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(12) Methods and data records of all chemical analyses of water quality parameters and test substance concentrations, including method validations and reagent blanks.

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§ 797.1600 Fish early life stage toxicity test.

(a) *Purpose.* This guideline is intended to be used for assessing the propensity of chemical substances to produce adverse effects to fish during the early stages of their growth and development. This guideline describes the conditions and procedures for the continuous exposure of several representative species to a chemical substance during egg, fry and early juvenile life stages. The Environmental Protection Agency (EPA) will use data from this test in assessing the potential hazard of the test substance to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in part 792—*Good Laboratory Practice Standards,* apply to this section. In addition, the following definitions are applicable to this specific test guideline:

(1) ''Acclimation'' physiological or behavioral adaptation of organisms to one or more environmental conditions associated with the test method (e.g., temperature, hardness, pH).

(2) ''Carrier'' solvent or other agent used to dissolve or improve the solubility of the test substance in dilution water.

(3) ''Conditioning'' exposure of construction materials, test chambers, and testing apparatus to dilution water or to the test solution prior to the start of the test in order to minimize the sorption of test substance onto the test facilities or the leachig of substances from test facilities into the dilution water or the test solution.

(4) ''Control'' an exposure of test organisms to dilution water only or dilution water containing the test solvent or carrier (no toxic agent is intentionally or inadvertently added).

(5) ''Dilution water'' the water used to produce the flow-through conditions of the test to which the test substance is added and to which the test species is exposed.

(6) ''Early life stage toxicity test'' a test to determine the minimum concentration of a substance which produces a statistically significant observable effect on hatching, survival, development and/or growth of a fish species continuously exposed during the period of their early development.

(7) ''Embryo cup'' a small glass jar or similar container with a screened bottom in which the embryos of some species (i.e., minnow) are placed during the incubation period and which is normally oscillated to ensure a flow of water through the cup.

(8) ''Flow through'' refers to the continuous or very frequent passage of fresh test solution through a test chamber with no recycling.

(9) ''Hardness'' the total concentration of the calcium and magnesium ions in water expressed as calcium carbonate (mg $CaCO_3$/liter).

(10) ''Loading'' the ratio of biomass (grams of fish, wet weight) to the volume (liters) of test solution passing through the test chamber during a specific interval (normally a 24-hr. period).

(11) ''No observed effect concentration (NOEC)'' the highest tested concentration in an acceptable early life stage test: (i) which did not cause the occurrence of any specified adverse effect (statistically different from the control at the 95 percent level); and (ii) below which no tested concentration caused such an occurrence.

(12) ''Observed effect concentration (OEC)'' the lowest tested concentration in an acceptable early life stage test: (i) Which caused the occurrence of any specified adverse effect (statistically different from the control at the 95 percent level); and (ii) above which all tested concentrations caused such an occurrence.

(13) ''Replicate'' two or more duplicate tests, samples, organisms, concentrations, or exposure chambers.

(14) ''Stock solution'' the source of the test solution prepared by dissolving the test substance in dilution water or a carrier which is then added to dilution water at a specified, selected concentration by means of the test substance delivery system.

(15) ''Test chamber'' the individual containers in which test organisms are maintained during exposure to test solution.

(16) ''Test solution'' dilution water with a test substance dissolved or suspended in it.

(17) ''Test substance'' the specific form of a chemical substance or mixture that is used to develop data.

(c) *Test Procedures*—(1) *Summary of test.* (i) The early life stage toxicity test with fish involves exposure of newly fertilized embryos to various concentrations of a test substance. Exposure continues for 28 days post hatch for the minnows and 60 days post hatch for the trout species. During this time various observations and measurements are made in a specific manner and schedule in order to determine the lowest effect and highest no-effect concentrations of the test substance.

(ii) A minimum of five exposure (treatment) concentrations of a test substance and one control are required to conduct an early life stage toxicity test. The concentration of the test substance in each treatment is usually 50 percent of that in the next higher treatment level.

(iii) For each exposure concentration of the test substance and for each control (i.e., regular control and carrier control is required) there shall be:

(A) At least two replicate test chambers, each containing one or more embryo incubation trays or cups; and there shall be no water connections between the replicate test chambers;

(B) At least 60 embryos divided equally in such a manner that test results show no significant bias from the distributions, between the embryo incubation trays or cups for each test concentration and control (i.e., 30 per embryo cup with 2 replicates);

(C) All surviving larvae divided equally between the test chambers for each test concentration and control (e.g., 30 larvae per test chamber with 2 replicates).

