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Method 1005.0: Sheepshead Minnow, *Cyprinodon variegatus,* Embryo-Larval Survival and Teratogenicity Test; Chronic Toxicity

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Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms

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SECTION 12

TEST METHOD

SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1005.0

12.1 SCOPE AND APPLICATION

12.1.1 This method, adapted in part from USEPA (1981) and USEPA (1987b), estimates the chronic toxicity of effluents and receiving waters to the sheepshead minnow, *Cyprinodon variegatus*, using embryos and larvae in a nine-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

12.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

12.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

12.2 SUMMARY OF METHOD

12.2.1 Sheepshead minnow, *Cyprinodon variegatus*, embryos and larvae are exposed in a static renewal system to different concentrations of effluent or to receiving water starting shortly after fertilization of the eggs through four days posthatch. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

12.3 INTERFERENCES

12.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

12.3.2 Adverse effects of low dissolved oxygen concentrations (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the effect of toxic substances.

12.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample after adjusting the sample salinity for use in marine testing.

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.3 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.3 pH units in pH-controlled tests (USEPA, 1996).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.5.1.1).

12.3.5.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992);

or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.4 SAFETY

12.4.1 See Section 3, Health and Safety.

12.5 APPARATUS AND EQUIPMENT

12.5.1 Facilities for holding and acclimating test organisms.

12.5.2 Sheepshead minnow culture unit -- see Subsection 12.6.12 below. To perform toxicity tests on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from an in-house sheepshead minnow culture unit. If necessary, embryos can be obtained from outside sources if shipped in well oxygenated water in insulated containers.

12.5.2.1 A test using 15 embryos per test vessel and four replicates per concentration, will require 360 newly-fertilized embryos at the start of the test. A test with a minimum of 10 embryos per test vessel and three replicates per concentration, and with five effluent concentrations and a control, will require a minimum of 180 embryos at the start of the test.

12.5.3 Brine Shrimp, *Artemia*, Culture Unit -- for feeding sheepshead minnow larvae in the continuous culture unit (see Subsection 12.6.12 below).

12.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L, and maintain sample temperature at 4°C.

12.5.5 Environmental Chamber or Equivalent Facility with Temperature Control (25 ± 1°C).

12.5.6 Water Purification System -- Millipore Milli-Q®, deionized water (DI) or equivalent.

12.5.7 Balance -- analytical, capable of accurately weighing to 0.00001 g. Note: An analytical balance is not needed for this test but is needed for other specified toxicity test methods with growth endpoints.

12.5.8 Reference Weights, Class S -- for checking the performance of the balance. The reference weights should bracket the expected weights of reagents, and the expected weights of the weighing pans and the weights of the weighing pans plus larvae.

12.5.9 Air Pump -- for oil free air supply.

12.5.10 Air Lines, and Air Stones -- for aerating water containing embryos, larvae, or supplying air to test solution with low DO

12.5.11 Meters, pH and DO -- for routine physical and chemical measurements.

12.5.12 Standard or Micro-Winkler Apparatus -- for determining DO (optional).

12.5.13 Dissecting microscope -- for examining embryos and larvae.

12.5.14 Light box -- for counting and observing embryos and larvae.

12.5.15 Refractometer -- for determining salinity.

12.5.16 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

12.5.17 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

12.5.18 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

12.5.19 Test Chambers -- four (minimum of three), borosilicate glass or non-toxic plastic labware per test concentration. Care must be taken to avoid inadvertently removing embryos or larvae when test solutions are decanted from the chambers. To avoid potential contanimation from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick). The covers are removed only for observation and removal of dead organisms.

12.5.20 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

12.5.21 Wash Bottles -- for deionized water, for washing embryos from substrates and containers, and for rinsing small glassware and instrument electrodes and probes.

12.5.22 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

12.5.23 Pipets, volumetric -- Class A, 1-100 mL.

12.5.24 Pipets, automatic -- adjustable, 1-100 mL.

12.5.25 Pipets, serological -- 1-10 mL, graduated.

12.5.26 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

12.5.27 Droppers and glass tubing with fire polished aperatures, 4 mm ID -- for transferring embryos and larvae.

12.5.28 Siphon with bulb and clamp -- for cleaning test chambers.

12.5.29 NITEX® or stainless steel mesh sieves, ($\leq$150 µm, 500 µm, and 3-5 mm) -- for collecting *Artemia* nauplii and fish embryos, and for spawning baskets, respectively.

12.6 REAGENTS AND CONSUMABLE MATERIALS

12.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.6.2 Data sheets (One set per test) -- for data recording (see Figure 1).

12.6.3 Tape, colored -- for labelling test chambers.

12.6.4 Markers, waterproof -- for marking containers, etc.

12.6.5 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for Standards and Calibration Check (see USEPA Method 150.1, USEPA, 1979b).

12.6.6 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

12.6.7 Laboratory quality assurance samples and standards -- for calibration of the above methods.

12.6.8 Reference toxicant solutions -- see Section 4, Quality Assurance.

12.6.9 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

12.6.10 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.10.1 Saline test and dilution water -- The salinity of the test water must be in the range of 5 to 32‰. The salinity should vary no more than ±2‰ among chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of the water should be similar.

12.6.10.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sheepshead minnow embryos to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition, it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities -- a hypersaline brine derived from natural seawater or artificial sea salts.

Test Dates: _____ Species: _____

Type Effluent: _____ Field: _____ Lab: _____ Test: _____

Effluent Tested: _____

Original pH: _____ Salinity: _____ DO: _____

CONCENTRATION:
Replicate I:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

CONCENTRATION:
Replicate II:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

Comments: _____

Note: Final endpoint for this test is total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) (see Subsection 12.10.9 and 12.13).

Figure 1. Data form for sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival/teratogenicity test. Daily record of embryo-larval survival/terata and test conditions.

CONCENTRATION:
Replicate III:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

CONCENTRATION:
Replicate IV:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

Comments:

Note: Final endpoint for this test is total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) (see Subsection 12.10.9 and 12.13).

Figure 1. Data form for sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival/teratogenicity test. Daily record of embryo-larval survival/terata and test conditions (CONTINUED).

12.6.10.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However if 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested using HSB is limited to 80% at 20‰ salinity, and 70% at 30‰ salinity.

12.6.10.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

12.6.10.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

12.6.10.3.3 High quality (and preferably high salinity) seawater should be filtered to at least l0 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

12.6.10.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on volume being generated) to ensure that salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

12.6.10.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 µm filter and poured directly into portable containers (20 L) cubitainers or polycarbonate water cooler jugs are suitable. The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained at room temperature until used.

12.6.10.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

12.6.10.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the HSB is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is one part in five (one part brine to four parts deionized water). To make 1 L of seawater at 20‰ salinity from a HSB of 100‰, divide 1 L (1000 mL) by 5.0. The result, 200 mL, is the quantity of HSB needed to make 1 L of sea water. The difference, 800 mL, is the quantity of deionized water required.

12.6.10.3.8 Table 1 illustrates the composition of test solutions at 20‰ if they are prepared by serial dilution of effluent with 20‰ salinity seawater.

