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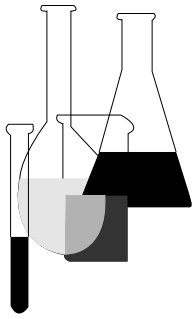
Ecological Effects Test Guidelines

OPPTS 850.1400 Fish Early-Life Stage Toxicity Test



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 850.1400 Fish early-life stage toxicity test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 797.1600 Fish Early Life Stage Toxicity Test; OPP 72–4 Fish Early Life-Stage and Aquatic Invertebrate Life-Cycle Studies (Pesticide Assessment Guidelines, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms) EPA report 540/09-82-024, 1982; and OECD 210 Fish Early-Life Stage Toxicity Test.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

(A) Cumulative mortality.

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

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

(D) Numbers of larvae hatching each day.

(E) Length and weight of surviving animals.

(F) Numbers of deformed larvae.

(G) Numbers of fish exhibiting abnormal behavior.

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

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

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

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

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

(B) Chemical identification data.

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

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

(B) Photoperiods.

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

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

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

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

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

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

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

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

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

(C) Days to hatch and numbers hatched.

(D) Data for length and weight.

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

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

(G) Statistical analysis and treatment of data.

(H) NOEC for each response assessed.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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