(iv) *Duration.* (A) For fathead minnow and sheepshead minnow a test begins when the newly fertilized minnow embryos (less than 48-hours old) are placed in the embryo cups and are exposed to the test solution concentrations. The test terminates following 28 days of post-hatch exposure, i.e., 28 days after the newly hatched fry are transferred from the embryo cups into the test chambers.

(B) For brook trout and rainbow trout a test begins when newly fertilized trout embryos (less than 96-hours old) are placed in the embryo trays or cups and are exposed to the test solution concentrations. The test terminates following 60 days of post-hatch exposure (for an approximate total exposure period of 90 days).

(C) For silverside a test begins with newly fertilized embryos (less than or equal to 48 hours old) and is terminated 28 days after hatching. The chorionic fibrils should be cut before randomly placing the embryos in the egg incubation cups.

(2) [Reserved]

(3) *Range-finding test.* (i) A range finding test is normally performed with the test substance to determine the test concentrations to be used in the early life stage toxicity test, especially when the toxicity is unknown. It is recommended that the test substance concentrations be selected based on information gained from a 4- to 10-day flow-through toxicity test with juveniles of the selected test species.

(ii) The highest concentration selected for the early life stage toxicity test should approximate the lowest concentration indicated in any previous testing to cause a significant reduction in survival. The range of concentrations selected is expected to include both observed effect and no-observed effect levels. The dilution factor between concentrations is normally 0.50, however, other dilution factors may be used as necessary.

(4) *Definitive test*—(i) *General.* (A) A test shall not be initiated until after the test conditions have been met and the test substance delivery system has been observed functioning properly for 48-hours. This includes temperature stability, flow requirements of dilution water, lighting requirements, and the function of strainers and air traps included in the water-supply system, and other conditions as specified previously.

(B) New holding and test facilities should be tested with sensitive organisms (i.e., juvenile test species or

daphnids) before use to assure that the facilities or substances possibly leaching from the equipment will not adversely affect the test organisms during an actual test.

(C) Embryos should be acclimated for as long as practical to the test temperature and dilution water prior to the initiation of the test.

(D) When embryos are received from an outside culture source (i.e., rainbow and brook trout) at a temperature at variance with the recommended test temperature they shall be acclimated to the test temperature. When eggs are received, they should be immediately unpacked and the temperature of the surrounding water determined. Sudden temperature changes should be avoided. Acclimation to the appropriate test temperature should be accomplished within a period of 6 hours, and should incorporate the use of dilution water.

(E) Embryos should be visually inspected prior to placement in the embryo cups or screen trays. All dead embryos shall be discarded. Dead embryos can be discerned by a change in coloration from that of living embryos (e.g., trout embryos turn white when dead). During visual inspection, empty shells, opaque embryos, and embryos with fungus or partial shells attached shall be removed and discarded. If less than 50 percent of the eggs to be used appear to be healthy, all embryos in such a lot shall be discarded.

(ii) *Embryo incubation procedures.* (A) Embryos can be distributed to the embryo cups or screen trays using a pipette with a large bore or a similar apparatus. Newly-hatched silverside fry are very sensitive to handling; the egg incubation cups should not be handled at all the first 5 days after hatching begins. Just before hatching is expected to begin, the embryos should be transferred to clean incubation cups. Trout embryos can be distributed by using a small container which has been precalibrated to determine the approximate number of embryos it can hold; embryos are measured volumetrically in this manner, and are then poured onto the screen tray (or embryo cup). Trout embryos should be separated on the screen tray so that they are not in contact with each other. A final count will ensure the actual number on the screen tray. After random assignment, the screen trays or embryo cups are placed in the test chambers.

(B) Each day until hatch the embryos are visually examined. Minnow embryos may be examined with the aid of a magnifying viewer. Trout embryos should not be touched. Trout embryos should be maintained in low intensity light or in darkness until 1-week post hatch, and are usually examined with the aid of a flashlight or under low intensity light. Dead embryos should be removed and discarded. Any embryos which are heavily infected with fungus shall be discarded and shall be subtracted from the initial number of embryos used as a basis for the calculations of percentage hatch.

(C) When embryos begin to hatch they should not be handled.

(iii) *Initiation of fry exposure.* (A) Forty-eight hours after the first hatch in each treatment level, or when hatching is completed, the live young fish shall be counted and transferred from each embryo cup into the appropriate test chamber. For silverside, all surviving fry are not counted until six days after hatching and are not transferred to embryo cups. All of the normal and abnormal fry shall be gently released into the test chamber by allowing the fry to swim out of each embryo cup; nets shall not be used. The trout embryos incubated on screen trays will hatch out in the test chambers, therefore handling of fish is not necessary.

(B) If necessary, fry can be transferred from one replicate embryo cup to the other replicate within a test concentration to achieve equal numbers in each replicate chamber.

