

Ecological Effects Test Guidelines

OCSP 850.1075: Freshwater and Saltwater Fish Acute Toxicity Test



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to <http://www.epa.gov/ocspp> and select "Test Methods & Guidelines" on the navigation menu. You may also access the guidelines in <http://www.regulations.gov> grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.1075: Freshwater and saltwater fish acute toxicity test

(a) Scope.

(1) **Applicability.** This guideline is intended for use in meeting 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, *et seq.*). It describes procedures that, if followed, would result in data that would generally be of scientific merit for the purposes described in paragraph (b) of this guideline.

(2) **Background.** The source materials used in developing this harmonized OCSPP 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 540/9-82-024, 1982) (see paragraph (j)(4) of this guideline); Standard Evaluation Procedure: Acute Toxicity Test for Freshwater Fish (see paragraph (j)(5) of this guideline); Standard Evaluation Procedure: Acute Toxicity Test for Estuarine and Marine Organisms (see paragraph (j)(6) of this guideline); EPA Pesticide Reregistration Rejection Rate Analysis: Ecological Effects, EPA 738-R-94-035, 1994 (see paragraph (j)(7) of this guideline); OECD 203 Fish Acute Toxicity Test, 1992 (see paragraph (j)(3) of this guideline); and ASTM E729-96 (07), Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians, 2007 (see paragraph (j)(1) of this guideline).

(b) **Purpose.** This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures (“test chemicals” or “test substances”) subject to environmental effects test regulations. This guideline describes an acute toxicity test in which freshwater and saltwater fish are exposed to a test substance in static, static-renewal, or flow-through systems. For freshwater testing, data on cold and warm water species are generally required (40 CFR 158). Rainbow trout, *Oncorhynchus mykiss*, and bluegill sunfish, *Lepomis macrochirus*, are the preferred species to meet this requirement for cold and warm water tests, respectively. These species are preferred because they are sensitive indicator species, and there is a large environmental contaminant-response database available on them. For saltwater tests, *Menidia sp.* (silversides) are the preferred species. Other species as identified in paragraph (e)(3)(i) of this guideline may be used. The Environmental Protection Agency will use data from this test in assessing the hazards and risks a test substance may present in the aquatic environment.

(c) **Definitions.** The definitions in OCSPP 850.1000 apply to this guideline. In addition, the following more specific definition also applies to this test guideline:

Death is defined as the lack of visible movement (*i.e.*, respiratory movements) or the lack of reaction when the caudal peduncle is touched.

(d) General considerations.

(1) **Summary of test.** Individuals of a particular species of fish are exposed to the test substance and to appropriate controls (*i.e.*, dilution water control and vehicle (solvent)

control, if a vehicle is used) for 96 hours, during which observations are made on organism survival and other toxic effects. The test is designed to determine the relationship between aqueous concentrations of the test substance and mortality of fish over the full concentration-response curve. The results of the test are expressed as the 96-hour median lethal concentration (96-h LC₅₀) and the slope of the concentration-response relationship. Although the 96-h LC₅₀ is the primary toxicity endpoint, information on other signs of toxicity such as abnormal appearance and behavior and concentration-response curves is useful in understanding the toxic response

(2) **General test guidance.** The general guidance in OCSPP 850.1000 applies to this guideline except as specifically noted herein.

(3) **Range-finding test.** A range-finding test is usually conducted to establish the appropriate test solution concentrations to be used in the definitive test. In the range-finding test, the test organisms are generally exposed to a series of widely-spaced concentrations of the test substance (*e.g.*, 1, 10, 100 milligrams per liter (mg/L)). The details of the range-finding test do not have to be the same as those of definitive testing in that the number of replicates, the number of test organisms, and duration of exposure may be less than that used in definitive testing. In addition, the types of observations made on test organisms may not be as detailed or as frequently observed as that of a definitive test.

(4) **Definitive test.** The primary goal of the definitive test is to determine the 96-hour concentration-response curve for mortality, the 96-h LC₅₀; its standard error and 95 percent (%) confidence interval; and the slope of the concentration-response curve, its standard error and 95% confidence interval. Where sufficient data are available, these values are also calculated for the 24-, 48-, and 72-h concentration-response curves. A minimum of 5 concentrations of the test substance, plus appropriate controls, should be tested. The selected test concentrations should bracket the 96-h LC₅₀. Clinical signs of toxicity such as abnormal appearance and behavior, if any, should be reported. Analytical confirmation of dissolved test concentrations should be performed as described in OCSPP 850.1000. Summaries of the test conditions are presented in Table 2 of this guideline. Test validity elements are listed in Table 3.

