grow and reproduce in the dilution water (e.g. in laboratory culture or a life-cycle toxicity test). The water should be characterized at least by pH, hardness, total solids, total organic carbon and, preferably also ammonium, nitrite and alkalinity and, for marine species, salinity. Although the parameters which

are important for optimal fish well-being are not fully known, the following Table 1. gives recommended maximum concentrations of a number of parameters for fresh and marine test waters.

Table 1.—Some chemical characteristics of an acceptable dilution water

Substance	Limit concentration
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Un-ionized ammonia	1 mg/L
Residual chlorine	10 mg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides	50 ng/L
plus polychlorinated biphenyls	25 ng/L
Total organic chlorine	1 µg/L
Aluminium	1 µg/L
Arsenic	1 µg/L
Chromium	1 µg/L
Cobalt	1 µg/L
Copper	1 µg/L
Iron	1 µg/L
Lead	1 µg/L
Nickel	1 µg/L
Zinc	1 µg/L
Cadmium	100 ng/L
Mercury	100 ng/L
Silver	100 ng/L

(ii) The water should be of constant quality during the period of a test. The pH value should be within the range 6.0 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test substance) or adversely affect the performance of the stock of fish, samples should be taken at intervals for analysis. Determination of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every 3 months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least 1 year, determinations can be less frequent and intervals extended (e.g. every 6 months).

(iii) The natural particle content as well as the total organic carbon (TOC) of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter which may reduce its bioavailability. The maximum acceptable value is 5 mg/L for particulate matter (dry matter, not passing a 0.45 µm filter) and 2 mg/L for total

organic carbon. If necessary, the water should be filtered before use. The contribution to the organic carbon content in water from the test fish (excreta) and from the food residues should be as low as possible. Throughout the test, the concentration of organic carbon in the test vessels should not exceed the concentration of organic carbon originating from the test substance and, if used, the solubilizing agent by more than 10 mg/L (±20 percent).

(4) **Test chemical.** Whether radiolabeled or not, the chemical purity of the test chemical should be as high as practical (preferably greater than or equal to 98 percent). If radiolabeled, the radiopurity should be greater than or equal to 95 percent.

(5) **Test chemical stock solutions.** Prepare a stock solution of the test substance at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersant (solubilizing agents) is not recommended; however this may occur in some cases in order to produce a suitably concentrated stock solution. Solvents which may be used are, ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersant which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01 percent and HCO-40. Care should be taken when using readily biodegradable agents as these can cause problems with bacterial growth in flow-through tests.

(6) **Test species.** (i) Important criteria in the selection of species are that they are readily available, can be obtained in convenient sizes and can be satisfactorily maintained in the laboratory. Other criteria for selecting fish species include recreational, commercial, ecological importance as well as comparable sensitivity, past successful use etc. Recommended test species and test conditions are given in the following Table 2. Other species may be used but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

Table 2.—Fish species recommended for testing

Species	Test temperature (°C)	Total length of test animal (cm)
Danio rerio [1] (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	20–25	3.0±0.5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20–25	5.0±2.0
Cyprinus carpio (Teleostei, Cyprinidae) (Linnaeus) Common carp	20–25	5.0±3.0
Oryzias latipes (Teleostei, Poecilliidae) (Temminck and Schlegel) Ricefish	20–25	4.0±1.0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters) Guppy	20–25	3.0±1.0
Lepomis macrochirus (Teleostei Centrarchidae) (Rafinesque) Bluegill	20–25	5.0±2.0
Oncorhynchus mykiss (Teleostei Salmonidae (Walbaum) Rainbow trout	13–17	8.0±4.0
Gasterosteus aculeatus (Teleostei, (Gasterosteidae) (Linnaeus) Three-spined stickleback	18–20	3.0±1.0

[1] Meyer A. and G. Orti. *Proceedings of the Royal Society of London* 252 (Series B):231 (1993).

(ii) Various estuarine and marine species have been used in different countries, for example: Spot (*Leiostomus xanthurus*); Sheepshead minnow (*Cyprinodon variegatus*); Silverside (*Menidia beryllina*); Shiner perch (*Cymatogaster aggregata*); English sole (*Parophrys vetulus*); Staghorn sculpin (*Leptocottus armatus*); Three-spined stickleback (*Gasterosteus aculeatus*); Sea bass (*Dicentracus labrax*); Bleak (*Alburnus alburnus*)

(iii) The fresh water fish listed are easy to rear and/or are widely available throughout the year, whereas the availability of marine and estuarine species is partially confined to the respective countries. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease-and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

(7) **Reference chemicals.** The use of reference compounds of known bioconcentration potential would be useful in checking the experimental procedure, when required. However, specific substances cannot yet be recommended.

(f) **Fish care and health**—(1) **Acclimation.** Acclimate the stock population of fish for at least 2 weeks in water at the test temperature and

feed throughout on a sufficient diet (see paragraph (f)(3) of this guideline) and of the same type to be used during the test.

(2) **Pretest mortality and health.** (i) Following a 48–h settling-in period (during acclimation), mortalities are recorded and the following criteria applied:

(A) Mortalities of greater than 10 percent of population in 7 days, reject the entire batch.

(B) Mortalities of between 5 and 10 percent of population in 7 days, acclimate for 7 additional days.

(C) Mortalities of less than 5 percent of population in 7 days, accept the batch. If more than 5 percent mortality during the second 7 days, reject the entire batch.

(ii) Ensure that fish used in tests are free from observable diseases and abnormalities. Discard any diseased fish. Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

(3) **Feeding.** (i) During the acclimation and test periods, feed an appropriate diet of known lipid and total protein content to the fish in an amount sufficient to keep them in a healthy condition and to maintain body weight. Feed daily throughout the acclimation and test periods at a level of approximately 1 to 2 percent of body weight per day; this keeps the lipid concentration in most species of fish at a relatively constant level during the test. The amount of feed should be recalculated, for example, once per week, in order to maintain consistent body weight and lipid content. For this calculation, the weight of the fish in each test chamber can be estimated from the weight of the fish sampled most recently in that chamber. Do not weigh the fish remaining in the chamber.

(ii) Siphon uneaten food and faeces daily from the test chambers shortly after feeding (30 min to 1 h). Keep the chambers as clean as possible throughout the test so that the concentration of organic matter is kept as low as possible (see paragraph (e)(3), since the presence of organic carbon may limit the bioavailability of the test substance under paragraph (k)(6) of this guideline.

(iii) Since many feeds are derived from fishmeal, the feed should be analyzed for the test substance. It is also desirable to analyze the feed for pesticides and heavy metals.

(g) **Exposure conditions during test**—(1) **Optional preliminary test to determine optimal conditions.** It may be useful to conduct a preliminary experiment in order to optimize the test conditions of the definitive test, e.g. selection of test substance concentrations, duration of the uptake and depuration phases.

(2) **Exposure concentrations of test chemical.** (i) During the uptake phase, expose fish under flow-through conditions to at least two concentrations of the test substance in water. Normally, select the higher (or highest) concentration of the test substance to be about 1 percent of its acute asymptotic LC50, and to be at least tenfold higher than its detection limit in water by the analytical method used. The highest test concentration can also be determined by dividing the acute 96–h LC50 by an appropriate acute/chronic ratio (e.g. appropriate ratios for some chemicals are about 3, but a few are above 100). If possible, choose the other concentrations such that it differs from the one above by a factor of 10. If this is not possible because of the 1 percent of LC50 criterion and the analytical limit, a lower factor than 10 can be used or the use of ^{14}C labeled test substance should be considered.

(ii) No exposure concentration used should be above the solubility in water of the test substance.

(iii) Where a solubilizing agent is used in the stock solution, its diluted concentration in the exposure water should not be greater than 0.1 mL/L and should be the same in all test vessels. Its contribution (together with the test substance) to the overall content of organic carbon in the test water should be known. However, every effort should be made to avoid the use of such materials.

(iv) Minimize results reported as ''not detected at the limit of detection'' by pretest method development and experimental design, since such results cannot be used for rate constant calculations. Pretest results can be used to determine the exposure concentrations necessary to ensure that concentrations in fish tissue are generally above method detection limits.

(3) **Duration of uptake phase.** (i) A prediction of the duration of the uptake phase and time required to reach steady state can be obtained from practical experience (e.g. from a previous study or an accumulation study on a structurally related chemical) or from certain empirical relationships utilizing knowledge of either the solubility in water or the octanol/water partition coefficient of the test substance (see paragraph (g)(5) of this guideline).

(ii) The uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier. If the steady-state has not been reached by 28 days, the uptake phase should be extended, taking further measurements, until steady-state is reached or 60 days, whichever is shorter. The depuration phase is then begun.

(4) **Duration of depuration phase.** (i) The depuration period is begun by transferring the fish to the same medium but without the test substance in another clean vessel. A depuration phase is always necessary unless uptake of the substance during the uptake phase has been insignificant (e.g. the BCF is less than 10).

(ii) A period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95 percent) reduction in the body burden of the substance to occur (see paragraph (g)(5) of this guideline for an explanation of the estimation). If the time required to reach 95 percent loss is impractically long, exceeding for example twice the normal duration of the uptake phase (i.e. more than 56 days) a shorter period may be used (e.g. until the concentration of test substance is less than 10 percent of steady-state concentration). However, for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model, yielding first order kinetics, allow longer depuration phases for determination of loss rate constants. The period may, however, be governed by the period over which the concentration of test substance in the fish remains above the analytical detection limit.

(5) **Prediction of the duration of the uptake and depuration phases**—(i) **Prediction of the duration of the uptake phase.** (A) Before performing the test, an estimate of k_2 and hence some percentage of the time needed to reach steady-state may be obtained from empirical relationships between k_2 and the *n*-octanol/water partition coefficient (P_{ow}) or k_2 and the aqueous solubilities.

(B) (*1*) An estimate of k_2 (day–1) may be obtained from the following empirical relationship (see paragraph (k)(20) of this guideline):

Equation 1

$$\log k_2 = -0.414 \log P_{ow} + 1.47 (r^2 = 0.95)$$

For other relationships see see paragraph (k)(14) of this guideline.

(*2*) If the partition coefficient (P_{ow}) is not known, an estimate can be made (see paragraph (k)(4) of this guideline) from a knowledge of the aqueous solubility (s) of the substance using:

Equation 2

$$\log P_{ow} = 0.862 \log(s) + 0.710 (r^2 = 0.994)$$

where s = solubility expressed as moles per liter: (n=36)

(*3*) These relationships apply only to chemicals with log P_{ow} values between 2 and 6.5 (see paragraph (k)(12) of this guideline).

The time to reach some percentage of steady-state may be obtained by applying the k_2-estimate, from the general kinetic equation describing uptake and depuration (first-order kinetics):

$$dC_f/dt = k_1 C_w - k_2 C_f$$

or, if C_w is constant:

Equation 3

$$C_f = k_1/k_2 \cdot C_w(1 - (\exp)^{-k2t}$$

When steady-state is approached (as t approaches infinity), equation 3 may be reduced (see paragraphs (k)(3) and (k)(9) of this guideline) to:

$$C_f = k_1/k_2 C_w$$

or

$$C_f/C_w = k_1/k_2 = BCF$$

Then $k_1/k_2 \cdot Cw$ is an approach to the concentration in the fish at steady-state ($C_{f,s}$). Equation 3 may be transcribed to:

$$C_f = C_{f,s}(1 - (\exp)^{-k2t}$$

or

Equation 4

$$C_f/C_{f,s} = 1 - e^{-k2t}$$

Applying equation 4, the time to reach some percentage of steady-state may be predicted when k_2 is preestimated using equation 1 or 2.

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BCFK) is that period which is required for the curve of the logarithm of the concentration of the test substance in fish plotted against linear time to reach its midpoint, or $1.6/k_2$, or 80 percent of steady-state but not more than $3.0/k_2$ or 95 percent of steady-state (see paragraph (k)(19) of this guideline).

The time to reach 80 percent of steady-state is (equation 4):

$$0.8 = 1 - e^{-k2t}$$

or

Equation 5

$$t_{80} = 1.6/k_2$$

Similarly 95 percent of steady-state is:

Equation 6

$$t_{95} - 3.0/k_2$$

For example, the duration of the uptake phase (up) for a test substance with log $P_{ow} = 4$ would be (using equations 1, 5, and 6):

$$\log k_2 = -0.414.(4) + 1.47$$

$$k_2 = 0.652 \text{ days}^{-1}$$

up (80 pct)= 1.6/0.652 i.e. 2.45 days (59 h)

or

up (95 pct)= 3.0/0.652 i.e. 4.60 days (110 h)

Similarly, for a test substance with s = 10^{-5} mol/L, (log(s) = – 5.0), the duration of *up* would be (using equations 1, 2 and 5, 6):

$\log (P_{ow}) = -0.862 (-5.0) + 0.710 = 5.02$

$\log k_2 = -0.414 (5.02) + 1.47$

$k2 = 0.246 \text{ days}^{-1}$

up (80 pct) = 1.6/0.246, i.e. 6.5 days (156 hours)

or

up (95 pct) = 3.0/0.246, i.e. 12.2 days (293 hours)

Alternatively, the expression:

$t_{eq} = 6.54 \times 10^{-3} P_{ow} + 55.31$ (hours)

may be used to calculate the time for effective steady-state to be reached (see paragraph (k)(12) of this guideline).

(ii) **Prediction of the duration of the depuration phase.** (A) A prediction of the time needed to reduce the body burden to some percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (first order kinetics) (see paragraphs (k)(13) and (k)(20) of this guideline.

For the depuration phase, C_w is assumed to be zero. The equation may then be reduced to:

$$dC_f/dt = -k_2 C_f$$

or

$$C_f = C_{f,0}(\exp)^{-k2t}$$

where $C_{f,0}$ is the concentration at the start of the depuration period.

50 percent depuration will then be reached at the time (t_{50}):

or

Similarly 95 percent depuration will be reached at:

$$t_{95} = 3.0/k_2$$

If 80 percent uptake is used for the first period ($1.6/k_2$) and 95 percent loss in the depuration phase ($3.0/k_2$), then depuration phase is approximately twice the duration of the uptake phase.

It is important to note, however, that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first order kinetics are obviously not obeyed, more complex models should be employed (e.g. paragraph (k)(16) of this guideline).

(6) **Numbers and characteristics of test fish.** (i) Select the numbers of fish per test concentration such that a minimum of four fish per sample are available at each sampling. If greater statistical power is required, more fish per sample will be necessary.

(ii) If adult fish are used, report whether male or female, or both are used in the experiment. If both sexes are used, differences in lipid content between sexes should be documented to be nonsignificant before the start of the exposure; pooling all male and all female fish may be necessary.

(iii) In any one test, select fish of similar weight such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values (see paragraph (k)(6) of this guideline) record these details accurately. It is recommended that a sub-sample of the stock of fish is weighed before the test in order to estimate the mean weight (see paragraph (h)(2) of this guideline).

(7) **Loading of fish.** (i) Use high water-to-fish ratios in order to minimize the reduction in C_w caused by the addition of the fish at the start of the test and also to avoid decreases in dissolved oxygen concentration. It is important that the loading rate is appropriate for the test species used. In any case, a loading rate of 0.1–1.0 g of fish (wet weight) per liter of water per day is normally recommended. High loading rates can be used if it is shown that the required concentration of test substance can be maintained within ±20 percent limits, and that the concentration of dissolved oxygen does not fall below 60 percent saturation.

(ii) In choosing appropriate loading regimes, take account of the normal habitat of the fish species. For example, bottom-living fish may demand a larger bottom area of the aquarium for the same volume of water than pelagic fish species.

(8) **Light and temperature.** The photoperiod is usually 12 to 16 h and the temperature (±2 °C) should be appropriate for the test species (see Table 3. under paragraph (e)(6)(i) of this guideline). The type and

characteristics of illumination should be known. Caution should be given to the possible phototransformation of the test substance under the irradiation conditions of the study. Appropriate illumination should be used avoiding exposure of fish to unnatural photoproducts. In some cases it may be appropriate to use a filter to screen out UV irradiation below 290 nm.

(9) **Water quality measurements.** During the test, dissolved oxygen, TOC, pH and temperature should be measured in all vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the higher (or highest) concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured 3 times—at the beginning, around the middle, and end of the uptake period—and once a week in the depuration period. TOC should be measured at the beginning of the test (24 h and 48 h prior to test initiation of uptake phase) before addition of the fish and, at least once a week, during both uptake and depuration phases. Temperature should be measured daily, pH at the beginning and end of each period and hardness once each test. Temperature should preferably be monitored continuously in at least one vessel.

(10) **Controls.** In addition to the two test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects observed in the bioconcentration test to a matching control group and to obtain background concentrations of test substance. One dilution water control and if relevant, one control containing the solubilizing agent should be run.

(11) **Validity of test.** For a test to be valid the following conditions apply:

(i) The temperature variation is less than ± 2 °C.

(ii) The concentration of dissolved oxygen does not fall below 60 percent saturation.

(iii) The concentration of the test substance in the chambers is maintained within ± 20 percent of the mean of the measured values during the uptake phase.

(iv) The mortality or other adverse effects/disease in both control and treated fish is less than 10 percent at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5 percent per month and not exceed 30 percent in all.

(h) **Sampling and analysis of fish and water**—(1) **Fish and water sampling schedule.** (i) Sample water from the test chambers for the determination of test substance concentration before addition of the fish and during both uptake and depuration phases. As a minimum, sample the

water at the same time as the fish and before feeding. During the uptake phase, the concentrations of test substance are determined in order to check compliance with the validity criteria (see paragraph (g)(11) of this guideline).

(ii) Sample fish on at least five occasions during the uptake phase and at least on four occasions during the depuration phase. Since on some occasions it will be difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples (especially when other than simple first-order depuration kinetics are indicated), it may be advisable to take samples at a higher frequency in both periods (see the following Table 3.) Store the extra samples as described in paragraph (h)(3) and analyze them only if the results of the first round of analyses prove inadequate for the calculation of the BCF with the desired precision.

(iii) An example of an acceptable sampling schedule is given in the following Table 3.

Table 3.—Theoretical example of sampling schedule for bioconcentration tests of substances with log P_{ow} = 4

Fish Sampling	Sample time schedule		No. of water samples**	No. of fish per sample**
	Minimal required frequency (days)	Additional sampling (days)		
Uptake phase	−1		2*	
	0		2	**Add 45-80 fish**
1st	0.3		2	4
	0.3		(2)	(4)
2nd	0.6		2	4
		0.9	(2)	(4)
3rd	1.2		2	4
		1.7	(2)	(4)
4th	2.4		2	4
		3.3	(2)	(4)
5th	4.7		2	6
Depuration phase				**Transfer fish to water free of test chemical**
6th	5.0			4
		5.3		(4)
7th	5.9			4
		7.0		(4)
8th	9.3			4
		11.2		(4)
9th	14.0			6
		17.5		(4)

*Sample water after minimum of 3 "chamber-volumes" have been delivered.

**Values in parentheses are numbers of samples (water, fish) to be taken if additional sampling is carried out.

Note: Pretest estimate of k_2 for log P_{ow} of 4.0 is 0.652 days^{-1}. The total duration of the experiment is set to 3 × *up* = 3 × 4.6 days = 14 days. For the estimation of *up* see paragraph (g)(5) of this guideline.

Other schedules can readily be calculated using other assumed values of P_{ow} to calculate the exposure time for 95 percent uptake.

(iv) Continue sampling during the uptake phase until a steady-state has been established or for 28 days, whichever is the shorter. If the steady-state has not been reached within 28 days continue until a steady-state has been attained or 60 days, whichever is shorter. Before beginning the depuration phase transfer the fish to clean tanks.

(2) **Sampling methodology.** (i) Obtain water samples for analysis by siphoning through inert tubing from a central point in the test chamber. Since neither filtration nor centrifuging appears always to separate the nonbioavailable fraction of the test substance from that which is bioavailable (especially for superlipophilic chemicals, those chemicals with a log P_{ow} greater than or equal to 5) (see paragraphs (k)(6) and (k)(8) of this guideline), samples may not be subjected to those treatments. Instead, measures should be taken to keep the tanks as clean as possible and the content of total organic carbon should be monitored during both the uptake and depuration phases (see paragraph (g)(9) of this guideline).

(ii) Remove an appropriate number of fish (normally a minimum of four) from the test chambers at each sampling time. Rinse the sampled fish quickly with water, blot dry, kill instantly, using the most appropriate and humane method, and then weigh.

(3) **Sample storage.** (i) It is preferable to analyze fish and water immediately after sampling in order to prevent degradation or other losses and to calculate approximate uptake and depuration rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

(ii) Failing immediate analysis, store the samples by an appropriate method. Obtain information on the proper method of storage for the particular test substance before the beginning of the study—for example, deep-freezing, holding at 4 °C, duration of storage, extraction, etc.

(4) **Analysis of fish samples.** (i) Radiolabeled test substances can facilitate the analysis of water and fish samples, and may be used to determine whether degradate identification and quantification should be made. BFCs based on total radiolabeled residues (e.g. by combustion or tissue solubilization) can serve as one of the criteria for determining if degrades identification and quantification is necessary. However, BCF determinations for the parent compound should be based upon the concentration of the parent compound in fish and water, not upon total radiolabeled residues.

(ii) If the BCF in terms of total radiolabeled residues is greater than or equal to 1,000, it may be advisable, and for certain categories of chemicals such as pesticides strongly recommended, to identify and quantify degradates representing greater than or equal to 10 percent of total residues in fish tissues at steady state. If degradates representing greater than or equal to 10 percent of total radiolabeled residues in the fish tissue are identified and quantified, then it is also recommended to identify and quantify degradates in the test water. The major metabolites may be characterized at steady-state or at the end of the uptake phase, whichever is the sooner. It is possible to combine a fish metabolism study with a bioconcentration study to identify and quantify residues in tissues.

(iii) The concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done but pooling does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of fish to accommodate the desired pooling, procedure and power, should be included in the test. See paragraphs (k)(7) and (k)(10) of this guideline for an introduction to relevant pooling procedures.

(5) **Determination of lipid content.** BCF should be expressed both as a function of total wet weight and, for high lipophilic substances, as a function of the lipid content. Determine the lipid content of the fish on each sampling occasion if possible. Suitable methods should be used for determination of lipid content (see paragraphs (k)(5) and (k)(15) of this guideline). Chloroform/methanol extraction technique may be recommended as standard method (see paragraph (k)(11) of this guideline). The various methods do not give identical values (see paragraph (k)(18) of this guideline), so it is important to give details of the method used. When possible, the analysis for lipid should be made on the same extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it can be analyzed chromatographically. The lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more ± 25 percent. The tissue percent solids should also be reported to allow conversion of lipid concentration from a wet to a dry basis.

(6) **Quality of analytical method.** Since the whole procedure is governed essentially by the accuracy, precision, and sensitivity of the analytical method used for the test substance, check the precision and reproducibility of the chemical analysis experimentally, as well as recovery of the test substance from both water and fish to ensure that they are satisfactory for the particular method. Also, check that the test substance is not detectable in the dilution water used. If necessary, correct the values of C_w and C_f obtained from the test for the recoveries and background values of controls. Handle the fish and water samples throughout in such a manner as

to minimize contamination and loss (e.g. resulting from adsorption by the sampling device).

(i) **Data analysis**—(1) **Determination of uptake and depuration rate constants.** (i) Obtain the uptake and depuration curves of the test substance by plotting its concentration in/on fish (or specified tissues) in the uptake and in the depuration phase against time on arithmetic scales. The depuration rate constant (k_2) is usually determined from the depuration curve (i.e. a plot of the decrease in test substance concentration in the fish with time). The uptake rate constant (k_1) is then calculated given k_2 and a value of C_f which is derived from the uptake curve. See paragraph (d)(2)(iv) of this guideline for a description of these methods. The preferred method for obtaining BCFK and the rate constants, k_1 and k_2, is to use nonlinear parameter estimation methods on a computer (see paragraph (k)(15) of this guideline). Otherwise, graphical methods may be used to calculate k_1 and k_2. If the depuration curve is obviously not first-order, then more complex models should be employed (see paragraphs (k)(3), (k)(4), (k)(9), (k)(12), (k)(13), (k)(14), (k)(19, and (k)(20) of this guideline) and advice sought from a biostatistician.

(ii) The uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor, and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water.

(iii) The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of the analytical method. Clearly defined uptake and loss curves are an indication of good quality bioconcentration data. The variation in uptake/depuration constants between the two test concentrations should be less than 20 percent. Observed significant differences in uptake/depuration rates between the two applied test concentrations should be recorded and possible explanations given. Generally the confidence limit of BCFs from well-designed studies approach ± 20 percent.

(2) **Determination of the steady state and kinetic BCFs.** (i) Obtain the uptake curve of the test substance by plotting its concentration in/on fish (or specified tissues) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis, calculate the steady state BCFs from the following relationship:

$$C_f \text{ at steady state (mean)}/C_w \text{ at steady state (mean)}$$

(ii) When no steady state is reached, it may be possible to calculate a BCFs of sufficient precision for hazard assessment from a steady-state at 80 percent (1.6/k2) or 95 percent (3.0/k2) of equilibrium.

(iii) Determine the concentration factor (BCFK) as the ratio k_1/k_2, the two first-order kinetic constants.

(iv) The BCF is expressed as a function of the total wet weight of the fish. However, for special purposes, specified tissues or organs (e.g. muscle, liver), may be used if the fish are sufficiently large or the fish may be divided into edible (fillet) and nonedible (viscera) fractions. Since, for many organic substances, there is a clear relationship between the potential for bioconcentration and lipophilicity, there is also a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Thus, to reduce this source of variability in test results for those substances with high lipophilicity (i.e. with log P_{ow} greater than or equal to 3), bioconcentration should be expressed in relation to lipid content in addition to whole body weight. The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance, when feasible.

(j) **Test report.** The test report must include the following information.

(1) **Summary.** Test chemical and test species, uptake and depuration rate constants, and steady state and kinetic BCFs

(2) **Materials.** (i) Exposure tanks and tubes--material and size of tanks.

(ii) Diluter-type and description.

(iii) Dilution water. Source, description of any pretreatment, and water characteristics including pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if appropriate) and any other measurements made.

(iv) Test substance. Physical nature and, where relevant, physicochemical properties; chemical identification data (including the organic carbon content, if appropriate); if radio-labeled, the precise position of the labeled atoms and the percentage of radioactivity associated with impurities.

(v) Stock solutions. Method of preparation of stock solutions and frequency of renewal (the solubilizing agent, its concentration and its contribution to the organic carbon content of test water must be given, when used).

(vi) Test species. Scientific name, strain, source, any pretreatment, age, size-range, etc.

(vii) Care of fish. Acclimation, pretest mortality and health, feeding (e.g. type of foods, source, composition—at least lipid and protein content if possible, amount given and frequency).

(3) **Test conditions**— (i) **Test design.** Number and size of test chambers, water volume replacement rate, number of replicates, number of fish per replicate tank, number of test concentrations, controls.

(ii) **Exposure concentrations.** The nominal concentrations, the means of the measured values and their standard deviations in the test vessels.

(iii) **Length of uptake and depuration phases.** Give the lengths of the uptake and depuration phases and the rationale behind them

(iv) **Light.** Type and characteristics of illumination used and photoperiods.

(v) **Water quality within test vessels.** pH, hardness, TOC, temperature and dissolved oxygen concentration.

(4) **Sampling and analysis.** (i) Sampling frequency for fish and water samples.

(ii) Sample storage.

(iii) Sample extraction and analysis.

(iv) Detection and quantification limits.

(v) Accuracy and precision—results of spike and replicate analyses

(5) **Results.** (i) Data obtained in any preliminary test.

(ii) Validity of the test. Fish mortality and/or abnormal behavior for exposed and control, variations in exposure concentrations, variations in temperature, and minimum dissolved oxygen with respect to test validity criteria.

(iii) Lipid content of the test fish.

(iv) Uptake and depuration curves of the test chemical in fish; graphical representation of data.