(C) The number of live fry, live normal fry, live embryos, dead embryos and unaccounted for embryos for each cup shall be recorded when hatching is deemed complete. Those fry which are visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal (either in swimming behavior or physical appearance) shall be counted. Late hatching embryos shall be left in the embryo cups to determine if they will eventually hatch or not. The range of time-to-hatch (to the nearest day) for each cup shall be recorded.

(iv) *Time to first feeding.* (A) The first feeding for the fathead and sheepshead minnow fry shall begin shortly after transfer of the fry from the embryo cups to the test chambers. Silversides are fed the first day after hatch. Trout species initiate feeding at swim-up. The trout fry shall be fed trout starter mash three times a day *ad libitum,* with excess food siphoned off daily. The minnow fry shall be fed live newly-hatched brine shrimp nauplii (*Artemia salina*) at least three times a day.

(B) For the first seven days, feeding shall be done at minimum intervals of four hours (i.e., 8 am, 12 noon, and 4 pm); thereafter the fry shall be fed as indicated below.

(v) *Feeding.* (A) The fathead and sheepshead minnow fry shall be fed newly hatched brine shrimp nauplii for the duration of the test at approximately 4-hour intervals three times a day during the week and twice on the weekend after the first week. Trout fry shall be fed at similar intervals and may receive live brine shrimp nauplii in addition to the trout starter food after the first week. Between days 1 and 8 after first hatching, silverside fry are fed the rotifer, *Brachionus plicatilis,* three times daily at a concentration of 5,000 to 10,000 organisms per egg cup (based on 15 fish/cup). From days 9 to 11, the fry shall be fed approximately 2,500 newly hatched brine shrimp (*Artemia*) nauplii and 5,000 to 10,000 rotifers twice daily. For the remainder of the test, the fish will be fed brine shrimp exclusively. The number of organisms used should be gradually increased to approximately 5,000 nauplii by test day 28.

(B) An identical amount of food should be provided to each chamber. Fish should be fed *ad libitum* for 30 minutes with excess food siphoned off the bottom once daily if necessary.

(C) Fish should not be fed for the last 24 hours prior to termination of the test.

(vi) *Carriers.* Water should be used in making up the test stock solutions. If carriers other than water are absolutely necessary, the amount used should be the minimum necessary to achieve solution of the test substance. Triethylene glycol and dimethyl formamide are preferred, but ethanol and acetone can be used if necessary. Carrier concentrations selected should be kept constant at all treatment levels.

(vii) *Controls.* Every test requires a control that consists of the same dilution water, conditions, procedures, and test organisms from the same group used in the other test chambers, except that none of the test substance is added. If a carrier (solvent) is used, a separate carrier control is required in addition to the regular control. The carrier control shall be identical to the regular control except that the highest amount of carrier present in any treatment is added to this control. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

(viii) *Randomization.* The location of all test chambers within the test system shall be randomized. A representative sample of the test embryos should be impartially distributed by adding to each cup or screen tray no more than 20 percent of the number of embryos to be placed in each cup or screen tray and repeating the process until each cup or screen tray contains the specified number of embryos. Alternatively, the embryos can be assigned by random assignment of a small group (e.g., 1 to 5) of embryos to each embryo cup or screen tray, followed by random assignment of a second group of equal number to each cup or tray, which is continued until the appropriate number of embryos are contained in each embryo cup or screen tray. The method of randomization used shall be reported.

(ix) *Observations.* During the embryo exposure period observations shall be made to check for mortality. During the exposure period of the fry, observations shall be made to check for mortality and to note the physical appearance and behavior of the young fish. The biological responses are used in combination with physical and chemical data in evaluating the overall lethal and sublethal effects of the test substance. Additional information on the specific methodology for the data obtained during the test procedure are discussed in the following sections.

(x) *Biological data.* (A) Death of embryos shall be recorded daily.

(B) When hatching commences, daily records of the number of embryos remaining in each embryo cup are required. This information is necessary to quantify the hatching success. A record of all deformed larvae shall be kept throughout the entire post-hatch exposure. Time to swim-up shall be recorded for the trout. Upon transfer of fry from the embryo cups to the test chambers, daily counts of the number of live fish should be made. At a minimum, live fish shall be counted on days 4, 11, 18, 25 and (weekly thereafter for the trout species) finally on termination of the test.

(C) The criteria for death of young fish is usually immobility, especially absence of respiratory movement, and lack of reaction to gentle prodding. Deaths should be recorded daily and dead fish removed when discovered.

(D) Daily and at termination of the test, the number of fish that appear (without the use of a magnifying viewer) to be abnormal in behavior (e.g., swimming erratic or uncoordinated, obviously lethargic, hyperventilating, or over excited, etc.) or in physical appearance (e.g., hemorrhaging, producing excessive mucous, or are discolored, deformed, etc.) shall be recorded and reported in detail.