(5) **Limit test.** In some situations, it is only necessary to ascertain that the 96-h LC₅₀ is above a certain limit (*i.e.*, 96-h LC₅₀ greater than (>) limit concentration). In a limit test, at least 7 fish are exposed to a single “limit concentration,” with the same number of organisms in appropriate controls. For most industrial chemicals, the lower of 100 mg/L or the limits of water solubility or dispersion is considered appropriate as the limit concentration. For pesticides, the lower of 100 milligrams active ingredient per liter (mg a.i./L), when estimated environmental concentrations are not expected to exceed 100 mg/L, or the limit of water solubility may be used as the limit concentration. Except for the number of test concentrations, limit tests should follow the same test procedures, have the same duration as the multiple-concentration definitive test (see Table 2 of this guideline), and have both a dilution water control and a vehicle (solvent) control, if a vehicle is used. Limit tests, like definitive tests, should include analytical confirmation of the dissolved concentration of the test substance. Clinical signs of toxicity such as abnormal appearance and behavior, if any, should be reported. For pesticides, at test

termination, if any fish dies in the limit test concentration, a multiple-concentration 96-hour test should be conducted.

(e) Test standards.

(1) **Test substance.** The substance to be tested should be technical or reagent grade unless the test is designed to evaluate a specific formulation, mixture, or end-use product. For pesticides, if more than one active ingredient constitutes a technical product, then the technical grade of each active ingredient should be tested separately, in addition to the combination, if applicable. OCSPP 850.1000 lists the type of information that should be known about the test substance before testing, and discusses methods for preparation of test solutions.

(2) **Test duration.** The test duration is a minimum of 96 hours.

(3) Test organisms.

(i) **Species.** Data on both a warm and a cold freshwater species are generally required for 40 CFR Part 158. If a marine or estuarine system may be affected, data on a saltwater species are also generally required. Freshwater and saltwater species that are recommended include:

(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 minnow, *Pimephales promelas*; guppy, *Poecilia reticulata*; rainbow trout, *Oncorhynchus mykiss*; medaka, *Oryzias latipes*; and zebrafish, *Danio rerio*.

(B) **Saltwater species.** Atlantic silverside, *Menidia menidia*; inland silverside, *Menidia beryllina*; tidewater silverside, *Menidia peninsulae*; and sheepshead minnow *Cyprinodon variegatus*;

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 are desired, a *Menidia spp.* (silverside) is preferred.

Juvenile fish less than (<) 3.0 grams, and old enough to be actively feeding, should be tested. It is recommended that the wet weight and length of at least 7 fish from the batch of fish used in a particular test be measured and that the mean values and ranges be reported. The longest fish should not be more than twice the length of the shortest fish. Since these fish should not be fed during the test and 24 to 48 hours prior to test initiation, they should be of sufficient size to survive and not exhibit signs of stress during this time period without food.

Fish may be cultured in the laboratory, purchased from culture facilities, or collected from the wild in a relatively unpolluted area. Wild caught fish should be quarantined 7 days in addition to the defined minimum holding and acclimation

periods (see paragraph (e)(3)(ii)). Whenever salmon or trout are used, they should be obtained from a hatchery that has been certified disease-free. Fish captured by electroshocking, chemical treatment, or gill nets should not be used. Fish used in a particular test should be obtained from the same source and population, be of similar age, and be of normal size and appearance. Records should be kept regarding the source of the initial stock and/or culturing techniques.

(ii) **Holding and acclimation.** Fish brought into the laboratory should be held for a minimum of 12 days prior to use. The recommended holding period, however, is 14 days. A minimum of 7 days of this period are used for acclimation to environmental conditions (*e.g.*, temperature, light intensity, temperature, dilution water) similar to those used in the test. To maintain organisms in good condition and avoid unnecessary stress, they should not be crowded or subjected to rapid changes in temperature or water quality. Acclimation water should be from the same dilution water source as used in the test; if not, acclimation to the dilution water should be done gradually over a 48-hour settling-in period. Within a 24-hour period, changes in water temperature during holding or acclimation should not exceed 3 degrees Celsius (°C), and for saltwater species, changes in salinity change should not exceed 2 parts per thousand (ppt).

