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Method 1004.0: Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival and Growth 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 11

TEST METHOD

SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS* LARVAL SURVIVAL AND GROWTH TEST METHOD 1004.0

11.1 SCOPE AND APPLICATION

11.1.1 This method, adapted in part from USEPA (1987b), estimates the chronic toxicity of effluents and receiving waters to the sheepshead minnow, *Cyprinodon variegatus*, using newly hatched larvae in a seven-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 species.

11.1.2 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).

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

11.1.4 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.

11.1.5 This method 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.

11.2 SUMMARY OF METHOD

11.2.1 Sheepshead minnow, *Cyprinodon variegatus*, larvae (preferably less than 24-h old) are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and weight of the larvae.

11.3 INTERFERENCES

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

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

11.3.3 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).

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

11.3.5 Food added during the test may sequester metals and other toxic substances and reduce the apparent toxicity of the test substance. However, in a growth test the nutritional needs of the organisms must be satisfied, even if feeding has the potential to confound test results.

11.3.6 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 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. 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.

11.3.6.1 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 11.3.6.2. 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.

11.3.6.1.1 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).

11.3.6.1.2 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).

11.3.6.1.3 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 11.3.6.1.1).

11.3.6.1.4 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 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 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.

11.4 SAFETY

11.4.1 See Section 3, Health and Safety.

11.5 APPARATUS AND EQUIPMENT

11.5.1 Facilities for holding and acclimating test organisms.

11.5.2 Brine shrimp, *Artemia*, culture unit -- see Subsection 11.6.14 below and Section 4, Quality Assurance.

11.5.3 Sheepshead minnow culture unit -- see Subsection 11.6.15 below. The maximum number of larvae required per test will range from a maximum of 360, if 15 larvae are used in each of four replicates, to a minimum of 240 per test, if 10 larvae are used in each of four replicates. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be obtained from other sources if shipped in well oxygenated saline water in insulated containers.

11.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

11.5.5 Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

11.5.6 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

11.5.7 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

11.5.8 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

11.5.9 Drying oven -- 50-105°C range, for drying larvae.

11.5.10 Air pump -- for oil-free air supply.

11.5.11 Air lines, and air stones -- for aerating water containing embryos or larvae, or for supplying air to test solutions with low DO.

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

11.5.13 Standard or micro-Winkler apparatus -- for determining DO (optional).

11.5.14 Dissecting microscope -- for checking embryo viability.

11.5.15 Desiccator -- for holding dried larvae.

11.5.16 Light box -- for counting and observing larvae.

11.5.17 Refractometer -- for determining salinity.

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

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

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

11.5.21 Test chambers -- four for each concentration and control. Borosilicate glass 1000 mL beakers or modified Norberg and Mount (1985) glass chambers used in the short-term inland silverside test may be used. It is recommended that each chamber contain a minimum of 50 mL/larvae and allow adequate depth of test solution (5.0 cm). To avoid potential contamination 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).

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

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

11.5.24 Crystallization dishes, beakers, culture dishes (1 L), or equivalent -- for incubating embryos.

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

11.5.26 Separatory funnels, 2-L -- two to four for culturing *Artemia* nauplii.

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

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

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

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

11.5.31 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

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

11.5.33 Forceps -- for transferring dead larvae to weighing boats.

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

11.6 REAGENTS AND CONSUMABLE MATERIALS

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

11.6.2 Data sheets (one set per test) -- for data recording.

11.6.3 Vials, marked -- 24 per test, containing 4% formalin or 70% ethanol, to preserve larvae (optional).

11.6.4 Weighing pans, aluminum -- 24 per test.

11.6.5 Tape, colored -- for labeling test chambers.

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

11.6.7 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).

11.6.8 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.l, USEPA, 1979b), or reagents -- for modified Winkler analysis.

11.6.9 Laboratory quality control samples and standards -- for calibration of the above methods.

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

11.6.11 Ethanol (70%) or formalin (4%) -- for use as a preservative for the fish larvae.

11.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

11.6.13 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.

11.6.13.1 Saline test and dilution water -- The salinity of the test water must be in the range of 20 to 32‰. The salinity should vary by 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. This test is not recommended for salinities less than 20‰.

11.6.13.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sheepshead minnow larvae 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.

11.6.13.3 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 will be 80% at 20‰ salinity and 70% at 30‰ salinity.

11.6.13.3.1 The ideal container for making brine 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.

11.6.13.3.2 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.

11.6.13.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

11.6.13.3.4 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 the 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.

11.6.13.3.5 After the required salinity is attained, the HSB should be filtered a second time through a l 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 HSB was generated and its salinity. Containers of HSB should be stored in the dark and maintained at room temperature until used.

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

11.6.13.3.7 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 brine is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is 1 part in 5 (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 brine needed to make 1 L of seawater. The difference, 800 mL, is the quantity of deionized water required.

11.6.13.4 Artificial sea salts: FORTY FATHOMS® brand sea salts have been used successfully at the EMSL-Cincinnati to maintain and spawn sheephead minnows and perform the larval survival and growth test (see Section 7, Dilution Water). HW MARINEMIX® sea salts have been used successfully at the USEPA Region 6 Houston Laboratory to maintain and spawn sheephead minnows and perform the larval growth and survival test and the embryo-larval survival and teratogenicity test. In addition, a slightly modified version of the GP2 medium (Spotte et al., 1984) has been successfully used to perform the sheepshead minnow survival and growth test (Table 1). Artifical sea salts may be used for culturing sheepshead minnows and for the larval survival and growth test if the criteria for acceptability of test data are satisfied (see Subsection 11.12).

11.6.13.4.1 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 the salts should be mixed in a separate container -- not in 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 air stone in the container, cover, and aerate the solution mildly for 24 h before use.

11.6.13.4.2 The GP2 reagent grade chemicals (Table 1) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be

between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

TABLE 1. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (l990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

11.6.14 BRINE SHRIMP, *ARTEMIA*, NAUPLII -- for feeding cultures and test organisms

11.6.14.1 Newly-hatched *Artemia* nauplii (see USEPA, 2002a) are used as food for sheepshead minnow larvae in toxicity tests and in the maintenance of continuous stock cultures. 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.

11.6.14.2 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, and 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 organochlorine 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.)

11.6.14.3 *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 (USEPA, 1985a; USEPA, 2002a; 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 5-10 minutes. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min 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.

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

11.6.14.4.1 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 (see Subsection 11.12). 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.

11.6.14.4.2 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.

11.6.14.4.3 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.

11.6.15 TEST ORGANISMS, SHEEPSHEAD MINNOWS, *CYPRINODON VARIEGATUS*

11.6.15.1 Brood Stock

11.6.15.1.1 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.

11.6.15.1.2 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.

11.6.15.1.3 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 (age one month) are fed flake food three or four times daily, supplemented with frozen adult brine shrimp.