(E) All physical abnormalities (e.g., stunted bodies, scoliosis, etc.) shall be photographed and the deformed fish which die, or are sacrificed at the termination of the test, shall be preserved for possible future pathological examination.

(F) At termination, all surviving fish shall be measured for growth. Standard length measurements should be made directly with a caliper, but may be measured photographically. Measurements shall be made to the nearest millimeter (0.1 mm is desirable). Weight measurements shall also be made for each fish alive at termination (wet, blotted dry, and to the nearest 0.01 g for the minnows and 0.1 g for the trout). If the fish exposed to the toxicant appear to be edematous compared to control fish, determination of dry, rather than wet, weight is recommended.

(G) Special physiological, biochemical and histological investigations on embryos, fry, and juveniles may be deemed appropriate and shall be performed on a case by case basis.

(5) *Test results.* (i) Data from toxicity tests are usually either continuous (e.g. length or weight measurements) or dichotomous (e.g. number hatching or surviving) in nature. Several methods are available and acceptable for statistical analysis of data derived from early life stage toxicity tests; however, the actual statistical methodology to analyze and interpret the test results shall be reported in detail.

(ii) The significance level for all statistical testing shall be a minimum of $P = 0.05$ (95 percent confidence level).

(A) *Example of statistical analysis.* (*1*) Mortality data for the embryonic stage, fry stage and for both stages in replicate exposure chambers should first be analyzed using a two-way analysis of variance (ANOVA) with interaction model. This analysis will determine if replicates are significantly different from each other. If a significant difference between replicates or a significant interaction exists, cause for the difference should be determined. Modification should then be made in the test apparatus or in handling procedures for future toxicity tests. Further calculations should incorporate the separation of replicates. If no significant difference is observed, replicates may be pooled in further analyses.

(*2*) After consideration of replicate responses, mortality data should then be subjected to one-way ANOVA. The purpose of this analysis is to determine if a significant difference exists in the percentage mortality between control fish and those exposed to the test material.

(*3*) If the one-way ANOVA results in a F ratio that is significant, it would be acceptable to perform t-tests on the control versus each concentration. A second technique is to identify treatment means that are significantly different; this method should involve the additional assumption that the true mean response decreases generally

with increasing concentration. The researcher may also be interested in determining significant differences between concentrations.

(*4*) Growth data should also be analyzed by one-way ANOVA with the inclusion of a covariate to account for possible differences in growth of surviving fry in embryo cup(s) that contain fewer individuals. This condition can occur in cases when the same amount of food is given to each test chamber regardless of the number of survivors.

(B) *Test data to be analyzed.* Data to be statistically analyzed are:

(*1*) Percentage of healthy, fertile embryos at 40–48 hours after initiation of the test. Percentage is based upon initial number used.

(*2*) Percentage of embryos that produce live fry for release into test chambers. Percentage is based on number of embryos remaining after thinning.

(*3*) Percentage of embryos that produce live, normal fry for release into test chambers. Percentage is based upon number of embryos remaining after thinning.

(*4*) Percentage of fry survival at swim-up for trout. Percentage is based upon number of embryos remaining after thinning.

(*5*) Percentage of embryos that produce live fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(*6*) Percentage of embryos that produce live, normal fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(*7*) Weights and lengths of individual fish alive at the end of the test.

(C) It is important that fish length and weight measurements be associated with individual test chambers since the density of the fish and available food should be considered in the growth of the organism.

(iii) *Acceptability criteria.* (A) An early life stage toxicity test is not acceptable unless at least one of the following criteria is significantly different ($p = 0.05$) from control organisms when compared with treated organisms, and the responses are concentration-dependent: mortality of embryos, hatching success, mortality of fry (at swim-up for trout), total mortality throughout the test, and growth (i.e. weight). If no significant effects occur, but the concentrations tested were the highest possible due to solubility or other physio-chemical limitations, the data will be considered for acceptance.

(B) In addition to obtaining significant effects on the exposed test species, a measure of acceptability in the response of control fish is also required.

(C) A test is not acceptable if the average survival of the control fish at the end of the test is less than 80 percent or if survival in any one control chamber is less than 70 percent. For silversides, a test is not acceptable if the average overall survival of the control embryos and fish at the end of the test is less than 60 percent.

(D) If a carrier is used, the criteria for effect (mortality of embryos and fry, growth, etc.) used in the comparison of control and exposed test organisms shall also be applied to the control and control with carrier chambers. For the test to be considered acceptable, no significant difference shall exist between these criteria.