Following a 48-hour settling-in period, mortalities should be recorded, and the following guidelines should be applied:

- (A) Mortalities of greater than 10% of the population in the 7 days of acclimation: rejection of entire batch;
- (B) Mortalities of between 5 and 10% of the population during the 7 days of acclimation: acclimation continued for additional 7 days;
- (C) Mortalities of less than 5% of the population during the 7 days of acclimation: acceptance of batch.

(iii) **Health status and condition.** Fish should not be used for a test:

- (A) If more than 5% of the culture or acclimating group dies or shows signs of stress (*e.g.*, disease, physical damage, or abnormalities) during the 48 hours preceding the test;
- (B) If they have been used in a previous test, either in a treatment or in a control group;
- (C) If disease treatments were administered within 48 hours of test initiation. Fish should not receive treatment for a disease during a test.

(iv) **Care and handling.** Organisms should be handled as little as possible, but when necessary, it should be done as carefully and quickly as possible. Any disturbance which might change the behavior of the test fish should be avoided. Detailed instructions for the care and handling of fish such as those described

under paragraph (j)(8) of this guideline can be followed during the culturing, holding, acclimating, and testing periods.

(v) **Diet and feeding.** During holding and acclimation, different food sources may be used depending on species and size (e.g., flake fish food, pelleted food, live *Artemia* brine shrimp).

Test fish should not be fed during testing. During the acclimation period, fish should be fed daily until 24 hours prior to test initiation. If fish are larger than 0.5 grams fish should be fed until 48 hours prior to test initiation.

(4) Administration of test substance.

(i) **Preparation of test solutions.** Preparation of test solutions depends on the solubility and stability of the test substance. Guidance for preparation of test solutions, especially for difficult or low solubility test substances, is provided in OCSPP 850.1000. Dilution water source and quality used in the test are described in OCSPP 850.1000 and paragraph (e)(7)(vi) of this guideline.

The concentration of vehicle solvent should not exceed 0.1 milliliters per liter (mL/L). A previous review recommends that solvent concentrations as low as 0.02 mL/L of dilution water be used (see paragraph (j)(2) of this guideline).

The pH of stock solutions may be adjusted to match the pH of dilution water or to a neutral pH if pH change does not affect the stability of the test substance in water. The pH of test solutions may be adjusted after the addition of the test substance or stock solution into the dilution water. However, all pH adjustments need to be made prior to the addition of test organisms. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) may be used for this adjustment if warranted.

See additional information about pH during testing in (e)(8)(ii).

(ii) **Exposure technique.** The test may be conducted using one of three basic exposure techniques: static, static-renewal, or flow-through. Guidance on the selection of the appropriate exposure technique is provided in OCSPP 850.1000.

(iii) **Treatment concentrations.** At least 5 test solution concentrations should be used for definitive testing, plus the appropriate control(s). A range-finding test can be used to establish the appropriate test solution concentrations for the definitive test (see paragraph (d)(3) of this guideline). For scientifically sound estimates of a given point estimate (e.g., LC₅₀), test substance concentrations should immediately bracket the point estimate(s) of concern. OCSPP 850.1000 provides guidance on selection of test concentrations. For a limit test, there is single treatment concentration, plus the appropriate control(s). Guidance on the limit concentration is provided in paragraph (d)(5) of this guideline.

(5) **Controls.** Every test includes a dilution water control and a vehicle (solvent) control, if a vehicle is used. Controls consist of the same dilution water, conditions, procedures, and test population as the test solutions, except that no test substance is added.

A test is not acceptable if more than 10% of the organisms in any control shows signs of disease, stress (*e.g.*, discoloration, unusual behavior, immobilization), and/or death.

(6) **Number of test organisms and replicates.** For definitive and limit tests, the minimum number of fish per treatment level is 7. The use of 10 fish per treatment level and 2 replicates per treatment level is preferred to obtain a more statistically accurate representation of the concentration-response curve. Each test vessel should contain an equal volume of test solution and an equal number of fish. Replicate test vessels should be physically separated, since the test vessel is the experimental unit.