(v) Concentrations of parent in fish tissue and exposure water. Tabular representation of data; C_f and C_w (with standard deviation and range, if appropriate) for all sampling times (C_f expressed in milligrams per gam of wet weight (parts per million) of whole body or specified tissues thereof e.g. lipid, and C_w expressed in milligrams per gam of wet weight (parts per million). C_w values for the control series (background should also be reported).

(vi) Uptake and depuration rate constants. Give values and 95 percent confidence limits for the uptake and depuration (loss) rate constants, describe the computation.

(vii) Steady state and kinetic BCFs. The BCFs and the BCFK (both expressed in relation to the whole body and the total lipid content, if measured, of the animal or specified tissues thereof), confidence limits and standard deviation (as available).

(viii) Degradate concentrations. Where radiolabeled substances are used, and when required, the accumulation of any major metabolites at steady state or at the end of the uptake phase.

(ix) Deviations and/or unusual observations. Report anything unusual about the test, any deviation from these procedures, and any other relevant information.

(k) **References.** The following references should be consulted for additional background material on this test guideline.

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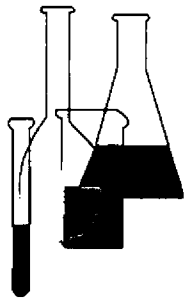
Ecological Effects Test Guidelines

OPPTS 850.1735 Whole Sediment Acute Toxicity Invertebrates, Freshwater



"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 850.1735 Whole sediment acute toxicity invertebrates, freshwater.

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

(2) [Reserved]

(b) **Objective.** This guideline may be used to determine the toxicity and bioaccumulation potential of chemicals in sediments in freshwater invertebrates. Natural sediment is spiked with different concentrations of test chemical and the results from the sediment toxicity tests can be used to determine causal relationships between the chemical and biological response. Reported endpoints from whole sediment toxicity tests may include the LC50 (median lethal concentration), EC50 (median effective concentration), NOEC (no-observable-effect-concentration), or the LOEC (lowest-observable-effect-concentration).

(c) **Definitions.**

Clean. Clean denotes a sediment or water that does not contain concentrations of test materials which cause apparent stress to the test organisms or reduce their survival.

Concentration. Concentration is the ratio of weight or volume of test material(s) to the weight or volume of sediment.

Contaminated sediment. Contaminated sediment is sediment containing chemical substances at concentrations that pose a known or suspected threat to environmental or human health.

Control sediment. Control sediment is sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test. Any contaminants in control sediment may originate from the global spread of pollutants and does not reflect any substantial input from local or non-point sources. Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination.

Effect concentration (EC). Effect concentration is the toxicant concentration that would cause an effect in a given percent of the test population. Identical to LC when the observable adverse effect is death. For example, the EC50 is the concentration of toxicant that would cause death in 50% of the test population.

Inhibition concentration (IC). Inhibition concentration is the toxicant concentration that would cause a given percent reduction in a non-quantal measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in growth for

the test population and the IC50 is the concentration of toxicant that would cause a 50% reduction.

Interstitial water or pore water. Interstitial water or pore water is water occupying space between sediment or soil particles.

Lethal concentration (LC). Lethal concentration is the toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observable adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

Lowest observable effect concentration (LOEC). Lowest observable effect concentration is the lowest concentration of a toxicant to which organisms are exposed in a test which causes an adverse effect on the test organisms (i.e., where the value for the observed response is statistically significant different from the controls).

No observable effect concentration (NOEC). No observable effect concentration is the highest concentration of a toxicant to which organisms are exposed in a test that causes no observable adverse effect on the test organisms (i.e., the highest concentration of a toxicant in which the value for the observed response is not statistically significant different from the controls).

Overlying water. Overlying water is the water placed over sediment in a test chamber during a test.

ppt. ppt is parts per thousand.

Reference sediment. Reference sediment is a whole sediment near an area of concern used to assess sediment conditions exclusive of material(s) of interest. The reference sediment may be used as an indicator of localized sediment conditions exclusive of the specific pollutant input of concern. Such sediment would be collected near the site of concern and would represent the background conditions resulting from any localized pollutant inputs as well as global pollutant input. This is the manner in which reference sediment is used in dredge material evaluations.

Reference-toxicity test. Reference-toxicity test is a test conducted in conjunction with sediment tests to determine possible changes in condition of the test organisms. Deviations outside an established normal range indicate a change in the condition of the test organism population. Reference-toxicity tests are most often performed in the absence of sediment.

Sediment. Sediment is particulate material that usually lies below water. Formulated particulate material that is intended to lie below water in a test.

Spiked sediment. Spiked sediment is a sediment to which a material has been added for experimental purposes.

Whole sediment. Whole sediment is sediment and associated pore water which have had minimal manipulation. The term bulk sediment has been used synonymously with whole sediment.

(d) **Test method.** (1) Whole sediment toxicity tests are outlined for the amphipod, *Hyalella azteca* and the midge, *Chironomus tentans*. Duration of whole sediment tests is 10 to 28 days and is accomplished in 300–mL test chambers containing 100 mL of sediment and 175 mL of overlying water. The overlying water may be renewed daily or a flow-through system may be used. Test organisms are fed during the toxicity test. The endpoint for *H. azteca* is survival, and for *C. tentans*, survival, growth and/or emergence.

(2) A range-finding test to establish a suitable range of test concentrations is recommended. A definitive test will not be required if no toxicity is observed at concentrations of 100 mg/kg dry weight of sediment.

(e) **Water, formulated sediment, reagents, and standards**—(1) **Water.** (i) Testing and culture water must be of uniform quality, and is acceptable if it allows satisfactory survival, growth, and reproduction of the test organisms. Disease or apparent stress (e.g. discoloration, unusual behavior) should not be prevalent. If problems occur during testing or culturing, water characteristics should be analyzed.

(ii) Natural water is considered to be of uniform quality if the ranges of hardness, alkalinity, and specific conductance are within 10 percent of the respective averages. The monthly pH range should be <0.4 units. Sources of natural water should be uncontaminated well or spring or surface water. Special considerations for surface water include minimizing quality and contamination variables, maximizing the levels of DO, and confirming that sulfides and iron levels are low. Chlorinated water should not be used for testing or culturing because chlorine-produced oxidants and residual chlorine are toxic to aquatic organisms. Tap water is acceptable if it is dechlorinated, deionized, and carbon filtered, but its use is not encouraged.

(iii) If source water is contaminated with facultative pathogens, it should be UV-irradiated using intensity meters and flow-controls, or filtered through 0.45 μm pore size.

(iv) The DO concentration of source water should be between 90 and 100 percent saturation. In some cases aeration may be required using air stones, surface aerators, or column aerators.

(v) High-purity distilled or deionized water may be reconstituted by adding specified amounts of reagent grade chemicals. The deionization

system should produce water with a resistance of 1 MΩ. For each batch of reconstituted water, the following parameters should be measured: Conductivity, pH, hardness, DO, and alkalinity. Aeration should be employed to maintain acceptable levels of pH and DO.

(vi) The preparation of 100 L of reconstituted water was developed at the USEPA EMSL-Cincinnati and has been tested with *H. azteca*, *C. tentans*, and *Chironomus riparius* in round-robin tests and is given as follows:

(A) Add approximately 75 L of deionized water to a properly cleaned container capable of holding 100 L.

(B) Add 5 g of $CaSO_4$ and 5 g of $CaCl_2$ to a 2–L aliquot of deionized water and mix (e.g., on a stir plate) for 30 min or until the salts dissolve.

(C) Add 3 g of $MgSO_4$, 9.6 g $NaHCO_3$, and 0.4 g KCl to a second 2-L aliquot of deionized water and mix on a stir plate for 30 min.

(D) Pour the two 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.

(E) Aerate the mixture for at least 24 h before use.

(F) The water quality of the reconstituted water should be approximately the following: Hardness, 90 to 100 mg/L as $CaCO_3$, alkalinity 50 to 60 mg/L as $CaCO_3$, conductivity 330 to 360 µS/cm, and pH 7.8 to 8.2.

(vii) Synthetic seawater may be prepared by adding commercial sea salts to deionized water. *H. azteca* may be cultured or tested at salinities up to 15 ppt.

(2) **Artificial sediment.** Artificial sediments consist of mixtures of materials designed to mimic natural sediments. Because artificial sediments have not been used routinely to assess the toxicity of contaminants in sediment, the use of uncontaminated natural sediment is recommended. If the use of artificial sediment is necessary, detailed information may be found in paragraph (l)(1) of this guideline.

(3) **Reagents.** All reagents and chemicals purchased from supply houses should be accompanied by appropriate data sheets. All test materials should be reagent grade. However, if specified as necessary, commercial product, technical-grade, or use-grade materials may be used. Dates for receipt, opening, and shelf-life should be logged and maintained for all chemicals and reagents. Do not use reagents beyond shelf-life dates.

(4) **Standards.** Acceptable standard methods for chemical and physical analyses should be used. When appropriate standard methods are not

available or lack the required sensitivity, other sources should be consulted for reliable methods.

(f) **Sample collection, storage, manipulation, and characterization—(1) Sample collection.** (i) Procedures for handling natural sediments should be established prior to collection. Pertinent data such as location, time, core depth, water depth, and collection equipment should be recorded.

(ii) Replicate sampling should be used for the collection of natural sediment to determine the variance in sediment characteristics. While some disruption of the sediment is inevitable regardless of the sampling equipment used, disruption of sediment should be kept to a minimum. Several devices are available for collecting sediment, but benthic grab or core samplers are recommended. The depth of sediment collected should reflect the expected exposure. During sediment collection, exposure to direct sunlight should be kept to a minimum. Cooling of sediment to 4 °C is recommended.

(2) **Storage.** Storage of sediment may affect bioavailability and toxicity. Although nonionic and nonvolatile organic contaminants in sediment may not result in substantive changes, metals and metalloids may affect redox, oxidation, or microbial metabolism in sediment. It is best to hold sediments at 4 °C in the dark and test within 2 to 8 weeks after collection. Long storage may result in changes of sediment properties. Sediment tests, and especially pore water tests, should be performed within 2 weeks of collection to minimize property changes in the sediment.

(3) **Manipulation.** (i) During homogenization, water above sediment that may have settled during shipment should be mixed back into the sediment. Sieving should not be used to remove indigenous microorganisms, unless an excessive number of oligochaetes are present. Because oligochaetes may inhibit the growth of the test organisms, it may be advantageous to remove them as well as other macroorganisms, rocks, wood, and the like by sieving. If sieving is used, sediment samples should be analyzed before and after sieving to document the influence of sieving on sediment characteristics. Sediments collected from multiple locations or sites may be pooled and mixed using suitable apparatus (e.g. stirring, rolling mill, feed mixer, etc.).

(ii) The preparation of test sediment may be accomplished by the spiking of natural or artificial sediments. Additional research is needed before artificial sediments may be used routinely. The responses of spiked sediment may be affected by mixing time and aging. Spiked sediment may be aged for at least 1 month to achieve equilibrium with the spiked chemicals, if the chemical is known to be persistent. Sediments spiked with industrial chemicals should be used as soon as possible. Point estimates of toxicity or minimum concentrations at which toxic effects are observed

may be determined by spiking natural sediments with a range of chemical concentrations. The test material should be reagent grade unless there is a specific need-to-use commercial product, technical-grade, or use-grade material. Specific information required for all test materials includes but is not limited to the following:

(A) Identity and concentration of major ingredients and impurities.

(B) Solubility in test water.

(C) Estimated toxicity to the test organism and to humans.

(D) When measured test concentrations are required, the precision and bias of the analytical method at the planned concentrations of test material.

(E) Recommended handling and disposal procedures.

(iii) Organic solvents should not be added to the sediment mixture because they may affect the concentration of dissolved organic carbon in pore water. and should not be used.

(4) **Characterization.** (i) The characteristics of all sediment should be determined, and at a minimum, the following factors should be measured: pH and ammonia concentration of pore water, organic carbon content (total organic carbon (TOC)), particle size distribution (percent sand, silt, clay), and percent water content. Additional analyses are suggested and include biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, total inorganic carbon, total volatile solids, acid volatile sulfides, metals, synthetic organic compounds, oil and grease, and petroleum hydrocarbons. Various physicochemical parameters should also be determined for interstitial water. Sediment characterization should also include qualitative parameters such as color, texture, and the presence of macrophytes or animals.

(ii) Standard analytical methods should be used to determine chemical and physical data. Precision, accuracy, and bias should be determined in sediment, water, and tissue for each analytical method. Analysis should include analytical standards and reagent blanks as well as recovery calculations.

(iii) Concentrations of spiked chemicals may be measured in sediment, interstitial water, and overlying water at the beginning and at the end of the test if so required. Measurement of degradation products may also be required. Sediment chemistry should be monitored during and at the end of a test. Separate replicates resembling the biological replicates and containing organisms should be specified for chemical sampling. The concentration of test material in water is measured by pipetting water samples from 1 to 2 cm above the sediment surface. Caution should be used to eliminate the presence of any surface debris, material from the sides

of the chamber, or sediment in the overlying water sample. At the end of the test, the test material may be removed for chemical analysis by siphoning (without disturbing sediment) the overlying water. Appropriate samples of sediment can then be removed for chemical analysis. The suggested method for isolation of interstitial water is by centrifugation without filtration.

(g) **Collection, culture, and maintainence of test organisms**—(1) **Hyalella azteca**—(i) **Life history.** (A) *H. azteca* are found throughout North and South America in permanent lakes, ponds, and streams. They are commonly found in mesotrophic or eutrophic lakes that are capable of supporting aquatic plants and that remain warm (20 to 30 °C) for most of the summer months. Densities may exceed 10,000 M^2 in optimal habitats. *H. azteca* are epibenthic detritivores that burrow into the sediment. They may be found in saline waters up to 29 percent, but are sensitive to hardness (e.g. they are not found in waters with calcium at <7 mg/L and DO at <2 mg/L).

(B) *H. azteca* reproduce sexually, averaging 18 eggs per brood and approximately 15 broods every 152 days. Hatching occurs approximately 5 to 10 days after fertilization at 24 to 28 °C. They proceed through a minimum of 9 instars, which are separated into 5 to 8 prereproductive instars and an indefinite number of postreproductive instars. Instars 1 through 5 form the juvenile life stage, instars 6 and 7 form the adolescent stage of development, instar 8 is the nuptial life stage, and later instars form the adult stages of the amphipod.

(C) *H. azteca* may be cultured under illumination of 500–1,000 lx. They feed during daylight and avoid bright light by hiding under litter.

(D) *H. azteca* is tolerable of a wide range of temperatures (0–33 °C), but are immobile at temperatures <10 °C and die at temperatures >33 °C. Reproduction can occur at temperatures of 10–18 °C, but the highest rate of reproduction occurs at temperatures between 26 and 28 °C.

(E) *H. azteca* can tolerate a wide range of substrates. Survival and growth of have not been shown to be negatively affected by either particle size (>90 percent silt and clay particles to 100 percent sand-sized particles) or grain size and organic matter in 10–day tests. In tests where organisms were not fed, survival decreased.

(ii) **Culturing procedures.** (A) To start a sediment test, 7– to 14–day–old amphipods must be produced. If growth is an endpoint, a narrower range, such as 1– to 2–day–old amphipods should be used. Details and further discussion of acceptable culture procedures for *H. azteca* are presented in paragraph (l)(1) of this guideline.

(B) *H. azteca* should be held and fed under the same conditions as the mass culture for at least 2 days prior to test initiation.

(2) **Chironomus tentans**—(i) **Life history.** (A) *C. tentans* are found in eutrophic ponds and lakes. In soft bottoms, approximately 95 percent of chironomid larvae are found in the upper 10 cm. Chironomid larvae are generally not found in sediments with hydrogen sulfide concentrations >0.3 mg/L.

(B) The aquatic phases of *C. tentans* include the larval and pupal stages. Female chironomids can oviposit eggs within 24 h of emergence, releasing a single gelatinous egg mass containing roughly 2,300 eggs. Hatch occurs in 2 to 4 days at 23 °C. The emergence of pupae as adults occurs after 21 days at 23 °C.

(C) *C. tentans* are able to tolerate a wide range of grain sizes and percentage organic matter. However, low percentage organic matter in conjunction with no feeding may result in decreased survival. Survival is best above pH 6.5. Poor control survival occurs at pH <6.5. Growth may also be impacted by coarser sediment.

(ii) **Culturing procedures.** (A) The third instar chironomids must be used to start a sediment test. Larvae should develop to the third instar within 9 to 11 days at a temperature of 23 °C. The instar stage of midges must be confirmed by head capsule width (~0.38 mm). Weight and height of midges should be monitored at the beginning of a sediment test. Details and further discussion of acceptable culture procedures are presented in paragraph (l)(1) of this guideline.

(B) The time to first emergence and the success of emergence should be recorded for all culture chambers. Growth may be monitored by periodically measuring the midge head capsule width.

(h) **Test method: Hyalella azteca 10– to 28–day sediment toxicity test**—(1) **Test conditions.** General test conditions required for a 10–day sediment toxicity test with *H. azteca* are presented in the following table XX. The 10–day sediment toxicity test must be conducted at 23 °C with a 16light:8dar photoperiod. Illumination should be approximately 500 to 1,000 lx. The recommended test chambers are 300–mL high-form beakers without lips containing 100 mL of sediment and 175 mL of overlying water. The test is started using 10 7– to 14–day–old amphipods. Eight replicates/treatment are recommended for routine testing. Because of potential impacts on study results, feed added to the test chamber should be kept to a minimum. Thoroughly mix food prior to removing aliquots. In order to prevent bacterial and fungal growth, feeding should be suspended for 1 to 2 days if food collects on sediment. Feeding should also be suspended if DO falls below 40 percent of saturation. When feeding is suspended in one treatment it should be suspended in all treatments. Feeding rates and appearance of sediment surface should be observed daily

and detailed records manitained. Each chamber should receive 2 volume additions per day or flow-through of overlying water. Sources of overlying water can be culture water, well water, surface water, site water, or reconstituted water.

Table XX.—General Test Conditions for 10-day Sediment Toxicity with *H. azteca*

Parameter	Conditions
1. Test type	Whole-sediment toxicity test with renewal of overlying water
2. Temperature	23± 1 °C
3. Light quality	Wide-spectrum fluorescent lights
4. Illuminance	500 to 1000 Lux
5. Photoperiod	16L:8D
6. Test chamber	300-mL high-form lipless beaker
7. Sediment volume	100 mL
8. Overlying water volume	175 mL
9. Renewal of overlying water	2 volume additions/d
10. Age of organisms	7- to 14- d old at start of test
11. Number of organisms/chamber	10
12. Number or replicate chambers/ treatment.	8
13. Feeding	Feed 1.5 mL daily to each test chamber
14. Aeration	None (unless D.O. drops below 40% of saturation)
15. Overlying water	Culture water, well water, surface water, site water or reconstituted water
16. Test chamber cleaning	Gently brush outside of screen when clogged
17. Overlying water quality	Hardness, alkalinity, conductivity, pH, and ammonia at beginning and end of test; temperature and D.O. daily
18. Test duration	10 - 28 d
19. Endpoints	Survival (growth optional)
20. Test acceptability	Minimum mean control survival of 80% and above conditions

(2) **Sediment into test chambers.** (i) Sediment should be thoroughly mixed and added to test chambers the day before (day−−1) the start of the test. The degree of homogeneity should be inspected visually. Homogeneity may be quantified by taking replicate subsamples and analyzing for TOC, chemical concentration, and particle size.

(ii) Equal amounts of sediments should be added to each test chamber on the basis of volume or dry weight. To minimize disturbance of sediment, overlying water should be poured gently along the sides of the test chambers or poured over a Teflon baffle (with handle) positioned above the sediment. The renewal of overlying water should commence on day−1. The test begins once organisms are added to the test chambers (day−0).

(3) **Renewal of overlying water.** Renewal or flow-through of overlying water is recommended during a test. Flow rates through any two test chambers should not differ by more than 10 percent at any time during the test. Each water-delivery system should be calibrated prior to test initiation to verify that the system is functioning properly. Renewal of overlying water is started on day−−1 before the addition of test organisms or food on day−0.

(4) **Acclimation.** Test organisms must be cultured and tested at 23 °C. The same water used for culture should be used for testing. Acclimation of test organisms to the test water is not required.

(5) **Placement of organisms in test chambers.** Handle test organisms as little as possible. Amphipods may be placed into test chambers by pipetting the organisms directly into the overlying water just below the air-water interface or by placing the organisms into 30–mL counting cups and floating them in the test chamber for 15 min prior to placement into the overlying water. Measurements of length or weight should be made on a subset of 20 organisms prior to test initiation.

(6) **Monitoring a test.** All test chambers should be checked daily. Test organisms should be observed for abnormal behavior, such as sediment avoidance. The exposure system should also be monitored daily to assure proper operation.

(7) **Measurement of overlying water-quality characteristics.** (i) Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test, and during any test should not vary more than 50 percent. Samples should be removed with a pipet from 1 to 2 cm above the sediment surface without disturbance. Caution is required to avoid removing test organisms when sampling.

(ii) DO should be measured daily, and should be maintained between 40 percent and 100 percent saturation. Both DO and pH may be measured in overlying water using a probe.

(iii) Temperature should be measured daily in one test chamber from each treatment. The mean and instantaneous temperatures should not vary from the desired temperature by more than 1 °C and 3 °C, respectively.

(8) **Feeding.** *H. azteca* may be fed with a mixture of yeast, Cerophyl, and trout chow (YCT) at a rate of 1.5 mL daily per test chamber. Food is required for proper maintenance of the test organisms but should be kept to a minimum to prevent alteration of contaminant availability or the growth of microbials such as fungus and bacteria. Collection of food on the bottom of the test chamber or reduced concentration of DO are indicators of possible overfeeding. Should either of the above conditions occur, feeding should be suspended in all test chambers until conditions have readjusted. Detailed records and observations should be made daily.

(9) **Ending a test.** Surviving amphipods may be pipetted from the test chamber prior to sieving the sediment. Immobile organisms isolated from either sediment or sieved material are considered dead. Sediment may be sieved by pouring one-half of the overlying water volume followed by one-half of the sediment through a #50 sieve (300 μm) into an examination pan. The coarser sediment remaining in the test chamber should be washed through a #40 (425 μm) sieve into a second examination pan.

Surviving organisms should be isolated and preserved (e.g. 8 percent sugar formalin) and measured for growth. The amount of time taken to recover test organisms should be consistent (e.g. 10 min per replicate). A recovery rate of 90 percent of organisms from the sediment is acceptable.

(10) **Test data.** (i) The primary endpoint for 10–day sediment toxicity test with *H. azteca* is survival.

(ii) Amphipod body length should be measured from the base of the first of antenna to the tip of the third uropod along the curve of the dorsal surface.

(iii) To determine dry weight of surviving amphipods:

(A) Pool all surviving organisms from a replicate.

(B) Dry the sample to constant weight at 60 to 90 °C.

(C) Bring sample to room temperature in a desiccator.

(D) Weigh the sample of organisms to the nearest 0.01 mg. This measure will give the mean weight of surviving organisms per replicate.

(11) **Interpretation of results**—(i) **Age sensitivity.** The relative sensitivity of *H. azteca* is comparable up to 24– to 26–day–old organisms. Amphipods 7– to 14–day–old represent sensitivity of *H. azteca* up to adult life stage.

(ii) **Grain size.** *H. azteca* tolerate a wide range of substrates. Neither grain size nor TOC correlate with the toxic response in sediment toxicity tests.

(iii) **Isolating organisms at the end of a test.** Quantitative recovery of amphipods <7–days–old is difficult. Starting testing with 7–day–old amphipods facilitates recovery.

(iv) **Influence of indigenous organisms.** The presence of oligochaetes does not reduce the survivability of amphipods in 28–day sediment tests. However, high density of oligochaetes does reduce the growth of amphipods. The number of oligochaetes and presence of predators in test sediment should be determined to improve the interpretation of growth data.

(i) **Interferences.** (1) Interferences are defined as those characteristics of sediment or sediment test systems that are unrelated to sediment-associated contaminants, but have the potential to affect the survival of test organisms. Interferences may lead to both Type I (false-positive) and Type II (false-negative) errors.

(2) Interferences may result from sediment characteristics that affect survival independently of chemical concentration, altered bioavailability

(e.g. sediment manipulation, storage, etc.), or when indigenous species are present.

(3) Test procedures and organism selection criteria were designed to minimize impacts due to interferences, and are suitable for providing direct measure of contaminant effects on benthic organisms.

(4) Several noncontaminant factors have the potential to affect sediment toxicity. These factors include but are not limited to avoidance, lighting, and geomorphological and physicochemical characteristics. Although laboratory sediment toxicity tests results may be used to predict effects in the field, extrapolations to the field may not prove valid in cases where motile organisms are able to avoid exposure.

(5) Toxicological responses of some chemicals may be altered by UV radiation contained in natural sunlight. Sediment testing with some chemicals, which are photoinduced by UV light, may not provide results useful for predicting field effects, because typical lighting (i.e. fluorescent) does not emit UV radiation.

(6) Natural geomorphological and physicochemical characteristics of sediment should be within the tolerance limits of the test organism. Factors such as texture, grain size, and organic carbon may influence the toxic response of the test organism.

(7) Sediment toxicity tests were designed to predict anticipated contaminant-related effects in the field or under natural conditions. However, sediment toxicity is related to bioavailability, which can be altered by physical manipulation, temperature, adjuncts, and organism uptake.

(8) In some cases bioavailability may differ between the laboratory and in situ. Sediment collection, handling, and storage are critical to preserving the integrity of contaminant equilibrium. The manipulation of sediment may disrupt the equilibrium with organic carbon and the pore water/particle system, resulting in the increased availability of organic compounds.

(9) The testing temperature is important to bioavailability. Temperature affects contaminant solubility, the partitioning coefficient, as well as the physical and chemical characteristics of sediment. Bioavailability may also be altered by interactions between sediment and overlying water.

(10) Adjuncts such as food, water, or solvents may alter bioavailability and promote the growth of microorganisms. While food addition is necessary, the quantity and composition of food added must be carefully considered.

(11) Uptake of contaminants by the test organisms or test chambers may influence bioavailability. Test organisms are sinks for contaminants, but to a lesser degree than sediments.

(12) The routes of exposure for sediment contaminants are not always known. In some cases, it may desirable to normalize sediment concentrations of contaminants to factors other than dry weight, such as organic-carbon for nonionic organic compounds or acid volatile sulfides for certain metals.

(13) The Agency recommends using natural sediments for spiking in sediment toxicity tests. However, indigenous species sometimes exist in field-collected sediments and their presence could negatively effect the growth rates of test organisms. Biological activity may be inhibited by gamma radiation, heat, sieving, mercuric chloride, or antibiotics, and their impact on sediment characteristics must be determined prior to the commencement of testing.

(j) **Test method—Chironomus tentans 10–day survival and growth test for sediments**—(1) **Test conditions.** The 10–day sediment toxicity test with *C. tentans* should be conducted at a temperature of 23 °C and photoperiod of 16 h light:8 h dark at 500 to 1,000 lx. The recommended test chambers are 300–mL high-form beakers without lips containing 100 mL of sediment and 175 mL of overlying water. Each test chamber is filled with 10 third-instar midges to begin the test. All organisms must be third-instar (50 percent of organisms) or younger. For routine testing, eight replicates are recommended. Midges should be fed 1.5 mL of a 4 g/L suspension of Tetrafin daily. Overlying water in each test chamber should receive two volume changes per day and can be culture water, well water, surface water, site water, or reconstituted water.

(2) **Sediment into test chambers.** Test sediment should be mixed thoroughly and placed into test chambers one day (day−−1) before commencement of the test. Sediment should be checked for homogeneity visually and quantitatively by analyzing TOC, chemical concentrations, and particle size. Equal volumes of sediment should be added to each test chamber, and on day−1 overlying water should be added by pouring water along a baffle to avoid any disturbance of the sediment. The test begins once the test organisms are added to the test chambers (day−0).

(3) **Renewal of overlaying water.** The renewal of overlying water is required and should be conducted on day−−1 prior to the addition of test organisms or food on day−0. Flow rates should not vary by more than 10 percent between any two test chambers at any time during the test. Proper system operation should be verified by calibration prior to initiation of the test.