11.6.15.1.3.1 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.

11.6.15.1.4 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.

11.6.15.1.5 The system is equipped with an undergravel or outside biological filter of shells (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.

11.6.15.2 Obtaining Embryos for Toxicity Tests (See USEPA, 1978)

11.6.15.2.1 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.

11.6.15.2.2 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 viable hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity. The coordination of spawning and hatching is further complicated by the fact that, even under the most ideal conditions, embryos spawned over a 24-h period may hatch over a 72-h period. Therefore, it is advisable (especially if natural spawning is used) to obtain fertilized eggs over several days to ensure that a sufficient number of newly hatched larvae (less than 24 h old) will be available to initiate a test.

11.6.15.2.3 Forced Spawning

11.6.15.2.3.1 HCG is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains l,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.

11.6.15.2.3.2 Each female is injected intraperitoneally with 50 IU HCG on two consecutive days, starting at least 10 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 ten females and five males are used per test to ensure that there is a sufficient number (400) of viable embryos.

11.6.15.2.3.3 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 days 4, 5, and 6. 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.

11.6.15.2.3.4 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.

11.6.15.2.3.5 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.

11.6.15.2.3.6 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 min, swirling occasionally.

11.6.15.2.3.7 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 min. After incubation, wash the eggs on a NITEX® screen and resuspend them in clean seawater. Examine the eggs periodically under a dissecting microscope until they are in the 2-8 cell stage. (The stage at which it is easiest to tell the developing embryos from the abnormal embryos and unfertilized eggs; see Figure 1). The eggs can then be gently rolled on a NITEX® screen and culled (see Section 6, Test Organisms).

11.6.15.2.4 Natural Spawning

11.6.15.2.4.1 Cultures of adult fish to be used for spawning are maintained at 18-20°C until embryos are required. When embryos are required, raise the temperature to 25°C in the morning, seven or eight days before the beginning of a test. That afternoon, transfer the adult fish (generally, at least five females and three males) to a spawning chamber (approximately, 20 × 35 × 22 cm high; USEPA, 1978), which is a basket constructed of 3-5 mm NITEX® mesh, made to fit a 57-L (15 gal) aquarium. Spawning generally will begin within 24 h or less. Embryos will fall through the bottom of the basket and onto a collecting screen (250-500 µm mesh) or tray below the basket. Allow the embryos to collect for 24 h. Embryos are washed from the screen, checked for viability, and placed in incubation dishes. Replace the screens until a sufficient number of embryos have been collected. One-to-three spawning aquaria can be used to collect the required number of embryos to run a toxicity test. To help keep the embryos clean, the adults are fed while the screens are removed.

11.6.15.2.5 Incubation

11.6.15.2.5.1 Four hours post-fertilization, the embryos obtained by natural or forced spawning are rolled gently with a finger on a 250-500 µm Nitex® screen to remove excess fibers and tissue. The embryos have adhesive threads and tend to adhere to each other. Gentle rolling on the screen facilitates the culling process described below. To reduce fungal contamination of the newly spawned embryos after they have been manipulated, they should be placed in a 250 µm sieve and briskly sprayed with seawater from a squeeze bottle.

11.6.15.2.5.2 Under a dissecting microscope, separate and discard abnormal embryos and unfertilized eggs. While they are checked, the embryos are maintained in seawater at 25°C. The embryos should be in Stages C-G, Figure 1.

11.6.15.2.5.3 If the test is prepared with four replicates of 15 larvae at each of six treatments (five effluent concentrations and a control), and the combined mortality of eggs and larvae prior to the start of the test is less than 20%, approximately 400 viable embryos are required at this stage.