(E) A test is not acceptable if the relative standard deviation (RSD = 100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

(6) *Analytical measurements*—(i) *Analysis of water quality.* Measurement of certain dilution water quality parameters shall be performed every 6 months, to determine the consistency of the dilution water quality. In addition, if data in 30-day increments are not available to show that freshwater dilution water is constant, measurements of hardness, alkalinity, pH, acidity, conductivity, TOC or COD and particulate matter should be conducted once a week in the highest test substance concentration. Measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable.

(ii) *Dissolved oxygen measurement.* The dissolved oxygen concentration shall be measured in each test chamber at the beginning of the test and at least once weekly thereafter (as long as live

organisms are present) in two replicates of the control and the high, medium, and low test substance concentrations.

(iii) *Temperature measurement.* Temperatures shall be recorded in all test chambers at the beginning of the test, once weekly thereafter and at least hourly in one test chamber. When possible, the hourly measurement shall be alternated between test chambers and between replicates.

(iv) *Test substance measurement.* (A) Prior to the addition of the test substance to the dilution water, it is recommended that the test substance stock solution be analyzed to verify the concentration. After addition of the test substance, the concentration of test substance should be measured at the beginning of the test in each test concentration and control(s), and at least once a week thereafter. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. If a malfunction in the delivery system is discovered, water samples shall be taken from the affected test chambers immediately and analyzed.

(B) The measured concentration of test substance in any chamber should be no more than 30 percent higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the test substance delivery system. If the difference is more than 30 percent, the concentration of test substance in the solution flowing into the exposure chamber (influent) should be analyzed. These results will indicate whether the problem is in the stock solution, the test substance delivery system or in the test chamber. Measurement of degradation products of the test substance is recommended if a reduction of the test substance concentration occurs in the test chamber.

(v) *Sampling and analysis methodology.* (A) Generally, total test substance measurements are sufficient; however, the chemical characteristics of the test substance may require both dissolved and suspended test substance measurements.

(B) For measurement of the test substance, water samples shall be taken midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides. Samples of test solutions shall be handled and stored appropriately to minimize loss of test substance by microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(C) Chemical and physical analyses shall be performed using standardized methods whenever possible. The analytical method used to measure the concentration of the test substance in the test solution shall be validated before the beginning of the test. At a minimum, a measure of the accuracy of the method should be obtained on each of two separate days by using the method of known additions, and using dilution water from a tank containing test organisms. Three samples should be analyzed at the next-to-lowest test substance concentration. It is also desirable to study the accuracy and precision of the analytical method for test guideline determination by use of reference (split) samples, or interlaboratory studies, and by comparison with alternative, reference, or corroborative methods of analysis.

(D) An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. In general, atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(E) In addition to analyzing samples of test solution, at least one reagent blank also should be analyzed when a reagent is used in the analysis. Also, at least one sample for the method of known additions should be prepared by adding test substance at the concentration used in the toxicity test.

(d) *Test conditions*—(1) *Test species.* (i) One or more of the recommended test species will be specified in rules under part 799 of this chapter requiring testing of specific chemicals. The recommended test species are:

(A) Fathead minnow (*Pimephales promelas* Rafinesque).

(B) Sheepshead minnow (*Cyprinodon variegatus*).

(C) Brook trout (*Salvelinus fontinalis*).

(D) Rainbow trout (*Salmo gairdneri*).

(E) Atlantic silverside (*Menidia menidia*).

(F) Tidewater silverside (*Menidia peninsulae*).

(ii) Embryos used to initiate the early life stage test shall be less than 48 hours old for the fathead and sheepshead minnows, silversides, and less than 96 hours old for the brook trout and rainbow trout. In addition, the following requirements shall be met:

(A) All embryos used in the test shall be from the same source. Embryos shall be obtained from a stock cultured in-house when possible, and maintained under the same parameters as specified for the test conditions. When it is necessary to obtain embryos from an external source, caution should be exercised to ensure embryo viability and to minimize the possibility of fungal growth. A description of the brood stock history or embryo source shall be made available to EPA upon request.

(B) Test species shall be cared for and handled properly in order to avoid unnecessary stress. To maintain test species in good condition and to maximize growth, crowding shall be prevented, and the dissolved oxygen level shall be maintained near saturation.

(C) Embryos and fish shall be handled as little as possible. Embryos shall be counted and periodically inspected until hatching begins. When larvae begin to hatch, they shall not be handled. Transfer of minnow larvae from embryo cups to test chambers shall not involve the use of nets. No handling is necessary following introduction into the test chambers until termination of the test.