(4) **Acclimation.** The required culture and testing temperature is 23 °C. The test organisms should be cultured in the same water to be used for testing. Acclimation of the test organisms to the test water is not required.

(5) **Placing organisms in test chambers.** Handle test organisms as little as possible. Midges may be placed into test chambers by pipetting the organisms directly into the overlying water just below the air-water interface or by placing the organisms into 30–mL counting cups and floating them in the test chamber for 15 min prior to placement into the overlying water. Measurements of length or weight should be made on a subset of 20 organisms prior to test initiation. Head capsule widths should be measured on midges to determine the instar used at test initiation.

(6) **Monitoring a test.** All test chambers should be checked daily. Test organisms should be observed for abnormal behavior, such as sediment avoidance. The exposure system should also be monitored daily to assure proper operation.

(7) **Measurement of overlying water-quality characteristics.** (i) Conductivity, hardness, pH, alkalinity, and ammonia concentration should be measured in all treatments at the beginning and end of a test, and during any test should not vary more than 50 percent. Samples should be removed with a pipet from 1 to 2 cm above the sediment surface without disturbance. Caution is required to prevent removing test organisms when sampling.

(ii) DO should be measured daily, and should be maintained between 40 and 100 percent saturation. Both DO and pH may be measured in overlying water using a probe.

(iii) Temperature should be measured in one test chamber from each treatment daily. The mean and instantaneous temperatures should not vary from the desired temperature by more than 1 and 3 °C, respectively.

(8) **Feeding.** Food is required for proper maintenance of the test organisms but should be kept to a minimum to prevent alteration of contaminant availability or the growth of microbials such as fungus and bacteria. Collection of food on the bottom of the test chamber or reduced concentration of DO are indicators of possible overfeeding. Should either of the these conditions occur, feeding should be suspended in all test chambers until conditions have readjusted. Detailed records and observations should be made daily.

(9) **Ending a test.** Surviving amphipods may be pipetted from the test chamber prior to sieving the sediment. Immobile organisms isolated from either sediment or sieved material are considered dead. Surviving organisms should be preserved (e.g. 8 percent sugar-formalin) and measured for growth. Specific sieving instruction may be found in paragraph (l)(1) of this guideline.

(10) **Test data.** (i) The endpoints measured in 10–day sediment tests with *C. tentans* are dry weight and survival. At the end of the test, *C. tentans* in control sediment should have an average size of 0.6 mg. Head

capsule width should be measured prior to dry weight. To determine dry weight of surviving midges:

(A) pool all surviving organisms from a replicate.

(B) Dry the sample at 60 to 90 °C to constant weight.

(C) Bring sample to room temperature in a desiccator.

(D) Weigh the sample of organisms to the nearest 0.01 mg. This measure will give the mean weight of surviving organisms per replicate.

(iv) Pupae and adults should be excluded from dry weight determinations. Length measurement is optional, but measurements should be from the anterior of the labrum to the posterior of the last abdominal segment.

(11) **Interpretation of results**—(i) **Age sensitivity.** First and second instar midges are more sensitive than third and fourth instar midges. Sediment tests should be initiated with midges of uniform size and age to avoid changes in sensitivity. Sediment tests are conducted with the third-instar midges because the greater size facilitates handling and isolation from sediment at test termination.

(ii) **Grain size.** *C. tentans* are tolerant of a wide range of substrates. The sensitivity of midges does not correlate with TOC or grain size. However, sensitivity may be influenced by artificial sediment when test organisms are not fed during the test.

(iii) **Isolating organisms at the end of a test.** Isolation and recovery of midges at the end of the test is not difficult. The midges are typically red and greater 5–mm in length.

(iv) **Influence of indigenous organisms.** There are no reports on the influence of indigenous organisms on *C. tentans* survival and response in sediment toxicity tests. However, survival of a congener, *Chironomus riparius*, was not reduced in the presence of oligochaetes, but growth was reduced in the presence of high numbers of oligochaetes. The number of oligochaetes and presence of predators in test sediment should be determined to improve the interpretation of growth data.

(k) **Reporting.** In addition to information meeting general reporting requirements, a report of the results of a whole sediment toxicity test should also include the following:

(1) Name of test and investigators, name and location of laboratory, and dates of start and end of test.

(2) Source of control or test sediment, method for collection, handling, shipping, storage and disposal of sediment.

(3) Source of test material, lot number if applicable, composition (identities and concentrations of major ingredient and impurities if known), known chemical and physical properties, and the identity and concentrations of any solvent used.

(4) Source and characteristics of overlying water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

(5) Source, history, and age of test organisms: Source, history, and age of brood stock, culture procedures and source and date of collection of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, age or life stage, means and ranges of weight or length, observed diseases or unusual appearance, treatments holding procedures.

(6) Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency and ration.

(7) Description of the experimental design and test chambers, the depth and volume of sediment and overlying water in the chambers, lighting, number of test chambers and number of test organisms/treatment, date and time test started and ended, temperature measurements, DO concentration (as percent saturation) and any aeration used before starting a test and during the conduct of a test.

(8) Methods used for physical and chemical characterization of sediment.

(9) Definitions of the effects used to calculate LC50 or EC50s, biological endpoints for tests, and a summary of general observations of other effects.

(10) A table of the biological data for each test chamber for each treatment including the controls in sufficient detail to allow independent statistical analysis.

(11) Methods used for statistical analyses of data.

(12) Summary of general observations on other effects or symptoms.

(13) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(14) Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

(l) **References.** The following references should be consulted for additional background material on this test guideline.

(1) U.S. Environmental Protection Agency. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates. EPA 600/R–94/024 (1994).

(2) [Reserved]

United States
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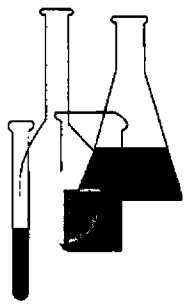
Ecological Effects Test Guidelines

OPPTS 850.1740 Whole Sediment Acute Toxicity Invertebrates, Marine



"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 850.1740 Whole sediment acute toxicity invertebrates, marine.

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

(2) [Reserved]

(b) **Objective.** This guideline may be used to determine the toxicity and bioaccumulation potential of chemicals in estuarine or marine sediments in marine invertebrates. Natural sediment is spiked with different concentrations of pesticide or contaminant and the results from the sediment toxicity tests can be used to determine causal relationships between the chemical and biological response. Reported endpoints from whole sediment toxicity tests include the LC50 (median lethal concentration), EC50 (median effective concentration), NOEC (no-observable-effect-concentration), or the LOEC (lowest-observable-effect-concentration).

(c) **Definitions.**

Clean. Clean denotes a sediment or water that does not contain concentrations of test materials which cause apparent stress to the test organisms or reduce their survival.

Concentration. Concentration is the ratio of weight or volume of test material(s) to the weight or volume of sediment.

Contaminated sediment. Contaminated sediment is sediment containing chemical substances at concentrations that pose a known or suspected threat to environmental or human health.

Control sediment. Control sediment is sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test. Any contaminants in control sediment may originate from the global spread of pollutants and does not reflect any substantial input from local or non-point sources. Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination.

Effect concentration (EC). Effect concentration is the toxicant concentration that would cause an effect in a given percent of the test population. Identical to LC when the observable adverse effect is death. For example, the EC50 is the concentration of toxicant that would cause death in 50% of the test population.

Inhibition concentration (IC). Inhibition concentration is the toxicant concentration that would cause a given percent reduction in a non-quantal measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in growth for

the test population and the IC50 is the concentration of toxicant that would cause a 50% reduction.

Interstitial water or pore water. Interstitial water or pore water is water occupying space between sediment or soil particles.

Lethal concentration (LC). Lethal concentration is the toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observable adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

Lowest observable effect concentration (LOEC). Lowest observable effect concentration is the lowest concentration of a toxicant to which organisms are exposed in a test which causes an adverse effect on the test organisms (i.e., where the value for the observed response is statistically significant different from the controls).

No observable effect concentration (NOEC). No observable effect concentration is the highest concentration of a toxicant to which organisms are exposed in a test that causes no observable adverse effect on the test organisms (i.e., the highest concentration of a toxicant in which the value for the observed response is not statistically significant different from the controls).

Overlying water. Overlying water is the water placed over sediment in a test chamber during a test.

Reference sediment. Reference sediment is a whole sediment near an area of concern used to assess sediment conditions exclusive of material(s) of interest. The reference sediment may be used as an indicator of localized sediment conditions exclusive of the specific pollutant input of concern. Such sediment would be collected near the site of concern and would represent the background conditions resulting from any localized pollutant inputs as well as global pollutant input. This is the manner in which reference sediment is used in dredge material evaluations.

Reference-toxicity test. Reference-toxicity test is a test conducted in conjunction with sediment tests to determine possible changes in condition of the test organisms. Deviations outside an established normal range indicate a change in the condition of the test organism population. Reference-toxicity tests are most often performed in the absence of sediment.

Sediment. Sediment is particulate material that usually lies below water. Formulated particulate material that is intended to lie below water in a test.

Spiked sediment. Spiked sediment is a sediment to which a material has been added for experimental purposes.

Whole sediment. Whole sediment is sediment and associated pore water which have had minimal manipulation. The term bulk sediment has been used synonymously with whole sediment.

(d) **Test method.** (1) Whole sediment toxicity tests are outlined for four species of estuarine/marine amphipods, *Ampelisca abdita*, *Eohaustorius estuarius*, *Rhepoxynius abronius*, and *Leptocheirus plumulosus*. Whole sediment tests last 10 or more days, and are conducted in in 1–L test chambers containing 175 mL (2 cm) of sediment and 800 mL of overlying water. The overlying water does not have to be renewed and test organisms do not have to be fed during the toxicity test. The endpoint is survival, but reburial for *E. estuarius*, *L. plumulosus*, and *R. abronius* is optional.

(2) A range-finding test to establish a suitable range of test concentrations is recommended. If no toxicity is observed at concentrations of 100 mg/kg dry weight of sediment, a definitive test is not required.

(e) **Water, reagents, and standards**—(1) **Water.** (i) Sea water used in sediment toxicity test should be of uniform quality and allow satisfactory survival, growth, or reproduction of the test organisms. Organisms cultured and tested in the selected sea water should not show signs of disease or stress.

(ii) Natural sea water should be from uncontaminated surface-water upstream of known discharges. Sea water should be collected at slack high tide or within 1 h of high tide. Full strength sea water should be collected from areas with salinities of 28 ppt. Sea water for estuarine test may be collected from areas with salinities close to the test salinity or diluted with freshwater. Water prepared from natural sea water should be covered, maintained at 4 °C, and used with 2 days.

(iii) Although natural sea water is preferable, reconstituted water is acceptable. Reagent grade chemicals should be added to high-purity distilled or deionized water (1 MΩ). Each batch of reconstituted water should be measured for salinity, pH, and dissolved oxygen (DO). Suspended particles should be removed by filtration (≤5 μm) from reconstituted water at least 24 h before use.

(2) **Reagents.** All reagents and chemicals purchased from supply houses should be accompanied by appropriate data sheets. All test materials should be reagent grade. However, if specified as necessary, commercial product, technical-grade, or use-grade materials may be used. Dates for receipt, opening, and shelf-life should be logged and maintained for all chemicals and reagents. Do not use reagents beyond shelf-life dates.

(3) **Standards.** Acceptable standard methods for chemical and physical analyses should be used. When appropriate standard methods are not

available or lack the required sensitivity, other sources should be consulted for reliable methods.

(f) **Sample collection, storage, manipulation, and characterization**—(1) **Sample collection.** (i) Procedures for handling natural sediments should be established prior to collection. Pertinent data such as location, time, core depth, water depth, and collection equipment should be recorded.

(ii) Replicate sampling should be used for the collection of natural sediment to determine the variance in sediment characteristics. While some disruption of the sediment is inevitable regardless of the sampling equipment used, disruption of sediment should be kept to a minimum. Several devices are available for collecting sediment, but benthic grab or core samplers are recommended. The depth of sediment collected should reflect the expected exposure. During sediment collection, exposure to direct sunlight should be kept to a minimum. Cooling of sediment to 4 °C is recommended.

(2) **Storage.** Storage of sediment may affect bioavailability and toxicity. Although nonionic and nonvolatile organic contaminates in sediment may not result in substantive changes, metals and metalloids may affect redox, oxidation, or microbial metabolism in sediment. It is best to hold sediments at 4 °C in the dark and test within 2 to 8 weeks after collection. Long storage may result in changes of sediment properties. Sediment tests, and especially pore water tests, should be performed within 2 weeks of collection to minimize property changes in the sediment.

(3) **Manipulation.** (i) During homogenization, water above sediment that may have settled during shipment should be mixed back into the sediment. Sieving should not be used to remove indigenous organisms, unless an excessive number of oligochaetes are present. Because oligochaetes may inhibit the growth of the test organisms, it may be advantageous to remove them by sieving. If sieving is used, sediment samples should be analyzed before and after sieving to document the influence of sieving on sediment characteristics. Sediments collected from multiple locations or sites may be pooled and mixed using suitable apparatus (e.g. stirring, rolling mill, feed mixer, etc.).

(ii) The preparation of test sediment may be accomplished by the spiking of natural or formulated sediments. Additional research is needed before formulated sediments may be used routinely. The responses of spiked sediment may be affected by mixing time and aging. Spiked sediment should be used immediately. Point estimates of toxicity or minimum concentrations at which toxic effects are observed may be determined by spiking natural sediments with a range of chemical concentrations. The test material should be reagent grade unless there is a specific need to use commercial product, technical-grade, or use-grade material. Specific

information required for all test materials include but is not limited to the following:

(A) Identity and concentration of major ingredients and impurities.

(B) Solubility in test water.

(C) Estimated toxicity to the test organism and to humans.

(D) When measured test concentrations are required, the precision and bias of analytical method at the planned concentrations of test material.

(E) Recommended handling and disposal procedures.

(iii) Organic solvents should not be added to the sediment mixture because they may affect the concentration of dissolved organic carbon in pore water.

(4) **Characterization.** (i) The characteristics of all sediment should be determined, and at a minimum the following factors should be measured: pH and ammonia concentration of pore water, organic carbon content (total organic carbon, TOC), particle size distribution (percent sand, silt, clay), and percent water content. Additional analyses are suggested and include biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, total inorganic carbon, total volatile solids, acid volatile sulfides, metals, synthetic organic compounds, oil and grease, and petroleum hydrocarbons. Various physicochemical parameters should also be determined for interstitial water. Sediment characterization should also include qualitative parameters such as color, texture, and the presence of macrophytes or animals.

(ii) Standard analytical methods should be used to determine chemical and physical data. For each analytical method, precision, accuracy, and bias should be determined in sediment, water, and tissue. Analysis should include analytical standards and reagent blanks as well as recovery calculations.

(iii) Concentrations of spiked chemicals should be measured in sediment, interstitial water, and overlying water at the beginning and at the end of the test. Degradation products should also be measured where appropriate. Sediment chemistry should be monitored during and at the end of a test. Separate replicates resembling the biological replicates and containing organisms should be specified for chemical sampling. The concentration of test material in water is measured by pipetting water samples from 1 to 2 cm above the sediment surface. Caution should be used to eliminate the presence of any surface debris, material from the sides of the chamber, or sediment in the overlying water sample. At the end of the test, the test material may be removed for chemical analysis by siphoning (without disturbing sediment) the overlying water. Appropriate samples

of sediment can then be removed for chemical analysis. The suggested method for isolation of interstitial water is by centrifugation without filtration.

(g) **Collection and maintainence of test organisms.** (1) The methods for collection of test organisms are species-specific. Subtidal species, such as *A. abdita* and *L. plumulosus*, may be collected with a small dredge or grab and may also be collected by skimming the sediment surface with a long-handled, fine-mesh net. *E. estuarius* and *R. abronius* are found both subtidally and intertidally. The aforementioned methods are suitable for subtidal populations. Intertidal populations may be collected using a shovel. Approximately one-third more organisms should be collected than are required for testing.

(2) All collecting, sieving, and transporting equipment should be clean and constructed of nontoxic material and clearly marked "live only". All apparatus should be cleaned and rinsed with an appropriate water source before use.

(3) Collected organisms should be handled gently, carefully, quickly, and no more than necessary. The sieving operation should be conducted by slowly immersing the sieve into collection site water. Sieved test organisms should be kept submerged in ambient collection water at all times. Direct exposure to sunlight of amphipods out of sediment must be avoided.

(4) *A. abdita* and *L. plumulosus* should be isolated from collection site sediment by using a 0.5 mm mesh sieve. *A. abdita* which remain in tubes must be left undisturbed for 20 to 30 min to allow for natural exit of the organisms. A 1.0 mm sieve should be used to isolate *E. estuarius* and *R. abronius*.

(5) *A. abdita* and *L. plumulosus* may be collected with a small dredge or grab apparatus (e.g. PONAR, Van Veen, etc.) and *E. estuarius* and *R. abronius* may be collected with a shovel. Collected amphipods should be sieved in the field by slow immersion in collection site water. Sieved amphipods should be separated from detritus and predators and transferred gently to transport containers containing 2 cm of collection site sediment. Mesh sizes of 0.5 to 1.0 mm should be utilized. Salinity and temperature of collection site sediment should be recorded surface and bottom locations. Amphipods should be transported in coolers with ice packs and held in the collection-site sediment at or below the temperature at the collection site. Aeration is recommended for transport times exceeding 1 h. Collection site sediment should be used as holding sediment in the laboratory and as control test sediment.

(h) **Ampelisca abdita, Eohaustorius estuarius, Leptocheirus plumulosus, or Rhepoxynius abronius 10– to 28–Day Survival Test For Sediments**—(1) **Recommended test method.** The recommended test temperatures for conducting sediment toxicity tests with *E. estuarius* and *R.*

abronius, *A. abdita*, and *L. plumulosus* are 15, 20, and 25 °C, respectively. *E. estuarius* and *L. plumulosus* should be tested at a salinity of 20 ppt and *A. abdita* and *R. abronius* at a salinity of 28 ppt. The recommended photoperiod is 24 h of light with illumination of 500 to 1,000 lx. Sediment (175 mL) and 800 mL of overlying seawater are placed in 1–L glass test chambers. Twenty organisms are placed in each test chamber to begin the test. Five replicates per treatment are recommended, however, this number may vary depending upon need. The size and life stage of amphipod required for testing varies from 2–4 mm for *L. plumulosus* to 3–5 mm for the three remaining species. Additionally, no mature male or female *A. abdita* or *L. plumulosus* should be used for testing. Overlying water does not have to be renewed and test organisms do not have to be fed during the test.

(2) **General procedure**—(i) **Introduction of sediment.** The test sediment should be homogenized one day before the test is to commence (day – –1) using a rolling mill, feed mixer, or other suitable apparatus. The sediment should be observed for homogeneity visually and quantitatively by measuring TOC, chemical concentrations, and particle size. A 175–mL aliquot of homogenized sediment should be added to each test chamber, and settled with the use of a nylon, fluorocarbon, or polyethylene spatula.

(ii) **Addition of overlying water.** A turbulence reducer (a disk cut from polyethylene, nylon, or Teflon, or a glass Petri dish attached to a glass pipet) should be used when adding overlying water. Turbulence reducers should be rinsed with seawater between replicates, and individual turbulence reducers used between treatments. Test chambers should be covered, immersed in a temperature bath, and gently aerated. The test commences once the test organisms are added to the test chambers (day – 0).

(iii) **Addition of amphipods.** Twenty amphipods are randomly added to each test chamber in batches of 5 to 10 on day – –0 following the addition of sediment and overlying water. One-third more amphipods than necessary are sieved from culture or control sediment and transferred to sorting trays. Recommended sieve sizes are 0.5 mm for *A. abdita* and *L. plumulosus* and 1.0 mm for *E. estuarius* and *R. abronius*. Isolated amphipods are transferred from the sorting tray to 150 mL of test sea water using pipets. The test organisms are observed for injury or stress after addition. If any *E. estuarius*, *L. plumulosus*, and *R. abronius* have not burrowed within 5 to 10 min, they should be replaced. *A. abdita* that have not burrowed within 1 h should also be replaced. Organisms expressing sediment avoidance, should be removed, recorded, but not replaced.

(3) **Test conditions**—(i) **Aeration.** Overlying sea water should be continuously aerated from day – 1 to day – 10 except when test organisms are being added. DO should be maintained at approximately 90 percent

saturation using gentle aeration without disturbing the sediment. Results are unacceptable if DO falls to below 60 percent saturation.

(ii) **Lighting.** Lights must be left on for the duration of the 10–day testing period. The constant light assures that the test organisms to remain burrowed during the test.

(iii) **Feeding.** None of the four test species need to be fed during the 10–day testing period.

(iv) **Water temperature.** The respective selected test temperatures are representative of the summertime thermal maximum for each species. *E. estuarius* and *R. abronius* (Pacific Coast amphipods) must be tested at 15 °C. *A. abdita* and *L. plumulosus* must be tested at 20 °C and 25 °C, respectively.

(v) **Salinity.** Overlying water salinity should be 28 ppt for *A. abdita* and *R. abronius* and 20 ppt for *E. estuarius* and *L. plumulosus*. Pore water salinity must be measured prior to the start of the test.

(4) **Measurements and observations.** (i) Temperature should be measured daily from at least one replicate from each treatment. Temperature of the water bath or exposure chamber must be monitored continuously.

(ii) Salinity, DO, and pH should be measured in overlying water daily in one test chamber in each treatment. These parameters should be measured in all test chambers at the beginning of the test and at termination.

(iii) Ammonia concentration should be measured near day−2 and day−8 during the 10–day test period. Ammonia concentration measurements should be accompanied by pH and temperature measurements. pH, temperature, and ammonia concentration should be measured in pore water at the beginning of the test.

(iv) Air-flow to overlying sea water must be monitored daily. Any test organisms trapped in air-water interface must be gently pushed back down using a glass rod or pipet.

(5) **Ending a test.** (i) Recovery of organisms from control sediment should equal or exceed 90 percent in a 10–day test or 80 percent in a 28–day test.

(ii) Test animals are isolated from the test chambers by sieving with sea water. Sieves should not exceed 0.5 mm. Test organisms should be washed into sorting trays containing sea water. Caution should be taken that no tube-dwelling organisms remain trapped on the sieve. Slapping the sieve forcefully against the surface of the water should successfully dislodge all *A. abdita*. The remaining species should be easily separated by the sieving process.

(iii) Small portions of material should be washed into sorting trays and should be examined carefully. The tubes of *A. abdita* should be teased apart under a dissecting microscope to ensure that all organisms are accounted for. The numbers of living, missing, or dead amphipods should be observed and recorded for all test chambers. Missing animals and all observed animals failing to respond to gentle prodding (i.e. neuromuscular twitch of pleopods or antennae) are recorded as dead.

(6) **Test data.** The primary endpoint for the 10–day sediment test is survival. Effective mortality (the sum of dead animals plus survivors that fail to rebury) may also be determined. To determine reburial, *E. estuarius*, *L. plumulosus*, and *R. abronius* should be transferred to a 2–cm layer of 0.5 mm sieved control sediment and overlying test sea water (2 cm).

(7) **Interpretation of results**—(i) **Influence of indigenous organisms.** Because test sediments collected from the field may contain indigenous species, data interpretation may be complicated by the presence of organisms similar to the test organism or predatory organisms.

(ii) **Effect of grain size.** While the four estuarine/marine test species are generally tolerable of a wide range of sediment types, grain size may adversely affect some species of amphipod. When this possibility exists, a clean control/reference sediment should be incorporated into the test design to facilitate distinction of contaminant effects versus particle size effects. Species-specific ranges of grain sizes are as follows.

(A) *A. abdita*: Survival may be impacted in sediments containing 95 percent or more sand. Test sediment should contain less than 95 percent sand.

(B) *L. plumulosus*: Survival should not be impacted in clean sediments containing 100 percent sand to 100 percent sand + clay.

(C) *E. estuarius*: Survival is unaffected by clean sediments containing 0.6 to 100 percent sand. However increased mortality may be associated with increased proportions of fine-grained sediment. In these cases an appropriate control/reference should be included.

(D) *R. abronius:* Very fine grains, particularly silts and clays, may reduce survival of this species. When test sediments contain silts and clays, the use of control/reference groups with particle sizes characteristic of the test sediment is recommended.

(iii) **Effects of pore water salinity.** The range of salinity tolerance is variable for the four amphipod species. For sediment testing, two scenarios for test salinity are acceptable given that appropriate conditions are met:

(A) *Salinity tolerance range* is the range of salinity in which a given species can survive for 10 days when the overlying water salinity is matched to that of the pore water salinity. In laboratory sediment testing, the overlying water salinity can be based on the standard salinity for each test species, or adjusted to match the salinity of pore water. It is critical that pore water salinity be measured prior to test initiation and that the appropriate species be used. Salinity tolerance ranges for *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* are 20–32 ppt, 2–34 ppt, 1.5–32 ppt and 25–32 ppt, respectively.

(B) *Salinity application range* is the range of pore water salinities in which a given species can survive for 10 days when using the species-specific overlying water salinity. Salinity application ranges for *A. abdita* with overlying water salinity of 28 to 32 ppt, *E. estuarius* with overlying water salinity of 20 ppt, *L. plumulosus* with overlying salinity of 20 ppt, and *R. abronius* with overlying water salinity of 28 to 32 ppt are 0 to 34, ≤2 to 34, ≤1.5 to 32, and 25 to 34, respectively.

(iv) **Effects of sediment-associated ammonia.** Ammonia concentrations in field-collected sediments may be toxic to amphipods. When ammonia concentration exceeds the water column no-effect levels, any mortality observed during the 10–day sediment test may be due to the ammonia. Ammonia levels should be measured approximately 1 cm above the sediment surface on day – 0 and, if necessary, reduced prior to the addition of test organisms by flushing the overlying water for up to two consecutive 24–h periods (six volume replacements per hour). Following flushing, the overlaying water ammonia concentration should be remeasured. If ammonia is at acceptable levels testing may be initiated but flushing at a rate of sic volume changes per 24–h period must be maintained throughout the test. Ammonia concentrations in overlaying water should be measured again on day – 10. If ammonia is not at acceptable levels, 24–h flushings must continue at the six–volume change per 24 h rate and ammonia concentration measured every 24 h.

(i) **Interferences.** (1) Interferences are characteristics of sediment or the sediment test system with potential to affect survival of test organisms independent of sediment-associated contaminant affects. Interferences are categorized into three categories: Noncontaminant factors causing reduced survival, changes in bioavailability due to manipulation or storage, and the presence of indigenous species. Noncontaminant factors can make extrapolation of laboratory test results to the field difficult. Specifically, the motility of organisms (i.e. escapism) and photoinduced toxicity due to UV light in from the sun (e.g. UV light absent from fluorescent light) may be markedly different between laboratory conditions and the natural environment. Other noncontaminant factors include sediment particle size, pore water salinity, and pore water ammonia concentration. The test conditions must be matched appropriately with the tolerance limits of the four amphipod test species (see paragraph (k)(1) of this guideline).

(2) Bioavailability of sediment-associated contaminants can be altered by collection, handling, and storage. The handling, storage, and preparation of test sediment should be as consistent as possible. Test sediments should be presieved and rehomogenized prior to introduction into the test chambers. Bioavailability may also be affected by temperature, salinity, the ratio of sediment to overlying water, and the depletion of contaminant due to organismal uptake. In some cases it is advantageous to normalize sediment concentrations to dry weight, organic-carbon content, or acid volatile sulfides.

(3) Test sediment collected from the field may contain indigenous organisms, and can potentially make interpretation of treatment effects difficult. If the presence of indigenous or predatory organisms is suspected, the test sediment should be press-sieved prior to test initiation.

(j) **Reporting.** In addition to information meeting the general reporting requirements of a toxicity test, a report of the results of a sediment toxicity test for estuarine and marine amphipods should include the following:

(1) Name of test and investigators, name and location of laboratory, and dates of start and end of test.

(2) Source of control or test sediment, method for collection, handling, shipping, storage, and disposal of sediment.