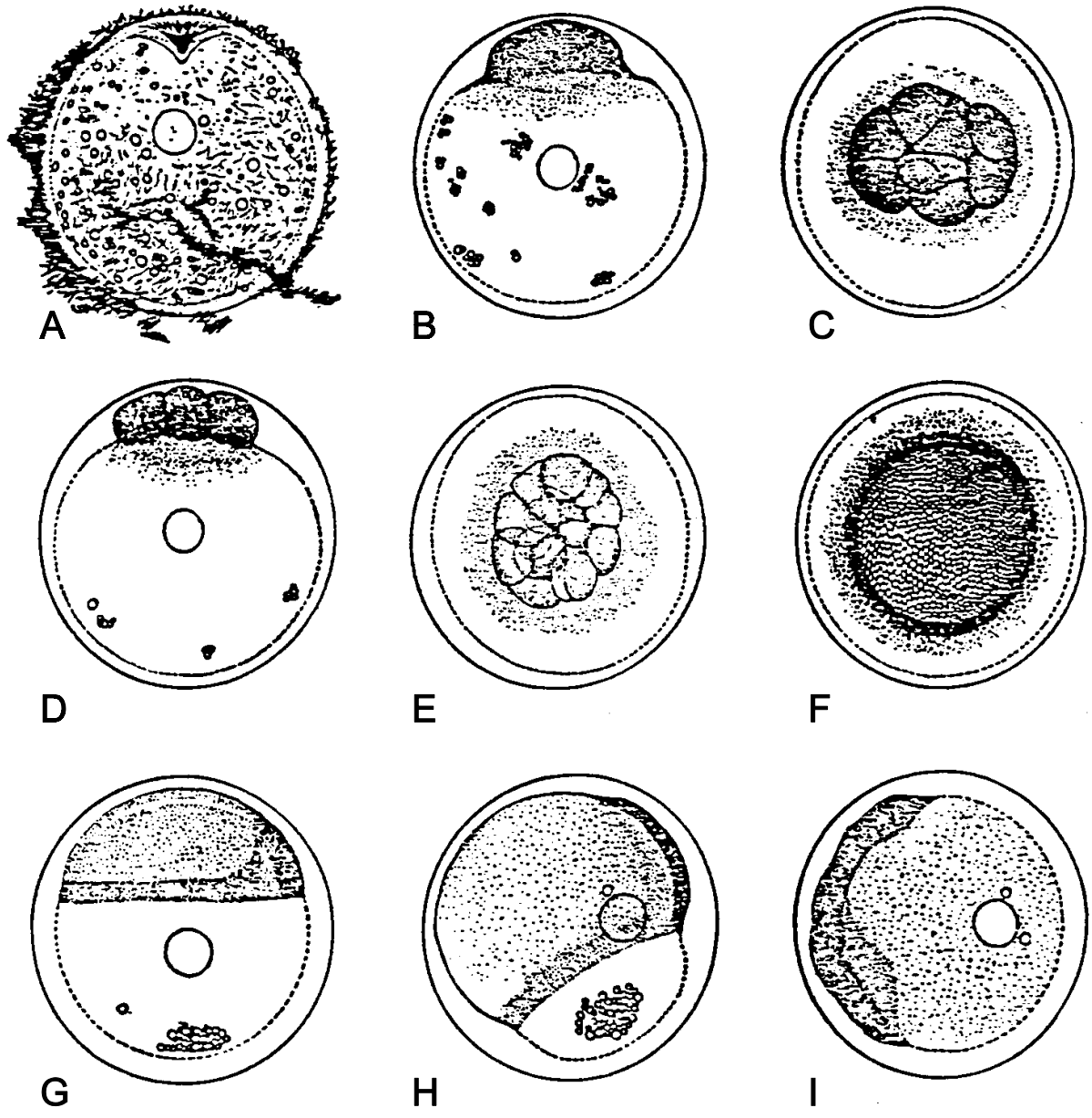

Figure 1. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: A. Mature unfertilized egg, showing attachment filaments and micropyle, X33; B. Blastodisc fully developed; C,D. Blastodisc, 8 cells; E. Blastoderm, 16 cells; F. Blastoderm, late cleavage stage; G. Blastoderm with germ ring formed, embryonic shield developing; H. Blastoderm covers over 3/4 of yolk, yolk noticeably constricted; I. Early embryo. From Kuntz (1916).

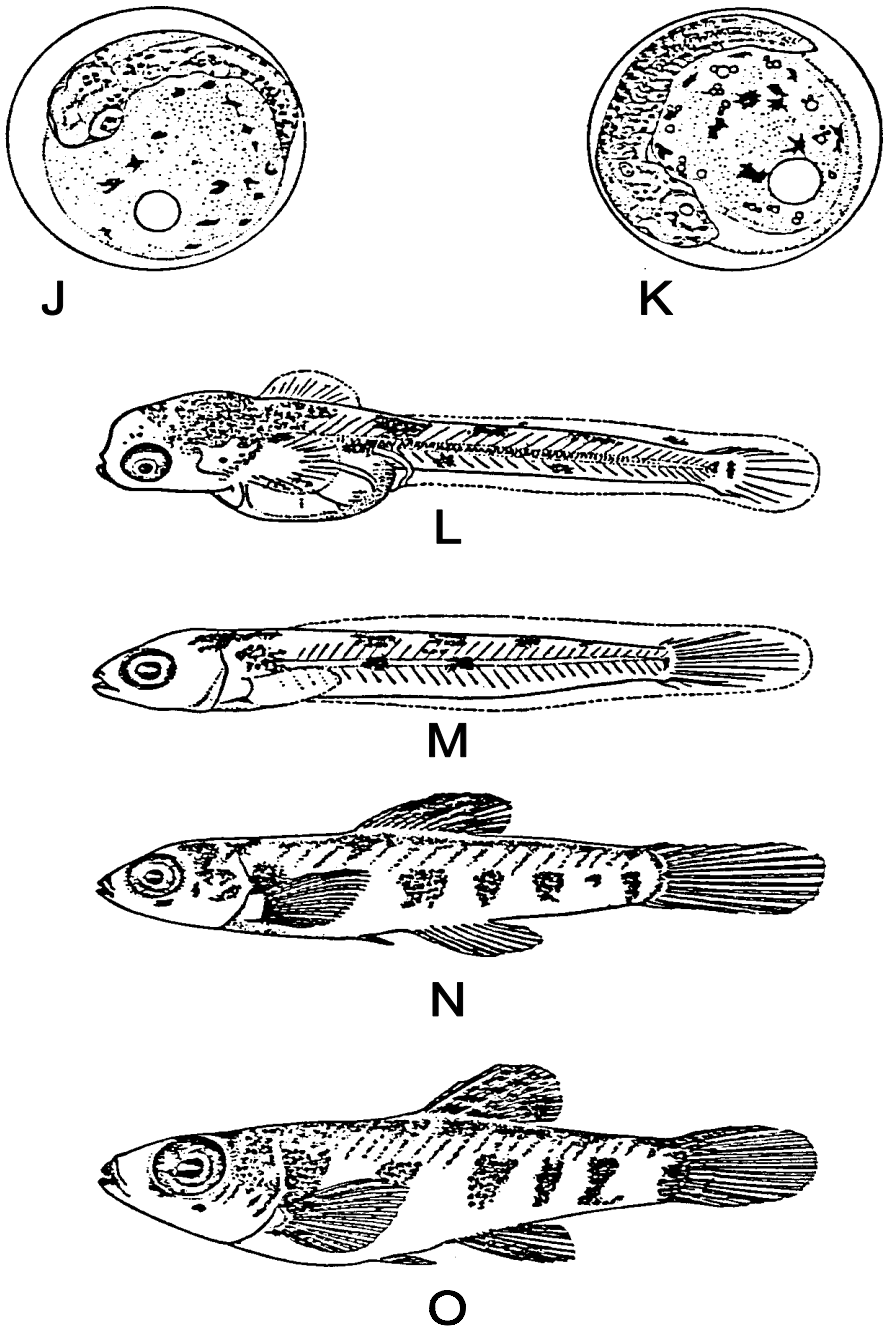

Figure 1. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: J. Embryo 48 h after fertilization, now segmented throughout, pigment on yolk sac and body, otoliths formed; K. Posterior portion of embryo free from yolk and moves freely within egg membrane, 72 h after fertilization; L. Newly hatched fish, actual length 4 mm; M. Larval fish 5 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).

11.6.15.2.5.4 Embryos are demersal. They should be aerated and incubated at 25°C, at a salinity of 20-30‰ and a 16-h photoperiod. The embryos can be cultured in either a flow-through or static system, using aquaria or crystallization dishes. However, if the embryos are cultured in dishes, it is essential that aeration and daily water changes be provided, and the dishes be covered to reduce evaporation that may cause increased salinity. One-half to three-quarters of the seawater from the culture vessels can be poured off and the incubating embryos retained. Embryos cultured in this manner should hatch in six or seven days.

11.6.15.2.5.5 At 48 h post-fertilization, embryos are examined under a microscope to determine development and survival. Embryos should be in Stages I and J, Figure 1. Discard dead embryos. Approximately 360 viable embryos are required at this stage.

11.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance.

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance.

11.10 TEST PROCEDURES

11.10.1 TEST SOLUTIONS

11.10.1.1 Receiving Waters

11.10.1.1.1 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 500-750 mL, and 400 mL for chemical analysis, would require approximately 2.4-3.4 L or more of sample per test per day.

11.10.1.2 Effluents

11.10.1.2.1 The selection of the effluent test concentrations 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%, 25.0%, 50.0%, 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‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity and 70% at 30‰ salinity.

11.10.1.2.2 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-to-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

11.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates per concentration for five concentrations of effluent and a control, each containing 750 mL of test solution, is approximately 5 L. Prepare enough test solution (approximately 3400 mL) at each effluent concentration to provide

400 mL additional volume for chemical analyses (Table 2).

