Ecological Effects Test Guidelines

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.

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 850.1000 Special considerations for conducting aquatic laboratory studies.


(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.

(1) 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 *Lemma 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 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.