(3) Source of test material, lot number if applicable, composition (identities and concentrations of major ingredients and impurities if known), known chemical and physical properties, and the identity and concentrations of any solvent used.

(4) Source and characteristics of overlying water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

(5) Source, history, and age of test organisms; source, history and age of brood stock, culture procedures; and source and date of collection of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, age or life stage, means and ranges of weight or length, observed diseases or unusual appearance, treatments, holding procedures.

(6) Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency and ration.

(7) Description of the experimental design and test chambers, the depth and volume of sediment and overlying water in the chambers, lighting, number of test chambers an number of test organisms/treatment, date and time test starts and ends, temperature measurements, DO concentration

(as percent saturation) and any aeration used before starting a test and during the conduct of a test.

(8) Methods used for physical and chemical characterization of sediment.

(9) Definitions of the effects used to calculate LC50s or EC50s, biological endpoints for tests, and a summary of general observations of other effects.

(10) A table of the biological data for each test chamber for each treatment including the controls in sufficient detail to allow independent statistical analysis.

(11) Methods used for statistical analyses of data.

(12) Summary of general observations on other effects or symptoms.

(13) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(k) **References.** The following references should be consulted for additional background material on this test guideline.

(1) U.S. Environmental Protection Agency. Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods. EPA 600/R--94/025 (1994).

(2) [Reserved]

United States
Environmental Protection
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Prevention, Pesticides
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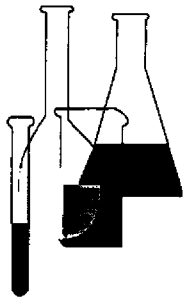
Ecological Effects Test Guidelines

OPPTS 850.1790 Chironomid Sediment 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 850.1790 Chironomid sediment 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 is 40 CFR 795.135 Chironomid Sediment Toxicity Test (proposed in the FEDERAL REGISTER of June 25, 1991 (56 FR 29149)).

(b) **Purpose.** This guideline may be used to develop data on the toxicity and bioavailability of chemical substances and mixtures (''chemicals'') in sediments subject to environmental effects test regulations under TSCA. This guideline prescribes tests to be used to develop data on the toxicity of chemicals present in sediments to chironomid larvae (midges). The EPA will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **Definitions.** The definitions in section 3 of TSCA and 40 CFR part 792, Good Laboratory Practice Standards (GLPS), apply to this test guideline. In addition, the following definitions also apply:

Bioconcentration factor (BCF) is the quotient of the concentration of a test substance in tissues of the chironomids at or over a specific time period of exposure divided by the concentration of test substance in the overlying water, interstitial water, or in the sediments at or during the same time period.

Cation exchange capacity (CEC) is the sum total of exchangeable cations that a sediment can absorb. The CEC is expressed in milliequivalents of negative charge per 100 g or milliequivalents of negative charge per gram of sediment (dry weight).

COD is chemical oxygen demand.

EC50 is an experimentally-derived concentration of test substance in the sediment that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time.

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

Geometric mean is the calculated mean between the highest test concentration with no statistically significant effects and the lowest concentration showing significant effects.

Interstitial water is liquid which is found in or directly adjacent to sediments and can be extracted from these sediments by several processes.

Loading is the ratio of chironomid biomass (grams wet weight) to the volume (liters) of test solution in a test chamber at a specified time or passing through the test chamber during a specific interval.

Lowest-observed-effect-concentration (LOEC) is the lowest treatment (i.e., test concentration) of a test substance that is statistically different in adverse effect on a specific population of test organisms from that observed in controls.

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

No-observed-effect-concentration (NOEC) is the highest treatment (i.e., test concentration) of a test substance that shows no statistical difference in adverse effect on a specific population of test organisms from that observed in controls.

Overlying water is liquid which is found above or placed over sediments. For purposes of this guideline, overlying water is equivalent to the term *water column.*

Partial life-cycle toxicity test is one which uses a sensitive portion of the life of a test organism (second instar of midges) to assess the effects of test substances.

Redox potential (Eh) means the oxidizing or reducing intensity or condition of a solution expressed as a current, referenced against a hydrogen electrode. Zero or negative Eh values may be exist due to reducing conditions within wet sediments.

Sediment is matter that settles to the bottom of a liquid in natural situations or a substrate prepared from a combination of natural sediments and artificial components. *Sediment* is equivalent to the term solid-phase sediments in this guideline.

Sediment partition coefficient is the ratio of the concentration of test substance on the sediment to the concentration in the overlying water. For the purposes of this guideline, this term is identical to *soil-water partition coefficient.*

Spiking is the addition of a test substance to a negative control and/or reference sediment so that the toxicity of a known quantity of test substance can be determined in a known nontoxic sediment. Often a solvent carrier is needed for low-water soluble test substances.

Subchronic toxicity test is a method used to determine the concentration of a test substance in water and for sediment which produces an adverse effect on chironomids over a partially extended period of time. In

this guideline, mortality and growth (expressed as change in wet weight of midges) are the criteria of toxicity.

TOC is total organic carbon.

(d) **Test procedures**—(1) **Summary of test.** (i) This flow-through test consists of three parts. Part 1. is a 14–day aqueous exposure test, with minimal sediments, with food, and with the test substance added to the overlying water. Part 2. is a 14–day sediment exposure test, with one or more sediments (4 to 6 cm in thickness) which may have varying amounts of organic carbon, with food, and with the test substance added to sediments. Part 3. is a 14–day interstitial exposure test, with one or more sediments (4 to 6 cm in thickness) which may have varying amounts of organic carbon, with food, and with the test substance added to overlying water. The flow-through test is illustrated in the following Table 1.

Table 1.—Experimental Design for the Chironomid Sediment Flow-Through Toxicity Test

Test system	Test substance concentrations (2 replicates each) [1]	Number of sediments (2 replicates each)	Number of Samples Analyzed (2 replicates each)			
			Overlying water P/C [2]	Interstitial water P/C [2]	Sediments	Midges [3]
Part 1						
14–Day Aqueous Exposure	5(10)	na [4]	5 (10)	na	na	5 (10)
Control (2 reps)	na	na	1 (2)	na	na	1 (2)
Solvent Control (2 reps)	na	na	1 (2)	na	na	1 (2)
Part 2						
14–Day Sediment Exposure	5 (10)	1-3 [5] (2-6)	5 (10)	na	5 (10)	5 (10)
Control (2 reps)	na	1 (2)	1 (2)	na	1 (2)	1 (2)
Solvent Control (2 reps)	na	1 (2)	1 (2)	na	1 (2)	1 (2)
Part 3						
14-Day Interstitial Water/Sediment Exposure	5 (10)	1-3 [5] (2-6)	5 (10)	5 (10)	5 (10)	
Control (2 reps)	na	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)
Solvent Control (2 reps)	na	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)

[1] Test substance concentration in all replicates measured at days 0 and 14 (reps = replicates)
[2] P/C = physical/chemical measurements (dissolved oxygen, temperature (in °C), and pH) on days 0, 4, 7, 10 and 14.
[3] Midges are observed throughout the test, dead chironomids recorded, removed, and weighed on days 4, 7, and 10. At end of each test, remaining midges from each replicate are removed, counted, and weighed.
[4] na = not applicable
[5] Number of sediment types tested will depend on range of TOC content tested; 1 to 3 types (low, medium, and high TOC levels) are recommended.

(ii) The day before the test is to be started, sediments (in treatments, and reference and negative controls) should be screened to remove large particles and endemic animals (especially midge predators) and added to the test chambers. The amount of sediments to be added to each test chamber will depend on the experimental design and test species. Only a minimum amount (to a depth of 2 mm) should be added in the aqueous exposure portion of the test. Each replicate test chamber should contain the same amount of sediments. Overlying water should be added to each test chamber.

(iii) In this flow-through test, the flow of dilution water through each chamber is begun and adjusted to the rate desired. The test substance should be introduced into each test chamber. The addition of test substance in the flow-through system should be done at a rate which is sufficient

to establish and maintain the desired concentration of test substance in the test chamber.

(iv) At the initiation of the test, chironomids which have been cultured or acclimated in accordance with the test design are randomly placed into the test chambers. Midges in the test chambers are observed periodically during the test. Immobile or dead larvae should be counted, removed, and weighed, and the findings recorded. "Floating" larvae are nonviable and should be replaced. Dissolved oxygen (DO) concentration, pH, temperature, the concentration (measured) of test substance, and other water quality parameters should be measured at specified intervals in selected test chambers, during all three parts of this test. (See Table 1 under paragraph (d)(1)(i) of this guideline.) Data should be collected during the test to determine any significant differences ($P<0.05$) in mortality and growth as compared to the controls. BCFs should be calculated at the end of the test based on route of exposure.

(2) **Range-finding test.** (i) A range-finding test should be conducted prior to beginning each of the three parts of the test to establish test solution concentrations for the three definitive parts of the test.

(ii) The chironomids should be exposed to a series of widely spaced concentrations of the test substance (e.g., 1, 10, 100 mg/L).

(iii) A minimum of 10 chironomids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate test concentrations. No replicates are required and nominal concentrations of the chemical are acceptable.

(3) Definitive test. (i) The purpose of the definitive portion of the test is to determine concentration-response curves, EC50 values, effects of a chemical on mortality and growth, and the determination of BCFs during subchronic exposure.

(ii) A minimum of 30 midges per concentration (15 midges per replicate test chamber) should be exposed in each part of the test to five or more concentrations of the test substance chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/L). An equal number of chironomids should be placed in two replicates. The concentration ranges should be selected to determine the concentration-response curves, EC50 values, and MATC. Solutions should be analyzed for chemical concentration prior to use and at designated times during the test.

(iii) Each test should include controls consisting of the same dilution water, sediments, conditions, procedures, and midges from the same population (same egg mass in culture container), except that none of the test substance is added.

(iv) The test duration is 14 days for each of the three parts of the test. The test is unacceptable if more than 20 percent of the control organisms die or are stressed or diseased during the test. A test period longer than 14 days may be necessary for high log K_{ow} chemicals.

(v) The number of dead chironomids in each test chamber should be recorded on days 4, 7, 10, and 14 of the test. At the end of the test, surviving midges are removed from the test chambers and weighed after being blotted dry. Concentration-response curves, EC50 values, and associated 95 percent confidence limits for mortality should be determined for days 4, 7, 10, and 14 in the aqueous exposure portion of the test. MATC, NOEC, and LOEC values should be determined for midge survival and growth.

(vi) In addition to survival and growth, any abnormal behavior or appearance of the chironomids should be reported.

(vii) Distribution of midges among the test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way that appropriate statistical analyses can be used to determine variation due to placement.

(viii) A control sediment and/or a reference sediment should be used in each part of this test. Use of these controls/references will help determine if the test is acceptable, serve to monitor the health of the chironomids used in the testing and the quality and suitability of test conditions, parameters and procedures, and aid in analyzing data obtained from this test. A negative control should be run in the test, using a sediment known to be nontoxic to the midges. A reference sediment can be run in the test in addition to or in place of the negative control. The reference sediment should be obtained from an area that is known to have low levels of chemical contamination and which is similar to or identical to the test sediments in physical and chemical characteristics.

(ix) In the first part of this test, the aqueous exposure, a minimal amount of sediment (<2mm) is placed in the test chambers. The presence of sediment is necessary to allow the midges to construct tubes, to reduce stress to the chironomids, and to reduce cannibalism.

(x) BCFs should be calculated at the end of each part of the test.

(4) **Analytical measurements**—(i) **Water quality analysis.** (A) The hardness, acidity, alkalinity, conductivity, TOC or COD, and particulate matter of the dilution water serving as the source of overlying water should be measured on days 0 and 14. The month-to-month variation of these values should be less than 10 percent and the pH should vary less than 0.4 units.

(B) During all three parts of the flow-through test, DO, temperature, and pH should be measured in each chamber on days 0, 4, 7, 10, and 14.

(ii) **Analysis of test substance.** (A) 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 of water and sediments. Radiolabeling of the test substance (e.g., by use of ^{14}C) may be necessary to measure quantities present in the sediments accurately. The analytical method used to measure the amount of test substance in the sample should be validated by appropriate laboratory practices before beginning the test. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interference which cannot be systematically identified and corrected mathematically. When radiolabeled test substances are used, total radioactivity should be measured in all samples. At the end of the test, water, sediments, and tissue samples should be analyzed using appropriate methodology to identify and estimate any major (at least 10 percent of the parent compound) degradation products or metabolites that may be present.

(B) The overlying water from each test chamber should be sampled for the test substance on days 0, 7, and 14 for all three aqueous exposure parts of this test.

(C) For the nonaqueous exposure parts of the test, the interstitial water from each test chamber should be analyzed for the test substance on days 0, 7, and 14. Interstitial water can be sampled by using a variety of methods, such as removal of overlying water and centrifugation, filtration of sediments, pressing the sediments, or using an interstitial water sample. Care should be taken during these measurements to prevent the biodegradation, transformation, or volatilization of the test substance.

(D) For the nonaqueous exposure portion of the test, the sediments from each test chamber should be analyzed for the test substance on days 0, 7, and 14.

(E) The sediment partition coefficient or soil-water partition coefficient is determined by dividing the average test substance concentration in sediment by the respective average concentration in the water column. Concentrations of test substance in the sediments to be used in this test can be chosen by measuring these partition coefficients. This sediment partition coefficient should be determined in triplicate by placing a quantity of a sediment with a known TOC content and spiked with the radiolabeled test substance into a quantity of dilution water. The ratio of sediment to dilution water should simulate the ratio present in the test. The sediment/dilution water mixture is shaken periodically, and the radiolabeled test sub-

stance measured. This shaking and sampling procedure is repeated until equilibrium is reached, as defined by the stage on the desorption curve.

(F) Overlying water samples should be filtered through a 0.45 μm filter to determine the concentration of dissolved test substance.

(G) BCFs should be calculated by determining the amount of test substance in the midge tissue and dividing by the concentration of test substance in the water column, interstitial water, and sediments. At test termination, the midges remaining in each test concentration are analyzed for test substance. Suitable methods are available, such as radiolabeling (^{14}C) the test substance, combusting the midges, trapping and counting the resulting radioactivity and the BCF calculated. If insufficient chironomid biomass is present at the conclusion of the test replicates may be pooled. BCFs cannot be calculated if after pooling there is insufficient biomass or if the accumulated test substance concentration is lower than the detection limit for the test substance.

(iii) **Numerical.** (A) The number of dead midge second instars should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for mortality concentration-response curves calculated on days 4, 7, 10, and 14. A 4–, 7–, 10–, and 14–day LC50 value based on second instar mortality, and with corresponding 95 percent confidence intervals, should be calculated. The methods recommended for calculating EC50s include probit, logit, binomial, and moving average.

(B) Appropriate statistical tests (e.g., analysis of variance and mean separation tests) should be used to test for significant chemical effects on growth (measured as wet weights) on days 4, 7, and 14. An MATC should be calculated using these test criteria.

(C) In no case should any analytical measurements be pooled except when calculating BCFs when there is insufficient biomass available for individual measurements as described under paragraph (d)(4)(ii)(G) of this guideline.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The midge, *Chironomus tentans* or *C. riparius*, should be used in this test. Both species are widely distributed throughout the United States, and the larvae and adult flies can be cultured in the laboratory. The larval portion life cycles of both species is spent in a tunnel or case within the upper layers of benthic sediments of lakes, rivers, and estuaries. Feeding habits of both species include both filter feeding and ingesting sediment particles.

(B) Second instar chironomids (< 10 days) of the same age and size are to be used in this test. Third and fourth instar are less desirable, as some evidence indicates they are less sensitive, at least to copper. Each instar is 4 to 7 days in duration.

(ii) **Acquisition.** (A) Chironomids to be used in this test should be cultured at the test facility. Adult flies are collected from the chironomid cultures and allowed to mate and lay egg masses. Two egg masses are collected and allowed to hatch. The larvae are fed daily. When the second instar stage (about 10 days after hatching) is reached, larvae are removed and placed in the test chambers. 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 population (culture container) and be the same age and size.

(B) Chironomids should not be used in a test if:

(*1*) During the final 48 hours of midge holding, obvious mortality is observed.

(*2*) The larvae are not in the second instar.

(iii) **Feeding.** (A) During the test, the chironomids should be fed the same diet at the same frequency as that used for culturing and acclimation. All treatments and controls should receive, as near as reasonably possible, the same amount of food on a per-animal basis.

(B) The food concentration depends on the type used and the nutritional requirements of the midges. The latter in turn is dependent upon their developmental stage.

(iv) **Loading.** The number of test organisms placed in a test chamber should not affect the test results. Loading should not exceed 30 chironomids per liter per 24 hours in the flow-through test. Loading should not affect test concentrations or cause the DO concentration to fall below the recommended level.

(v) **Care and handling of test organisms.** (A) Chironomids should be cultured in dilution water under similar environmental conditions as those in the test. Food such as Tetra Conditioning Food has been demonstrated to be adequate for chironomid cultures.

(B) Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and as quickly as possible. During culturing and acclimation, midges should be observed for any signs of stress, physical damage, and mortality. Dead and abnormal individuals should be discarded. Organisms that are damaged or dropped during handling should be discarded.

(C) Wide-bore, smooth glass tubes or pipets equipped with a rubber bulb can be used for transferring midges.

(vi) **Acclimation.** (A) Midges should be maintained in 100 percent dilution water at the test temperature for at least 4 days prior to the start of the test. This is easily accomplished by culturing them in the dilution

water at the test temperature. Chironomids should be fed the same food during the test as is used for culturing and acclimation.

(B) Midges should be maintained in facilities similar to those of the testing area during culturing and acclimation to the dilution water.

(2) **Test system**—(i) **General.** (A) Facilities needed to perform this test include:

(*1*) Containers for culturing and acclimating the chironomids.

(*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 to ensure that the test solution flows regularly into and out of the test chamber.

(*4*) Test chambers can be small aquaria capable of holding 3 L of water or test solution, 5.7–L clear glass battery jars, or 1–L beakers made of borosilicate glass. Each chamber should be equipped with screened overflow holes, standpipes, or U-shaped notches covered with Nitex screen.

(B) Construction materials and commercially purchased equipment that may contact dilution water should not contain substances that can be leaked or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact test solutions should be chosen to minimize sorption of test substances.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water by evaporation, and to minimize the entry of dust or other particulates into the solutions.

(ii) **Test substance delivery.** (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 delivery system should be calibrated before and after 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 the 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 or from one time to any other.

(iii) **Cleaning of test system.** All test equipment and test chambers should be cleaned before each test following standard laboratory proce-

dures. Cleaning of test chambers may be necessary during the testing period.

(iv) **Dilution water.** (A) Surface or ground water, reconstituted water, or dechlorinated tap water are acceptable as dilution water if chironomids 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 2.:

Table 2.—Specifications For Dilution Water

Substance	Maximum Concentration
Particulate matter	20 mg/L
TOC or COD	2 mg/L or 5 mg/L, respectively
Boron, fluoride	100 µg/L
Un-ionized ammonia	10 µg/L
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc..	1 µg/L
Residual chlorine	3 µg/L
Cadmium, mercury, silver	100 ng/L
Total organophosphorus pesticides.	50 ng/L
Total organochlorine pesticides and polychlorinated biphenyls (PCBs) or organic chlorine..	50 ng/L or 25 ng/L respectively

(B) The water quality characteristics listed in Table 2. 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, hardness, alkalinity, pH, acidity, particulate matter, TOC or COD, and particulate matter 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 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 such as triethylene glycol (CAS No. 112–27–6), dimethylformamide (CAS No. 68–12–2), or acetone (CAS No. 67–64–1) should be used. The concentration of such carriers should not exceed 0.1 mL/L.

(v) **Sediments.** (A) **Preparation and source.** (*1*) Sediments used in this test may contain low (<1 percent) to high (>15 percent) amounts of organic carbon because they are derived from variable natural sediments. Prior to use, the sediments should be sieved to remove larger particles. The should be characterized for particle size distribution (sand, silt, clay percentages), percent water holding capacity, total organic and inorganic carbon, total volatile solids, COD, BOD, cation exchange capacity, redox

potential (Eh), oils and greases, petroleum hydrocarbons, organophosphate pesticide concentrations, organochlorine pesticide and polychlorinted biphenyl (PCB) concentrations, toxic metal concentrations, and pH.

(*2*) The source of the sediments used in this test should be known and the characteristics listed above should be measured every time additional sediments are obtained. The sediments should not contain any endemic organisms, as these may be chironomid predators.

(*3*) Sediments should not be resuspended during the test.

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

(A) Temperature of 20±1 °C for *C. tentans* and 22±1 °C for *C. riparius.*

(B) DO concentration of the dilution water should be 90 percent of saturation or greater. The DO concentrations of the test solutions should be 60 percent or greater of saturation throughout the test. Aeration may be necessary, and if this is done, all treatment and control chambers should be given the same aeration treatment.

(C) A photoperiod of 16 h light/and 8 h dark with a 15– to 30–minute transition period.

(ii) Additional measurements include:

(A) The concentration of dissolved test substance (that which passes through a 0.45 μm filter) 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 and 14 of the test.

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

(C) Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary by more than 20 percent at any time or 30 percent during the test.

(D) The dissolved oxygen concentration, temperature, and pH should be measured at the beginning of the test and on days 7 and 14 in each chamber.

(f) **Calculated values**—(1) **Sediment partition coefficient.** (A) The sediment or soil-water partition coefficient (K_p) is defined as the ratio of

the concentration of the test substance in the sediment (C_s) to the concentration in the water or interstitial water (C_w) as given in the follwing expression:

$$K_p = C_s/C_w$$

The resultant K_p values for the sediment or sediments tested are used to select test substance concentrations for the sediment test.

(B) The K_p value is equivalent or related to the sediment organic carbon sorption coefficient multiplied by the percent organic carbon content of the sediment.

(C) The sediment partition coefficient should be determined in triplicate for each sediment type at equilibrium by spiking with the radiolabeled test substance and shaking. The test substance concentration in the water is measured radiometrically at intervals and the data used to create a desorption curve. The process is repeated until an equilibrium is reached, as defined by the shape of the curve.

(2) **BCFs.** BCFs should be calculated for each part of the test. These values are computed as the amount of test substance present in the midge tissues divided by test substance concentrations in the water column, interstitial water, and sediments. At test termination, the chironomids remaining in each test concentration are analyzed for radiolabeled test substance.

(g) **Reporting.** The sponsor should submit all data developed by the test that are suggestive and predictive of toxicity and all associated toxicologic manifestations to the Agency. In addition to the reporting requirements prescribed in the GLPS, 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 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, TOC or COD, and particulate matter) and a description of any pretreatment.

(4) The source of the sediment, its physical and chemical characteristics (e.g., particle size distribution, TOC, pesticide and metal concentrations), and a description of any pretreatment.

(5) Detailed information about the chironomids used as a stock, including the scientific name and method of verification, age, source, treat-

ments, feeding history, acclimation procedures, and culture methods. The age (in days) and instar stage of the midges used in the test should be reported.

(6) A description of the test chambers, the volume of solution in the chambers, and the way the test was begun (e.g., conditioning and test substance additions). The number of test organisms per test chamber, the number of replicates per treatment, the lighting, the test substance delivery system, flow rates expressed as volume additions per 24 hours for the flow-through subchronic test, the method of feeding (manual or continuous), and type and amount of food.

(7) The concentration of the test substance in the water, interstitial water, and sediments in test chambers at times designated in the flow-through tests.

(8) The number and percentage of organisms that show any adverse effect in each test chamber at each observation period, and wet weights of midges in each test chamber at days 7 and 14.

(9) BCFs for all three parts of the test (i.e., overlying water or water column, sediment, and interstitial water modes of exposure).

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

(11) The data records of the culture, acclimation, and test temperatures. Information relating to calculation of sediment (or soil-water) partition coefficients (K_p).

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

(13) An LC50 value based on mortality and an EC50 value based on adverse effects on growth (wet weights), with corresponding 95 percent confidence limits, when sufficient data are present for days 4, 7, and 14. These calculations should be made using the average measured concentration of the test substance.

(14) Concentration-response curves utilizing the average measured test substance concentration should be fitted to both number of midges that show adverse effects (mortality) and effects on growth or wet weights of midges at days 4, 7, and 14. A statistical test of goodness-of-fit should be performed and the results reported.

(15) The MATC to be reported is calculated as the geometric mean between the lowest measured test substance concentrationthat had significant ($P < 0.05$) effect and the highest measured test substance concentration that had no significant ($P > 0.05$) effect on days 4, 7, and 14 of the test. 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 and mean separation tests should be used to test for significant test substance effects. The statistical tests employed and the results of these tests should be reported.

(h) **References.** The following references should be consulted for further background information on this test guideline.

(1) Adams, W.J. et al. *Aquatic safety assessment of chemicals sorbed to sediments.* R.D. Cardwell, R. Purdy, and R.C. Bahner, eds. In: Aquatic Toxicology and Hazard Assessment. ASTM STP 854. American Society for Testing and Materials, Philadephia, PA (1985).

(2) Nebeker, A.V. et al. Relative sensitivity of *Chironomus tentans* life stages to copper. *Environmental Toxicology and Chemistry* 3:151–158. (1984).

(3) Nebeker, A.V. et al. Biological methods for determining toxicity of contaminated freshwater sediments to invertebrates. *Environmental Toxicology and Chemistry* 3:617–630. (1984).

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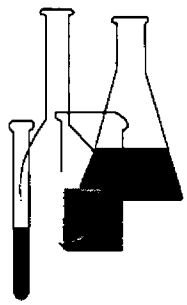
Ecological Effects Test Guidelines

OPPTS 850.1800 Tadpole/Sediment Subchronic 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 850.1800 Tadpole/sediment subchronic 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 is 40 CFR 797.1995 Tadpole/sediment subchronic toxicity test.

(b) **Purpose.** This guideline may be used to develop data on the subchronic toxicity of chemical substances and mixtures subject to environmental effects testing. This guideline prescribes tests to be used to develop data on the subchronic toxicity of chemicals sorbed to natural sediments to bullfrog tadpoles. The EPA will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **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 for physical, chemical, persistence, and ecological effects testing apply to this test guideline. The following definitions also apply:

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

Carrier means a solvent or dispersant used to dissolve a test substance.

Cation exchange capacity (CEC) means the sum total of exchangeable cations that a sediment can absorb. The CEC is expressed in milliequivalents of negative charge per 100 g or milliequivalents of negative charge per gram of sediment (dry weight).

Clay mineral analysis means the estimation or determination of the kinds of clay-size minerals and the amount present in a sediment.

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

Control means the exposure of test organisms to uncontaminated sediments.

Death means the total lack of movement by a test tadpole.

EC50 means that test substance concentration calculated from experimentally-derived growth or sublethal effects data that has affected 50 per-

cent of a test population during continuous exposure over a specified period of time.

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

LC50 means the test substance concentration calculated from experimentally-derived mortality data that is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

Loading means the ratio of tadpole biomass (in grams, wet weight) to the volume (in liters) of test solution in a test chamber or passing through it in a 24–h period.

Lowest-observed-effect-concentration (LOEC) means the lowest treatment (i.e., test concentration) of a test substance that is statistically different in adverse effect on a specific population of test organisms from that observed in controls.

No-observed-effect-concentration (NOEC) means the highest treatment (i.e., test concentration) of a test substance that shows no statistical difference in adverse effect on a specific population of test organisms from that observed in controls.

Organic matter is the organic fraction of the sediment; it includes plant and animal residues at various stages of decomposition, cells and tissues of sediment-based organisms, and substances synthesized by the microbial community.

Particle size analysis is the determination of various amounts of different particle sizes in a sample (i.e., sand, silt, and clay), usually by sedimentation, sieving, micrometry, or combinations of these methods. The names and diameter ranges commonly used in the United States are provided in the following Table 1.:

Table 1.—Particle Size

Name	Diameter range
Very coarse sand	2.0 to 1.0 mm
Coarse sand	1.0 to 0.5 mm
Medium sand	0.5 to 0.25 mm
Fine sand	0.25 to 0.125 mm
Very fine sand	0.125 to 0.052 mm
Silt	0.052 to 0.002 mm
Clay	< 0.002 mm

Sediment is the unconsolidated inorganic and organic material that is suspended in and being transported by surface water, or has settled out and has deposited into beds.