(i) **Loading.** The number of fish placed in each test vessel should not be so large as to cause the dissolved oxygen concentration to fall below the recommended levels or affect the results of the test. In static or static-renewal tests, loading should not exceed 0.8 grams wet weight of organism per liter (g/L). In flow-through tests, loading requirements will vary depending upon the flow rate of dilution water, but should not exceed 0.5 g/L of test solution passing through the test vessel in 24 hours or 5 g/L at any time.

(ii) **Introduction of test organisms.** The test should be started by introducing juvenile fish, which have been acclimated to the test conditions, into the test vessels after the test substance has been added. Test vessels for treatment levels should be randomly or indiscriminately located within the test area, and test organisms should be randomly or indiscriminately distributed among test vessels. Further guidance is provided in OCSPP 850.1000.

(7) **Facilities, apparatuses, and supplies.** Normal laboratory equipment should be used, especially the following:

(i) **Facilities.** Facilities for culturing, holding, acclimating, and testing fish that are well ventilated and free of fumes and disturbances which may affect the test organisms. There should also be flow-through or recirculation tanks for culturing and acclimating fish. Equipment for culturing and/or handling food sources for fish.

(ii) **Environmental control equipment.** Mechanisms for controlling and maintaining the water temperature and lighting during the culturing, holding, acclimation, and test periods. Apparatus for aerating dilution water and removing gas bubbles as necessary. For flow-through tests, apparatus for aerating the dilution water in the head box before mixing with the test substance or delivery to test vessels. An apparatus providing a 30-minute lighting transition period may be needed.

(iii) **Water quality testing instruments.** Equipment for determination of water quality characteristics (pH, hardness, temperature, *etc.*)

(iv) **Cleaning of test system.** Test substance delivery systems and test vessels should be cleaned before each test. See OCSPP 850.1000 for further information.

(v) **Test containers and delivery system.** 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 affect the test results. Construction materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test substances. Refer to OCSPP 850.1000 for additional information on appropriate construction materials. Test vessels, which should be constructed of chemically inert material, should be of a capacity to maintain the loading rate and environmental conditions. Test vessels should be loosely covered to reduce the loss of test solution or dilution water due to evaporation, to minimize the entry of dust or other particulates into solutions, and to prevent loss of test fish. A flow-through system, if used, should contain an appropriate test substance delivery system.

Many different sizes of test vessels have been used successfully. The size, shape, and depth of the test vessel is appropriate if the specified flow rate and loading requirements can be achieved.

(vi) **Dilution water.** Clean surface water, ground water, reconstituted water, or natural or artificial seawater (for saltwater species) are acceptable as dilution water if the test species will survive in it for the duration of the culturing, holding, acclimation, and testing periods without showing signs of stress.

Natural seawater should be filtered through a filter with a pore size of <20 micrometers (μm) prior to use in a test. Artificial seawater can be prepared by adding commercially available formulations or specific amounts of reagent-grade chemicals to reagent water (deionized, distilled, or reverse osmosis water), surface water, or ground water. For saltwater species, a salinity should be selected from a range of 15 and 25 ppt. For artificial seawater or natural seawater that is diluted with freshwater, salinity should be maintainable within a weekly range of 2 ppt.

Dechlorinated tap water is not recommended (either as the freshwater source, preparation of artificial seawater, or dilution of natural seawater) because some forms of chlorination are difficult to remove adequately. If dechlorinated tap water is used, recommended maximum chlorine levels as well as other ways to demonstrate suitability as a dilution water source are in OCSPP 850.1000.

Dissolved oxygen in the dilution water (prior to use in a test) should be between 90 and 100% saturation. If necessary, the dilution water can be aerated before the addition of the test substance.

For freshwater testing, hardness, alkalinity, and conductivity should be measured in the dilution water at the beginning of the test. For saltwater testing, salinity should be measured in the dilution water at the beginning of the test.

Measurement of total organic carbon (TOC) or chemical oxygen demand (COD) in the dilution water at the beginning of the test is recommended, but at a minimum, TOC and COD should be analyzed periodically in the dilution water source to document and characterize their magnitude and variability. For tests with cationic substances, TOC or COD should be measured at the beginning of the test.

Specifications for dilution water quality and constancy are described in OCSPP 850.1000.

(8) **Environmental conditions.** Environmental parameters during the test should be maintained as specified below. The number and frequency of measurements recommended for documenting and confirming the magnitude and variability of water quality parameters (e.g., temperature, dissolved oxygen, pH, and salinity) in test solutions during the test are described in detail in OCSPP 850.1000.