12.6.10.4 Artificial sea salts: HW MARINEMIX® brand sea salts have been used successfully at the USEPA, Region 6, Houston laboratory to culture sheepshead minnows and perform the embryo-larval survival and teratogenicity test. EMSL-Cincinnati has found FORTY FATHOMS® artificial sea salts to be suitable for culturing sheepshead minnows and for performing the larval survival and growth test and embryo-larval test. Artificial sea salts may be used for culturing sheepshead minnows and for the embryo larval test if the criteria for acceptability of test data are satisfied (see Subsection 12.11).

12.6.10.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and salts should be mixed in a separate container -- not the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte et al., 1984; Bower, 1983) before it is used for culturing or testing. After adding the water, place an airstone in the container, cover, and aerate the solution mildly for at least 24 h before use.

12.6.11 BRINE SHRIMP, *ARTEMIA*, CULTURE -- for feeding cultures.

12.6.11.1 Newly-hatched *Artemia* nauplii are used as food in the sheepshead minnow culture, and a brine shrimp culture unit should be prepared (USEPA, 2002a). Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

12.6.11.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985; Leger, et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A sample of newly hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organic chlorine pesticides exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982).

TABLE 1. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 20‰, USING 20‰ NATURAL OR ARTIFICAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICAL SEA SALTS

		Solutions To Be Combined		
Effluent Solution	Effluent Conc. (%)	Volume of Effluent Solution		Volume of Diluent Seawater (20‰)
1	100[1,2]	4000 mL		- - -
2	50	2000 mL Solution 1	+	2000 mL
3	25	2000 mL Solution 2	+	2000 mL
4	12.5	2000 mL Solution 3	+	2000 mL
5	6.25	2000 mL Solution 4	+	2000 mL
Control	0.0			2000 mL
Total				10000 mL

[1] This illustration assumes: (1) the use of 400 mL of test solution in each of four replicates and 400 mL for chemical analysis (total of 2000 mL) for the control and five concentrations of effluent (2) an effluent dilution factor of 0.5, and (3) the effluent lacks appreciable salinity. A sufficient initial volume (4000 mL) of effluent is prepared by adjusting the salinity to the desired level. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 20‰ seawater (natural seawater, hypersaline brine, or artificial seawater). The salinity of the initial 4000 mL of 100% effluent is adjusted to 20‰ by adding 80 g of dry artificial sea salts (HW MARINEMIX or FORTY FATHOMS®), and mixing for 1 h. Test concentrations are then made by mixing appropriate volumes of salinity-adjusted effluent and 20‰ salinity dilution water to provide 4000 mL of solution for each concentration. If hypersaline brine alone (100‰) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be achieved would be 80% at 20‰ salinity, and 70% at 30‰ salinity.

[2] The same procedures would be followed in preparing test concentrations at other salinities between 20‰ and 30‰: (1) The salinity of the bulk (initial) effluent sample would be adjusted to the appropriate salinity using artificial sea salts or hypersaline brine, and (2) the remaining effluent concentrations would be prepared by serial dilution, using a large batch (10 L) of seawater for dilution water, which had been prepared at the same salinity as the effluent, using natural seawater, hypersaline and deionized water.

12.6.11.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L of deionized water, to a 2 L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985d, USEPA, 2002a; and ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for five to 10 minutes. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 minutes without aeration.

4. Drain the nauplii into a beaker or funnel fitted with a ≤150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

12.6.11.4 Testing *Artemia* nauplii as food for toxicity test organisms.

12.6.11.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the sheepshead minnow larvae. The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

12.6.11.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

12.6.11.4.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

12.6.11.4.4 The average seven-day survival of larvae should be 80% or greater, and (2) the average dry weight of larvae should be 0.60 mg or greater, if dried and weighed immediately after the test, or (3) the average dry weight of larvae should be 0.50 mg or greater, if the larvae are preserved in 4% formalin before drying and weighing. The above minimum weights presume that the age of the larvae at the start of the test is not greater than 24 h.

12.6.12 TEST ORGANISMS, SHEEPSHEAD MINNOWS, *CYPRINODON VARIEGATUS*

12.6.12.1 Brood stock

12.6.12.1.1 Adult sheepshead minnows for use as brood stock may be obtained by seine in Gulf of Mexico and Atlantic coast estuaries, from commercial sources, or from young fish raised to maturity in the laboratory. Feral brood stocks and first generation laboratory fish are preferred, to minimize inbreeding.

12.6.12.1.2 To detect disease and to allow time for acute mortality due to the stress of capture, field-caught adults are observed in the laboratory a minimum of two weeks before using as a source of gametes. Injured or diseased fish are discarded.

12.6.12.1.3 Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. The larvae, juvenile, and adult fish should be kept in appropriate size rearing tanks, maintained at ambient laboratory temperature. The larvae should be fed sufficient newly hatched *Artemia* nauplii daily to assure that live nauplii are always present. Juveniles are fed frozen adult brine shrimp and a commercial flake food, such as TETRA SM-80® or MARDEL AQUARIAN® Tropical Fish Flakes, or equivalent. Adult fish are fed flake food three or four times daily, supplemented with frozen adult brine shrimp.

12.6.12.1.3.1 Sheepshead minnows reach sexual maturity in three-to-five months after hatch, and have an average standard length of approximately 27 mm for females and 34 mm for males. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity and are to be used for natural spawning, the temperature should be controlled at 18-20°C.

12.6.12.1.4 Adults can be maintained in natural or artificial seawater in a flow-through or recirculating, aerated system consisting of an all-glass aquarium, or equivalent.

12.6.12.1.5 The system is equipped with an undergravel or outside biological filter of shells (see Spotte, 1973; Bower, 1983) for conditioning the biological filter, or a cartridge filter, such as a MAGNUM® Filter, or an EHEIM® Filter, or equivalent, at a salinity of 20-30‰ and a photoperiod of 16 h light/8 h dark.

12.6.12.2 Obtaining Embryos for Toxicity Tests

12.6.12.2.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory. Ripe eggs can be obtained either by natural spawning or by intraperitoneal injection of the females with human chorionic gonadotrophin (HCG) hormone. If the culturing system for adults is temperature controlled, natural spawning can be induced. Natural spawning is preferred because repeated spawnings can be obtained from the same brood stock, whereas with hormone injection, the brood stock is sacrificed in obtaining gametes.

12.6.12.2.2 It should be emphasized that the injection and hatching schedules given below are to be used only as guidelines. Response to the hormone varies from stock to stock and with temperature. Time to hatch and percent hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity.

12.6.12.2.3 Forced Spawning

12.6.12.2.3.1 HCG is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains 1,000 IU to be reconstituted in 10 mL of saline. Freeze-dried HCG which comes with premeasured and sterilized saline is the easiest to use. Use of a 50 IU dose requires injection of 0.05 mL of reconstituted hormone solution. Reconstituted HCG may be used for several weeks if kept in the refrigerator.

12.6.12.2.3.2 Each female is injected intraperitoneally with 50 IU HCG on two consecutive days, starting at least four days prior to the beginning of a test. Two days following the second injection, eggs are stripped from the females and mixed with sperm derived from excised macerated testes. At least 10 females and five males are used per test to ensure that there is a sufficient number of viable embryos.