Static means the test solution is not renewed during the period of the test.

Subchronic toxicity test means a method used to determine the concentration of a substance that produces adverse effects on a specified percentage of test organisms in a specified period of time (e.g., 30 days) which is a significant portion of the organism's life cycle. In this guideline, survival (i.e., death) and growth is used as the measure of toxicity.

Test slurry means the test substance and the natural sediment on which the test substance is sorbed. This sediment/test substance slurry is dosed directly into the tadpole.

(d) **Test procedures**—(1) **Summary of the test.** (i) Test chambers are filled with appropriate volumes of dilution water, or appropriate amounts of contaminated natural sediments and dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired.

(ii) This toxicity test may be performed by either of two methods:

(A) Dosing the tadpole directly with a sediment/test substance slurry and maintaining tadpoles in test chambers with only clean dilution water.

(B) Maintaining tadpoles in test chambers containing contaminated sediments and allowing tadpoles to ingest contaminated sediments ad libitum.

(iii) Tadpoles which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Tadpoles in the test and control chambers should be observed daily during the test. Dead tadpoles should be removed at least twice each day and the findings recorded.

(v) Live tadpoles in the test and control chambers should be weighed at least every 3 days.

(vi) The dissolved oxygen (DO) concentration, pH, temperature, and the concentration of test substance in contaminated sediments and/or water should be measured at intervals in selected test chambers.

(vii) Concentration-response curves, LC50, EC50, LOEC, and NOEC values for the test substance are developed from the survival and growth data collected during the test.

(2) **Range finding test.** If the toxicity of the test substance is not already known, a range-finding test should be performed to determine the range of concentrations to be used in the definitive test.

(3) **Definitive test.** (i) This toxicity test may be conducted by either of two methods:

(A) Dosing the tadpole directly with a sediment/test substance slurry and maintaining tadpoles in test chambers with only clean dilution water.

(B) Maintaining tadpoles in test chambers containing sediments and allowing tadpoles to ingest contaminated sediments ad libitum.

(ii) If this test is to be performed by dosing the tadpoles directly, the sediment/test chemical slurry should be placed directly into the buccal cavity of the tadpole with a pipet. The slurry should be shaken or mixed and 50 µL of the slurry should be placed directly into the posterior portion of the buccal cavity. The dosed tadpole should be held out of the water for about 1 minute after dosing to ensure ingestion and then returned to the test chamber. The test slurry should be prepared by adding 5 mL of distilled water to 1 g of dry sediment; the test chemical is added and the final volume is brought to 10 mL. This test slurry should be mixed on a mechanical shaker for at least 8 h before dosing.

(iii) If this test is to be conducted by maintaining tadpoles in test chambers containing contaminated sediments and allowing tadpoles to ingest contaminated sediments ad libitum, appropriate amounts of contaminated sediments sufficient to cover the bottom of each test chamber with about 3 to 5 cm of the contaminated sediment should be prepared. An appropriate amount of clean dilution water (i.e., about 10 to 20 cm above the sediment) should be added carefully to each chamber followed by tadpoles.

(iv) It is recommended that this test be performed three times, each time with a different natural sediment depending on the organic carbon content: Low (0.1 to 0.2 percent), medium (0.5 to 1.0 percent), and high (2.0 to 3.0 percent) organic carbon content (refer to OPPTS guideline 835.1220). However, natural sediments with a medium organic carbon content should be used if this test is to be done only once. Sediments selected for testing should be characterized by sampling location, general clay fraction mineralogy, percent sand, silt, and clay (particle size analysis), percent organic matter, percent organic carbon, pH (1:1 solids:water), and CEC.

(v) A minimum of 20 tadpoles should be exposed to each of five or more test substance concentrations (i.e., treatments) and a control. Test concentrations should be chosen in a geometric series in which the ratio is between 1.5 and 2.0 mg/kg (e.g., 2, 4, 8, 16, 32, and 64 mg/kg). All test concentrations should be based on milligrams of test chemical (100 percent active ingredient (AI)) per kilogram of sediment (dry weight). The concentration range should be selected to determine the concentration-response relationship, EC50 values, LOEC, and NOEC values for survival, sublethal effects, and growth.

(vi) An equal number of tadpoles should be placed in two or more replicates. The distribution of individual tadpoles among the test chambers should be randomized. Test concentrations in sediment and/or dilution water should be analyzed for test chemical concentrations prior to the start of the test and at designated times during the test.

(vii) Every test should include a control consisting of uncontaminated sediments, the same dilution water, conditions, procedures, and tadpoles from the same group used in the test, except that none of the test substance is added.

(viii) The test duration is 30 days.

(ix) It is recommended that this test be performed under flow-through conditions.

(x) The number of dead tadpoles should be recorded daily. In addition, the number of tadpoles showing sublethal effects and the type of effect (e.g., any abnormal behavior or appearance) should also be recorded daily. Each tadpole should be weighted every 3 days. Data on survival, sublethal effects, and growth which are collected during the test are used to calculate the LC50 value for survival, the EC50 value for sublethal effects, the EC50 value for growth, and to determine the LOEC and NOEC values on days 10, 20, and 30.

(xi) Tadpoles should be fed a suitable food every day. Food which sinks to the bottom should be used; food which floats on the water surface should not be used. In tests in which the tadpoles are dosed with a sediment/test chemical slurry and held in dilution water without sediments, any excess food or fecal material should be removed when observed. In tests in which tadpoles are allowed to feed ad libitum on contaminated sediments, excess food should not be given.

(4) **Test results.** (i) Survival and growth should be the primary criteria used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance or physiology, such as discoloration (e.g., reddened leg), excessive mucous production, opaque eyes, curved spine, or hemorrhaging should be recorded.

(iii) Each test and control chamber should be checked for dead or effected tadpoles and observations recorded every 24 h after the beginning of the test or within 1 h of the designated times. Dead tadpoles should be removed at least twice a day.

(iv) Live tadpoles in the test and control chambers should be weighted at least every 3 days and fresh weights recorded.

(v) The mortality data should be used to calculate LC50 values and their 95 percent confidence limits, and to plot concentration-response curves at 10, 20, and 30 days. The statistical methods recommended for use in calculating LC50 values include probit, logit, moving average angle, and binomial.

(vi) The sublethal effects and growth (i.e., fresh weight) data should be used to plot concentration-response curves, calculate EC50 values, and determine LOEC and NOEC values. The statistical methods recommended for use in calculating the EC50 values include probit, logit, moving average angle, and binomial. Appropriate statistical methods (e.g., analysis of variance and multiple comparison test) should be used to test for significant differences between treatment means and determine LOEC and NOEC values.

(vii) A test is unacceptable if:

(A) More than 20 percent of the control tadpoles die or appear to be stressed, or are seen to be diseased during the test.

(B) The tadpoles in the control lose a significant amount of weight during the test, i.e. 30 percent.

(5) **Analytical measurements**—(i) **Water quality analysis.** (A) The hardness, acidity, alkalinity, pH, conductivity, total organic carbon (TOC) or chemical oxygen demand (COD), and particulate matter of the dilution water should be measured in the control test chambers at the beginning of each static test and at the beginning and end of each flow-through test. The month-to-month variation of the above values should be less than 10 percent and the pH should vary less than 0.4 units.

(B) During static tests, the DO concentration, temperature, and pH should be measured in each test chamber at the beginning of the test, and as often as needed thereafter, to document changes from the initial levels. The dilution water volume should not be reduced by more than 10 percent as a result of these measurements.

(C) During flow-through tests, the DO, temperature, and pH measurements should be made in each chamber at the beginning of the test and every 48 hours thereafter until the end of the test. It is recommended that this test be done under flow-through conditions.

(ii) **Collection of samples for measurement of test substance.** Samples of sediment to be analyzed for the test substance should be taken with a coring device. Samples of dilution water to be analyzed for desorbed test substance should be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples should be analyzed immediately or handled and stored in a manner which mini-

mizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(iii) **Measurement of test substance.** (A) The concentration of test substance in sediment and/or dilution water should be measured at a minimum in each test chamber at the beginning (zero-hour, before tadpoles are added) and every 10 days thereafter.

(B) The analytical methods used to measure the amount of test substance in a sample should be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions. This involves adding a known amount of the test substance to three samples of dilution water or sediment taken from a chamber containing dilution water and the same number of tadpoles as are used in the test. The nominal concentration of the test substance in those samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least 2 separate days prior to starting the test.

(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(D) In addition to analyzing samples of dilution water and sediment, at least one reagent blank, containing all reagents used, should also be analyzed.

(E) Among replicate test chambers, the measured concentrations in sediment should not vary more than 20 percent. The measured concentration of the test substance in sediment in any chamber during the test should not vary more than 30 percent from the measured concentration prior to initiation of the test.

(F) The mean measured concentration of test substance in sediment (dry weight) should be used to plot all concentration-response curves and to calculate all LC50, EC50, LOEC, and NOEC values.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** The test species for this test is the bullfrog tadpole (*Rana catesbeiana*).

(ii) **Age and condition of tadpoles.** (A) Tadpoles having the morphological characteristics of premetamorphic stages VI through IX as described by Taylor and Kollros (1946) under paragraph (g)(3) of this guideline, characterized by the emergence of hind paddles and respiration by gills, should be used. Tadpoles used in a test should be the same age, weight (i.e., 2 to 5 g), and be of normal size and appearance for their age. The longest tadpole should not be more than twice the length of the shortest tadpole.

(B) All newly acquired tadpoles should be quarantined and observed for at least 14 days prior to use in a test.

(C) Tadpoles should not be used for a test if they appear stressed or if more than 5 percent die during the 48 h immediately prior to the test.

(iii) **Acclimation of test tadpoles.** (A) If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48–h period and tadpoles should be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed about 1 °C per hour or 3 °C per day. Tadpoles should be held for a minimum of 7 days at the test temperature prior to testing.

(B) During the final 48 h of acclimation, tadpoles should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(2) **Facilities**—(i) **General.** Facilities needed to perform this test include:

(A) Flow-through tanks for holding and acclimating tadpoles.

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation, and test periods.

(C) Apparatus for straining particulate matter, removing gas bubbles, or insufficiently dissolved oxygen, respectively.

(D) Apparatus for providing a 16–h light/8–h dark photoperiod with a 15– to 30–min transition period.

(E) Chambers for exposing test tadpoles to the test substance.

(F) A dilution water delivery system for flow-through tests.

(ii) **Construction materials.** Construction materials and commercially purchased equipment that may contact the stock solution or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, no. 316 stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be thoroughly conditioned before use. If cast iron pipe is used in freshwater supply systems, colloidal iron may leach into the dilution water and strainers should be used to remove rust particles. Rubber, copper, brass, galvanized metal,

epoxy glues, and lead should not come in contact with the dilution water or stock solution.

(iii) **Dilution water delivery system.** In flow-through tests, the system used should be calibrated before each test. Calibration includes determining the flow rate of dilution water through each chamber. The general operation of the dilution water delivery system should be checked twice daily during a test. The 24–h flow rate through a test chamber should be a minimum of six tank volumes. During a test, the flow rates should not vary more than 10 percent from one test chamber to another or from one time to any other.

(iv) **Test chambers.** Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(v) **Cleaning of test system.** Dilution water delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and rinsed in sequence with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) **Dilution water.** (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if the test tadpoles will survive in it for the duration of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration (i.e., reddened leg), hemorrhaging, disorientation, or other unusual behavior. The quality of the dilution water should be constant and should meet the specifications in the following Table 2. when analyzed (at least twice a year).

Table 2.—Specifications for Dilution Water

Substance	Maximum Concentration
Particulate matter	20.0 mg/L
Total organic carbon (TOC)	20 mg/L
Chemical oxygen demand (COD)	50 mg/L
Un-ionized ammonia	1.0 µg/L
Residual chlorine	1.0 µg/L
Total organochlorine pesticides	50.0 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50.0 ng/L
Organic chlorine	25.0 ng/L

(B) The concentration of DO in the dilution water should be between 90 and 100 percent saturation, or >5 mg/L at sea level. If necessary, the dilution water can be aerated before the addition of the test substance.

All reconstituted water should be aerated before use. Hardness should be <180 mg/L as $CaCO_3$; pH should be 6.5 to 8.5.

(C) If disease organisms (e.g., pathogenic bacteria) are present in the dilution water in sufficient numbers to cause infection, they should be killed or removed by suitable equipment.

(D) Glass distilled or carbon filtered deionized water with a conductivity less than 1 μS/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity, and TOC or COD should be measured on each batch.

(vii) **Carriers.** (A) Distilled water should be used in making stock solutions of the test substance. If a carrier is absolutely necessary to dissolve the test substance, the volume used should be minimal. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution. Concentrations of stock solution should be based on 100 percent AI of the test chemical.

(B) Triethylene glycol and dimethyl formamide are the preferred carriers, but acetone can also be used.

(3) **Test parameters**—(i) **Loading**. The number of tadpoles placed in a test chamber should not be so great as to affect the results of the test. The loading should not be so great that the test substance concentrations in treated sediments are decreased by more than 20 percent due to uptake by the tadpoles. Loading should not exceed one tadpole per liter of dilution water in the test chamber at any time. Loading rates should be adjusted to maintain the DO concentration above the recommended levels and the ammonia concentration below 20 μg/L.

(ii) **Dissolved oxygen concentration.** The DO in each test chamber should be greater than 5.0 mg/L.

(iii) **Temperature.** The test temperature should be about 18 °C. The temperature should be measured at least hourly in one test chamber.

(iv) **Light.** A 16–h light/8–h dark photoperiod with a 15– to 30–minute transition period should be maintained.

(e) **Reporting**. (1) The final report should include, but not necessarily be limited to, the following information.

(i) Name and address of the facility performing the study, and the dates on which the study was initiated and was completed, terminated, or discontinued.

(ii) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(iii) Statistical methods used for analyzing the data. A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

(iv) The test substance identified by name, Chemical Abstracts Service (CAS) registry number or code number, source, lot or batch number, strength, purity, and composition, or other appropriate characteristics.

(v) Stability of the test and, if used, control substances under the conditions of administration.

(vi) A description of the methods used, which should include the following:

(A) Description of the test chambers, the depth and volume of solution in the chamber, the specific way the test was begun (e.g., conditioning and test substance additions), and for flow-through tests, a description of the dilution water delivery system including a diagram if the design is complex.

(B) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter.

(C) The source of the natural sediment (i.e., sampling location), sediment physical-chemical properties, percent sand, silt, and clay (particle size analysis), percent organic matter, percent organic carbon, pH (1:1 solids:water), CEC, general clay fraction mineralogy, and procedures used to determine the above properties.

(D) Methods used to determine the placement of test chambers and the assignment of treatment concentrations to particular test chambers to ensure randomization of exposure.

(E) Frequency, duration, and methods of observations.

(F) Detailed information about the test tadpoles, including the scientific name and method of verification, source of test species, histories of the species, average fresh weight (grams), average size, age, observed diseases, treatments and mortalities, acclimation procedures, and food used.

(G) The number of treatments and replicates used, the number of organisms per replicate, the loading rate, and the flow rate of dilution water for flow-through tests.

(H) A description of the preparation of the sediment/test substance slurry or the treated sediments. A description of the dosing procedures if tadpoles were dosed directly.

(I) The concentration of the test substance in the test slurry or in sediments and/or dilution water in each test chamber just before the start of the test and at all subsequent sampling periods. The concentration of the test substance in the stock solution, if used, and the type and concentration of carrier solvent, if used.

(vii) The measured DO, pH, and temperature and the lighting regime.

(viii) The reported results should include:

(A) The results of the preliminary test and measurements. The number of tadpoles and concentrations of test substance used and observed effects on tadpoles should be stated.

(B) For the definitive test, in each untreated control and for each treatment concentration used:

(*1*) The number of dead and live tadpoles.

(*2*) The percentages of tadpoles that died or showed adverse sublethal effects.

(*3*) The number that showed any abnormal effects.

(*4*) The fresh weights of live tadpoles.

(*5*) The LC50, EC50, LOEC, and NOEC values at days 10, 20, and 30.

Results of the data analysis should include the concentration-response curves with 95 percent confidence limits and the results of a goodness-of-fit (e.g., X^2-square test).

(ix) A description of all circumstances that may have affected the quality or integrity of the data.

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

(xi) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(xii) The signed and dated reports of each of the individual scientists or other professionals involved in the study including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(xiii) The locations where all specimens, raw data, and the final report are stored.

(xiv) The quality control statement prepared and signed by the quality assurance unit.

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

(1) National Research Council. Amphibians: Guidelines for the Breeding, Care, and Management of Laboratory Animals. National Academy of Sciences, Washington, DC (1974).

(2) Perkins, K. W. et al. Reptiles and Amphibians: Care and Culture. Carolina Biological Supply Co., Burlington, NC (1981).

(3) Taylor, A. C. and Kollros, J. J. Stages in the Normal Development of *Rana pipiens*. *Anatomy Records* 94:2 (1946).

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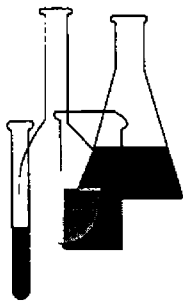
Ecological Effects Test Guidelines

OPPTS 850.1850 Aquatic Food Chain Transfer



"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 850.1850 Aquatic Food Chain Transfer

(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 is OPP 72–6 Aquatic Organism Accumulation Tests (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation: Wildlife and Aquatic Organisms) EPA report 540/09–82–025 (1982).

(b) **Test standards.** Data sufficient to satisfy the requirements in 40 CFR 158.145 should be derived from tests which comply with the following test standards:

(1) **Test substance**. Data should be derived from testing conducted with the technical grade of each active ingredient in the product (studies using radioisotopes require analytical grade) or the purest available form of the principal degradatioc products, whichever has a water solubility of less than 0.5 mg/L, an octanol/water partition coefficient greater than 1,000, and is persistent in water (i.e., a half-life greater than 4 days).

(2) **Test organisms.** (i) Consultation with the Agency is advised before selection of species is made. One or more of the following species may be used in accumulation testing:

(A) A typical bottom-feeding fish (e.g., catfish or carp).

(B) A cold-water fish, a warm-water fish, or marine fish (e.g. brrok trout, rainbow trout, bass, bluegill, northern pike, walleye, or sheepshead minnow).

(C) Molluscs (e.g., oyster or freshwater clams).

(D) Crustaceans (e.g., *Daphnia* spp., shrimp, or crayfish).

(E) Insect nymphs (e.g., mayfly).

(ii) The following factors should be considered in selecting species:

(A) The use pattern of the formulated product.

(B) The relative sensitivity of the different species to toxic effects.

(C) Data on route of exposure and method of uptake.

(c) **Reporting and evaluation of data.** Specific data reporting and evaluation guidance should be determined by consultation with the Agency.

(d) **References.** The following references can provide usefull background information on developing protocols. The conditions under which an accelerated aquatic organism test may be an acceptable substitute for a full-length test should be determined by consulting with the Agency.

(1) Johnson, B.T. and R.A. Schoettger. A biological model for estimating the uptake, transfer, and degradation of xenobiotics in a food chain. FEDERAL REGISTER 40(123):26906–26909. (June 25, 1975).

(2) Macek, K.J. et al. Bioconcentration of ^{14}C pesticides by bluegill sunfish during continuous exposure. Pp. 119–142 in Structure-activity correlations of studies of toxicity and bioconcentration with aquatic organisms. Proceedings of a symposium held at Burlington, Ontario, March 11–13, 1975. G.D. Veith and D.E. Konasewich, eds. Sponsored by the Standing Committee on Scientific Basis for Water Quality Criteria of the International Joint Commission's Research Advisory Board. (1975).

(3) Schimmel, S.C. et al. Acute toxicity to and bioconcentration of endosulfan by estuarine animals. Pp. 241–252 in Aquatic Toxicology and Hazard Evaluation. F.L. Mayer and J.L. Hamelink, eds. STP no. 634, American Society for Testing and Materials, Philadelphia, PA (1977).

(4) Branson, D.R. et al. Bioconcentration of 2,2',4,4'-tetrachlorobiophenyl in rainbow trout as measured by an accelerated test. *Transactions of the American Fish Society.* 104:785–792 (1975).

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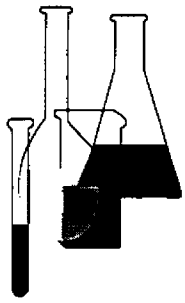
Ecological Effects Test Guidelines

OPPTS 850.1900 Generic Freshwater Microcosm Test, Laboratory



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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 805 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 850.1900 Generic freshwater microcosm test, laboratory.

(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 is 40 CFR 797.3050 Generic freshwater microcosm test (proposed in the FEDERAL REGISTER of September 28, 1987 (52 FR 36344)). This guideline may be used with OPPTS 850.7100.

(b) **Purpose**. This guideline is intended for use in developing data on the chemical fate and/or ecological effects of chemical substances and mixtures (''test substances'') subject to environmental effects testing 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 methodologies to predict the potential fate and/or effects of a chemical substance in freshwater ecosystems using various types of microcosms, i.e., standardized aquatic microcosm, naturally derived mixed-flask culture microcosm, or naturally derived pond microcosm, with and without sediment. The microcosms contain freshwater algae and zooplankton with an assortment of unidentified bacteria and fungi. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the potential hazard of a chemical substance to freshwater ecosystems.

(c) **Definitions.** The definitions in second 3 of TSCA and the definitions in Part 792—Good Laboratory Practice Standards apply to this guideline. The following definitions also apply to this guideline:

Aseptic means free from contaminating organisms, e.g., aseptic transfer of an algal culture into a sterilized tube via a sterile inoculating loop.

Axenic means free from other living organisms. An axenic culture (pure culture) of algae contains only one species of algae, no bacteria, and no fungi.

Batch culture means a culture of organisms that use only the initial supply of nutrients in the culture medium. Without replenishment of nutrients, concentrations of nutrients decline and waste products accumulate in the culture medium with the increase in numbers of organisms.

Bioconcentration factor (BCF) means the ratio of the concentration of the test substance in an organism or tissue (i.e., the biota) to the concentration in microcosm water or sediment, as specified.

Carrier means the organic solvent, solubilizer and/or other substance used to disperse the test substance into microcosm water.

Detritivore means an organism (e.g., ostracod) that feeds on detritus, i.e., dead organic matter.

Ecosystem means a community of organisms and its interrelated physical and chemical environment functioning as a unit.

ECX means the experimentally derived test substance concentration, in the aqueous phase, that is calculated to affect X percent of the test species.

Generic microcosm means a general representation of an aquatic ecosystem in which a microcosm is maintained under constant laboratory conditions and no attempt is made to simulate the physical/chemical environment of the natural system.

Gnotobiotic means a culture or community containing only known species or organisms.

Grazer means an animal that grazes or feeds on growing plants, e.g., daphnids, rotifers, and some protozoa.

Herbivore means an animal that feeds on plants, synonymous with grazer.

Linear contrast means the statistical comparison of the means of two treatment groups, e.g., the control and another treatment group.

Medium means the chemically-defined culture solution used in the microcosms.

Microcosm means a miniaturized model of a natural ecosystem.

Naturally-derived means using an assortment of organisms and/or water and sediment collected from one or more natural freshwater ecosystems.

Net daytime production means the increase in dissolved oxygen (DO) concentration in microcosm water during the light phase of the photoperiod.

Nighttime respiration means the decline in DO concentration during the dark phase.

Semicontinuous culture means an algae culture that is periodically harvested by partial draining and replenished with an equal volume of fresh nutrient solution.

Standardized aquatic microcosm (SAM) means a culture of a community containing known species of algae and aquatic invertebrates, but containing uncharacterized species of protozoa and microorganisms.

Treatment group means the replicate microcosms that receive the same amount (if any) of the test substance; controls are treatment groups that receive none of the test substance.

Unialgal culture means the cultivation or growth of a single species of algae; each species of algae is established and maintained in a separate culture.

Xenic means a culture or community containing one or more kinds of unidentified organisms.

(d) **Test procedures**—(1) **Summary of the test.** (i) In preparation for the test, a sufficient number of containers for the test plus an appropriate number of extra containers should be filled with appropriate volumes of nutrient medium or natural water, numbers and types of organisms, and, in some cases, natural or artificial sediment. Microcosm components should be allowed to interact and adjust to one another for a specified period of time. After culling microcosms which deviate most from the group as a whole, microcosms should be randomly assigned to treatment groups and to specific locations in the test area.

(ii) The test should be started by applying the test substance to the microcosms. Appropriate control groups should be established. Microcosms should be sampled and/or monitored for changes in one or more attributes at specified intervals during the exposure period or the recovery period or both. The means of the attributes should be compared using suitable statistical methods to assess the fate or effects of the test substance. Dose-response curves should be plotted for appropriate attributes.

(iii) Microcosms should be monitored for at least 6 weeks after the test substance is applied. Monitoring may be terminated earlier if all test parameters in the treatment microcosms treated with the test substance remain the same as the control microcosms for 2 weeks after the application of test substance (the last application in the case of multiple applications).

(2) **Administration of test substance**. (i) When possible, it is preferred that a test substance be radiolabeled so that its residues can be rapidly and accurately measured by radioassay.

(ii) A test substance that is soluble in water should be dissolved in distilled water to make a stock solution of known concentration; a nominal concentration of test substance could be established in the microcosm by adding a measured volume of stock solution and thoroughly dispersing it by adequate stirring.

(iii) A test substance that is insoluble in water, but that is soluble in relatively non-toxic, water-miscible solvents, such as acetone, should be dissolved in the minimum volume of carrier solvent required to form a homogenous stock solution of known concentration. At the beginning of the test, a measured portion of stock solution should be added to microcosm water and dispersed to form a homogeneous suspension. Carrier controls should be included in the experimental design and monitored simultaneously with microcosms treated with test substance.

(iv) A test substance that is insoluble in both water and water-miscible solvents should be dissolved in more than one carrier, for example, consisting of a lipophilic solvent and an emulsifier, and a measured portion of stock solution should be dispersed into microcosm water to form a homogeneous suspension.

(v) In the pond microcosm, where stirring is hampered by the macrophyte vegetation and the potential siltation of natural sediment, the stock solution of test substance may be mixed thoroughly with 1 or 2 L of water taken from the microcosm, and poured slowly back into the microcosm while the microcosm water is gently stirred.

(vi) Sufficient quantities of stock solution should be made as needed to minimize storage time and disposal volume.

(vii) If the test substance is a formulated preparation, the strength of the active ingredient (AI) in the preparation and the concentration of the test substance in microcosm water should be specified in terms of percent AI.

(viii) The nominal concentration of test substance in both stock solution and microcosm water should be confirmed by chemical analyses at the beginning of the exposure period.

(3) **Range-finding test**. (i) A range-finding test may be conducted to establish if definitive testing is necessary and, if it is necessary, to establish concentrations of the test substance for the definitive test.

(ii) Culled, old control, or newly established microcosms should be exposed for 2 weeks to a series of test substance concentrations (e.g., 0.1, 1.0, 10, and 100 mg/L). Controls should also be used. The exposure period may be shortened if sufficient data are gathered in a shorter time.

(iii) The lowest test substance concentration in a test series, exclusive of controls, should be the lowest concentration which can be analytically quantified. The highest concentration should be 100 mg/L or the maximum water solubility of the test substance at ambient temperature. Replicates are not needed, and nominal concentrations of the test substance are acceptable for range-finding. If all calculated EC50s for all species are greater than 100 mg/L or less than the analytical detection limit, definitive testing is not necessary. However, replicates and measured concentrations of the appropriate dose are needed to substantiate this result.

(iv) A range-finding test is not necessary if data on environmental concentrations of the test substance are available from monitoring studies, or environmental releases of the test substance are known or can be predicted from models, and the objective of the test is to bracket environmental concentrations which result from the releases. Otherwise, a range-

finding test is advisable since microcosm response can differ significantly from single species tests.

(4) **Definitive test**—(i) **Purpose.** The purpose of the definitive test is to determine the potential ecological effects and/or fate of a test substance released into the freshwater environment.