11.10.1.2.4 The salinity of effluent and receiving water tests for sheepshead minnows should be between 20‰ and 30‰. 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 between 20‰ and 30‰, whichever comes closest to the salinity of the receiving waters. Table 2 illustrates the quantities of effluent, artificial sea salts, hypersaline brine, or seawater needed to prepare 3 L of test solution at each treatment level for tests performed at 20‰ salinity.

11.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of sufficient quantity of the sample 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.

11.10.1.2.6 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.

11.10.1.2.7 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.

11.10.1.3 Dilution Water

11.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 1 and Section 7, Dilution Water). Other artificial sea salts may be used for culturing sheepshead minnows and for the larval survival and growth test if the control criteria for acceptability of test data are satisfied.

11.10.2 START OF THE TEST

11.10.2.1 Tests should begin as soon as possible, preferably within 24 h after sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests 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).

TABLE 2. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 20‰ , USING 20‰ SALINITY DILUTION WATER PREPARED FROM NATURAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICIAL SEA SALTS

Effluent Solution	Effluent Conc. (%)	Solutions To Be Combined	
		Volume of Effluent Solution	Volume of Diluent Seawater (20‰)
1	100[1,2]	6800 mL	----
2	50	3400 mL Solution 1	+ 3400 mL
3	25	3400 mL Solution 2	+ 3400 mL
4	12.5	3400 mL Solution 3	+ 3400 mL
5	6.25	3400 mL Solution 4	+ 3400 mL
Control	0.0		3400 mL
Total			17000 mL

[1] This illustration assumes: (1) the use of 750 mL of test solution in each of four replicates and 400 mL for chemical analysis (total of 3,400 mL) for the control and each of five concentrations of effluent (2) an effluent dilution factor of 0.5, and (3) the effluent lacks appreciable salinity. A sufficient initial volume (6,800 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). Following addition of salts, the effluent is stirred for 1 h to ensure that the salts have dissolved. The salinity of the initial 6,800 mL of 100% effluent is adjusted to 20‰ by adding 136 g of dry artificial sea salts (FORTY FATHOMS®). Test concentrations are then made by mixing appropriate volumes of salinity-adjusted effluent and 20‰ salinity dilution water to provide 6,800 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. When dry sea salts are used to adjust the salinity of the effluent, it may be desirable to use a salinity control prepared under the same conditions and used to determine survival and growth.

[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 (17,000 mL) of seawater for dilution water, which had been prepared at the same salinity as the effluent, using natural seawater, or hypersaline or artificial sea salts and deionized water.

11.10.2.2 If the embryos have been incubating at 25°C, 30‰ salinity, and a 16-h photoperiod, for 5 to 6 days with aeration and daily water renewals, approximately 24 h prior to hatching, the salinity of the seawater in the incubation chamber may be reduced from 30‰ to the test salinity, if lower than 30‰. In addition to maintaining good water quality, reducing the salinity and/or changing the water may also help to initiate hatching over the next 24 h. A few larvae may hatch 24 h ahead of the majority. Remove these larvae and reserve them in a separate dish, maintaining the same culture conditions. It is preferable to use only the larvae that hatch in the 24 h prior to starting the test. However, if sufficient numbers of larvae do not hatch within the 24-h period, the larvae that hatch prior to 24 h are added to the test organisms. The test organisms are then randomly selected for the test. When eggs or larvae must be shipped to the test site from a remote location, it may be necessary to use larvae older than 24-h because of the difficulty in coordinating test organism shipments with field operations. However, in the latter case, the larvae should not be more than 48-h old at the start of the test and should all be within 24-h of the same age.

11.10.2.3 Label the test chambers with a marking pen. Use of color coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each test. Each treatment (including

controls) must have a minimum of four replicates. For exposure chambers, use 1000 mL beakers, non-toxic disposable plasticware, or glass chambers with a sump area as illustrated in the inland silverside test method (see Section 13).

11.10.2.4 Prepare the test solutions and add to the test chambers.

11.10.2.5 The test is started by randomly placing larvae from the common pool into each test chamber until each chamber contains a minimum of 10 larvae, for a total of a minimum of 40 for each concentration (see Appendix A). The amount of water added to the chambers when transferring the larvae should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.10.2.6 The chambers may be placed on a light table to facilitate counting the larvae.

11.10.2.7 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.

11.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

11.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C. The test salinity should be in the range of 20 to 30‰ to accommodate receiving waters that may fall within this range. Conduct of this test at salinities less than 20‰ may cause an unacceptably low growth response and thereby invalidate the test. The salinity should vary by 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.

11.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain a satisfactory DO. The DO should be measured on new solutions at the start of the test (Day 0) and before daily renewal of test solutions on subsequent days. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, 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 aeration does not cause undue stress on the fish.

11.10.5 FEEDING

11.10.5.1 *Artemia* nauplii are prepared as described above.

11.10.5.2 Sheepshead minnow larvae are fed newly-hatched (less than 24-h old) *Artemia* nauplii once a day from hatch day 0 through day 6; larvae are not fed on day 7. Feed 0.10 g nauplii per test chamber on days 0-2, and 0.15 g nauplii per test chamber on days 3-6. Equal amounts of *Artemia* nauplii must be added to each replicate test chamber to minimize the variability of larval weight. Sufficient numbers of nauplii should be fed to ensure that some remain alive overnight in the test chambers. An adequate but not excessive amount should be provided to each replicate on a daily basis. Feeding excessive amounts of nauplii will result in a depletion in DO to a lower than acceptable level (below 4.0 mg/L). Siphon as much of the uneaten *Artemia nauplii* as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

11.10.5.3 On days 0-2, weigh 4 g wet weight or pipette 4 mL of concentrated, rinsed *Artemia* nauplii for a test with five treatments and a control. Resuspend the 4 g *Artemia* in 80 mL of natural or artificial seawater in a 100 mL beaker. Aerate or swirl *Artemia* to maintain a thoroughly mixed suspension of nauplii. Dispense 2 mL *Artemia* suspension by pipette or adjustable syringe to each test chamber. Collect only enough *Artemia* in the pipette or syringe for one test

chamber or settling of *Artemia* may occur, resulting in unequal amounts of *Artemia* being distributed to the replicate test chambers.

11.10.5.4 On days 3-6, weigh 6 g wet weight or pipette 6 mL *Artemia* suspension for a test with five treatments and a control. Resuspend the 6 g *Artemia* in 80 mL of natural or artificial seawater in a 100 mL beaker. Aerate or swirl as 2 mL is dispensed to each test chamber.

11.10.5.5 If the survival rate in any test replicate on any day falls below 50%, reduce the volume of *Artemia* added to that test chamber by one-half (i.e., from 2 mL to 1 mL) and continue feeding one-half the volume through day 6. Record the time of feeding on data sheets (Figure 2).