(i) **Temperature.** Recommended test temperatures by species are shown in Table 1. During a given test, the selected temperature should be constant within plus or minus ($\pm$) 2 °C.

Table 1.—Recommended Test Temperatures by Species in the Freshwater and Saltwater Fish Acute Toxicity Test

Species	Temperature (°C)
Atlantic salmon (<i>Salmo salar</i>)	12
Atlantic silverside (<i>Menidia menidia</i>)	22
Bluegill sunfish (<i>Lepomis macrochirus</i>)	22
Brook trout (<i>Salvelinus fontinalis</i>)	12
Channel catfish (<i>Ictalurus punctatus</i>)	22
Coho salmon (<i>Oncorhynchus kisutch</i>)	12
Common carp (<i>Cyprinus carpio</i>)	22
Fathead minnow (<i>Pimephales promelas</i>)	22
Guppy (<i>Poecilia reticulata</i>)	22
Inland silverside (<i>Menidia beryllina</i>)	22
Medaka (<i>Oryzias latipes</i>)	22
Rainbow trout (<i>Oncorhynchus mykiss</i>)	12
Sheepshead minnow (<i>Cyprinodon variegatus</i>)	22
Tidewater silverside (<i>Menidia peninsulae</i>)	22
Zebrafish (<i>Danio rerio</i>)	22

(ii) **pH and salinity.** The pH should be between 6.0 and 8.5 for freshwater species and between 7.5 and 8.5 for saltwater species and should vary less than 1 pH unit during the test within a test vessel and between test concentrations (including

control(s)). During a given test, the salinity (selected from a range of 15 to 25 ppt) should be constant within ± 2 ppt.

(iii) **Lighting and photoperiod.** A photoperiod should be selected from regimes of 12 hours light:12 hours dark to 16 hours light:8 hours dark. For any given test, the light regime should be constant. Light intensity should range from 540 to 1080 lux (approximately 50-100 foot-candles (ft-c)). A 15- to 30-minute transition period between light and dark is recommended.

(iv) **Dissolved oxygen.** The dissolved oxygen concentration should be between 60 and 100% saturation during the test. If aeration is needed to achieve an appropriate dissolved oxygen level, it should be done before addition of the test substance. For flow-through exposures, the dilution water may be aerated vigorously prior to delivery to the test vessels (*e.g.*, in the diluter head box) such that the dissolved oxygen concentration is at or near 90 to 100% saturation. If the water is heated, precautions should be taken to ensure that supersaturation of dissolved gases is avoided. Aeration of the test solutions during the test is not recommended. Gentle aeration of test vessels during the exposure period is permitted only in cases where the dissolved oxygen levels are in danger of dropping below 60% saturation. In such cases, assurances should be made that the use of aeration does not stress the test organisms; test substance concentrations should be measured during the test to ensure that they are not affected by the use of aeration; and all treatment and control vessels should be given the same aeration treatment.

(v) **Flow in a flow-through system.** During a test, the flow rates should not vary more than 10% between any one replicate and another. The minimum number of test vessel volume replacements should be five per 24-hour period. It is recommended that diluter systems be monitored for proper adjustment and operation at least twice daily throughout the test period to better ensure that the target test concentrations are achieved and maintained. The flow rate to each test vessel should be measured at the beginning and end of the test.

(9) Observations.

(i) **Measurement of test substance.** OCSPP 850.1000 describes the recommended sampling methods, frequency of sampling, and sample processing (especially of low solubility test substances) for analytical confirmation of dissolved test concentrations and characterization of test substance stability throughout the test. The analytical methods used to measure the amount of dissolved test substance in a sample should be validated before beginning the test, as described in OCSPP 850.1000, and the relevant method detection limit(s) and limit(s) of quantification should be reported.

(ii) **Test solution appearance.** Observations on test solution appearance and test substance solubility should be made daily and at the beginning and end of the test. The appearance of surface slicks, precipitates, or material adhering to the sides of

the test vessels or in any part of the mixing and delivery system should be recorded at a minimum at the beginning and end of the test and during the test when the test solution appearance changes.

(iii) **Measures of effect.**

(A) **Mortality.** The number of dead fish in each test vessel should be counted and recorded at 24, 48, 72, and 96 hours. An observation period at <12 hours is desirable. Dead fish should be removed from the test vessels at the time of observation.