12.6.12.2.3.3 HCG is injected into the peritoneal cavity, just below the skin, using as small a needle as possible. A 50 IU dose is recommended for females approximately 27 mm in standard length. A larger or smaller dose may be used for fish which are significantly larger or smaller than 27 mm. With injections made on days one and two, females which are held at 25°C should be ready for stripping on Day 4. Ripe females should show pronounced abdominal swelling, and release at least a few eggs in response to a gentle squeeze. Injected females should be isolated from males. It may be helpful if fish that are to be injected are maintained at 20°C before injection, and the temperature raised to 25°C on the day of the first injection.

12.6.12.2.3.4 Prepare the testes immediately before stripping the eggs from the females. Remove the testes from three to five males. The testes are paired, dark grey organs along the dorsal midline of the abdominal cavity. If the head of the male is cut off and pulled away from the rest of the fish, most of the internal organs can be pulled out of the body cavity, leaving the testes behind. The testes are placed in a few mL of seawater until the eggs are ready.

12.6.12.2.3.5 Strip the eggs from the females, into a dish containing 50-100 mL of seawater, by firmly squeezing the abdomen. Sacrifice the females and remove the ovaries if all the ripe eggs do not flow out freely. Break up any clumps of ripe eggs and remove clumps of ovarian tissue and underripe eggs. Ripe eggs are spherical, approximately 1 mm in diameter, and almost clear.

12.6.12.2.3.6 While being held over the dish containing the eggs, the testes are macerated in a fold of NITEX® screen (250-500 µm mesh) dampened with seawater. The testes are then rinsed with seawater to remove the sperm from tissue, and the remaining sperm and testes are washed into the dish. Let the eggs and milt stand together for 10-15 minutes, swirling occasionally.

12.6.12.2.3.7 Pour the contents of the dish into a beaker, and insert an airstone. Aerate gently, such that the water moves slowly over the eggs, and incubate at 25°C for 60-90 minutes. After incubation, wash the eggs on a NITEX® screen and resuspend them in clean seawater.

12.6.12.2.4 Natural Spawning

12.6.12.2.4.1 Short-term (Demand) Embryo Production

12.6.12.2.4.1.1 Adult fish should be maintained at 18-20°C in a temperature controlled system. To obtain embryos for a test, adult fish (generally, at least eight to 10 females and three males) are transferred to a spawning chamber, with a photoperiod of 16 h light/8 h dark and a temperature of 25°C, two days before the beginning of the test. The spawning chambers are approximately 20 x 35 x 22 cm high (USEPA, 1978), and consist of a basket of 3-5 mm NITEX® mesh, made to fit into a 57-L (15 gal) aquarium. Spawning generally will begin within 24 h or less. The embryos will fall through the bottom of the basket and onto a collecting screen (250-500 µm mesh) or tray below the basket. The collecting tray should be checked for embryos the next morning. The number of eggs produced is highly variable. The number of spawning units required to provide the embryos needed to perform a toxicity test is determined by experience. If the trays do not contain sufficient embryos after the first 24 h, discard the embryos, replace the trays, and collect the embryos for another 24 h or less. To help keep the embryos clean, the adults are fed while the screens are removed.

12.6.12.2.4.1.2 The embryos are collected in a tray placed on the bottom of the tank. The collecting tray consists of 250-500 µm NITEX® screen attached to a rigid plastic frame. The collecting trays with newly-spawned, embryos are removed from the spawning tank, and the embryos are collected from the screens by washing them with a wash bottle or removing them with a fine brush. The embryos from several spawning units may be pooled in a single container to provide a sufficient number to conduct the test(s). The embryos are transferred into a petri dish or equivalent, filled with fresh culture water, and are examined using a dissecting microscope or other suitable magnifying device. Damaged and infertile eggs are discarded (see Figure 2). It is strongly recommended that the embryos be obtained from fish cultured in-house, rather than from outside sources, to eliminate the uncertainty of damage caused by shipping and handling that may not be observable, but which might affect the results of the test.

12.6.12.2.4.1.3 After sufficient embryos are collected for the test, the adult fish are returned to the (18-20°C) culture tanks.

12.6.12.2.4.2 Sustained Natural Embryo Production

12.6.12.2.4.2.1 Sustained (long-term), daily, embryo production can be achieved by maintaining mature fish in tanks, such as a (285 L or 75 gal) LIVING STREAM® tank, at a temperature of 23-25°C. Embryos are produced daily, and when needed, embryo "collectors" are placed on the bottom of the tank on the afternoon preceding the start of the test. The next morning, the embryo collectors are removed and the embryos are washed into a shallow glass culture dish using artificial seawater.

12.6.12.2.4.2.2 Four embryo collectors, approximately 20 cm x 45 cm, will approximately cover the bottom of the 285 L tank. The collectors are fabricated from plastic fluorescent light fixture diffusors (grids), with cells approximately 14 mm deep X 14 mm square. A screen consisting of 500 µm mesh is attached to one side (bottom) of the grid with silicone adhesive. The depth and small size of the grid protects the embryos from predation by the adult fish.

12.6.12.2.4.2.3 The brood stock is replaced annually with feral stock.

12.6.12.2.5 Test Organisms

12.6.12.2.5.1 Embryos spawned over a less than 24-h period, are used for the test. These embryos may be used immediately to start a test or may be placed in a suitable container and transported for use at a remote location. When overnight transportation is required, embryos should be obtained when they are no more than 8-h old. This permits the tests at the remote site to be started with less than 24-h old embryos. Embryos should be transported or shipped in clean, insulated containers, in well aerated or oxygenated fresh seawater or aged artificial sea water of correct salinity, and should be protected from extremes of temperature and any other stressful conditions during

transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution, should be less than 2°C. Instantaneous changes of pH, dissolved ions, osmotic strength, and DO should also be kept to a minimum.

12.6.12.2.5.2 The number of embryos needed to start the test will depend on the number of tests to be conducted and the objectives. If the test is conducted with four replicate test chambers (minimum of three) at each toxicant concentration and in the control, with 15 embryos (minimum of 10) in each test chamber, and the combined mortality of embryos prior to the start of the test is less than 20%, 400 viable embryos are required for the test.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

12.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests

12.8 CALIBRATION AND STANDARDIZATION

12.8.1 See Section 4, Quality Assurance

12.9 QUALITY CONTROL

12.9.1 See Section 4, Quality Assurance

12.10 TEST PROCEDURES

12.10.1 Test Solutions

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 400-500 mL, and 400 mL for chemical analysis, would require approximately 2.0-2.5 L or more of sample per test per day.

12.10.1.2 Effluents

12.10.1.2.1 The selection of the effluent test concentration should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ±100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥0.5 dilution factor.** If 100‰ salinity HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ and 70% at 30‰ salinity.

12.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

12.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates (minimum of three) per concentration for five concentrations of effluent and a control, each containing 400 mL of test solution, is approximately 4 L. Prepare enough test solution (approximately 3000 mL) at each effluent concentration to refill the test chambers and provide at least 400 mL additional volume for chemical analyses.

12.10.1.2.4 Maintain the effluent at 0-6°C. Plastic containers such as 8-20 L cubitainers have proven successful for effluent collection and storage.

12.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample(s) to make the test solutions should be adjusted to the test temperature (25 ± 1°C) and maintained at that temperature during the addition of dilution water.