11.10.6 DAILY CLEANING OF TEST CHAMBERS

11.10.6.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, dead fish larvae, and other debris are removed from the bottom of the test chambers with a siphon hose. As much of the uneaten *Artemia* as possible should be siphoned from each chamber to ensure that the larvae principally eat newly hatched nauplii. Alternately, a large pipet (50 mL), fitted with a safety pipet filler or rubber bulb, can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of live larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the live larvae caught in the siphon can be retrieved and returned to the appropriate test chamber. Any incidence of removal of live larvae from the test chambers by the siphon during cleaning, and subsequent return to the chambers, should be noted in the test records.

11.10.7 OBSERVATIONS DURING THE TEST

11.10.7.1 Routine Chemical and Physical Determinations

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

11.10.7.1.2 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, 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 vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

11.10.7.1.3 The pH is measured in the effluent sample each day.

11.10.7.1.4 Record all the measurements on the data sheet (Figure 2).

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____Lab _____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									**REPLICATE:**								**REPLICATE:**								**REPLICATE:**							
DAYS	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
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SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
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SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			

TIME FED							

COMMENTS:

Figure 2. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Daily record of larval survival and test conditions. (From USEPA, 1987b).

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____Lab _____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									REPLICATE:								REPLICATE:								REPLICATE:							
DAYS	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
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DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			

TIME FED							

COMMENTS:

Figure 2. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Daily record of larval survival and test conditions. (CONTINUED) (From USEPA, 1987b).

11.10.7.2 Routine Biological Observations

11.10.7.2.1 The number of live larvae in each test chamber are recorded daily (Figure 2), and the dead larvae are discarded.

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

11.10.8 TEST SOLUTION RENEWAL

11.10.8.1 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 one, three, and five. Maintain the samples at 0-6°C until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (750 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the larvae.

11.10.9 TERMINATION OF THE TEST

11.10.9.1 The test is terminated after 7-d of exposure. At test termination, dead larvae are removed and discarded. The surviving larvae in each test chamber (replicate) are counted and immediately prepared as a group for dry weight determination, or are preserved as a group in 4% formalin or 70% ethanol. Preserved organisms are dried and weighed within 7 days. For safety, formalin should be used under a hood.

11.10.9.2 For immediate drying and weighing, siphon or pour live larvae onto a 500 µm mesh screen in a large beaker to retain the larvae and allow *Artemia* and debris to be rinsed away. Rinse the larvae with deionized water to wash away salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of deionized water.

11.10.9.3 Small aluminum weighing pans can be used to dry and weigh the larvae. Mark for identification an appropriate number of small aluminum weighing pans (one per replicate). Weigh to the nearest 0.01 mg, and record the weights (Figure 3).

Test Dates: _____ Species: _____

Pan No.	Conc. & Rep.	Initial Wt. (mg)	Final Wt. (mg)	Diff. (mg)	No. Larvae	Av. Wt./ Larvae (mg)

Figure 3. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Dry weights of larvae (from USEPA 1987b).

11.10.9.4 Immediately prior to drying, rinse the preserved larvae in distilled (or deionized) water. The rinsed larvae from each test chamber are transferred to a tared weighing pan and dried at 60°C for 24 h or at 105°C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator until weighed, to prevent the absorption of moisture from the air. Weigh to the nearest 0.01 mg all weighing pans containing dried larvae and subtract the tare weight to determine the dry weight of larvae in each replicate. Record the weights (Figure 3). For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight, and record (Figure 3). For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptable criteria (see Subsection 11.12). Complete the summary data sheet (Figure 4) after calculating the average measurements and statistically analyzing the dry weights and percent survival. Average dry weights should be expressed to the nearest 0.001 mg.

11.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

11.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 The tests are acceptable if (l) the average survival of control larvae equals or exceeds 80%, and (2) the average dry weight per surviving unpreserved control larvae is equal to or greater than 0.60 mg, or (3) the average dry weight per surviving preserved control larvae is equal to or greater than 0.50 mg. The above minimum weights presume that the age of the larvae at the start of the test is less than or equal to 24 h.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is listed in Table 4.

11.13.1.2 The endpoints of toxicity tests using the sheepshead minnow larvae are based on the adverse effects on survival and growth. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25 and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50, IC25 and IC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

Test Dates: _____ Species: _____

Effluent Tested: _____

TREATMENT						
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WT/ LARVAE (MG) ± SD						
SIGNIF. DIFF. FROM CONTROL (o)						
MEAN TEMPERATURE (°C) ± SD						
MEAN SALINITY ‰ ± SD						
AVE DISSOLVED OXYGEN (MG/L) ± SD						

COMMENTS:

Figure 4. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Summary of test results (from USEPA, 1987b).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1004.0)[1]

1.	Test type:	Static renewal (required)
2.	Salinity:	20‰ 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 termperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory illumination (recommended)
5.	Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6.	Photoperiod:	16 h light, 8 h darkness (recommended)
7.	Test chamber size:	600 mL - 1 L beakers or equivalent (recommended)
8.	Test solution volume:	500-750 mL/replicate (loading and DO restrictions must be met) (recommended)
9.	Renewal of test solutions:	Daily (required)
10.	Age of test organisms	Newly hatched larvae (less than 24 h old; less than or equal to 24-h range in age) (required)
11.	No. larvae per test chamber:	10 (required minimum)
12.	No. replicate chambers per concentration	4 (required minimum)
13.	No. larvae per concentration:	40 (required minimum)
14.	Source of food:	Newly hatched *Artemia* nauplii, (less than 24-h old) (required)
15.	Feeding regime:	Feed once a day 0.10 g wet weight *Artemia* nauplii per replicate on Days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on Days 3-6 (recommended)
16.	Cleaning:	Siphon daily, immediately before test solution renewal and feeding (required)
17.	Aeration:	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/minimum (recommended)

[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 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1004.0) (CONTINUED)

18.	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)
19.	Test concentrations:	Effluent: 5 and a control (required) Receiving Waters: 100% receiving water (or minimum of 5) and a control (recommended)
20.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None, or ≥ 0.5 (recommended)
21.	Test duration:	7 days (required)
22.	Endpoints:	Survival and growth (weight) (required)
23.	Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers must be 0.60 mg or greater, if unpreserved, or 0.50 mg or greater after no more than 7 days in 4% formalin or 70% ethanol (required)
24.	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)
25.	Sample volume required:	6 L per day (recommended)

TABLE 4. SUMMARY OF SURVIVAL AND GROWTH DATA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAE EXPOSED TO AN EFFLUENT FOR SEVEN DAYS[1]

Effl. Conc. (%)	Proportion of Survival in Replicate Chambers				Mean Prop. Surv	Avg Dry Wgt (mg) in Replicate Chambers				Mean Dry Wgt (mg)
	A	B	C	D		A	B	C	D	
0.0	1.0	1.0	1.0	1.0	1.00	1.29	1.32	1.59	1.27	1.368
6.25	1.0	1.0	0.9	1.0	0.98	1.27	1.00	0.97	0.97	1.053
12.5	1.0	1.0	1.0	1.0	1.00	1.32	1.37	1.35	1.34	1.345
25.0	1.0	1.0	1.0	0.8	0.95	1.29	1.33	1.20	0.94	1.190
50.0	0.8	0.8	0.7	0.6	0.73	1.62	0.56	0.46	0.46	0.525
100.0	0.0	0.0	0.0	0.0	0.00	---	---	---	---	---

[1] Four replicates of 10 larvae each.