(ii) **Concentration**. At least three concentrations of test substance, exclusive of controls, should be tested. The concentration range selected should define the dose-response curves for major microcosm species between EC10 and EC90, unless a known environmental or release concentration is being bracketed. A minimum of six replicate microcosms should be used for each concentration.

(iii) **Controls**—(A) **General requirements**. Each test should include controls consisting of the same nutrient medium or natural water, types of biological groups, kind and amount of sediment (if present), and otherwise should be treated the same as exposed groups, except that none of the test substance is added. If a carrier is used to dissolve or suspend the test substance, additional controls containing the carrier should also be included in the test to determine any effect of the carrier on the microcosms.

(B) **Standardized aquatic microcosm**. To demonstrate the health of standardized microcosms in use, untreated controls should meet the criteria specified below; otherwise, test data may be rejected by EPA, unless adequately justified.

(*1*) One day 28, the following criteria should be met in the static microcosms:

(*i*) At least 90 percent reduction in nitrate (NO_3) concentration.

(*ii*) Algal biomass in each mL of medium has exceeded $2{,}000 \times 10^4$ $(\mu m)^3$.

(*iii*) Oxygen gain has exceeded 4 mg/L (ppm).

(*iv*) Population density of daphnids, including members of all size groups, has exceeded 85 *Daphnia* per 100 mL.

(*v*) Coefficient of variation for each microcosm attribute within ± 0.5 more than 50 percent of the time except as noted; coefficient of variation should not be calculated for any nitrate concentration below 2 µM or for oxygen gain below 1 mg/L (ppm).

(*vi*) pH values in late-afternoon between 6 and 10; coefficient of variation among replicate microcosms within ± 0.05 more than 50 percent of the time.

(*2*) From day 28 to the conclusion of the test, the performance of control microcosms should always meet the following criteria:

(*i*) Algal biomass exceeds 100×10^4 $(\mu m)^3$/mL per mL.

(*ii*) Positive oxygen gain in daytime.

(*iii*) Daphnid population density exceeds 15 Daphnia/100 mL.

(*iv*) More than 50 percent of the time, the coefficient of variation is within ± 0.5 among replicates of control microcosms for algal biomass, daphnid population density, and for oxygen gain above 1.00 mg/L (ppm).

(*v*) pH values in late-afternoon between 6 and 9, and coefficient of variation for pH values among control replicates within ± 0.05 more than 50 percent of the time.

(*3*) When control microcosms fail to meet the above criteria, adequate statistical justification is required for EPA acceptance of test data.

(iv) **Initiation and maintenance of microcosms—(A) Standardized aquatic microcosm.** The standardized microcosm should be initiated and maintained as follows:

(*1*) At least 36 glass jars (or more if extra controls are needed) should be filled with 3 L of culture medium, 200 g of acid-washed silica sand, 0.5 g of rinsed chitin, and 0.5 g of cellulose powder, and sterilized in an autoclave as specified in paragraph (e)(2)(ii)(A)(*2*) of this guideline.

(*2*) On day 0, at least 30 of the 36 autoclaved jars containing sterilized culture media should be inoculated with 10 species of algae at 10^3 cells/ mL for each species. Algal cultures are covered and incubated on a white table under adequate illumination.

(*3*) On day 4, algae cultures should be examined for algal abundance, pH, oxygen gain, and other variables and each jar of algal culture should be stocked with five species of animals, which include both grazers and detritivores. The numbers of microinvertebrates to be added to each liter of algal culture are 110 *Hypotrich* protozoans and 30 *Philodina* rotifers. The volume of media with protozoa and rotifers should not exceed 5 mL. The macroinvertebrates to be stocked into each microcosm include:

(*i*) Sixteen daphnids consisting of 3 adults with embryos, 3 adults without embryos, and 10 juveniles.

(*ii*) Six ostracods.

(*iii*) Twelve amphipods consisting of three mating pairs (if possible) and six juveniles.

(*4*) On day 7, the 30 microcosms should be reexamined and any outliers should be culled. At least 24 microcosms should be selected for the test. The following attributes of microcosms should be used in the selection of the 24 microcosms:

(*i*) Dissolved oxygen gain in the daytime.

(*ii*) pH value (pre-light).

(*iii*) Abundance of daphnids and the presence of ostracods and amphipods.

(*iv*) Abundance of *Selenastrum* and *Chlamydomonas.*

(*5*) Selected microcosms should be randomly assigned to one of the treatment groups including the controls, and located on the support table in a six-block design as follows:

(*i*) Each of the 24 selected microcosms (the number of microcosms for a typical test) should be randomly assigned to one of the four treatment groups (including the control), appropriately labeled, and treated with appropriate concentrations of the test substance except that the control microcosm does not receive the test substance.

(*ii*) Each of the six microcosms in each of the four treatment groups should be randomly assigned to one of the six block groups on the table; therefore, each block group has four microcosms, one from each treatment group.

(*iii*) Finally, each of the four microcosms in each block group should be randomly assigned to one of the four specific locations within that block on the table.

(*iv*) To facilitate the handling of microcosms during the test, a series of new numbers should be assigned to the microcosms according to their ordered locations on the table.

(*6*) The test substance should be added after sampling on experiment day 7 (see paragraph (c)(4)(iv)(A)(*4*) of this guideline).

(*7*) The standardized microcosm should be sampled and examined at least once every 7 days after the test substance is added and reinoculated as follows:

(*i*) After sampling and enumeration on each Friday, any microcosm that is underpopulated (less than three individuals) with mature macroinvertebrates should be reinoculated with reproductive age adults so that each microcosm contains at least three individual amphipods, daphnids, and ostracods.

(*ii*) About 0.05 mL (1 drop) of dense *Hypotrich* protozoan culture and the same volume of dense *Philodina* rotifer culture should be added to each microcosm after each examination.

(*iii*) Each microcosm should be reinoculated every 7 days with about 0.05 mL of an algal mixture that is prepared by pooling equal volumes of monoculture from each of the 10 algal species.

(B) **Naturally-derived mixed-flask microcosm.** The mixed-flask microcosm should be initiated and maintained as follows:

(*1*) A culture medium should be prepared from fresh refrigerated stock solution (warmed to ambient temperature before measuring) in sufficient volume to fill each container with 950 mL of culture medium from the same stock solution.

(*2*) Stock cultures, which are derived from biotic samples collected from a variety of ecosystems, should be at least 3 months old before they are inoculated into the microcosms.

(*3*) Each microcosm should contain 50 mL of inoculum, 950 mL of culture medium, and 50 mL of acid-washed sand, and should be randomly assigned to one of the four treatment groups, including controls.

(*4*) Inoculum in each 50–mL beaker should be placed under microcosm water with the beaker and decanted into the microcosm water to avoid exposing the zooplankton to the air during inoculation and cross-seeding.

(*5*) Microcosms are placed in the environmental chamber according to a randomized block design.

(*6*) All microcosms should be cross-seeded at least twice per week for 3 weeks following inoculation. Cross-seeding should be performed by collecting a 50–mL aliquot of a homogeneous suspension from each microcosm, carefully pooling and mixing them together and returning a 50–mL aliquot of the mixture to each microcosm.

(*7*) Each microcosm should be reinoculated weekly with a 50–mL inoculum.

(*8*) Following weekly reinoculation, distilled water should be added to each microcosm to return the volume to 1 L to compensate for loss of water through evaporation.

(*9*) The test substance (and carrier, if needed) should be introduced into appropriate microcosms 6 weeks following initial inoculation of the system.

(C) **Naturally-derived pond microcosm**. The pond microcosms should be initiated and maintained as follows:

(*1*) All microcosm components, including water, sediment and biota, should be collected from a single ecosystem, preferably on the same day they are to be used. A shouldow pond is the best source of material for pond microcosms, but littoral zones of lakes, or slow-moving rivers, may be acceptable alternatives.

(*2*) Water should be collected before sediment. At least 60 L of water should be collected from the pond for each microcosm.

(*3*) Sediment should be collected from the upper 26 cm of the pond bottom and placed in appropriate containers for transportation. Stones, twigs, and other large debris should be removed before the sediment is placed in microcosm containers. At least 12 L of sediment are required for each microcosm.

(*4*) If a macrophyte community is present in the pond, a portion should be collected from the bottom and placed in an appropriate container. All organisms naturally associated with the macrophyte community may be included in the samples except crayfish. At least 100 gm of the macrophytes is needed for each microcosm. If macrophyte communities are unavailable in the pond, filamentous algae communities may be collected instead if present.

(*5*) Water, sediment, and biota should be protected from bright sunlight and extreme temperatures, and placed, as soon as possible, in an environmental chamber that is set at a temperature equal to that of the pond water.

(*6*) The glass aquaria should be positioned in the environmental chamber before filling.

(*7*) Approximately 12 L of sieved sediment should be placed in each aquarium, resulting in a layer of sediment about 6.7 cm thick. Sediment in each transportation container should be equally divided among all microcosms.

(*8*) If interstitial water sampling is planned, two suitable water collectors, such as a glass diffuser, should be embedded in the sediment of each microcosm. The fritted-glass disk of the air diffusers should be positioned 4 cm below the sediment surface which is leveled and smoothed.

(*9*) Approximately 55 L of pond water should be added slowly to each aquarium. Pond water in each transportation container should be equally divided among all microcosms. To minimize resuspension of sediment during water filling, a plastic film may be used to cover the sediment layer and a simple diffuser should be used to dissipate the kinetic force of the water flow. The diffuser may be made of the bottom half of a 4–L polyethylene jug with holes punched around the perimeter.

(*10*) One hundred grams of drained macrophytes or filamentous algae from the source, such as *Elodea canadensis*, should be planted in the sediment in each microcosm.

(*11*) After macrophytes are planted, 1 to 2 L of water remaining in the macrophyte collection container should be added to each microcosm as an additional source of biota.

(*12*) The microcosm should be incubated in the environmental chamber for at least 4 weeks before the test substance is applied.

(*13*) Distilled water should be added to the microcosms periodically to compensate for the loss of water through evaporation. If a significant volume of microcosm water is removed in sampling, it should be replaced with an equal volume of dechlorinated tap water or well water.

(v) **Sampling procedures**—(A) **Ecological effects.** Sampling of microcosms for routine monitoring and final sampling can be performed as follows:

(*1*) Each species of macroinvertebrates, including daphnids, ostracods, and amphipods, in the microcosm can be counted visually if the numbers of animals are less than 20 and the water is clear enough for counting. When a dense population or turbid water hampers direct counting of all macroinvertebrates in the microcosm, a series of 100–mL subsamples should be taken out of the standardized microcosm for enumeration of each macroinvertebrate species until 20 of each invertebrate are counted or 6 subsamples are removed, whichever occurs first. Water samples should be quickly captured and confined in a wide-mouth sampler before removal. Periphyton should be scraped from the glass surface and thoroughly dispersed into the culture media preceding sampling of the water column. Zooplankton should be counted in the mixed-flask microcosm by removing a series of 25 mL subsamples. Four such samples are usually sufficient. In the pond microcosm, zooplankton population should be measured twice per week. They are captured with a 2–L beaker that is submerged rapidly into the microcosm water, concentrated on a 80–μm mesh plankton bucket, stained, and preserved. Population density for three groups of zooplankton, (i.e., cladocera, copepod, and rotifers) should be counted in the pond microcosm: major groups of zooplankton should be identified according to genus, or species if possible.

(*2*) The population density of protozoa and rotifiers should be determined in standardized aquatic microcosms, a water sample of up to 2 mL should be dispersed in a 0.01–, 0.1– or 0.2–mL aliquot on counting plates (e.g., Palmer cell with water depth of 4 mm) at 12× magnification under a stereomicroscope. The total volume of aliquots examined should contain at least 50 individuals per species.

(*3*) The population density of each algal species can be counted twice per week. In the standardized aquatic microcosm, at least 50 cells should be counted for each known algal species from a series of up to 35 fields on the counting chamber under the microscope. If species cannot be identified, the major genus of the phytoplankton and periphyton should be identified for the following groups of algae: diatoms, green algae, euglenoid, and blue-green algae.

(*4*) Filamentous algae in the algal mat should be examined every 7 days with a microscope to detect the potential extinction of any inoculated algae and the possible presence of contaminant algal species.

(*5*) The biomass of primary producers should be estimated twice per week with in vivo fluorescence or optical density of chlorophyll *a* in acetone solution.

(*6*) The rate of uptake of dissolved inorganic carbon-14 by phytoplankton should be measured every 7 days as follows:

(*i*) Primary productivity in each microcosm should be measured in duplicate bottles under the same light intensity as that intensity over the microcosm, with a set of two duplicate bottles placed in the dark as controls.

(*ii*) Dissolved inorganic ^{14}C should be kept sterile before the test. For example, it may be kept in a sealed ampule and autoclaved.

(*iii*) About 100 mL of water should be taken from each microcosm, sieved through a 440–μm nylon screen and placed in a 125–mL conical flask.

(*iv*) The sieved phytoplankton suspension in each flask should be shaken vigorously and poured into a set of four 16.5 mL test tubes until water rises to the rim of each tube, which are then sealed with a serum stopper.

(*v*) About 10 μCi of ^{14}C-labeled $NaHCO_3$ (specific activity about 1.0 μCi/1.0 μg) per milliliter of alkaline aqueous solution should be maintained at pH 9.5, packed in a glass ampule, and sterilized after the ampule is sealed.

(*vi*) About 1 μCi of $NaH^{14}CO_3$ in 0.1 mL aqueous solution should be injected into each of the four 16.5–mL test tubes. Two of the tubes should be immediately placed in the dark inside a light-tight box while the other two should be exposed to the same level of light intensity as that prevailing over the microcosms. All tubes should be vigorously shaken during the 2–h incubation.

(*vii*) After incubation, the phytoplankton culture in each tube should be filtered through a 0.45 μm filter membrane over a vacuum flask. The

membrane filter and the phytoplankton retained on its surface should be dried and stored in a desiccator over silica gel before the radioassay.

(*viii*) Immediately before liquid scintillation counting, each filter membrane with the phytoplankton materials should be fumed over concentrated hydrochloric acid for at least 90 seconds to remove remaining inorganic ^{14}C, and placed in a counting vial for radioassay.

(*ix*) The counting rate for each liquid scintillation counting vial that holds the particulate matter from one of the incubation tubes should be properly calibrated for quenching effects.

(*x*) If the absolute rate of carbon assimilation (besides the relative ^{14}C uptake) is desired, the total dissolved inorganic carbon should be determined. The total content of dissolved inorganic carbon in the microcosm, which affects the specific activity of ^{14}C (added as $NaHCO_3$) in the incubation tube, should be measured simultaneously with measurement of ^{14}C uptake rate. Total CO_2 content is usually calculated from measured values of total carbonate alkalinity and pH in the microcosm water. It can also be measured by gas chromatography if the buffering capacity of the microcosm medium interferes with the alkalinity-pH method.

(*7*) The content of chlorophyll *a* in microcosm water should be measured weekly as follows:

(*i*) A sample of microcosm water, from 30– to 60–mL depending on the standing crop of algae, should be sieved through a 0.3–mm nylon screen to remove any macroinvertebrates among the phytoplankton.

(*ii*) Sieved microcosm water should be filtered under suction through a 0.45 μm filter pad, which is covered with a fine powder of $MgCO_3$ at about 10 mg/cm^2 of filter area. Following filtration, phytoplankton on the filter pad should be immediately extracted for chlorophyll *a* or temporarily stored at –30 °C.

(*iii*) Retained on the filter pad, the phytoplankton and magnesium carbonate should be placed in a glass, pestle-type homogenizer with 3 to 5 mL of 90 percent acetone and homogenized at 500 rpm for about 1 min.

(*iv*) After each homogenate is transferred to a graduated centrifuge tube equipped with a cap, the homogenizer and its pestle should be rinsed 2 to 3× with 90 percent acetone before its next use. The final volume of pooled homogenate and washes should be adjusted to 15.0 mL.

(*v*) After the cap is fastened, the centrifuge tube with its contents should be allowed to stand in a dark, cold (below 10 °C) place for at least 1 h, and centrifuged at 4,000–5,000 g for approximately 10 minutes. Any turbid supernatant should be recentrifuged if its absorbance at 750 nm exceeds 0.005 at 1 cm of light path.

(*vi*) Without disturbing the precipitate, the supernatant in the centrifuge tube should be poured or pipetted into a tube, capped, placed in a dark place, and warmed to room temperature before quantification of chlorophyll *a* by a fluorometric or spectrophotometric method.

(*vii*) In spectrophotometry, the band-width of each monochromatic light should be 3 nm or less. The absorbance (A) of the acetone extract should be measured at 750, 663, 645, and 630 nm against a 90 percent acetone blank. The concentration of chlorophyll *a* (x) in the acetone extract (in micrograms per milliliter) should be calculated from the length of the optical path (in centimeters) and the absorbance at each of the four wave lengths using the formula:

$$[x] = m11.64(A_{663} - A_{750}) - 2.16(A_{645} - A_{750}) + 0.10(A_{630} - A_{750}) \%$$ (light path).

(*viii*) The concentration of chlorophyll *a* in a water sample (in micrograms per milliliter) is calculated by multiplying the concentration in the extract by the volume of the extract (in milliliters), and dividing the product by the total volume of the water sample (in liters).

(*8*) At least twice each week, the peak and troughs on the diel curve of DO in microcosm water can be measured to estimate oxygen gain and loss resulting from daytime photosynthesis and nighttime respiration, respectively. The morning measurement of DO should be taken immediately before the light is turned on, while the afternoon measurement should be taken in the late afternoon or evening after the DO concentration in each microcosm has reached the peak in its diel cycle. At least once during the early part of the study, DO readings should be taken hourly during the light cycle to determine when the peak occurs. The net daytime community production, which is the gain in DO during the 12–hour photophase, should be calculated as the difference between the DO concentration at the end of the photophase and the DO concentration at the end of the preceding dark phase. The net nighttime community respiration, which is the loss of DO in the microcosm during the dark phase, should be calculated as the difference between the DO concentration at the end of the photophase and the DO concentration at the end of the following dark phase.

(*9*) The pH values of microcosm water should be read to 0.01 unit after the pH meter is calibrated with standard buffers of pH 7 and pH 10, and the pH probe should be rinsed very thoroughly between readings. The pH value should be taken at the same time day on scheduled sampling dates after addition of the test substance to the microcosm as, for example, 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, 35, and 42 days after addition of the test substance. It is preferable to take the pH reading at the end of the dark phase to reflect community respiration or at the end of the photophase to reflect photosynthetic activity.

(*10*) Dissolved nutrients in the microcosms should be monitored at least twice each week for the first 4 weeks and at least once every 7 days thereafter; the samples should be filtered through a 0.45 μm membrane and kept frozen before they are analyzed by standard analytical methods for soluble reactive phosphorus, ammonia, nitrite, and nitrate.

(*11*) Net daytime community production and net nighttime community respiration should be measured on scheduled sampling dates as, for example, days 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, 35, and 42 after addition of the test substance.

(12) Biomass decomposition rate, represented by the decomposition rate of ^{14}C-glucose in 15 mL of microcosm suspension, can be measured on scheduled sampling dates as, for example, days 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, and 35, after addition of the test substance to the microcosms. Sampling for biomass decomposition (^{14}C-glucose decomposition) should precede reinoculation if both occur on the same day. The ^{14}C-glucose decomposition should be performed as follows:

(*i*) A 15 mL water sample should be collected in a 50–mL flask.

(*ii*) A glucose solution that contains 0.15 μCi radioactivity in 0.3 mL of distilled water should be added to the flask.

(*iii*) The flask should be immediately sealed with a specially designed serum stopper, fitted with a plastic center well containing a 2 × 5 cm strip of chromatographic paper, and shaken gently for approximately 15 min in the dark.

(*iv*) The heterotrophic activity should be stopped by injecting 1.0 mL of 2N H_2SO_4 into the flask. A CO_2 trapping agent, such as carbosorb, should be immediately injected onto each filter paper under the stopper after the acidification to collect the evolving CO_2.

(*v*) The flask should be gently shaken for at least 2 h, and the ^{14}C activity in the filter paper should be counted with a liquid scintillation counter.

(*vi*) The percentage deviation in the counts per minute (CPM) of the treatment from the control should be calculated.

(*13*) Total alkalinity, dissolved organic carbon, and specific conductivity of microcosm water can be measured weekly.

(*14*) Interstitial water in the sediment, if present, can be collected weekly to be analyzed for ammonium-nitrogen content. The first 5–mL water sample from the embedded gas diffuser, as specified in the pond microcosm, should be discarded, and the second sample of 10 mL should be filtered before chemical analysis.

(*15*) Any extinction of macrophytes, such as *Elodea canadensis* in the pond microcosm, in treated microcosms can be noted during the test, and biomass of macrophytes should be determined at the end of the test.

(*16*) The extinction and reappearance of benthic fauna, such as insects, snails, and oligochaetes, can be observed weekly in those microcosms containing natural sediments.

(*17*) Water-borne bacteria can be counted weekly.

(B) **Chemical fate.** Sampling should be performed according to the following procedures:

(*1*) The initial concentration of test substance in microcosm water should be determined by chemical analysis of samples that are taken immediately after the test substance is thoroughly dispersed in microcosm water.

(*2*) The dissolved test substance, its total residue, or both should be measured in the filtrate of microcosm water semiweekly immediately after the test substance is applied and at least once more during the first week, measured at least once during the second week, and measured biweekly until the end of the test. The filtrate may be substituted with unfiltered microcosm water if the test substance is partitioned into the particulate fraction in such a high proportion that the chemical concentration in the filtrate fraction falls below the analytical detection limit for the test substance using the most practical analytical method.

(*3*) The concentration of test substance in macrophyte shoots, if present, can be measured biweekly if the sample is less than 5 percent of biomass.

(*4*) Distribution of the test substance among compartments of microcosms can be determined at the end of the test; the components may include:

(*i*) Macrophytes, subdivided into roots, shoots, and leaves.

(*ii*) Phytoplankton.

(*iii*) Zooplankton.

(*iv*) Benthic fauna.

(*v*) Sediment core, sectioned into 1–cm subcores.

(*vi*) Periphyton, if any.

(5) **Analytical measurements**—(i) **Chemical.** Standard analytical methods should be used in performing analyses. The analytical method used to measure the amount of test substance in a sample should be vali-

dated by appropriate laboratory practices before beginning the test. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis or oxidation products, give positive or negative interference which cannot be systematically identified and mathematically corrected.

(ii) **Numerical**. (A) The following data should be obtained from the standardized microcosm test:

(*1*) Abundance of each species of alga and macroinvertebrate.

(*2*) Abundance of each type of microscopic animal (i.e., protozoa and rotifers).

(*3*) Net daytime production.

(*4*) Net nighttime respiration.

(*5*) Chlorophyll *a* concentration.

(*6*) Water pH.

(*7*) Nutrients (at least nitrate) in water.

(B) The following data should be obtained from the mixed-flask, microcosm test:

(*1*) Abundance of phytoplankton and zooplankton.

(*2*) Net daytime production (DO gain).

(*3*) Net nighttime respiration (DO loss).

(*4*) Chlorophyll *a* concentration.

(*5*) Water pH.

(*6*) ^{14}C glucose decomposition rate.

(C) The following data should be obtained for the pond microcosm:

(*1*) Abundance of phytoplankton and zooplankton.

(*2*) Abundance of each type of benthic fauna.

(*3*) Net daytime production.

(*4*) Net nighttime respiration.

(*5*) Chlorophyll *a* concentration.

(*6*) Water pH, alkalinity, conductivity, and dissolved oxygen.

(*7*) Concentrations of the test substance in each compartment of the microcosm.

(*8*) Bioconcentration factor.

(D) Means and standard deviations of each chemical and biological attribute specified in this test rule should be calculated for the replicates of each treatment and control groups.

(E) EC50 values and their 95–percent confidence limits should be calculated for each of the appropriate attributes for the time between application of the test substance and recovery from test substance treatment, if data are adequate for statistical analysis. Otherwise, ECX should be calculated as the percent deviation of an attribute in a treatment group from that in the control.

(F) Appropriate statistical analyses (e.g., Dunnett's procedure) should be performed to determine whether significant differences in attributes exist between the carrier (if appropriate) and carrier-free controls and between the control and treated groups, and between microcosms receiving different concentrations of test substance.

(G) For the pond microcosm, appropriate statistical analyses should be performed to determine whether significant differences in the amount and in the bioconcentration factor of the test substance exist between treated different compartments within treated microcosms and between treated microcosms receiving different treatments.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** (A) The organisms inoculated into the standardized microcosm should include 10 algal species; 1 each of protozoa, rotifer, daphnid, ostracod, and amphipod species; and an uninvited assortment of unidentified heterotrophs, such as bacteria and fungi.

(*1*) The following 10 species of algae should be included: *Anabaena cylindrica*, *Ankistrodesumus* sp., *Chlamydomonas reinhardi*, *Chlorella vulgaris*, *Lyngbya* sp., *Nitzschia kutzigiana*, *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Stigeeclonium* sp., *Udotheric* sp.,

(*2*) *Daphnia magna* should be included. Species identity of the test daphnids should be verified using appropriate systematic keys.

(*3*) Amphipods, *Hyaletia azteca*, also named *H. knickerbockeri*, should be used in the test. Mating pairs and the young are inoculated into the microcosm.

(*4*) Ostracods chosen should be either *Cypridopsis* or *Cyprinotus* sp. Only adults should be used.

(*5*) Protozoa should belong to the order *Hypotrichida*, and the culture should be 72–h–old when it is inoculated into the microcosm.

(*6*) Rotifers should belong to the *Philodina* sp.

(B) Inoculum for the mixed-flask microcosm test should at least contain the following:

(*1*) Two species of single-celled green algae or diatoms.

(*2*) One species of filamentous green alga.

(*3*) One species of nitrogen-fixing blue-green alga.

(*4*) One species of grazing macroinvertebrate.

(*5*) One species of benthic, detritus-feeding macroinvertebrate.

(*6*) Bacteria and protozoa.

(C) The following broad groups of biota should be included in the pond microcosm: Macrophyte, phytoplankton, periphyton, zooplankton, and benthic animals.

(ii) **Source.** (A) Each unialgal culture that is a part of the 10-species composite inoculum for all standardized microcosms in a test should be of the same batch that in turn is subcultured to the exponential growth phase from a single source. Before the test, at least two successive subcultures outside the microcosm are required to acclimate the algal monoculture from agar slant to microcosm medium. A semicontinuous culture system is recommended for culture of unicellular algae.*Anabaena, Ankistrodesumus, Selenastrum,* and *Lyngbya* should be cultured in batch culture before they are inoculated into microcosms. Recommended procedures for culturing algae as well as the other organisms used in this test are described by Taub and Read under paragraph (g)(2) of this guideline.

(B) The original stock culture for the mixed-flask microcosm should be collected from a variety of natural ecosystems. New stock culture should be added to the old stock cultures at least twice each year. To prepare the inoculum for microcosms, samples from several different aged stock cultures should be mixed together. Stock cultures should be at least 3 mon old to be used as a source of microcosm inoculum. Distilled water should be added to the stock cultures in the open aquaria as needed to replace losses by evaporation. Aquatic organisms collected from a variety of natural ecosystems should be inoculated into culture medium to start stock cultures.

(C) Organisms for the pond microcosm should be obtained from the same natural ponds that supply the water and sediment used in the microcosm.

(2) **Facilities**—(i) **Apparatus.** (A) The environmental chambers or room housing the microcosms should provide adequate environmental controls to meet temperature, irradiation, photoperiod, and air circulation requirements. Chambers should be designed to prevent escape of contaminated internal air into the external environment by using appropriate filtering devices to prevent contamination of the external environment with the test substance.

(B) Laboratory facilities where the test substance is handled should have nonporous floor covering, absorbent bench covering with impermeable backing, and adequate disposal facilities to accommodate liquid waste (e.g., test and waste solutions containing the test substance at the end of each test), and solid wastes (e.g., bench covering, lab clothing, disposable glassware, or other contaminated materials).

(C) The test substance should be stored in a room separate from stock cultures and microcosms.