(D) If fathead minnow embryos are obtained from in-house culture units, the embryos should be gently removed from the spawning substrate. The method for separating the fertilized eggs from the substrate is important and can affect the viability of the embryos; therefore the finger-rolling procedure is recommended.

(E) Disease treatment. Chemical treatments to cure or prevent diseases should not be used before, and should not be used during a test. All prior treatments of brood stock should be reported in detail. Severely diseased organisms should be destroyed.

(2) *Test facilities*—(i) *Construction materials.* Construction materials and equipment that contact stock solutions, test solutions, or dilution water into which test embryos or fish are placed should not contain any substances that can be leached or dissolved into aqueous solutions in quantities that can affect test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals from dilution water. Glass, #316 stainless steel, nylon screen and perfluorocarbon plastic (e.g., Teflon#) are acceptable materials. Concrete or rigid (unplasticized) plastic may be used for holding and acclimation tanks, and for 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 should be used to remove rust particles. Natural rubber, copper, brass, galvanized metal, epoxy glues, and flexible tubing should not come in contact with dilution water, stock solutions, or test solutions.

(ii) *Test chambers* (exposure chambers). (A) Stainless steel test chambers should be welded or glued with silicone adhesive, and not soldered. Glass should be fused or bonded using clear silicone adhesive. Epoxy glues are not recommended, but if used ample curing time should be allowed prior to use. As little adhesive as possible should be in contact with the water.

(B) Many different sizes of test chambers have been used successfully. The size, shape and depth of the test chamber is acceptable if the specified flow rate and loading requirements can be achieved.

(C) The actual arrangement of the test chambers can be important to the statistical analysis of the test data. Test chambers can be arranged totally on one level (tier) side by side, or on two levels with each level having one of the replicate test substance concentrations or controls. Regardless of the arrangement, it shall be reported

in detail and considered in the data analysis.

(iii) *Embryo incubation apparatus.* (A) Recommended embryo incubation apparatus include embryo cups for the minnow species and screen trays for the trout species, although embryo cups can be used for the trout species. Embryo cups are normally constructed from approximately 4–5 cm inside diameter, 7–8 cm high, glass jars with the end cut off or similar sized sections of polyethylene tubing. One end of the jar or tubing is covered with stainless steel or nylon screen (approximately 40 meshes per inch is recommended). Embryo cups for silversides are normally constructed by using silicone adhesive to glue a 10-cm high, 363-um nylon mesh tube inside a 9-cm I.D. glass Petri dish bottom. The embryo cups shall be appropriately labeled and then suspended in the test chamber in such a manner as to ensure that the test solution regularly flows through the cup and that the embryos are always submerged but are not agitated too vigorously. Cups may be oscillated by a rocker arm apparatus with a low rpm motor (e.g., 2 rpm) to maintain the required flow of test water. The vertical-travel distance of the rocker arm apparatus during oscillation is normally 2.5–4.0 cm. The water level in the test chambers may also be varied by means of a self-starting siphon in order to ensure exchange of water in the embryo cups.

(B) The trout embryo incubation trays can be made from stainless steel screen (or other acceptable material such as plastic) of about 3–4 mm mesh. The screen tray should be supported above the bottom of the test chamber by two folds of screen or other devices which function as legs or supports. The edges of the screen tray should be turned up to prevent bump spills and to prevent the embryos from rolling off in the event of excessive turbulence. Suspending or supporting the screen tray off the bottom ensures adequate water circulation around the embryos and avoids contact of embryos with possible bottom debris.

(iv) *Test substance delivery system.* (A) The choice of a specific delivery system depends upon the specific properties and requirements of the test substance. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to the test chambers. The system selected shall be calibrated before each test. Calibration includes determining the flow rate through each chamber, and the proportion of stock solution to dilution water delivered to each chamber. The general operation of the test substance delivery system shall be checked at least twice daily for normal operation throughout the test. A minimum of five test substance concentrations and one control shall be used for each test.

(B) The proportional diluter and modified proportional diluter systems and metering pump systems have proven suitable and have received extensive use.

(C) Mixing chambers shall be used between the diluter and the test chamber(s). This may be a small container or flow-splitting chamber to promote mixing of test substance stock solution and dilution water, and is positioned between the diluter and the test chambers for each concentration. If a proportional diluter is used, separate delivery tubes shall run from the flow-splitting chamber to each replicate test chamber. Daily checks on this latter system shall be made.

(D) Silverside fry are injured easily and are susceptible to impingement on the mesh of the incubation cups. Consequently, water flow into and out of the cups when counting fry must be at a slow rate. This can be accomplished by using small diameter (e.g., 2 mm I.D.) capillary tubes to drain the test solution from spitter boxes into the replicate test chambers. The use of a self-starting siphon to gradually lower (i.e., less than or equal to 1 min.) the water level approximately 2 cm in the test chamber is recommended. A minimum water depth of 5 cm should be maintained in the cups. Although it may be satisfactory, a rocker-arm type apparatus has not yet been used with silversides.