12.10.1.2.6 Higher effluent concentrations (i.e., 25%, 50%, and 100%) may require aeration to maintain adequate dissolved oxygen concentrations. However, if one solution is aerated, all concentrations must be aerated. Aerate effluent as it warms and continue to gently aerate test solutions in the test chambers for the duration of the test.

12.10.1.2.7 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labelled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

12.10.1.3 Dilution Water

12.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artifical seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 3 in Section 7, Dilution Water). Other artificial sea salts may be used for culturing sheepshead minnows if the control criteria for acceptability of test data are satisfied.

12.10.2 START OF THE TEST

12.10.2.1 Tests should begin as soon as possible, preferably within 24 h after sample collection. For on-site toxicity studies, no more than 24 h should elapse between collection of the effluent and use in an embryo-larval study. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity studies unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.2.2 Label the test chambers with a marking pen. Use color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each concentration (including controls) is to have four replicates (minimum of three). Use 500 mL beakers, crystallization dishes, nontoxic disposable plastic labware, or equivalent for test chambers.

12.10.2.3 Prepare the test solutions (see Table 1) and add to the test chambers.

12.10.2.4 Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are evenly dispersed.

12.10.2.5 The test is started by randomly placing embryos from the common pool, using a small bore (2 mm), fire polished, glass tube calibrated to contain approximately the desired number of embryos, into each of four replicate test chamber, until each chamber contains 15 embryos (minimum of 10), for a total of 60 embryos (minimum of 30) for each concentration (four replicates recommended, three minimum) (see Appendix A). The amount of water added to the chambers when transferring the embryos should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with undamaged embryos. It may be more convenient and efficient to transfer embryos to intermediate containers of dilution water for examination and counting. After the embryos have been examined and counted in the intermediate container, assign them to the appropriate test chamber and transfer them with a minimum of dilution water.

12.10.2.7 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

12.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

12.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 µE/m^2/s, or 50-100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The test water temperature should be maintained at 25 ± 1°C. The salinity should be 5 to 32‰ ± 2‰ to accommodate receiving waters that may fall within this range. The salinity should vary no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Holding, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® Serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the fish.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

12.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period at each test concentration and in the control.

12.10.6.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine the temperature variation in the environmental chambers.

12.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

12.10.6.1.4 Record all measurements on the data sheet (Figure 1).

12.10.6.2 Routine Biological Observations

12.10.6.2.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine and count the embryos. Remove the dead embryos (milky colored and opaque) and record the number. If the rate of mortality or fungal infection exceeds 20% in the control chambers, or if excessive nonconcentration related mortality occurs, terminate the test and start a new test with new embryos. If the above mortality conditions do not occur, continue the test for the full nine days.

12.10.6.2.2 At 25°C, hatching begins on about the sixth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed and/or debilitated larvae, daily (see Figure 2 for illustrations of morphological development of embryo and larva). Deformed larvae are those with gross morphological abnormalities such as curved spines, lack of appendages, lack of fusiform shape (non-distinct mass),

a colored beating heart in an opaque mass, lack of mobility, abnormal swimming, or other characteristics that preclude survival. Remove dead embryos and dead and deformed larvae as previously discussed and record the numbers for all test observations (see Figure 2).

12.10.6.2.3 Protect the embryos and larvae from unnecessary disturbance during the test by carefully carrying out the daily test observations, solution renewals, and removal of dead organisms. Make sure the test organisms remain immersed during the performance of the above operations.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the tank causes a problem.

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 The test solutions are renewed daily using freshly prepared solution, immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent and receiving water samples used in toxicity tests should be collected daily, and no more than 24 h should elapse between collection of the sample and use in the test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples must be collected, preferably on days 1, 3, and 5. Maintain the samples at 0-6°C until used.

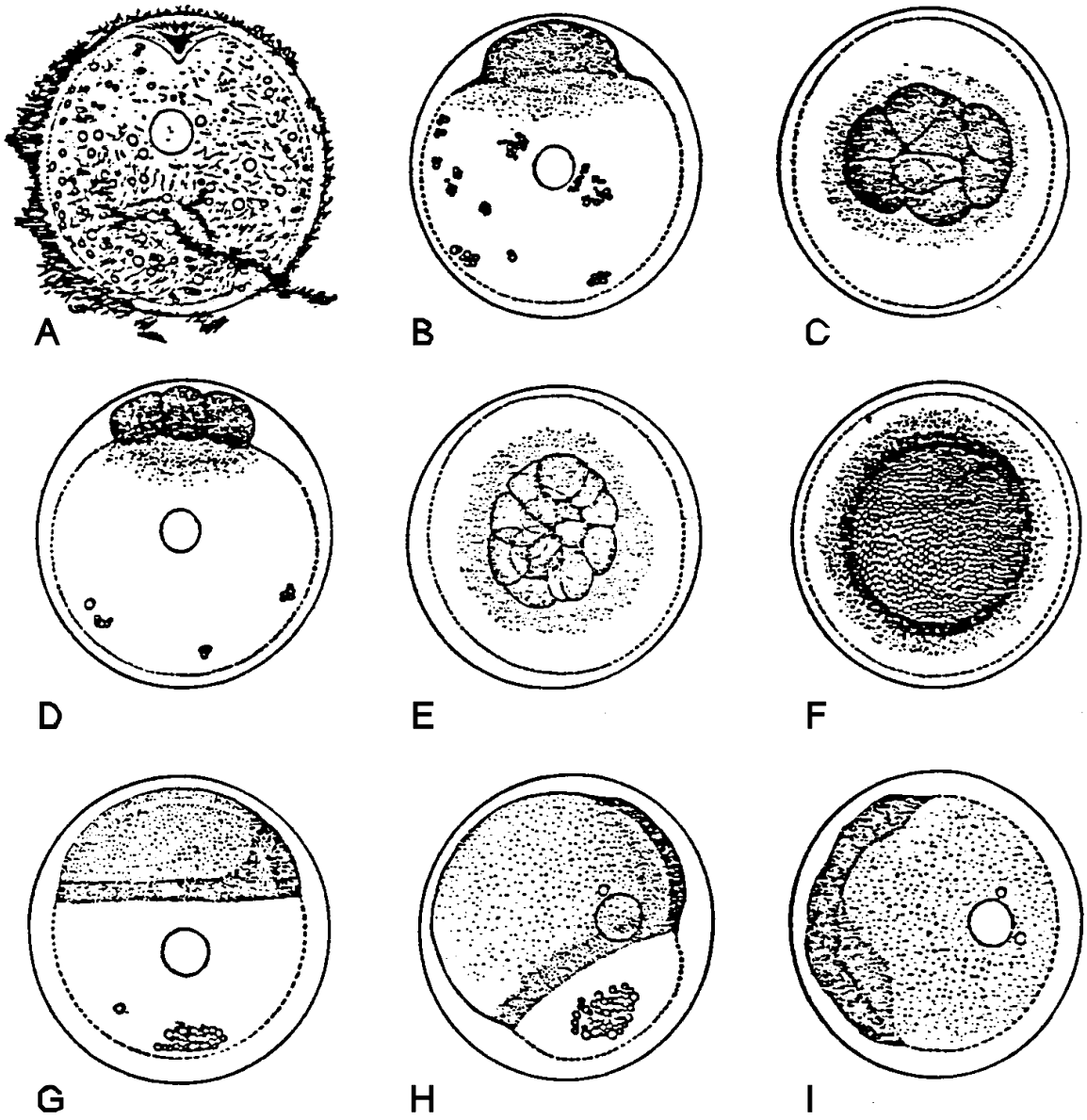

Figure 2 Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: A. Mature unfertilized egg, showing attachment filaments and micropyle, X33; B. Blastodisc fully developed; C,D. Blastodisc, 8 cells; E. Blastoderm, 16 cells; F. Blastoderm, late cleavage stage; G. Blastoderm with germ ring formed, embryonic shield developing; H. Blastoderm covers over ¾ of yolk, yolk noticeably constricted; I. Early embryo. From Kuntz (1916).