(D) A large autoclave capable of sterilizing several 1-gal microcosm containers should be used. An autoclave large enough for sterilizing culture medium in a 20–L (5–gal) carboy is desirable.

(E) The dimensions of the bench space for the gnotobiotic microcosms should be at least 2.6 × 0.85 m and should have a white top or white covering.

(F) Standard laboratory equipment and, if the test substance is radiolabeled, a liquid scintillation counter for radioassays is required.

(G) For the standardized and mixed-flask microcosm tests, a special sampler should be used to capture macroinvertebrates from the microcosm. The sampler should be taller than the microcosm to reach the bottom of the jar, have a large diameter for free passage of water into the sampler, and a rubber stopper attached to a long glass rod to stir the water before sampling and to seal the bottom of the sampler for transferring water out of the microcosm after the sample is taken.

(ii) **Containers and media**—(A) **Standardized microcosm.** (*1*) The containers used in each standardized microcosm test should be new glass jars with the capacity of at least 1 gal (3.8 L). The jars should be at least 25 cm in height and 16.0 cm in diameter, with an opening 10.6 cm in diameter. The new jars should be washed with diluted (1:10) HCl, flushed with tap water, and rinsed with distilled water before use.

(*2*) Each standardized microcosm should contain at least 3 L of a medium, such as Taub's T82MV, in addition to an artificial sediment made of silica sand (200 g) enriched with chitin (0.5 g) and cellulose (0.5 g). Before use, the sand should be washed with diluted (1:10) HCl for 2 h, repeatedly rinsed with clean water until the pH rises to 7, and dried in

an oven at 120 °C. The crude chitin from commercial sources should be rinsed with distilled water, air-dried, ground in a blender, and sifted through a 0.4 mm sieve. The cellulose powder, which is also packing material for chromatographic columns, is commercially available.

(B) **Naturally derived mixed-flask microcosm.** Hard-glass containers (e.g., 1–L Pyrex beakers), should be selected for testing organic substances in mixed-flask microcosms. Polypropylene beakers may be used for testing inorganic substances.

(C) **Naturally derived pond microcosm.** For the pond microcosm test, 72–L glass aquaria (60 cm long by 30 cm wide by 40 cm deep) should be used as containers. About 12 L of sieved sediment and 55 L of pond water should be added to each aquarium.

(D) **Materials and equipment.** Materials and equipment that contact test solutions should be selected to minimize sorption of test substances from the microcosm and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(iii) **Cleaning and sterilization.** Microcosm containers, stock culture containers, nutrient storage containers, and all other glassware should be cleaned before use. All glassware and equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or previous use. Dichromate solution should not be used for cleaning glassware. In the standardized microcosm, all glass containers and equipment for culturing and testing organisms should be sterilized by autoclave where possible. DO and pH probes may be cleaned with ethanol and thoroughly rinsed with distilled water before use. All sampling devices should be sterilized before each test; sampling devices in contact with lake water or sediment should be sterilized after each use.

(iv) **Nutrient media.** (A) Taub's T82MV (see paragraph (g)(2) of this guideline) medium is recommended for use in the standardized microcosm. Its composition is given in the following Table 1.

Table 1.—Nutrient Medium, Taub T82MV

[pH adjusted to 7.0 with dilute HCl (1:10)]

Compound	Molecular weight	Concentration		
		Units	Element of concern	mg/L
		mM		
$NaNO_3$	85.0	0.5	N	7.0
$MgSO_4 \cdot 7H_2O$	246.5	0.1	Mg	2.43
KH_2PO4	136.0	0.04	P	1.23
NaOH	40.0	0.099	Na	2.27
$CaCl_2 \cdot 2H_2O$	147.0	1.0	Ca	40.0
NaCl	58.5	1.5	Na	34.5
$Al_2(SO_4)_3 \cdot 18H_2O$	666.5	0.0048	Al	0.26
$Na_2SiO_3 \cdot 9H_2O$	284.0	0.80	Na	36.8
sand			Si	22.4
Trace Metals:		µM		
$FeSO_4 \cdot 7H_2O$	278.0	1.12	Fe	0.0625
H_3BO_3	61.8	0.75	B	0.008
$ZnSO_4 \cdot 7H_2O$	287.5	0.025	Zn	0.0015
$MnCl_2 \cdot 4H_2O$	197.9	0.25	Mn	0.0135
$Na_2MoO_4 \cdot 2H_2O$	242.0	0.025	Mo	0.0024
$CuSO_4 \cdot 5H_2O$	249.7	0.005	Cu	0.00032
$Co(NO_3)_2 \cdot 6H_2O$	291.0	0.0025	Co	0.00015
EDTA	292.0	1.42	EDTA	0.4145
Vitamins:				
Calcium pantothenate	476.5	1.47	–	0.70
Cyanocobalamin (B_{12})	1,355.4	0.000022	–	0.00003
Thiamin (B_1)	337.3	0.18	–	0.06
Riboflavin (B_2)	376.4	0.11	–	0.04
Nicotinamide	122.1	1.06	–	0.13
Folic Acid	441.4	0.75	–	0.33
Biotin	244.3	0.12	–	0.03
Putrescine	161.1	0.19	–	0.03
Choline	181.7	2.75	–	0.50
Inositol	216.2	5.09	–	1.10
Pyridoxine monohydrochloride	205.7	2.43	–	0.50

(B) The recommended medium for growth and establishment of stock cultures for the mixed-flask microcosm is Taub's T82, which is the same as T82MV without vitamins. The modified Taub's no. 36 medium (Leffler 1981) under paragraph (g)(1) of this guideline used in the early protocol development is also adequate.

(C) There is no need to add nutrients to pond microcosms.

(3) **Test parameters.** Environmental conditions for the microcosms should be maintained as follows:

(A) Temperature within 21 to 25 °C (23 ± 2 °C).

(B) Photoperiod of 12 h light/12 h darkness.

(C) Standard deviation of light intensities among the microcosms within ±10 percent of the mean and a light intensity of 150 $\mu Em^{-2}sec$ for this test.

(e) **Reporting.** (1) The final report should include, but not necessarily be limited to, the following information:

(i) Name and address of the facility performing the study and the dates on which the study was initiated and completed, terminated, or discontinued.

(ii) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(iii) Statistical methods used for analyzing the data.

(iv) The test substance identified by name, Chemical Abstract Service (CAS) Registry number or code number, source, lot or batch number, strength, purity, and composition, or other appropriate characteristics.

(v) Stability of the test substance under the conditions of administration.

(vi) A description of the methods used, including the facilities and supporting equipment.

(vii) A description of the test system used, including: Microcosm dimensions and water volume, sediment type and volume if used, temperature, photoperiod, and light intensity over the water surface.

(viii) A description of the organisms included in the microcosms representing various functional groups that are essential for the maintenance of a healthy microcosm.

(ix) A description of the nutrient media, if applicable.

(x) A description of the experimental design, treatment concentrations and media, and pattern of administration.

(xi) The materials, the methods, and the results of any range-finding test.

(xii) For the definitive test, reported results should include:

(A) For the standardized microcosm, a description of the following ecological effects and the fate of the test substance in the biota:

(*1*) Phytoplankton abundance, in numbers per milliliter, for each species.

(*2*) Population density of rotifers and protozoans, in numbers per milliliter, for each species.

(*3*) Abundance of daphnids, in numbers per liter, for each size group (small, medium, and large).

(*4*) Abundance of amphipods, in numbers per microcosm, for each size group (small and large).

(*5*) Abundance of ostracods, in numbers per microcosm.

(*6*) Relative abundance of phytoplankton in microcosms.

(*i*) Absorbance density at 440 nm, as an index of the particulate materials, including phytoplankton.

(*ii*) Content of chlorophyll *a*.

(*iii*) In vivo fluorescence.

(*7*) Concentrations of major mineral nutrients, such as orthophosphate, ammonia, nitrite, and nitrate in the filtrate of microcosm water.

(*8*) Primary productivity, as measured by ^{14}C-uptake methods.

(*9*) Community production and respiration, measured by the three-point methods (the net gain in dissolved oxygen during the photophase is the photosynthetic production of phytoplankton, while the loss of DO during the dark phase is an indicator of community respiration).

(*10*) Carrier effects when a carrier is used. Assessed by comparing biological variables in carrier controls to those in plain-water controls.

(*11*) Chemical effects assessed by comparing biological data in treated microcosms to that in plain-water controls or in combined controls for both the carrier and plain water.

(B) For the mixed-flask microcosm, a description of the following ecological effects and the fate of the test substance in biota:

(*1*) Phytoplankton abundance for the entire community or standing crop for each of the major species, in number of plants per milliliter.

(*2*) Zooplankton abundance for the community or standing crop for each life stage of the major species, in numbers of animals per liter.

(*3*) Type and total number of the benthic organisms, or the standing crop for each species of benthic organism, in numbers of organisms per square meter.

(*4*) Carrier effects when carrier is used.

(*5*) Chemical effects assessed by comparing treated microcosms to controls.

(*6*) EC50 values for the test substance expressed in terms of pH change, net daytime community production, net nighttime community respiration, and decomposition rate of organic matter.

(*7*) Concentration of test substance residues in aquatic organisms or in specific tissues.

(*8*) The bioconcentration factors of the test substance or its total residues.

(*9*) Effect of the initial concentration of the test substance on its bioconcentration factor.

(C) For the pond microcosm, a description of the following ecological effects and fate of the test substance in biota:

(*1*) Phytoplankton abundance for the entire community or standing crop for each of the major species, number of phytoplankton per milliliter or chlorophyll *a* concentration.

(*2*) Chlorophyll *a* content of periphyton and the major groups of periphytons, such as diatoms, green algae, blue-green algae, and euglenoid, if possible, genus or species names.

(*3*) Abundance of macrophytes in the microcosm calculated by estimating the volume of microcosm water occupied by the macrophytes and determining the standing crop of the macrophytes, including tops and roots.

(*4*) Zooplankton abundance for the community or standing crop for each life stage of the major species, in number of animals per liter.

(*5*) Type and total number of benthic organisms, or standing crop for each species of benthic organism, in number of organisms per square meter.

(*6*) Concentration of major dissolved nutrients, such as ammonium-nitrogen, nitrate and nitrite, and orthophosphate, in microcosm water.

(*7*) Carrier effects when carrier solvent is used.

(*8*) Chemical effects assessed by comparing treated microcosms to controls.

(*9*) The median effect concentration (EC50) and its 95–percent confidence limit if the concentration of test substance causes partial reduction in any biological attribute in enough treatment groups. If the partial reduction occurs in only a few treatment groups, indicate the percentage reduction of biological abundance caused by the concentration of test substance (ECX).

(*10*) Element cycling such as ammonium-nitrogen content, in micrograms per liter.

(*11*) Maximum and minimum diel DO concentration on sampling date.

(*12*) Net daytime production and net nighttime respiration, in milligrams per liter of DO change.

(*13*) Ratio of production to respiration (P/R ratio).

(*14*) Concentrations of the test substance in both particulate and dissolved fractions of the water column.

(*15*) Concentration of test substance in representative species of zooplankton and benthos.

(*16*) Concentration of test substance in periphyton.

(*17*) Vertical distribution of the test substance in the sediment core.

(*18*) Concentrations of the test substance in total biota.

(*19*) Concentrations of the test substance which may include its transformation products, at steady state in the water column and sediment profile, and the amount in the periphyton on the glass surface.

(*20*) Effect of the test substance concentration applied to the microcosm on the residual concentration of the test substance in each compartment.

(*21*) Bioconcentration factors of the test substance or its total residues.

(*22*) Effect of the initial concentration of test substance on its bioconcentration factors.

(D) A description of any circumstance that may have affected the quality or integrity of the data, including reporting and explaining any significant excursions from normal for microcosm parameters during the test.

(xiii) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(xiv) A description of the transformations, calculations, or operations performed on the data, and a statement of the conclusion drawn from the analysis.

(xv) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted

an analysis or evaluation of data or specimens from the study after data generation was completed.

(xvi) The locations where all specimens, raw data, and the final report are stored.

(xvii) The statement prepared and signed by the quality assurance unit.

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

(1) Leffler, J.W. *Tentative protocol of an aquatic microcosm screening test for evaluating ecosystem-level effects of chemicals.* Final report, EPA Contract No. 68–01–5043 (Subcontract No. T6411(7197)025 with EPA Office of Toxic Substances, Washington, DC (1981)). Available from J.V. Nabholz, 7403, Environmental Effects Branch, Health and Environmental Review Division, Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, 401 M St., SW., Washington, DC 20460–0001.

(2) Taub, F.B., and Read, P.L. *Standardized aquatic microcosm protocol.* Draft final report, U.S. Food and Drug Administration Contract No. 223–83–7000 with FDA, Washington, DC 20005 (1986). Available from Dr. B.L. Hoffmann, U.S. FDA, HFF–304, Environmental Impact Staff, 1110 Vermont Ave., NW., Suite 710, Washington, DC 20005.

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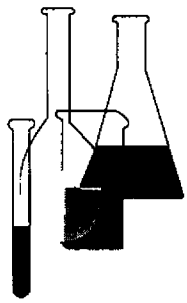
Ecological Effects Test Guidelines

OPPTS 850.1925 Site-Specific Aquatic Microcosm Test, Laboratory



"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 850.1925 Site-specific aquatic microcosm test, laboratory.

(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 is 40 CFR 797.3100 Site-Specific Aquatic Microcosm Test (proposed in the FEDERAL REGISTER of September 28, 1987 (52 FR 36344)).

(b) **Purpose.** This guideline is intended for use in developing site-specific data on the chemical fate and ecological effects of chemical substances and mixtures (''test substances'') subject to environmental effects test regulations. This guideline prescribes methodologies to predict the potential fate and/or effects of either organic or inorganic substances in a natural aquatic ecosystem using a microcosm made of an indigenous water column and sediment core. This test system is capable of evaluating organic chemical substances, either soluble or insoluble, which may form either air-water surface films or aggregates which sink to bottom sediments. The EPA will use data from this test in assessing the potential hazard of a chemical substance to a particular natural aquatic system (natural system).

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

Benthic community or benthos means numbers, species composition, size range, and feeding types of organisms present in the sediment of the natural system.

Benthic subsystem means an undisturbed core collected from the natural system and placed in the microcosm.

Bioaccumulation factor or *bioconcentration factor* means the ratio of the concentration of the test substance in an aquatic organism (i.e., biota) to the associated exposure concentration of the test substance from the food particles and the surrounding exposure medium (i.e., water or sediments).

Carrier means the organic solvent, solubilizer and/or other substance used to disperse the test substance into microcosm water.

Chemical residues means the test substance and its transformation products retained in the water, sediment, surface film, biota, and glass surfaces of the microcosm during the experiment period.

Exposure concentration means the concentration of test substance in the water or the sediment in which the aquatic organisms live.

Natural aquatic system or *natural system* means a particular geographic location consisting of a water column and its associated benthic component.

Radioactivity budget or *radioactivity mass balance* means a quantitative relationship among the input, retention, and export of radioactivity in a microcosm after applying a radiolabeled test substance into the microcosm. The amount of radioactivity added to the microcosm during the test usually is equal to the sum of the radioactivity remaining in the microcosm compartments and the radioactivity exported from the microcosm with the departed water, surface film, and exhaust air.

Ratio of benthic surface area to water volume means the ratio obtained by dividing the calculated benthic surface area of the natural system by the best estimate of water volume of the system.

Sediment means the bottom substrate existing at the mean water depth within the natural system during the period of the test.

Site-specific aquatic microcosm means a miniaturized mimic of a specific natural aquatic system.

Slick protector means a partially submerged glass cylinder within which surface film is removed.

Water column means the water within the natural system or the microcosm tank.

Water flow rates over the sediment surface means the rate of average water flow over the surface of the sediment as measured in the natural system or in the microcosm tank.

Water replacement or *replacement water* means the natural water added to the microcosm at specific intervals to simulate water turnover rate.

Water turbulence means the average water motion in the water column of the natural system or the microcosm tank during the test.

Water turnover rate or *residence time* means the time required for one complete water replacement or exchange within the natural system.

(d) **Test procedures**—(1) **Summary of the test.** A site-specific microcosm is constructed with an indigenous water column and the intact sediment core associated with it. The water and sediment retain their associated organisms in the pelagic and benthic components, respectively, of the natural aquatic system. Environmental variables such as temperature, water turbulence, and water turnover rate are manipulated to be similar to the conditions found in the natural aquatic system. After the test substance is initially introduced into the microcosm, the fate of the test sub-

stance as well as properties indicative of the structure and function of the microcosm are monitored for at least 30 days. Effects of the test substance on the abundance and diversity of aquatic life, and on elemental cycling in the microcosm are determined by comparisons with microcosms that do not contain the test substance.

(2) **Administration of test substance.** (i) Only test substances that are resistant to photolysis (i.e., those having a half-life greater than or equal to 30 days) should be tested in this microcosm system.

(ii) All the test substance added to the microcosms during the study should be accounted for by mass balance. If the test substance is degradable (not persistent), it is recommended that the test substance be radiolabeled.

(iii) Test substances can be either gases, liquids, or solids and may or may not be soluble in water.

(A) If the test substance is soluble in water, it should be dissolved in distilled water to make a stock solution of known concentration. Measured portions of the stock solution should be added to the water in the microcosms and thoroughly dispersed by adequate stirring.

(B) If the test substance is insoluble in water but soluble in a relatively nontoxic, water-miscible solvent such as acetone, it should be dissolved in the minimum volume of carrier solvent required to form a homogeneous stock solution of known concentration. A measured portion of the stock solution should be dispersed into the microcosm water at the beginning of the test to form a homogeneous suspension. Carrier controls should be included in the experimental design and monitored simultaneously with the microcosms treated with the test substance.

(C) If the test substance is a solid and is insoluble in either water or a suitable carrier, it should be ground to a fine powder, weighed to achieve the mass required, and added to a 1–L aliquot of the test water contained in a 2–L separatory funnel. The separatory funnel should be shaken vigorously to achieve as homogeneous a suspension as possible and the suspension should be added to the microcosm water.

(D) If the test substance is a liquid, the measured portion should be added to 1 L of the microcosm water contained in a 2–L separatory funnel, and shaken to achieve as homogenous a suspension as possible. The suspension should be mixed and added to the microcosm tanks.

(E) The amount of test substance remaining in the separatory funnel must be determined by suitable solvent extraction and analyses to accurately determine the amount added to the microcosms.

(iv) Sufficient quantities of the stock solution should be made as needed to minimize storage time and disposal volume.

(v) A test substance that is insoluble in both water and water-miscible carriers should be dissolved in more than one carrier, for example, consisting of a lipophilic solvent and an emulsifier, and a measured portion of stock solution should be dispersed into the microcosm water to form a homogeneous suspension.

(vi) The method and pattern of applying a test substance to microcosms should reasonably reflect the release pattern expected in the natural system. If the input of the test substance to the natural system is other than a one dose application (i.e., multiple application, runoff), the test substance must be added to the microcosm tank in the same manner as the initial dose and each time there is a microcosm water replacement, but only in quantities sufficient to achieve the desired test concentrations in the replacement water.

(3) **Selection of treatment concentration**. (i) Range-finding tests are not recommended, but may be needed to determine treatment concentrations.

(ii) Initially, the microcosms should be treated with concentrations of the test substance that are 0.1, 1, and 10× as high as the average ambient concentration of the test substance observed or predicted in the natural system.

(iii) The test substance should be tested in concentrations of 1, 10, and 100 μg/L, if reliable data on observed or predicted average ambient concentrations are not available.

(4) **Definitive test.** (i) The purpose of the definitive test is to determine the potential fate and ecological effects of a test substance in a specific aquatic ecosystem.

(ii) At least three concentrations of the test substance, exclusive of controls, should be tested for at least 30 days. A minimum of five replicate microcosms should be used for each concentration. All tanks within a given airtight compartment should be treated with the same concentration of the test substance.

(iii) A minimum of five control microcosms should be used in the test for each water-soluble test substance. For those test substances that require a carrier, two of the five control microcosms should be designated carrier controls and treated with the carrier leaving the remaining microcosms as carrier-free controls.

(iv) Two tests are recommended for each test substance. One should be performed in the summer and another in the winter if the fate and ecological effects of the test substance are expected to vary significantly with seasons.

(v) Microcosms should be installed and maintained in the following manner:

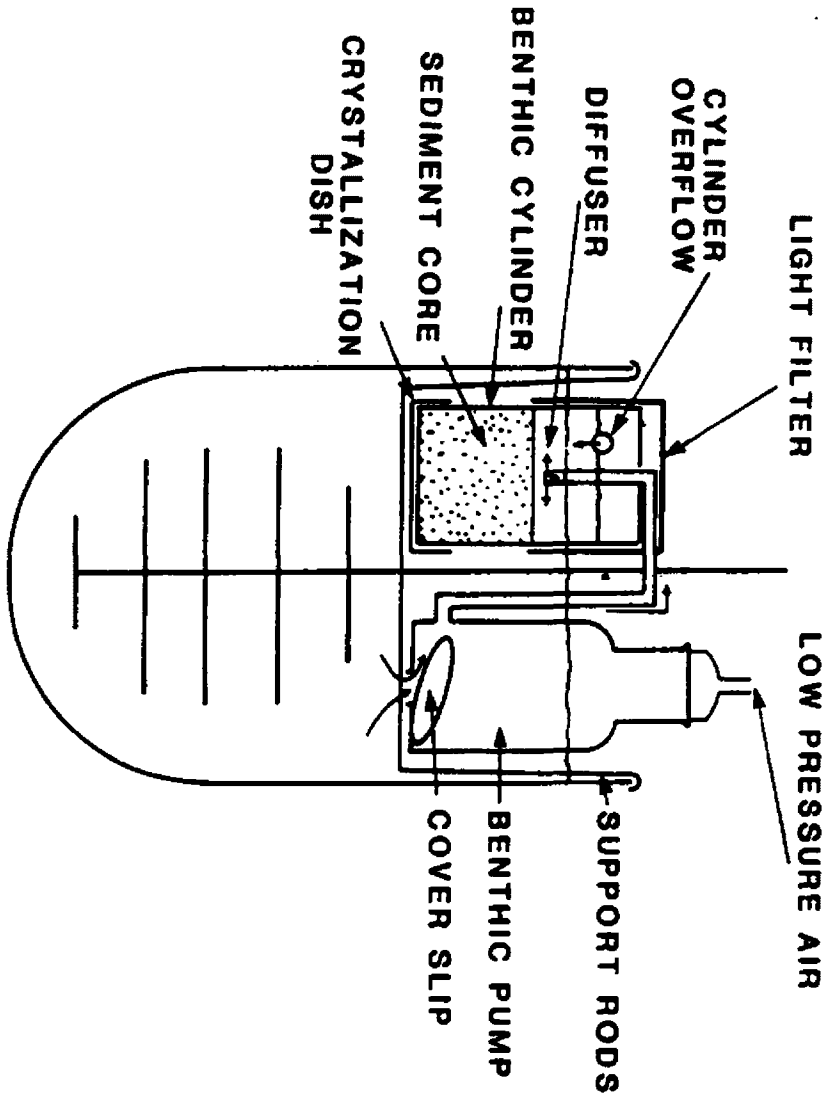
(A) All microcosm tanks should be placed in a water bath maintained within ± 1 °C of the ambient water temperature in the natural system. Water may be pumped from the natural system into the water bath to regulate the temperature in the microcosms if the test laboratory is nearby.

(B) Water for the microcosm should be collected from the natural system, at mid-tide for estuaries, by hand bucketing or nondestructive pumping, e.g., diaphragm pump. If the natural water column in the natural system is stratified, the microcosm water should contain subsamples taken from various depths.

(C) Water samples should be transported to the test facility in glass containers. On arrival at the test facility, water in each container should be distributed equally among microcosms to a prescribed volume of approximately 140 L. Plankton samples must be collected from each microcosm tank and analyzed to ensure homogeneous distribution.

(D) Each sediment core should be collected undisturbed from the natural system by inserting a glass cylinder into the sediment and extracting the core from a prescribed location. The bottom of the core is sealed by seating it in a crystallization dish slightly larger than the cylinder in the following Figure 1. It is desirable to use scuba divers to inspect the uniformity of the benthic component in the natural system, to select representative cores of appropriate length to preserve intact habitats, and to collect the cores with as little disturbance as possible.

FIGURE 1.—EXPERIMENTAL MICROCOSM (NOT DRAWN TO SCALE)



(E) The ratio of benthic surface area to water volume in the microcosm should be made equal to that ratio in the natural system being simulated. Because the water volume in the microcosm is fixed, the desired ratio is obtained by selecting benthic cylinders with the appropriate inner diameter.

(F) The benthic cylinder housing the sediment core should be mounted in the microcosm tank so that the overflow port of the box is 5 cm above the water level in the tank (see Figure 1. in paragraph (d)(3)(v)(D) of this guideline). Any disturbed sediment should be allowed to settle for at least 30 minutes before starting water circulation in the benthic box and water turbulence in the microcosm tank.

(G) The benthic pump should be mounted beside the benthic cylinder with the outlet diffuser of the pump submerged below the surface of the water (overflow port of the cylinder) but above the sediment surface (see Figure 1. in paragraph (d)(3)(v)(D) of this guideline). The rate of water flow over the sediment surface in the microcosm tank should be adjusted to be equivalent to the average water flow rate over the sediment surface in the natural system.

(H) The light intensity over the microcosms should be adjusted to produce an abundance of phytoplankton statistically equivalent to that in the natural system. Preliminary tests should be performed to establish the proper light intensity over the microcosms and should be done with all the microcosm equipment and facilities (i.e., water bath, tank paddle, benthic cylinder and pump) in place. The preliminary tests should be performed at several light intensities for at least 14 days. The photoperiod in both preliminary and definitive tests should be set once every 7 days to match the actual photoperiod within 0.5 h in the location of the natural system.

(I) The light intensity on the surface of the sediment core in the microcosms should be adjusted to the level that is equivalent to the average light intensity on the sediment surface in the natural system. Light intensity can be adjusted by covering the upper portion of the benthic cylinder with a screen, such as a nylon net, or other spectrally-neutral light filters.

(J) The speed of the stirring paddle installed in the microcosm tanks should be adjusted to generate a water turbulence level statistically equivalent to that in the natural system, as measured in the gypsum dissolution method. This method measures the turbulence level by the average dissolution of pure gypsum. Weight loss should be at least 5 to 10 percent. This may take several hours depending on temperature and turbulence. Dissolution rates should be measured and water turbulence adjusted in the microcosms before each test.

(K) Any resuspended sediment that settles on the bottom of a microcosm tank should be collected with a tubing pump and returned to the benthic cylinders when water turnover is simulated.

(L) Water turnover in the natural system should be simulated in the microcosm as follows:

(*1*) A measured portion of the water in each microcosm tank should be replaced at least three times every 7 days with water newly collected from the natural system.

(*2*) The water replacement should match the water turnover rate observed in the natural system.

(*3*) Water replacement should be scheduled immediately after sampling of microcosm water and should occur on the same day.

(*4*) The volume of microcosm water to be removed each time should be the difference between the calculated volume to be replaced and the total volume of water samples removed to keep the water volume at 140 L.

(M) If the test substance accumulates in a thin film on the surface of water in the microcosm tank, a portion of the film should be removed with a filter pad or other absorbent material prior to removal of the volume of water to be replaced. This simulates the surface film advective transport from the natural system. The area (in squarecentimeters) of surface film to be removed should be equal to the product of the ratio of the replacement water volume to total tank volume ratio and the surface area of the tank water, minus the area displaced by the benthic pump and cylinder.

Film area removed =

$$\frac{\text{Replacement water}}{\text{Total tank volume}} \times [\text{Tank water surface area} - (\text{Benthic pump area} + \text{Benthic cylinder area})]$$

(vi) Sampling procedures for the study of chemical fate should be performed as follows:

(A) Water samples should be taken at approximately 0, 1, 2, 3, 6, 12, and 24 h after the initial application of the test substance. Therefore, samples should be taken before each water replacement. Water samples should be collected through a slick protector within which the surface film has been removed. Samples may be taken more frequently to follow the fate of a chemical substance that is disappearing from the system at a relatively rapid rate. The samples should be collected at a location at least

3 cm from the side of the tank and 10 cm below the water surface while both the stirring paddle and the benthic pump are in operation.