11.13.2 EXAMPLE OF ANALYSIS OF SHEEPHEAD MINNOW, *CYPRINODON VARIEGATUS* SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 5 and 6. The response used in the analysis is the proportion of animals surviving 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 LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoint.

11.13.2.2 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.

11.13.2.3 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.

11.13.2.4 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).

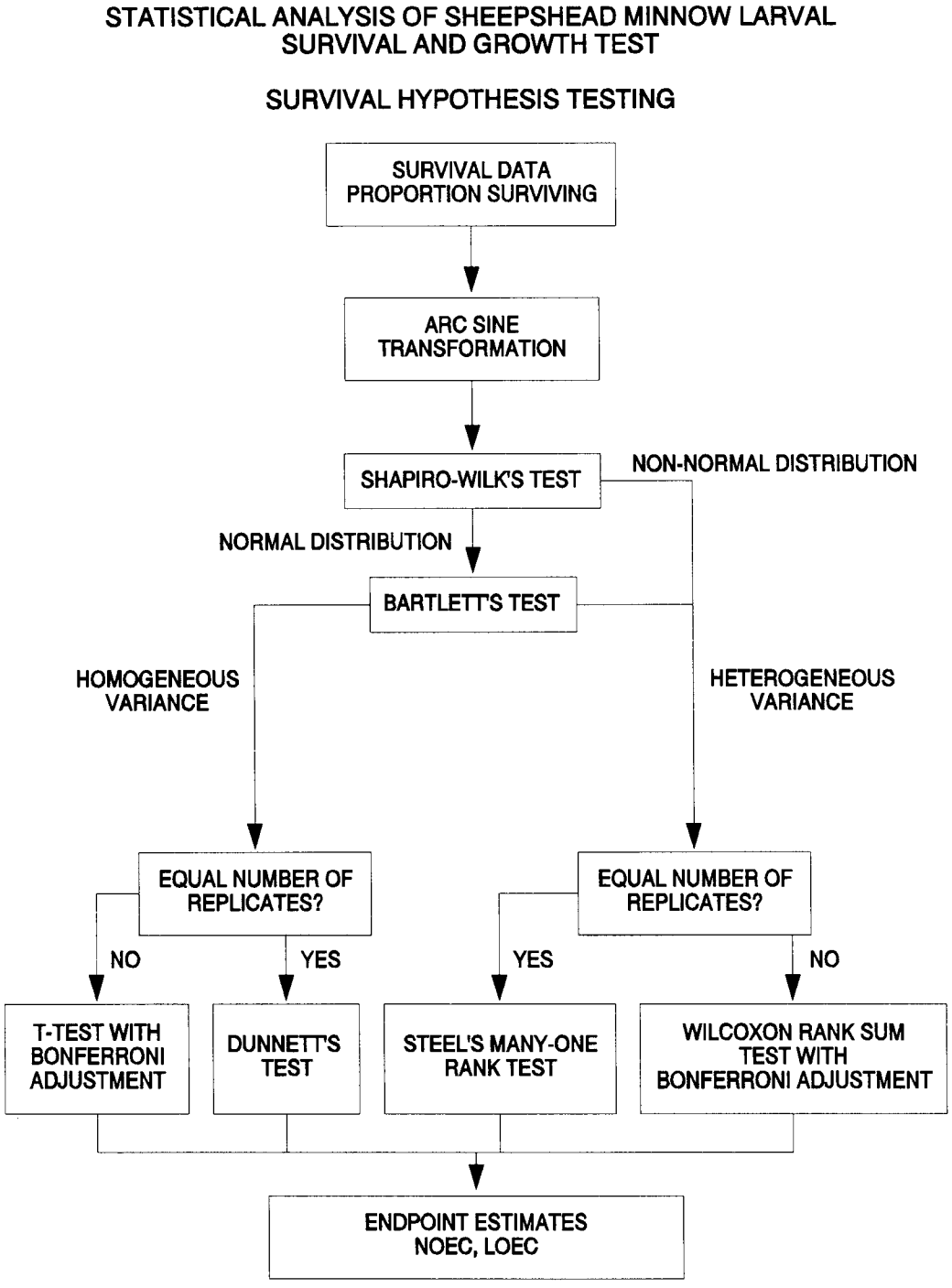

Figure 5. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval survival data by hypothesis testing.