(v) *Other equipment required.* (A) An apparatus for removing undesirable organisms, particulate matter and air bubbles.

(B) An apparatus for aerating water.

(C) A suitable magnifying viewer for examination of minnow embryos.

(D) A suitable apparatus for the precise measurement of growth of the fish, including both length (e.g., with metric or ruler caliper or photographic equipment) and weight.

(E) Facilities for providing a continuous supply of live brine shrimp nauplii (*Artemia salina*).

(F) For silversides, facilities for providing a supply of rotifers (*Brachionus plicatilis*) for approximately 11 days.

(G) Facilities (or access to facilities) for performing the required water chemistry analyses.

(vi) *Cleaning of equipment.* (A) Test substance delivery systems and test chambers should be cleaned before use. Test chambers should be cleaned during the test as needed to maintain the dissolved oxygen concentration, and to prevent clogging of the embryo cup screens and narrow flow passages.

(B) Debris can be removed with a rubber bulb and large pipette or by siphoning with a glass tube attached to a flexible hose. Debris should be run into a bucket light enough to observe that no live fish are accidentally discarded.

(vii) *Dilution water*—(A) *General.* (*1*) A constant supply of acceptable dilution water should be available for use throughout the test. Dilution water shall be of a minimum quality such that the test species selected will survive in it for the duration of testing without showing signs of stress (e.g., loss of pigmentation, disorientation, poor response to external stimuli, excessive mucous secretion, lethargy, lack of feeding, or other unusual behavior). A better criterion for an acceptable dilution water for tests on early life stages should be such that the species selected for testing will survive, grow, and reproduce satisfactorily in it.

(*2*) The concentration of dissolved oxygen in the dilution water (fresh or salt) shall be between 90 percent and 100 percent saturation. When necessary, dilution water should be aerated by means of airstones, surface aerators, or screen tubes before the introduction of the test substance.

(*3*) Water that is contaminated with undesirable microorganisms (e.g., fish pathogens) shall not be used. If such contamination is suspected, the water should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter before use. Efficacy of the sterilizer can be determined by using standard plate count methods.

(B) *Freshwater.* (*1*) Natural water (clean surface or ground water) is preferred, however, dechlorinated tap water may be used as a last resort. Reconstituted freshwater is not recommended as a practical dilution water for the early life stage toxicity test because of the large volume of water required.

(*2*) Particulate and dissolved substance concentrations should be measured at least twice a year and should meet the following specifications:

Substance	Concentration maximum
Particulate matter	<20 mg/liter.
Total organic carbon (TOC)	<2 mg/liter.
Chemical oxygen demand (COD)	<5 mg/liter.
Un-ionized ammonia	<1 µg/liter.
Residual chlorine	<1 µg/liter.
Total organoposphorus pesticides	<50 ng/liter.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs).	<50 ng/liter.
Total organic chlorine	<25 ng/liter.

(*3*) During any one month, freshwater dilution water should not vary more than 10 percent from the respective monthly averages of hardness, alkalinity and specific conductance; the monthly pH range should be less than 0.4 pH units.

(C) *Saltwater.* (*1*) Marine dilution water is considered to be of constant quality if the minimum salinity is greater than $15^0/_{00}$ and the weekly range of the salinity is less than $15^0/_{00}$. The monthly range of pH shall be less than 0.8 pH units. Saltwater shall be filtered to remove larval predators. A pore size of ≤20 micrometers (µm) is recommended. For silversides, the recommended salinity is 20 ppt and shall be maintained between 15 and 25 ppt throughout testing.

(*2*) Artificial sea salts may be added to natural seawater during periods of low salinity to maintain salinity above $15^0/_{00}$.

(3) *Test parameters*—(i) *Dissolved oxygen concentration.* It is recommended that the dissolved oxygen concentration be maintained between 90 and 100

percent saturation; but it shall be no less than 75 percent saturation at all times for both minnow species and between 90 and 100 percent saturation for the trout species in all test chambers. Dilution water in the head box may be aerated, but the test solution itself shall not be aerated.

(ii) *Loading and flow rate.* (A) The loading in test chambers should not exceed 0.1 grams of fish per liter of test solution passing through the test chamber in 24 hours. The flow rate to each chamber should be a minimum of 6 tank volumes per 24 hours. During a test, the flow rates should not vary more than 10 percent from any one test chamber to any other.