(B) **Appearance and behavior.** In addition to mortality, any abnormal behavior or appearance, and the number of individuals exhibiting these characteristics, should be counted and recorded at the same time as observations of mortality.

(f) **Treatment of results.**

(1) **Summary statistics.**

(i) **Mortality.** The number of fish exposed at test initiation in each treatment and replicate and the cumulative number of dead fish should be summarized in tabular form by time of observation, treatment, and replicate.

(ii) **Appearance and behavior.** The number of fish exhibiting abnormal appearance or behavioral symptoms should be summarized in tabular form by time of observation, treatment, and replicate.

(2) **Percent mortality.** The percent mortality at each treatment level and in the controls at 24, 48, and 72 hours and at test termination (96 hours) should be calculated.

(3) **Evaluation of limit test results.** For pesticides, at test termination, if any fish dies in the limit concentration, a multiple-concentration acute 96-hour test should be conducted.

(4) **Evaluation of multiple-concentration definitive test.**

(i) **Concentration-response curve, slope, and LC₅₀.** Statistical procedures should be employed to calculate the 96-h LC₅₀ (standard error and 95% confidence interval) based upon mortality. If a concentration-response curve model (*e.g.*, probit) was fit to the data to determine the LC₅₀, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded. The 24-, 48-, and 72-h LC₅₀ values should also be calculated if the magnitude of the mortality allows.

(ii) **No observed effect concentration (NOEC).** While calculation of the NOEC and lowest observed effect concentration (LOEC) is usually not part of the experimental design for the regression-based definitive test, reporting these values

when possible is useful when testing industrial and pesticide chemicals for understanding the toxic response.

(iii) **Statistical methods.** Statistical procedures for modeling quantal data should be used. Additional discussion about endpoints and statistical procedures can be found in OCSPP 850.1000.

(g) **Tabular summary of test conditions.** Table 2 lists the important conditions that should prevail during the multiple-concentration definitive test. The same conditions are recommended for a limit test, except for differences in the number of test concentrations. Meeting these test conditions will help ensure the satisfactory performance of the test.

Table 2.—Summary of test conditions for freshwater and saltwater fish acute toxicity test

Test type	Static, static-renewal, or flow-through
Test duration	96 hours
Temperature	12 °C for Atlantic salmon, Brook trout, Coho salmon, Rainbow trout 22 °C for Atlantic silverside, Bluegill sunfish, Channel catfish, Common carp, Sheepshead minnow, Inland silverside, Tidewater silverside, Fathead minnow, Guppy, Medaka, Zebrafish Constant during test within ± 2 °C
Light quality	Ambient laboratory illumination
Light intensity	540-1080 lux (approximately 50-100 ft-c)
Photoperiod	Selected from among 12 hours light:12 hours dark to 16 hours light:8 hours dark schemes
pH	Between 6.0 and 8.5 for freshwater testing; between 7.5 and 8.5 for saltwater testing (constant during test within ± 1 pH unit)
Water hardness (as CaCO ₃) (freshwater tests)	For freshwater: <250 mg/L (preferably <180 mg/L); 40-50 mg/L for testing with metals
Salinity (saltwater tests)	Selected from a range of 15 to 25 ppt (constant during test within ± 2 ppt for selected salinity)
Total organic carbon (TOC)	≤ 2 mg/L
Age/size of test organisms	Juvenile fish <3.0 grams
Number of test organisms per concentration	7 minimum, 10 preferred
Number of replicate test vessels per concentration	1 minimum, 2 preferred
Loading	Static or static-renewal tests: ≤ 0.8 g wet weight per liter; Flow-through test: ≤ 0.5 g/L per 24 hours and <5 g/L at any time
Feeding regime	No feeding during test

Test vessel aeration	Not recommended; gentle aeration of test vessels may only be used in cases where the dissolved oxygen levels are in danger of dropping below 60% saturation. In such cases, assurances should be made that the use of aeration does not stress the test organisms; test substance concentrations should be measured during the test; and all treatment and control vessels should be given the same aeration treatment.
Test concentrations	Definitive test: minimum of 5 test concentrations chosen in a geometric series plus a dilution water control and a vehicle (solvent) control, if a vehicle is used
Vehicle concentration, if used	≤0.1 mL/L for recommended solvents (see OCSPP 850.1000)
Measure of effect or measurement endpoint	96-h LC ₅₀ based on mortality

(h) **Test validity elements.** This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 3 occurred. These parameters are not the only elements considered when evaluating the acceptability of a test, and it is possible that a test could be found unacceptable or invalid based on other considerations. However, except for the conditions listed in Table 3 and in OCSPP 850.1000, it is unlikely that a test will be rejected when there are only slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline (such as deviating from the organism size), the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) may have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for the changes given, and any resulting effects on test endpoints noted and discussed.