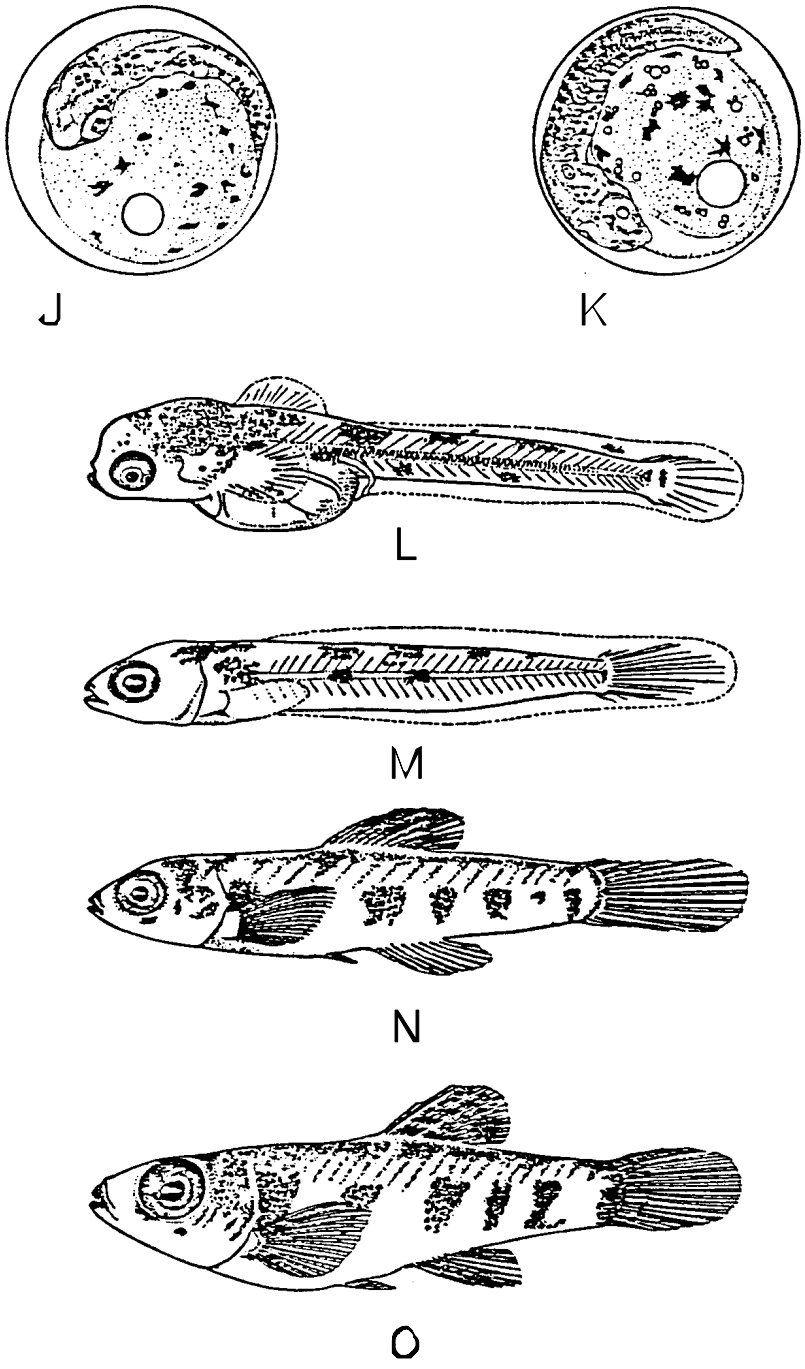

Figure 2. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: J. Embryo 48 h after fertilization, now segmented throughout, pigment on yolk sac and body, otoliths formed; K. Posterior portion of embryo free from yolk and moves freely within egg membrane, 72 h after fertilization; L. Newly hatched fish, actual length 4 mm; M. Larval fish five days after hatching, actual length 5 mm; N. Young fish 9 mm in length; O. Young fish 12 mm in length (CONTINUED). From Kuntz (1916).

12.10.8.2 The test solutions are adjusted to the correct salinity and renewed daily using freshly collected samples. During the daily renewal process, 7-10 mm of water is left in the chamber to ensure that the embryos and larvae remain submerged during the renewal process. New test solution (400 mL) should be added slowly by pouring down the side of the test chamber to avoid exposing the embryos and larvae to excessive turbulence.

12.10.8.3 Prepare test solutions daily, making a minimum of five concentrations and a control. If concurrent effluent and receiving water testing occurs, the effluent test salinity should closely approximate that of the receiving water test. If an effluent is tested alone, select a salinity which approximately matches the salinity of the receiving waters. Table 1 illustrates the quantities of effluent, seawater, deionized water, and artificial sea salts needed to prepare 3 L of test solution at each effluent concentration for tests conducted at 20‰ salinity.

12.10.9 TERMINATION OF THE TEST

12.10.9.l The test is terminated after nine days of exposure, or four days post-hatch, whichever comes first. Count the number of surviving, dead, and deformed and/or debilitated larvae, and record the numbers of each. The deformed larvae are treated as dead. Keep a separate record of the total number of deformed larvae for use in reporting the teratogenicity of the test solution.

12.11 ACCEPTABILITY OF TEST RESULTS

12.11.1 For the test results to be acceptable, survival in the controls must be at least 80% or better.

12.12 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

12.12.1 A summary of test conditions and test acceptability criteria is listed in Table 2.

12.13 DATA ANALYSIS

12.13.1 General

12.13.1.1 Tabulate and summarize the data.

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, dead larvae, and deformed larvae. The EC endpoints are calculated using Probit Analysis (Finney, 1971). LOEC and NOEC values, for total mortality, are obtained using a hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

TABLE 2. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1005.0)[1]

1. Test type:	Static renewal (required)
2. Salinity:	5‰ to 32‰ (±2‰ of the selected test salinity) (recommended)
3. Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory light (recommended)
5. Light intensity:	10-20 µE/m^2/s, or 50-100 ft-c (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	400-500 mL (recommended)
8. Test solution volume:	250-400 mL per replicate (loading and DO restrictions must be met) (recommended)
9. Renewal of test solutions:	Daily (required)
10. Age of test organisms:	Less than 24 h old (required)
11. No. of embryos per chamber:	15 (recommended) 10 (required minimum)
12. No. replicate test chambers per concentration:	4 (recommended) 3 (required minimum)
13. No. embryos per concentration:	60 (recommended) 30 (required minimum)
14. Feeding regime:	Feeding not required
15. Aeration:	None unless DO falls below 4.0 mg/L (recommended)
16. Dilution water:	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2, or equivalent) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 2. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1005.0)[1]

17. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
18. Dilution factor:	Effluent: ≥0.5 (recommended) Receiving waters: None, or ≥0.5 (recommended)
19. Test duration:	9 days (required)
20. Endpoints:	Percent hatch; percent larvae dead or with debilitating morphological and/or behavior abnormalities such as: gross deformities; curved spine; disoriented, abnormal swimming behavior; surviving normal larvae from original embryos (required)
21. Test acceptability criteria:	80% or greater survival in controls (required)
22. Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23. Sample volume required:	5 L per day (recommended)

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined in Figure 3. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% mortality in all of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC endpoints.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's

Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

12.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method or the Graphical Method may be used (see Appendices H-K).