(B) If the test substance accumulates in a thin film on the water surface, it should be sampled with a filter pad before each water replacement. The quantity of a radiolabeled test substance absorbed onto the filter membrane can be easily determined with liquid scintillation counting assuming all radioactivity represents the original form of the test substance. If the test substance has degraded, the percentage of the total radioactivity that is the test substance should be determined.

(C) Samples of selected zooplankton species in the microcosm should be collected once every 7 days to be analyzed for the test substance and, if practical, for its transformation products.

(D) Air samples should be collected once every seven days with a suitable sampler. For example, and inverted crystallization dish equipped with inlet and outlet tubes on the side may be placed above the water surface to collect air samples for chemical analysis; fresh air could be drawn by a vacuum pump at the end of the sampling train, entering the modified dish through the inlet tube, sweeping over the water surface, and carrying any volatilized forms of the test substance through the outlet tube to a suitable trap for subsequent quantification. Under the inverted dish, air flow over the water surface should be adjusted to match the flow rate over the rest of the water surface in the microcosm. The duration for each sample collection should be kept as short as possible.

(E) The quantity of test substance adsorbed onto the glass surfaces of the microcosm above and below the water surface should be sampled and estimated as follows:

(*1*) For estimates of the test substance adsorbed onto the glass of the microcosm tanks below the surface, glass rods of known surface area should be suspended in the water column, and removed periodically from the water and placed in a scintillation counting vial for radioassay. If a surface film is present, glass rods should be removed through a slick protector. If possible, the estimated quantity of the radiolabeled chemical substance on the glass surfaces using the glass rod method should be verified with extraction of the test substance from all subsurface glass surfaces whenever a microcosm is sacrificed during the test.

(*2*) A portion of the interior microcosm tank wall extending from the water surface to the lip of the tank should have an appropriate absorbent material attached to it. This material should be removed and extracted at the conclusion of the test to provide an estimate of the amount of the test substance adsorbed to the tank walls above the water.

(*3*) Any unlabeled test substance on the glass surface should be thoroughly extracted and quantified after the water and sediment are removed from the microcosm.

(F) The quantities of the test substance in the benthic component should be determined as follows:

(*1*) One of the five replicate microcosm tanks for each of the three treatments should be randomly selected for sampling and samples of the core contents should be collected on day 10; another of the remaining replicate microcosm tanks should be selected for sampling and samples should be collected on day 20. The three remaining treated replicates and the controls should be sampled at the end of the test on day 30.

(*2*) Three sediment subcores, at least 25 cm in diameter by 7 cm in depth, should be collected from each benthic component to determine the vertical distribution of the test substance in the benthic component, i.e., concentration of test substance in each centimeter of the sediment core.

(*3*) Before triplicate sediment subcores are taken, the surface film (if present) on both the microcosm tank and the benthic cylinder should be removed with suitable tools such as a suction skimmer or a sheet of absorbent material, and the water in both the tank and the benthic cylinder should be drained.

(*4*) Samples of each of the major animal species in the benthic component should be analyzed for the test substance and its transformation products, if possible.

(vii) Sampling procedures for ecological effects study should be performed as follows:

(A) Water samples from microcosms should be taken as described in paragraphs (d)(4)(vi)(A) and (d)(4)(vi)(B) of this guideline.

(B) When water replacement and ecological effects sampling occur on the same day, biological samples should be taken first.

(C) Samples of at least 2 mL of water should be collected daily from the microcosms and such samples should be analyzed for enumeration and identification of phytoplankton.

(D) Samples of at least 2 L of water should be collected from the microcosms at least twice each week and such samples should be analyzed for enumeration and identification of zooplankton and transient larval forms. The water samples should be collected at a rate sufficient to overcome the zooplankters' avoidance reaction and should be screened through a 20–μM plankton net. The retained organisms should be rinsed into a

Petri dish and preserved for subsequent determination of population density and species composition.

(E) The ammonium-nitrogen concentration in the water column of the microcosms and the natural system should be determined once every 7 days.

(F) Population densities of phytoplankton and zooplankton in the natural system should be determined at least twice each week, and ammonium-nitrogen concentration in natural water should be measured at least once every 7 days. This can be done conveniently at the time for water replacement.

(G) The flux rate of ammonium-nitrogen between the benthic component and its associated water column should be determined weekly by stopping the benthic pump for a period of 1 to 3 h. Ammonia concentrations in water above the benthic component should be measured at the beginning and end of this period. The flux rate should be expressed as the weight of ammonium-nitrogen produced by each square meter of sediment surface area per hour.

(H) The abundance and diversity of benthos should be determined. Benthic animals should be captured by sieving the wet sediment through a 0.5 mm screen. All animals retained on the screen should be identified and counted. Similar characterization of the benthic community of the natural system should be established at the time of the experiment.

(5) **Analytical measurements**—(i) **Instrumental methods**. Atomic absorption and gas chromatography are preferable to colorimetric methods for quantitative analyses of metals and organic compounds, respectively. Liquid scintillation counting is recommended for quantitative analysis of radiolabeled test substances, and high-pressure liquid chromatography is recommended in conjunction with liquid scintillation counting for separation and quantification of the test substance and its transformation products.

(ii) **Chemical.** (A) A stock solution of the test substance should be prepared just before use, and its nominal concentration and purity should be confirmed by chemical analysis. Standard analytical methods, if available, should be used to determine the chemical concentration in microcosm samples and stock solution. The analytical methods used to measure all environmental samples should be validated before the beginning of the test.

(B) Concentrations of the test substance, and its transformation products, if possible, should be measured for the following components of the microcosm:

(*1*) Air.

(*2*) Surface film, if present.

(*3*) Water column, both particulate and dissolved fractions.

(*4*) Various layers of the benthic component.

(*5*) Representative species of zooplankton.

(*6*) Representative benthic organisms.

(*7*) Glass surfaces above and below the water surface.

(C) If a radiolabeled test substance is used, a complete budget of all radioactivity should be calculated, including the amount of radioactivity added to the microcosm, removed by gas transport and water replacement, and remaining among the compartments of the microcosm.

(iii) **Numerical.** (A) Mean and standard deviations of biological attributes should be calculated for each treatment and control. The following information should be determined: Abundance of phytoplankton, zooplankton, and each type of benthic fauna. If the species of plankton can be identified, abundance should be calculated for each one.

(B) Statistical analyses should be performed to determine:

(*1*) Whether significant differences exist in biological attributes between:

(*i*) The control microcosms and the natural system.

(*ii*) The carrier control and the carrier-free control.

(*iii*) The control and the microcosms treated with the test substance.

(*2*) Whether significant differences exist in the amount, export, and bioconcentration of the test substance among:

(*i*) Different compartments of the microcosms receiving the same treatment, and

(*ii*) The microcosms receiving different treatments.

(e) **Test conditions**—(1) **Test species.** (i) The organisms tested should include the indigenous fauna and flora representing both the pelagic and benthic communities of the natural system, except the macrofauna.

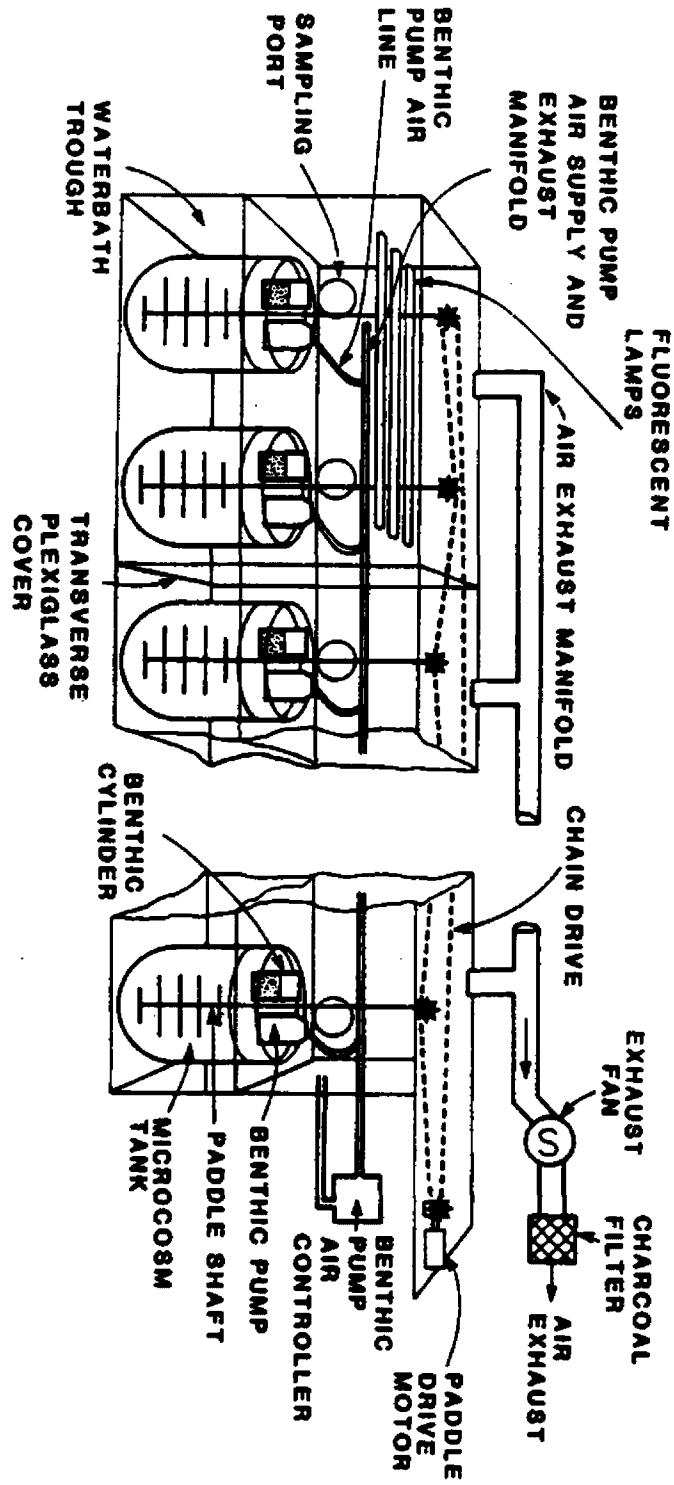
(ii) Neither acclimation nor supplemental food is necessary for the test organisms.

(2) **Facilities**—(i) **Supporting equipment.** (A) The capacity of the water bath used to maintain the water temperature and the flow rate of the water through the water bath should be such that the water temperature

in all microcosms will be kept within ± 1 °C of the ambient water temperature in the natural system.

(B) Cool white fluorescent light should uniformly illuminate the water surface of all microcosms. The fluorescent lights should be mounted on a canopy above the microcosm tanks, (see Figure 2). The desired, uniform light intensity is achieved by wrapping the fluorescent lamps with aluminum foil.

FIGURE 2.—EXPERIMENTAL MICROCOSM FACILITY



(C) In the room containing the microcosms, no light source except that specifically for the microcosms should be allowed.

(D) To match the water turbulence in the natural system, the water turbulence level in the microcosms should be controlled by the speed of an electric motor that is mounted with its chain drive and drive shafts above the canopy and controls the speed of all stirring paddles (see Figure 2 in paragraph (e)(2)(i)(B) of this guideline).

(E) The gypsum dissolution method measures the water turbulence level by the average dissolution rate (i.e., weight loss/time) of cubes (2.5 cm × 1.5 cm × 1.0 cm) of pure gypsum ($CaSO_4$) suspended in the microcosm tank or in the natural system. Gypsum cubes from the same source and lot should be used for the entire set of dissolution tests in the microcosms and in the natural system.

(F) The airspace between the canopy and water bath should be enclosed and sealed with acrylic plastic sheets to facilitate containment of the test substance transported into the gas phase (atmosphere) from the water (see Figure 2 in paragraph (e)(2)(i)(B) of this guideline).

(*1*) The enclosed volume under the canopy and above the water bath should be divided into relatively airtight compartments with Plexiglas panels mounted transversely to the module and extending approximately 5 cm below the water surface of the water bath.

(*2*) Each airtight compartment should have its own air outlet to the exhaust, a removable front cover to facilitate setting up and filling the microcosm tanks, and hinged ports in the front cover to provide access to the tanks during testing.

(G) Airflow over the water surface (microcosms and water bath) in each compartment should be maintained by a manifold connected to an exhaust fan which draws the air from all compartments through its outlet tube and vents the exhaust air through a charcoal filter and a stack outside the laboratory building (see Figure 2 in paragraph (e)(2)(i)(B) of this guideline).

(ii) **Microcosm.** Each microcosm is a multitrophic level model that combines pelagic and benthic communities similar to those existing in the natural system.

(A) Hard glass (e.g., Pyrex) containers are preferred to soft glass or plastic ones for the testing of organic chemicals.

(B) For each experiment, at least 20 microcosm tanks should be required. Each tank, about 140 L in capacity should hold enough water and sediment to support the quantity of benthic invertebrates present in the benthic subsystem, such as a medium-sized shellfish, for 30 days or more.

(C) The benthic cylinder, up to 30 cm tall, should have an inner diameter that makes the ratio of the sediment surface area to water volume in the microcosm equal to that in the natural system.

(D) The benthic cylinder, which holds the sediment core, should be sealed at the bottom end with a crystallization dish.

(E) The benthic pump (see Figure 1, in paragraph (d)(3)(v)(D) of this guideline) should be an all-glass, air displacement pump. It should be large enough to provide the appropriate water flow rate over the sediment surface.

(F) To minimize disturbance of the sediment core by the discharge from the benthic pump, a diffuser should be attached to the water outlet tube of the benthic pump to direct the outgoing water into several horizontal streams over the sediment surface.

(G) If the test substance forms a thin film covering the microcosm water surface, a 6–cm length of glass cylinder, or surface film protector, should be partially submerged in the water to provide a sampling port for uncontaminated water samples after the surface film inside the cylinder is removed.

(iii) **Cleaning**. Microcosm tanks, benthic cylinders, crystallization dishes, benthic pumps, support rack, slick protectors, and glass rods should be cleaned before use. All equipment should be washed according to standard laboratory practices to remove any residues remaining from manufacturing or previous use. A dichromate solution should not be used for cleaning glass containers. Solvents and/or high temperature (450 °C for 8 h) combustion may be necessary to ensure the ultimate cleanliness of the microcosms and associated glass components. If cleansing solvents are used, disposal should conform to existing Federal regulations.

(3) **Test parameters**. Environmental conditions in the microcosm should simulate the natural aquatic system as closely as possible.

(f) **Reporting**. The final report should include, but not necessarily be limited to, the following information:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing data.

(4) The test substance identified by name, Chemical Abstracts Service (CAS) registry number or code number, source, lot or batch number, strength, purity, and composition or other appropriate characteristics.

(5) Stability of the test substance under the conditions of administration.

(6) A description of the methods used, including:

(i) Description of microcosm facilities and supporting equipment; and

(ii) Description of natural system being simulated, including boundaries of natural system, pelagic community, benthic community, sediment type, water quality, history of natural system, light regime, ratio of benthic surface area to the water volume, water turbulence rate, water flow rate over sediment surface, water turnover rate, light intensity over sediment surface, seasonal attributes (e.g., water temperature), and ecological attributes (e.g., productivity).

(7) A description of the test system used, including: microcosm tank size, sediment core size, ratio of benthic surface area to water volume, light intensity on water surface, light intensity on sediment surface, water flow rate over sediment surface, and water turbulence.

(8) A description of the experimental design, treatment concentrations, and methods and pattern of administration. The report results should include:

(i) The results of the preliminary tests.

(ii) For the definitive test, various ecological effects and chemical fate parameters may include:

(A) **Ecological effects.** (*1*) Phytoplankton abundance, in numbers per mL, for the community or for each species.

(*2*) Zooplankton and transient larval forms abundances, in numbers per liter, for the community or for each life stage of each species.

(*3*) Number of organisms in the benthic community or, if known, in each species, expressed in numbers per m^3. Indicate the categories of benthic organisms if species identification is not feasible.

(*4*) Concentrations of major nutrients, such as ammonium-nitrogen, in the water column.

(*5*) Carrier effects when a carrier solvent is used.

(*6*) Assessment of microcosm realism by comparing the biological attributes in the natural system to that in the control microcosms.

(*7*) Effects of the test substance are assessed by comparing the treated microcosms to carrier controls.

(B) **Chemical fate**. (*1*) The concentrations of test substance in representative species of zooplankton and benthic organisms.

(*2*) The amount of test substance transported to the atmosphere.

(*3*) The amount of test substance adsorbed onto the glass surface of the microcosm.

(*4*) The vertical distribution of the test substance in the sediment core of the benthic component.

(*5*) The uptake and biotransformation of the test substance in biota.

(*6*) A mass balance consisting of the total quantity of the test substance added to the microcosm, the quantities exported from the microcosm and the quantities remaining in the microcosm.

(*7*) Concentrations of the test substance and its transformation products, at steady state in the water column and sediment core, and the amount on the glass surfaces both above and below the water surface and on the surface film, if present.

(*8*) The effect of treatments on the residual concentrations of the test substance in each ecosystem compartment.

(C) **Transport of test substance and its transformation products**. (*1*) Amount of test substance and transformation products exported from the microcosm through the air, water replacement, and removal of surface film.

(*2*) The effect of the treatments on the export rate of test substance and transformation products from each ecosystem compartment and on the total amount of test substance being exported.

(D) **Bioaccumulation potential of test substance in aquatic organisms**. (*1*) The concentrations of test substance residues in aquatic organisms (mass of test substance per kilogram wet weight).

(*2*) The bioaccumulation factor for selected benthos as well as water column species, such as zooplankton.

(*3*) The effect of the treatments concentration on the bioaccumulation factor.

(*4*) A description of all circumstances that may have affected the quality or integrity of the data.

(*5*) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(*6*) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

(*7*) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(*8*) The locations where all specimens, raw data, and the final report are stored.

(*9*) The statement prepared and signed by the quality assurance unit.

United States
Environmental Protection
Agency

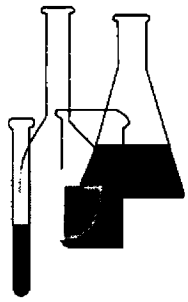
Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712-C-96-135
April 1996

EPA

Ecological Effects Test Guidelines

OPPTS 850.1950
Field Testing for Aquatic Organisms



"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.

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OPPTS 850.1950 Field testing for aquatic organisms.

(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 OPP 72–7 Simulated or Actual Field Testing for Aquatic Organisms (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982 and subsequent guidance on aquatic mesocosm tests under paragraph (e)(3) of this guideline.

(b) **Test standards**—(1) **Test substance.** Unless specified otherwise, data should be derived from testing conducted with an end-use product. An end-use product may be the applicant's own product or a typical end-use product.

(2) **Concentration analysis.** The concentration of the test substance in the water should be determined at the start of the study and samples should be collected periodically for analysis to verify concentrations.

(3) **Test conditions.** The test conditions for conducting field tests should resemble the conditions likely to be encountered under actual use. Specifically, the pesticide should be applied according to the rate, frequency, and method specified on the label.

(4) **Endangered species.** Studies should not be conducted in critical habitats or areas containing, or suspected to contain, endangered or threatened plants or animals which may be threatened by the tests to be conducted.

(5) **Residue levels.** When the test substance is applied under simulated or actual field condition testing, residues should be determined in appropriate vegetation, soil, water, sediments, and other environmental components, and in selected tissues of test organisms.

(6) **Other standards.** Any additional standards for conducting these tests will be provided by the Agency in writing following consultation between the applicant and the Agency, and will take into account the mechanisms by which a pesticide may enter the environment, and the food sources and habitats that may be affected.

(c) **Simulated field studies (mesocosm)**—(1) **Physical description**—(i) **Experimental design.** (A) One acceptable design is a minimum of four experimental treatments consisting of a control which receives no test compound, an *X* treatment level representing expected exposures, an *X*+ treatment level representing an upper bound, and an *X*– treatment level representing a lower bound. At least three replicates per treatment level

are needed to provide the requisite resolution of effects and probability of their occurrence. However, it is recommended that the number of replicates be dictated as a function of the parameters of interest and the sensitivity of their analysis.

(B) Alternative designs which emphasize regression analysis and utilize more treatment levels with fewer or no replicates may also be appropriate. Regression designs are most useful for determining maximum exposure conditions which provide no significant impacts or a specified level of effect in test systems.

(ii) **Mesocosm number.** A minimum of 12 mesocosms is required, with additional mesocosms added as replicates or treatments when needed to increase the sensitivity of analysis for specific parameters.

(iii) **Mesocosm size.** Dimensions of a mesocosm must be large enough to accommodate a viable finfish population. Depth should be sufficient to provide a representative open water area, and sloped sides should provide a littoral area for macrophyte growth and finfish reproduction. An acceptable design would occupy approximately 0.1 acre surface area with a volume of at least 300 m^3 and a maximum depth of 2 m. Sides of the mesocosm should be sloped approximately 1 unit of drop for every 2–3 units of linear distance.

(iv) **Mesocosm features.** (A) Mesocosms can be constructed as dugout ponds or enclosures of existing impoundments. The mesocosms should be lined with an impervious material of known adsorption for the test compound. The sediment used should be well-defined and representative in composition (percent clay, silt and sand, organic carbon, and organic nitrogen and ion exchange capacity) to pond sediments in the intended use area of the pesticide. The sediment depth at the bottom of the systems should be a minimum of 15 cm. Sediments may consist of natural pond sediment or top soil. If top soil is used, the complete mesocosm should be seasoned for 1 year prior to experimental use. This time is necessary to develop benthic biota. If pond sediments are used, a shorter seasoning period (e.g. 6 mon) is adequate. Organic content of the top soil should be at least 2 percent.

(B) A means of interchange (circulation, fill-drain-refill, etc.) of the water between the systems during initial establishment is desirable to ensure even distribution of biota among the mesocosms. Once the systems have become established or at initiation of a test the circulation should be stopped and each system kept separate from all other systems. The required precautions to ensure no cross contamination from pond overflow during rainstorms, leakage in the circulation system, etc., should be taken from the outset.

(v) **Mesocosm biota.** (A) The mesocosms must contain a representative pond biota. It is recommended that an established pond with diverse

biota act as a parent pond. The water in the mesocosm should be equivalent to the water of the parent pond and biota collected from the parent pond should be evenly distributed to each mesocosm to act as a starter base. Biota from other sources may be used to augment a natural assemblage to ensure adequate representation of important taxa.

(B) Phytoplankton are expected to reach a concentration consistent with the nutrient levels of the system prior to introduction of macroinvertebrates. Nutrient levels should be within a mesotrophic classification. The macroinvertebrate fauna should include representatives of the rotifers, annelids, copepods, cladocerans, amphipods, aquatic insects, and gastropods. Introduced macroinvertebrates, if necessary to augment naturally colonized populations, should not exceed 10 g wet-weight/m^3 and finfish should not be introduced at more than 2 g wet-weight/m^3. Fish species used in the test must be of known sensitivity to the test compound (determined from acute toxicity tests) and appropriate to small pond enclosures. Finfish species used must be native North American species (bluegill sunfish alone or in combination with largemouth bass are recommended).

(vi) **Mesocosm treatment.** Treatment levels of the mesocosms should be based on exposure models and residue monitoring data if available. In a three-replicate by four-treatment design, the three experimental treatments should be separated into a low, intermediate, and high treatment (dosed) and a control treatment (undosed). The intermediate treatment should approximate the estimated environmental concentration determined through modeling and experiential data for the intended pesticide use. It is recommended that the low treatment be 1/10 and the high treatment 10× the intermediate concentration. Regression designs should bracket expected exposures and expected response concentrations. Loading of pesticide into the mesocosms is to be by direct overspray to simulate drift and aerial deposition and with a sediment/water slurry channeled into the system at predetermined points to simulate runoff. Model predictions with available monitoring data will dictate the timing, frequency, and mode of introduction of the test material.

(2) **Measured parameters**—(i) **Chemical/physical properties.** (A) Mesocosm water should be monitored for pH, temperature, transparency (turbidity), dissolved oxygen, alkalinity, total nitrogen, total phosphorus, conductivity (total hardness), and particulate and dissolved organic carbon at appropriate intervals (e.g., biweekly). Observations are to be made at several locations throughout the mesocosm (which will be dictated by the physical design of the mesocosm) and at appropriate depths to allow quantification of vertical and horizontal variations. A complete water analysis should be conducted at test initiation and termination, and at significant periods during the test (i.e., pesticide inputs, substantial changes in other observed parameters, etc.). Temperature, pH, and dissolved oxygen should be monitored on a continuous basis for 24 h on a biweekly schedule and

at significant periods during the test to provide an estimate of gross production and community respiration.

(B) Mesocosm sediment must be analyzed for pesticide content, particle size, cation exchange capacity, organic content, and pH at the initiation of the test.

(ii) **Biological structure.** (A) Biota will be identified to species or lowest taxonomic unit practical. The schedule for sampling and collection of biological samples will depend on the design and composition of the mesocosm and must be determined prior to the initiation of the test. Collections should not be so frequent as to disrupt the system.

(B) Phytoplankton are to be collected from the water column, dominant species identified, and biomass determined by measuring chlorophyll a and phaeophytin. All samples should be preserved for archival reference. Periphyton are to be collected from glass slide substrates placed in the mesocosm and exposed for a minimum of 2 weeks. Periphyton should be analyzed for chlorophyll and ash-free weight. Macrophytes are to be identified to species, biomass determined by dry weight, and percent cover of the mesocosm determined.

(C) Zooplankton will be collected weekly with tube cores of the water column and vertical net tows. All samples are to be archived for future reference. Zooplankton samples will be analyzed biweekly by enumerating and identifying dominant species. Cladocerans should be identified to genus and differentiated by size (e.g., measured for length of muon). Macroinvertebrates, at a minimum, should be collected from emergent insect traps and artificial substrates. Sampling of sediment directly (e.g., Ekman dredge), should be employed cautiously, if necessary for tracking benthic community parameters, to minimize disruption to the benthic community. Samples should be enumerated, identified to lowest practical taxon, and archived.

(D) Finfish will be identified to species, enumerated, sexed (when possible) and measured in length and weight (wet) at introduction into the mesocosms and at test termination. Also at test termination, females will be assessed for fecundity and all collected fish will be examined for gross pathology. Spawning substrates will be placed in the systems and periodically surveyed for number of deposited eggs.

(E) Toxicity testing and bioassays with indigenous fauna on-site and in the laboratory may be used to assist in confirming cause and effect relationships.

(iii) **Residue analysis.** Residues of the test material and major degradates/metabolites will be analyzed at appropriate intervals to the environmental properties of the compound in the water, sediments, and biota at a sensitivity consistent with concentrations of concern.

(iv) **Meteorological conditions.** Continuous monitoring of air temperature, wind velocity, precipitation, evaporation, and solar radiation are required within 1 mile of the mesocosm test facility.

(d) **Actual field studies.** Data from an actual field study are required on a case-by-case basis to support registration of an end-use product intended for outdoor application. Consultation with the Agency is advised before undertaking these tests. Whenever data are required, the determination will be made in writing by the Agency and will state which properties and use patterns of the product were used in the determination.

(e) **References.** The following references can provide useful background information for conducting a simulated or actual field study for aquatic organisms.

(1) Graney, R.L. et al. (Eds.). *Aquatic Mesocosm Studies in Ecological Risk Assessment*, Lewis, Boca Raton, FL (1994).

(2) Hill, I.R. et al. (Eds). *Freshwater Field Tests for Hazard Assessment of Chemicals*, Lewis, Boca Raton, FL (1994).

(3) Touart, L.W. Aquatic Mesocosm Tests to Support Pesticide Registrations. U.S. Environmental Protection Agency, Hazard Evaluation Division; Technical Guidance Document. National Technical Information Service, Springfield, VA) (1988).

(4) Voshell, Jr., J.R. (Ed.). *Using Mesocosms to Assess the Aquatic Ecological Risk of Pesticides: Theory and Practice.* MPPEAL 75 (1989).