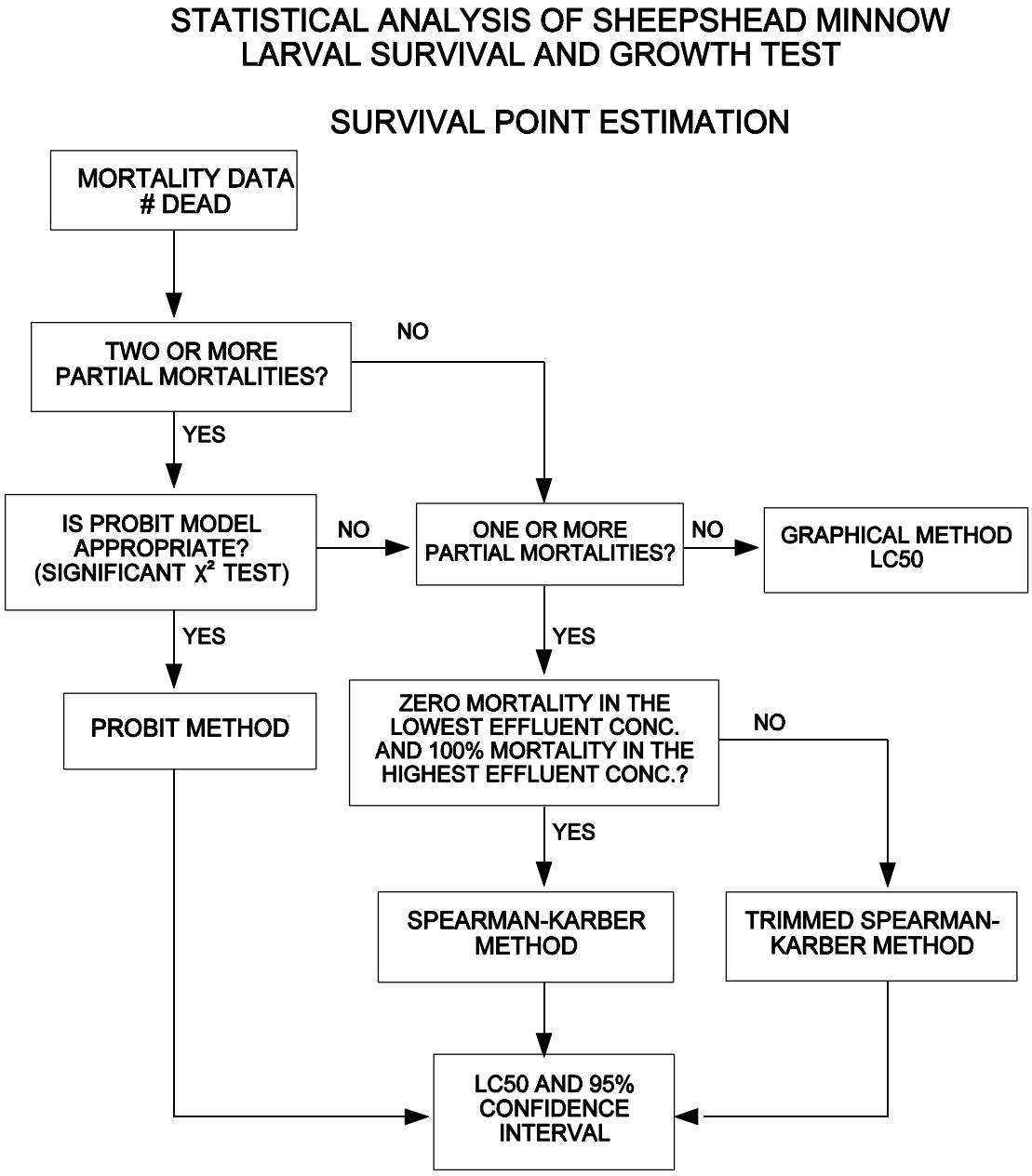

Figure 6. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval survival data by point estimation.

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Sheepshead Minnow Larval Survival and Growth Test. The proportion surviving 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 variances of the transformed observations at each effluent concentration and control are listed in Table 5. A plot of the survival proportions is provided in Figure 7. Since there was 100% mortality in all four replicates for the 100% concentration, it was not included in the statistical analysis and was considered a qualitative mortality effect.

11.13.2.6 Test for Normality

11.13.2.6.1 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 6.

11.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

TABLE 5. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, SURVIVAL DATA

	Replicate	Control	Effluent Concentration (%)			
			6.25	12.5	25.0	50.0
RAW	A	1.0	1.0	1.0	1.0	0.8
	B	1.0	1.0	1.0	1.0	0.8
	C	1.0	0.9	1.0	1.0	0.7
	D	1.0	1.0	1.0	0.8	0.6
ARC SINE	A	1.412	1.412	1.412	1.412	1.107
TRANSFORMED	B	1.412	1.412	1.412	1.412	1.107
	C	1.412	1.249	1.412	1.412	0.991
	D	1.412	1.412	1.412	1.107	0.886
Mean ($\bar{Y}_i$)		1.412	1.371	1.412	1.336	1.023
S_i^2		0.0	0.007	0.0	0.023	0.011
i		1	2	3	4	5

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Effluent Concentration (%)			
		6.25	12.5	25.0	50.0
A	0.0	0.041	0.0	0.076	0.084
B	0.0	0.041	0.0	0.076	0.084
C	0.0	-0.122	0.0	0.076	-0.032
D	0.0	0.041	0.0	-0.229	-0.137

11.13.2.6.3 For this set of data,

$$n = 20$$

$$\bar{X} = \frac{1}{20}(-0.001) = 0.000$$

$$D = 0.1236$$

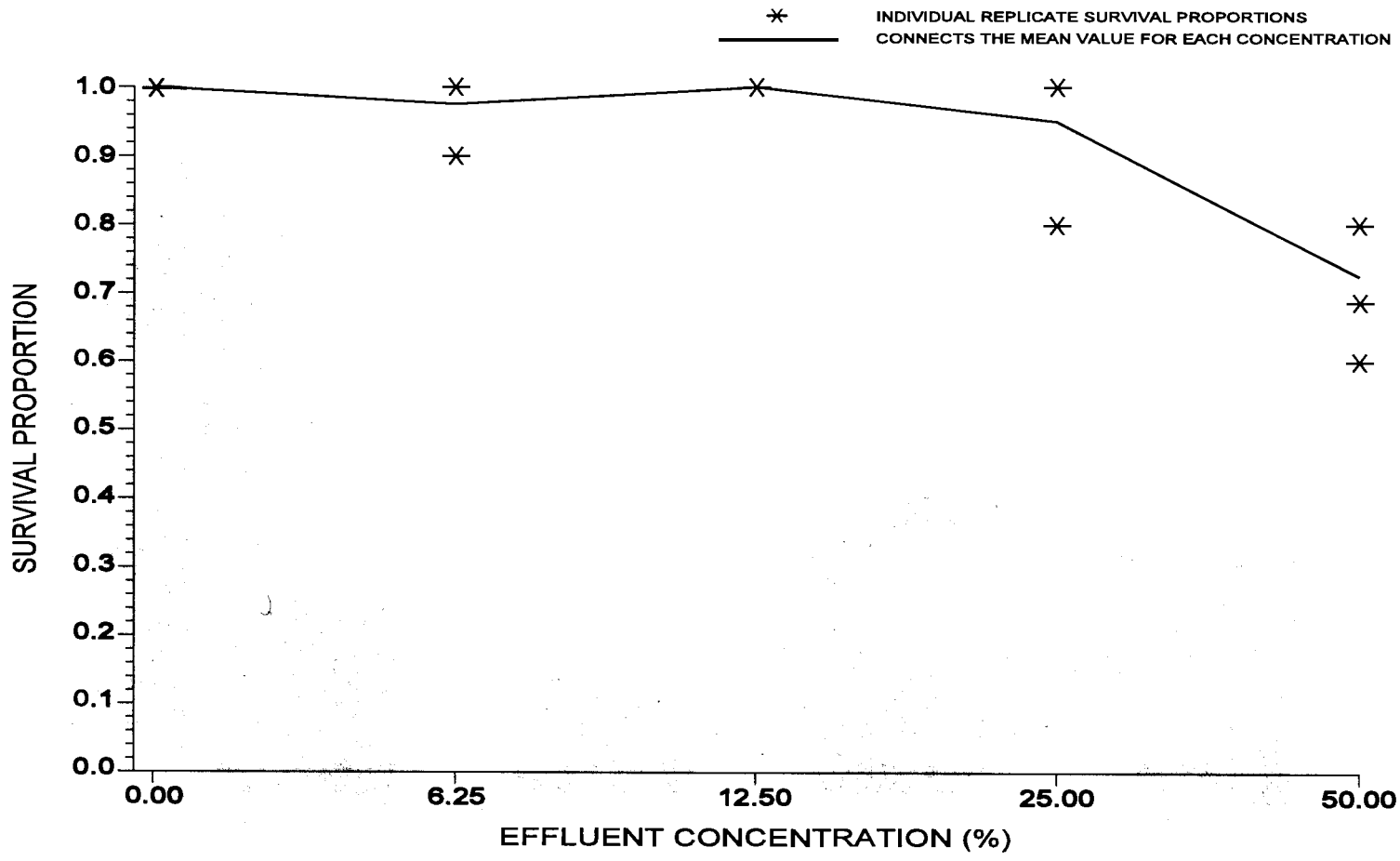

Figure 7. Plot of mean survival proportion data in Table 5.

