



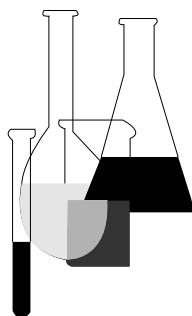
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# Ecological Effects Test Guidelines

## 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 <sub>3</sub> 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  $\mu\text{S}/\text{cm}$  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  $\mu\text{g}/\text{L}$ .

(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  $\pm 2$  °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.