12.13.2.5 Example of Analysis of Survival Data

12.13.2.5.1 The data for this example are listed in Table 3. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variences of the transformed observations at each SDS concentration and control are listed in Table 3. A plot of the data is provided in Figure 4. Since there is 100% mortality in all replicates for the 8.0 mg/L concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

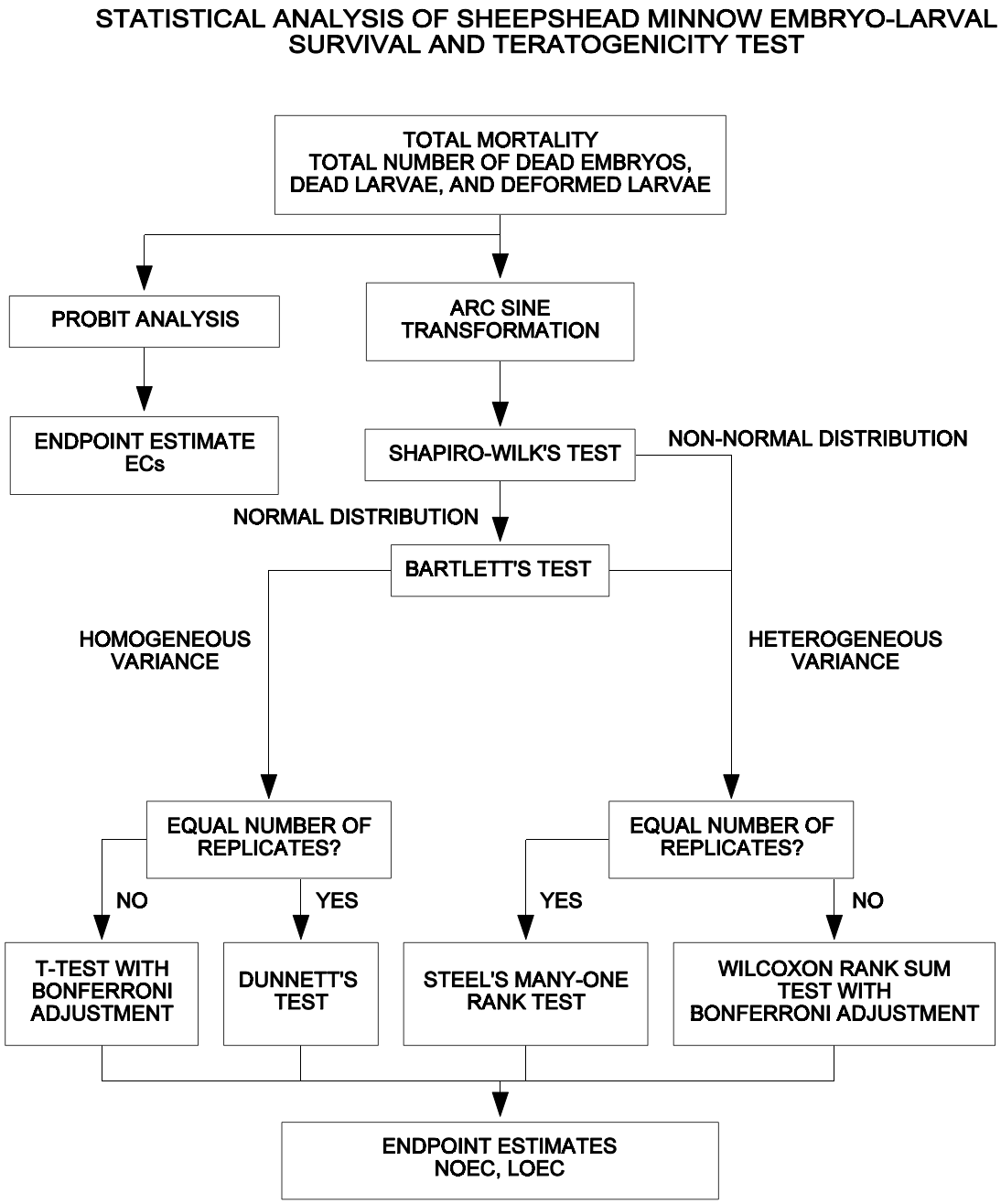

Figure 3. Flowchart for statistical analysis of sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival and teratogenicity test. Survival and terata data.

TABLE 3. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL TOTAL MORTALITY DATA

			SDS Concentration (mg/L)				
	Replicate	Control	0.5	1.0	2.0	4.0	8.0
RAW	A	0.1	0.0	0.0	0.3	0.9	1.0
	B	0.0	0.2	0.1	0.1	0.7	1.0
	C	0.1	0.2	0.1	0.2	0.8	1.0
	D	0.0	0.1	0.2	0.4	0.8	1.0
ARC SINE	A	0.322	0.159	0.159	0.580	1.249	–
TRANS-	B	0.159	0.464	0.322	0.322	0.991	–
FORMED	C	0.322	0.464	0.322	0.464	1.107	–
	D	0.159	0.322	0.464	0.685	1.107	–
Mean ($\overline{Y}_i$)		0.241	0.352	0.317	0.513	1.114	
S^2_i		0.009	0.021	0.016	0.024	0.011	
i		1	2	3	4	5	

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

TABLE 4: CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		SDS Concentration (mg/L)				
Replicate	Control	0.5	1.0	2.0	4.0	8.0
A	0.081	-0.193	-0.158	0.067	0.135	–
B	-0.082	0.112	0.005	-0.191	-0.123	–
C	0.081	0.112	0.005	-0.049	-0.007	–
D	-0.082	-0.030	0.147	0.172	-0.007	–

