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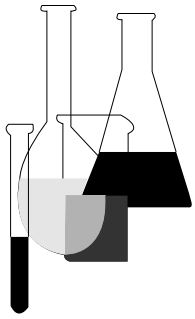
Ecological Effects Test Guidelines

OPPTS 850.1075
Fish Acute Toxicity Test, Freshwater and 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.

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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).