Table 2.—Test validity elements for the Freshwater and Saltwater Fish Acute Toxicity Test

1. All test vessels were not identical.
2. Treatments were not randomly or indiscriminately assigned to individual test vessel locations, or individual test organisms were not randomly or indiscriminately assigned to test vessels.
3. A dilution water control (and vehicle (solvent) control, if a vehicle was used) was not included in the test.
4. More than 10% of the organisms in either the dilution water or vehicle (solvent) controls showed signs of disease, stress (<i>e.g.</i> , discoloration, unusual behavior, immobilization), and/or death.
5. Fish were fed during the test.
6. A surfactant or dispersant was used in the preparation of a stock or test solution. (However, adjuvants may be used when testing pesticide typical end-use products.)

(i) Reporting.

(1) **Background information.** Paragraph (k)(1) of OCSPP 850.1000 describes the minimum background information to be supplied in the report.

(2) **Guideline deviations.** Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences that may have influenced the results of the test, the reasons for these changes, and any resulting effects on test endpoints noted and discussed.

(3) **Test substance.**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, purity (*i.e.*, for pesticides, the identity and concentration of active ingredient(s)), and radiolabeling, if any, including the location of label(s) and radiopurity.

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods of preparation of the test substance and the treatment concentrations used in the range-finding and definitive tests, or limit test. Identify whether the nominal concentrations are corrected or uncorrected for purity of the test substance.

(iv) Physicochemical properties of the test substance such as water solubility, vapor pressure, UV absorption, pKa, and K_{ow} .

(v) If a vehicle (solvent) is used to prepare stock or test substance provide: the name and source of the vehicle, the nominal concentration(s) of the test substance in the vehicle in stock solutions or mixtures, and the vehicle concentration(s) used in the treatments and vehicle control. If different vehicle concentrations are used at different treatment levels, the report should, at a minimum, identify the maximum vehicle concentration used. It is helpful to support the vehicle choice by including a description of any measures that were taken to identify an appropriate vehicle for use in the test, such as the types and concentrations of vehicles used and their corresponding effects on solubility during any preliminary work.

(vi) If a positive control is used, provide the name and source of positive control and the nominal concentration(s) of the positive control material in stock solutions or mixtures.

(4) **Test organism.**

(i) Scientific name and common name.

(ii) Method for verifying the species.

(iii) Information about the fish used in the test: source, culture practices, and holding and acclimation procedures and conditions, including acclimation period, water used, feeding history, and health status (mortality before test initiation and

any preventative or disease treatments). Feed should be analyzed periodically to identify background contaminants such as heavy metals (*e.g.*, arsenic, cadmium, lead, mercury, and selenium) and persistent pesticides, especially chlorinated insecticides.

(iv) Age of test organisms at test initiation (mean and range).

(v) Weight of test organisms at test initiation (mean and range).

(vi) Length of test organisms at test initiation (mean and range).

(5) Test system and conditions. Provide a description of the test system and conditions used in the definitive or limit test and any preliminary range-finding tests.

(i) Description of the test vessels: size, type, material, and fill volume.

(ii) Description of the exposure technique: static, static-renewal, flow-through, open or closed system. If static-renewal, the frequency of test solution renewal, and if flow-through, a description of the flow-through system, including flow rate and test vessel turnover rate. For closed systems, a description of the closed system design. For all systems, a description of the calibration and validation methods.

(iii) Description of the dilution water and any water pretreatment: source/type; temperature; salinity (saltwater); pH; hardness and alkalinity (freshwater); dissolved oxygen; total organic carbon or chemical oxygen demand; particulate matter; conductivity; metals, pesticides, and residual chlorine concentrations (mean, standard deviation, range). Describe the frequency and sample date(s) for documenting dilution water quality and consistency.