(B) A lower loading or higher flow rate or both shall be used if necessary to meet the following three criteria at all times during the test in each chamber containing live test organisms:

(*1*) The concentration of dissolved oxygen shall not fall below 75 percent saturation for the fathead and sheepshead minnows and 90 percent for the rainbow and brook trout;

(*2*) The concentration of un-ionized ammonia should not exceed 1 μg/1; and

(*3*) The concentration of toxicant should not be lowered (i.e., caused by uptake by the test organisms and/or materials on the sides and bottoms of the chambers) more than 20 percent of the mean measured concentration.

(iii) *Temperature.* (A) The recommended test temperatures are:

(*1*) Fathead minnow—25 °C for all life stages.

(*2*) Sheepshead minnow—30 °C for all life stages.

(*3*) Rainbow and brook trout—10 °C for embryos. 12 °C for fry and alevins.

(*4*) Atlantic and tidewater silversides—25 °C for all life stages.

(B) Excursions from the test temperature shall be no greater than ±2.0 °C. It is recommended that the test system be equipped with an automatic alarm system to alert staff of instantaneous temperature changes in excess of 2 °C. If the water is heated (i.e., for minnow species), precautions should be taken to ensure that supersaturation of dissolved gases is avoided. Temperatures shall be recorded in all test chambers at the beginning of the test and weekly thereafter. The temperature shall be recorded at least hourly in one test chamber throughout the test.

(iv) *Light.* (A) Brook and rainbow trout embryos shall be maintained in darkness or very low light intensity through one week post-hatch, at which time a 14-hour light and 10-hour dark photoperiod shall be provided.

(B) For fathead and sheepshead minnows, a 16-hour light and 8-hour dark (or 12:12) photoperiod shall be used throughout the test period.

(C) For silversides, a 14-hour light and 10-hour dark photoperiod shall be used throughout the test period.

(D) A 15-minute to 30-minute transition period between light and dark is optional.

(E) Light intensities ranging from 30 to 100 lumens at the water surface shall be provided; the intensity selected should be duplicated as closely as possible for all test chambers.

(e) *Reporting.* A report of the results of an early life stage toxicity test shall include the following:

(1) Name of test, sponsor, investigator, laboratory, and dates of test duration.

(2) Detailed description of the test substance including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers (solvents) or other additives used.

(3) The source of the dilution water, its chemical characteristics, and a description of any pretreatment.

(4) Detailed information about the test organisms including scientific name and how verified and source history, observed diseases, treatments, acclimation procedure, and concentration of any contaminants and the method of measurement.

(5) A description of the experimental design and the test chambers, the depth and volume of the solution in the chambers, the way the test was begun, the number of organisms per treatment, the number of replicates, the loading, the lighting, a description of the test substance delivery system, and the flow rate as volume additions per 24 hours.

(6) Detailed information on feeding of fish during the toxicity test, including

type of food used, its source, feeding frequency and results of analysis (i.e., concentrations) for contaminants.

(7) Number of embryos hatched, number of healthy embryos, time to hatch, mortality of embryos and fry, measurements of growth (weight and length), incidence of pathological or histological effects and observations of other effects or clinical signs, number of healthy fish at end of test.

(8) Number of organisms that died or showed an effect in the control and the results of analysis for concentration(s) of any contaminant in the control(s) should mortality occur.

(9) Methods used for, and the results of (with standard deviation), all chemical analyses of water quality and test substance concentration, including validation studies and reagent blanks; the average and range of the test temperature(s).

(10) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(11) A description of any abnormal effects and the number of fish which were affected during each period between observations in each chamber, and the average concentration of test substance in each test chamber.

(12) Reference to the raw data location.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19064, May 20, 1987]

§ 797.1930 Mysid shrimp acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes a test using mysid shrimp as test organisms to develop data on the acute toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in part 792—*Good Laboratory Practice Standards* of this chapter, apply to this test guideline. The following definitions also apply to this guideline.

(1) ''Death'' means the lack of reaction of a test organism to gentle prodding.

(2) ''Flow-through'' means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

(3) ''LC_{50}'' means that experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of time.

(4) ''Loading'' means the ratio of test organisms biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

(5) ''Retention chamber'' means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating loss of organisms in outflow water.

(6) ''Static system'' means a test chamber in which the test solution is not renewed during the period of the test.

(c) *Test procedures*—(1) *Summary of the test.* In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. In a flow-through test, the rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into the test chambers. Mysids in the test chambers are observed periodically during the test, the dead mysids removed and the findings recorded. Dissolved oxygen concentration, pH, temperature, salinity, the concentration of test substance, and other water quality characteristics are measured at specified intervals in test chambers. Data collected during the test are used to develop concentration-response curves and LC_{50} values for the test substance.

(2) [Reserved]