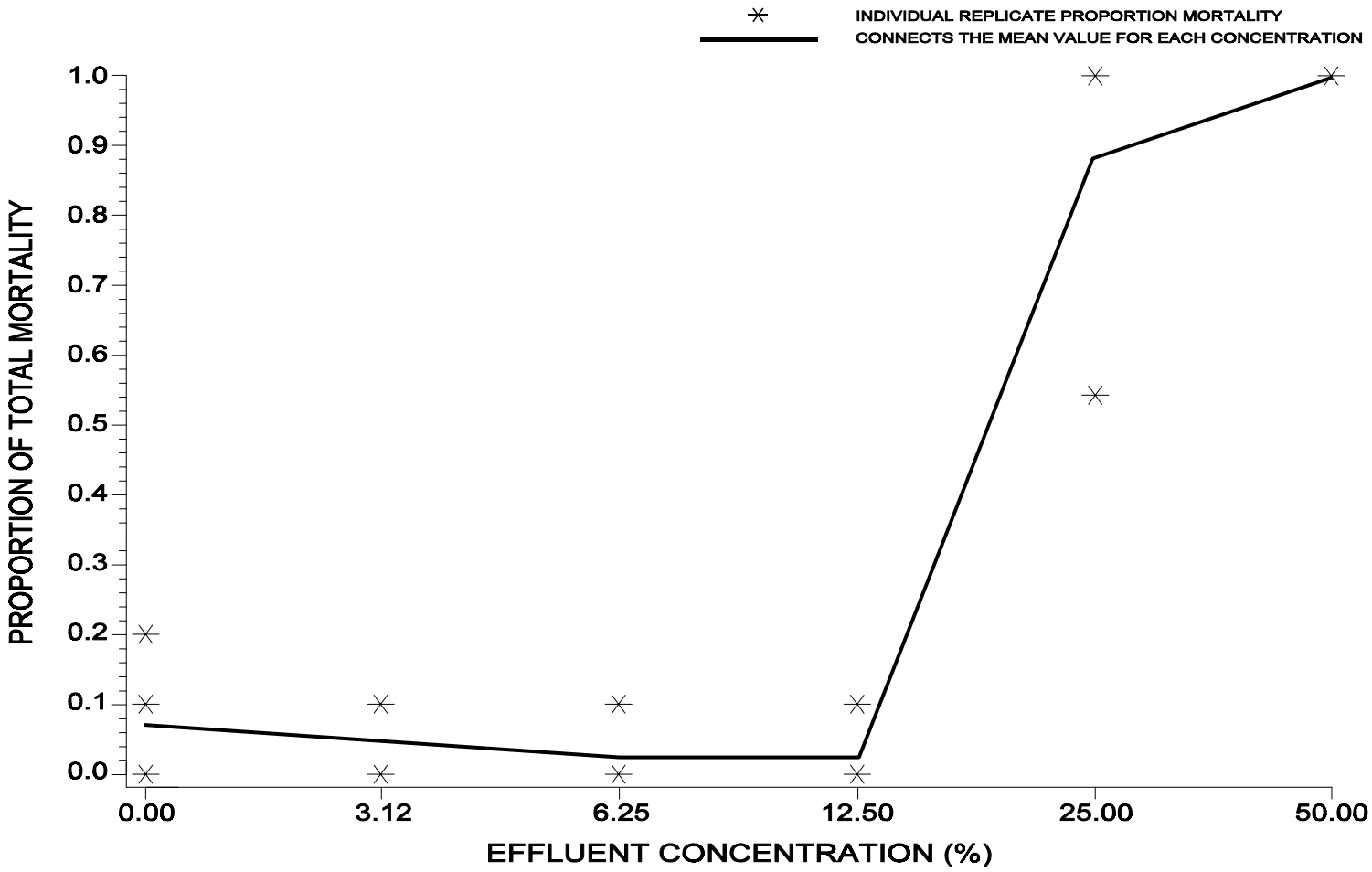

Figure 4. Plot of Sheepshead minnow, *Cyprinodon variegatus*, total mortality data from the embryo-larval test

12.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

12.13.2.6.3 For this set of data, n = 20

$$\bar{X} = \frac{1}{20} (-0.005) = 0.000$$

$$D = 0.2428$$

12.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where: $X^{(i)}$ = the ith ordered observation

The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.193	11	0.005
2	-0.191	12	0.005
3	-0.158	13	0.067
4	-0.123	14	0.081
5	-0.082	15	0.081
6	-0.082	16	0.112
7	-0.049	17	0.112
8	-0.030	18	0.135
9	-0.007	19	0.147
10	-0.007	20	0.172

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 6.

TABLE 6. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.365	$X^{(20)} - X^{(1)}$
2	0.3211	0.338	$X^{(19)} - X^{(2)}$
3	0.2565	0.293	$X^{(18)} - X^{(3)}$
4	0.2085	0.295	$X^{(17)} - X^{(4)}$
5	0.1686	0.194	$X^{(16)} - X^{(5)}$
6	0.1334	0.163	$X^{(15)} - X^{(6)}$
7	0.1013	0.130	$X^{(14)} - X^{(7)}$
8	0.0711	0.097	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.012	$X^{(11)} - X^{(10)}$

12.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D} \left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.2428} (0.4807)^2 = 0.952$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 12.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.952 is greater than the critical value, conclude that the data are normally distributed.

12.13.2.7 Test for Homogeneity of Variance

12.13.2.7.1 The test used to examine whether the variation in mean proportion mortality is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{P} V_i\right) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i

$$\bar{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i)^2}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3\ (p-1)]^{-1}\ [\sum_{i=1}^{p} i/v_i\ -\ (\sum_{i=1}^{p} v_i)-1]$$

12.13.2.7.2 Since B is approximately distributed as chi-square with p-1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p-1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

12.13.2.7.3 For the data in this example, $V_i = 3$, p=5, $\bar{S}^2 = 0.0162$, and C = 1.133. The calculated B value is:

$$B = \frac{(15)\ [\ln\ (0.01262)]-3\sum_{i=1}^{p} \ln(S_i^2)}{1.33}$$

$$= \frac{15\ (-4.1227)\ -\ 3\ (-20.9485)}{1.33}$$

$$= 0.886$$

12.13.2.7.4 Since B is approximately distributed as chi-square with p-1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.277 for a significance level of 0.01. Since B = 0.886 is less than the critical value of 13.277, conclude that the variances are not different.

12.13.2.8 Dunnett's Procedure

12.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 7.

TABLE 7. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

12.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

N = 20

$$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 0.962$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 1.409$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 1.267$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.051$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.454$$

$$G = T_1 + T_2 + T_3 + T_4 = 10.143$$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(28.561) - \frac{(10.143)^2}{20} = 1.996$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7.383 - \frac{(10.143)^2}{20} = 1.996$$

$$SSW = SST - SSB = 2.239 - 1.996 = 0.243$$

$$S_w^2 = SSB/(p-1) = 1.996/(5-1) = 0.499$$

$$S_w^2 = SSW/(N-p) = 0.243/(20-5) = 0.016$$

12.13.2.8.3 Summarize these calculations in the ANOVA table (Table 8).

TABLE 8. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	4	1.996	0.499
Within	15	0.243	0.016
Total	19	2.239	

12.13.2.8.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion surviving for concentration i

$\bar{Y}_1$ = mean proportion surviving for the control

S_w = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

Since we are looking for an increased response in percent of total mortality over control, the control mean is subtracted from the mean at a concentration.

12.13.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 0.5 mg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{0.352 - 0.241}{[0.1265\sqrt{(1/4) + (1/4)}]} = 1.241$$

12.13.2.8.6 Since the purpose of this test is to detect a significant increase in total mortality, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 15 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.36. The mean proportion of total mortality for concentration "i" is considered significantly less than the mean proportion of total mortality for the control if t_i is greater than the critical value. Therefore, the 2.0 mg/L and the 4.0 mg/L concentrations have significantly higher mean proportions of total mortality than the control. Hence the NOEC is 1.0 mg/L and the LOEC is 2.0 mg/L.