(iv) Use of aeration, if any, and location within exposure system of aeration (*e.g.*, test solution or dilution water prior to test substance addition).

(v) Number of test organisms added to each test vessel at test initiation.

(vi) Number of test vessels (replicates) per treatment level and control(s).

(vii) Methods used for treatment randomization and assignment of test organisms to test vessels.

(viii) Date of introduction of test organism to test solutions and test duration.

(ix) Loading rate.

(x) Photoperiod and light source.

(xi) Methods and frequency of environmental monitoring performed during the definitive or limit test for test solution temperature, dissolved oxygen, pH, salinity (if applicable), and light intensity.

(xii) Methods and frequency of measuring the dissolved test substance to verify exposure concentrations.

(xiii) Methods and frequency of counting number of dead test organisms and measuring any other toxic symptoms.

(xiv) For the definitive and limit tests, description of all analytical procedures, accuracy of the method, method detection limit, and limit of quantification.

(6) Results.

(i) Nominal exposure concentrations and a tabulation of test substance analytical results by treatment group and test vessel (provide raw data) and descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation).

(ii) Environmental monitoring data results (test solution temperature, dissolved oxygen, pH, salinity (if applicable), and light intensity) in tabular form (provide raw data for measurements not made on a continuous basis) and descriptive statistics (mean, standard deviation, minimum, maximum).

(iii) For preliminary range-finding test, if conducted, a tabulation of the number and percentage of dead fish in each test vessel, for all treatment levels and control(s), at each observation period. A description and count of any other appearance or behavioral effects, if recorded, at each treatment level and in the control(s).

(iv) For limit test, a tabulation of the number and percentage of dead fish in each test vessel, for the limit concentration and control(s), at each observation period (provide the raw data) and descriptive statistics (mean, standard deviation, minimum, maximum).

(v) For definitive test, a tabulation of the number and percentage of dead fish, for all treatment levels and control(s), at each observation period (provide the raw data) and descriptive statistics (mean, standard deviation, minimum, maximum).

(vi) For limit and definitive tests, a description and tabulation of abnormal appearance and behavioral signs of toxicity by test vessel, treatment, and observation time (provide raw data).

(vii) Graphs of the concentration-response data for percent mortality.

(viii) For limit test, conclusion about the 96-h LC_{50} being above the limit concentration.

(ix) For definitive test, where sufficient data exist to fit a model (*e.g.*, probit) a tabulation of the 96-hour slope of the concentration-response curve, its standard error and 95% confidence interval, and any goodness-of-fit results.

(x) For definitive test, the 96-h LC₅₀ value, its standard error and 95% confidence interval.

(xi) For definitive test, results for the 24-, 48-, and 72-h LC₅₀ values if the magnitude of the mortality allows.

(xii) For the definitive tests, the 96-hour NOEC for mortality, if determined.

(xiii) Description of statistical method(s) used for point estimates, including the software package for determining LC₅₀ values and fitting the concentration-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(xiv) Description of statistical method(s) used for NOEC and LOEC determination, including the software package, and the basis for the choice of method.

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

(1) American Society for Testing and Materials. ASTM E729-96, Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. In Annual Book of ASTM Standards, ASTM, West Conshohocken, PA. Current edition approved October 1, 2014.

(2) Hutchinson, T.H., N. Shillabeer, M.J. Winter and D.B. Pickford, 2006. Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. *Aquatic Toxicology*, 76, 69-92.

(3) Organization of Economic Cooperation and Development, 1992. Guidelines for Testing of Chemicals, Guideline 203, Fish Acute Toxicity Test, Adopted July 17, 1992. Updated version October 12, 2009.

(4) U.S. Environmental Protection Agency, 1982. Pesticide Assessment Guidelines, Subdivision E, Hazard Evaluation, Wildlife and Aquatic Organisms, EPA 540/09-82-024, U.S. Environmental Protection Agency, Washington, DC.

(5) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division 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.

(6) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division Standard Evaluation Procedure: 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.

(7) U.S. Environmental Protection Agency, 1994. Pesticides Reregistration Rejection Rate Analysis: Ecological Effects, EPA 738-R-94-035, Office of Prevention, Pesticides and Toxic Substances, December, 1994.

(8) U.S. Environmental Protection Agency, 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, Fifth edition, October 2002. EPA-821-R-02-012.