TABLE 9. CALCULATED T VALUES

SDS Concentration (mg/L)	i	t_i
0.5	2	1.241
1.0	3	0.850
2.0	4	3.041
4.0	5	9.760

12.13.2.8.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's procedure

S_W = the square root of the within mean square

n_1 = the number of replicates in the control

n = The common number of replicates at each concentration (this assumes equal replication at each concentration)

12.13.2.8.8 In this example:

$$MSD = 2.36\ (0.1265)\ \sqrt{(1/4) + (1/4)}$$

$$= 2.36\ (0.1265)\ (0.7071)$$

$$= 0.211$$

12.13.2.8.9 The MSD (0.450) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Add the MSD to the transformed control mean.

   0.241 + 0.211 = 0.452

2. Obtain the untransformed values for the control mean and the sum calculated in 1.

   $[\ \text{Sine}\ (0.241)\ ]^2 = 0.057$

   $[\ \text{Sine}\ (0.452)\ ]^2 = 0.191$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from Step 2.

   $MSD_u = 0.191 - 0.057 = 0.134$

12.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion of total mortality between the control and any SDS concentration that can be detected as statistically significant is 0.134.

12.13.2.8.11 This represents a 268% increase in mortality from the control.

12.13.2.9 Calculation of the LC50

12.13.2.9.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix H.

TABLE 10. DATA FOR PROBIT ANALYSIS

	SDS Concentration (mg/L)					
	Control	0.5	1.0	2.0	4.0	8.0
Number Dead	2	5	4	10	32	40
Number Exposed	40	40	40	40	40	40

12.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears appropriate for this set of data.

12.13.2.9.3 Figure 5 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Probit Analysis of Sheepshead Minnow Embryo-Larval Survival and Teratogenicity Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	2	0.5000	0.0000
0.5000	40	5	0.1250	0.0369
1.0000	40	4	0.1000	0.0094
2.0000	40	10	0.2500	0.1745
4.0000	40	32	0.8000	0.7799
8.0000	40	40	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.782
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Sheephead Minnow Embryo-Larval Survival and Teratogenicity Data

Estimated LC/EC Values and Confidence Limits

Point		Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC	1.00	1.187	0.643	1.601
LC/EC	50.00	2.912	2.432	3.361

Figure 5. Output for USEPA Probit Analysis Program, Version 1.5

12.14 PRECISION AND ACCURACY

12.14.1 PRECISION

12.14.1.1 Single-Laboratory Precision

12.14.1.1.1 Data on the single-laboratory precision of the Sheepshead Minnow Embryo-larval Survival and Teratogenicity test are available for eight tests with copper sulfate and five tests with sodium dodecyl sulfate (USEPA, 1989a). The data for the first five tests show that the same NOEC and LOEC, 240 µg Cu/L and 270 µg Cu/L, respectively, were obtained in all five tests, which is the maximum level of precision that can be attained. Three additional tests (6-8) were performed with narrower (20 µg) concentration intervals, to more precisely identify the threshold concentration. The NOEC and LOEC for these tests are 200 µg and 220 µg Cu/L, respectively. For sodium dodecyl sulfate, the NOEC's and LOEC's for all tests are 2.0 and 4.0 mg/L, respectively. The precision, expressed as the coefficient of variation (CV%), is indicated in Tables 11-12. For copper (Cu), the coefficient of variation, depending on the endpoint used, ranges from 2.5 to 6.1% which indicates excellent precision. For sodium dodecyl sulfate (SDS), the coefficient of variation, depending on the endpoint used, ranges from 11.7 to 51.2%, indicating acceptable precision.

12.14.1.2 Multilaboratory Precision

12.14.1.2.1 Data on the multilaboratory precision of this test are not yet available.

12.14.2 Accuracy

12.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 11. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST PERFORMED IN HW MARINEMIX® ARTIFICIAL SEAWATER, USING EMBRYOS FROM FISH MAINTAINED AND SPAWNED IN HW MARINEMIX® ARTIFICIAL SEAWATER USING COPPER (CU) SULFATE AS REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	EC1 (µg/L)	EC5 (µg/L)	EC10 (µg/L)	EC50 (µg/L)	NOEC (µg/L)
1	173	189	198	234	240
2	*	*	*	*	240
3	*	*	*	*	240
4	182	197	206	240	240
5	171	187	197	234	240
6	*	*	*	*	< 200
7	*	*	*	*	220
8	195	203	208	226	220
n:	4	4	4	4	7
Mean:	180	194	202	233	NA
CV (%):	6.1	3.8	2.8	2.5	NA

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Terry Hollister, Aquatic Biologist, Houston Facility, Environmental Services Division, Region 6, USEPA, Houston, Texas.

3 *Cyprinodon variegatus* embryos used in the tests were less than 20 h old when the tests began. Two replicate test chambers were used for the control and each toxicant concentration. Ten embryos were randomly added to each test chamber containing 250 mL of test or control water. Solutions were renewed daily. The temperature and salinity of the test solutions were 24 ± 1°C and 20‰, respectively.

4 Copper test concentrations were prepared using copper sulfate. Copper concentrations for Tests 1-5 were: 180, 210, 240, 270, and 300 µg/L. Copper concentrations for Test 6 were: 220, 240, 260, 280, and 300 µg/L. Copper concentrations for Tests 7-8 were: 200, 220, 240, 260, and 280 µg/L. Tests were conducted over a two-week period.

5 Adults collected in the field.

6 NOEC Range: 200-240 µg/L (this represents a difference of two exposure concentrations).

7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

* = Data did not fit the Probit model.

TABLE 12. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST PERFORMED IN HW MARINEMIX® ARTIFICIAL SEAWATER, USING EMBRYOS FROM FISH MAINTAINED AND SPAWNED IN HW MARINEMIX® ARTIFICIAL SEAWATER USING SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	EC1 (mg/L)	EC5 (mg/L)	EC10 (mg/L)	EC50 (mg/L)	NOEC (mg/L)
1	1.7	2.0	2.2	3.1	2.0
2	*	*	*	*	4.0
3	0.4	0.7	0.9	2.5	2.0
4	1.9	2.2	2.4	3.3	2.0
5	1.3	1.7	1.9	3.0	2.0
n:	4	4	4	4	5
Mean:	1.3	1.6	1.9	2.9	NA
CV (%):	51.2	41.6	35.0	11.7	NA

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Terry Hollister, Aquatic Biologist, Houston Facility, Environmental Services Division, Region 6, USEPA, Houston, Texas.

3 *Cyprinodon variegatus* embryos used in the tests were less than 20 h old when the tests began. Two replicate test chambers were used for the control and each toxicant concentration. Ten embryos were randomly added to each test chamber containing 250 mL of test or control water. Solutions were renewed daily. The temperature and salinity of the test solutions were 24 ± 1°C and 20‰, respectively.

4 SDS concentrations for all tests were: 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L. Tests were conducted over a three-week period.

5 Adults collected in the field.

6 NOEC Range: 2.0-4.0 mg/L (this represents a difference of two exposure concentrations).

7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

* = Data did not fit the Probit model.