11.13.2.6.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

11.13.2.6.5 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 8.

11.13.2.6.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For the data in this example,

$$W = \frac{1}{0.1236} (0.3178)^2 = 0.8171$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.229	11	0.0
2	-0.137	12	0.0
3	-0.122	13	0.041
4	-0.032	14	0.041
5	0.0	15	0.041
6	0.0	16	0.076
7	0.0	17	0.076
8	0.0	18	0.076
9	0.0	19	0.084
10	0.0	20	0.084

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 11.13.2.6.6 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 = 0.817 is less than the critical value, conclude that the data are not normally distributed.

11.13.2.6.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the survival data.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.313	$X^{(20)} - X^{(1)}$
2	0.3211	0.221	$X^{(19)} - X^{(2)}$
3	0.2565	0.198	$X^{(18)} - X^{(3)}$
4	0.2085	0.108	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.041	$X^{(15)} - X^{(6)}$
7	0.1013	0.041	$X^{(14)} - X^{(7)}$
8	0.0711	0.041	$X^{(13)} - X^{(8)}$
9	0.0422	0.0	$X^{(12)} - X^{(9)}$
10	0.0140	0.0	$X^{(11)} - X^{(10)}$

11.13.2.7 Steel's Many-one Rank Test

11.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

11.13.2.7.2 An example of assigning ranks to the combined data for the control and 6.25% effluent concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are next summed for each effluent concentration, as shown in Table 11.

11.13.2.7.3 For this example, determine if the survival in any of the effluent concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the survival at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates is 10 (see Table 5, Appendix E).

11.13.2.7.4 Since the rank sum for the 50% effluent concentration is equal to the critical value, the proportion surviving in the 50% concentration is considered significantly less than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have a significantly lower proportion surviving than the control. Hence, the NOEC and the LOEC are the 25% and 50% concentrations, respectively.

11.13.2.8 Calculation of the LC50

11.13.2.8.1 The data used for the calculation of the LC50 is summarized in Table 12. For estimating the LC50, the data for the 100% effluent concentration with 100% mortality is included.

11.13.2.8.2 Because there are at least two partial mortalities in this set of test data, calculation of the LC50 using Probit Analysis is recommended. For this set of data, however, the test for heterogeneity of variance was significant. Probit Analysis is not appropriate in this case. Inspection of the data reveals that, once the data is smoothed and adjusted, the proportion mortality in the lowest effluent concentration will not be zero although the proportion mortality in the highest effluent concentration will be one. Therefore, the Spearman-Karber Method is appropriate for this data.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 6.25% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Surviving	Effluent Concentration (%)
1	1.249	6.25
5	1.412	6.25
5	1.412	6.25
5	1.412	6.25
5	1.412	Control
5	1.412	Control
5	1.412	Control
5	1.412	Control

TABLE 10. TABLE OF RANKS

		Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0	50.0
A	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.412 (5)	1.107 (3.5)
B	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.412 (5)	1.107 (3.5)
C	1.412 (5,4.5,5,6.5)	1.249 (1)	1.412 (4.5)	1.412 (5)	0.991 (2)
D	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.107 (1)	0.886 (1)

TABLE 11. RANK SUMS

Effluent Concentration (%)	Rank Sum
6.25	16
12.5	18
25.0	16
50.0	10

11.13.2.8.3 Before the LC50 can be calculated the data must be smoothed and adjusted. For the data in this example, because the observed proportion mortality for the 12.5% effluent concentration is less than the observed response proportion for the 6.25% effluent concentration, the observed responses for the control and these two groups must be averaged:

$$p_o^s = p_1^s = p_2^s = \frac{0.00+0.025+0.00}{3} = \frac{0.025}{3} = 0.0083$$

Where: p^s_i = the smoothed observed mortality proportion for effluent concentration i

11.13.2.8.3.1 Because the rest of the responses are monotonic, additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table 12.

11.13.2.8.4 Because the smoothed observed proportion mortality for the control is now greater than zero, the data in each effluent concentration must be adjusted using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: p^s_0 = the smoothed observed proportion mortality for the control

$p^a_i = (p^s_i - p^s_0) / (1 - p^s_0)$

p^s_i = the smoothed observed proportion mortality for effluent concentration i

11.13.2.8.4.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0083 - 0.0083}{1 - 0.0083} = \frac{0.00}{0.9917} = 0.0$$

$$p_3^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{0.05 - 0.0083}{1 - 0.0083} = \frac{0.0417}{0.9917} = 0.042$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.275 - 0.0083}{1 - 0.0083} = \frac{0.2667}{0.9917} = 0.269$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.0083}{1 - 0.0083} = \frac{0.9917}{0.9917} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table 12.

TABLE 12. DATA FOR EXAMPLE OF SPEARMAN-KARBER ANALYSIS

Effluent Concentration %	Number of Deaths	Number of Organisms Exposed	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0	40	0.000	0.0083	0.000
6.25	1	40	0.025	0.0083	0.000
12.5	0	40	0.000	0.0083	0.000
25.0	2	40	0.050	0.0500	0.042
50.0	11	40	0.275	0.2750	0.269
100.0	40	40	1.000	1.0000	1.000

11.13.2.8.5 Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_i^a + 1)\ (X_i + X_{i+1})}{2}$$

Where: p^a_i = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.5.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
[(0.042 - 0.000) (1.0969 + 1.3979)]/2 +
[(0.269 - 0.042) (1.3979 + 1.6990)]/2 +
[(1.000 - 0.269) (1.6990 + 2.0000)]/2

= 1.755873

11.13.2.8.6 Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1-p_i^a)(X_{i+1}+X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p^a_i = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.6.1 For this example, the estimated variance of m, V(m), is calculated as follows:

V(m) = (0.000)(1.000)(1.3979 - 0.7959)^2/4(39) +
(0.042)(0.958)(1.6990 - 1.0969)^2/4(39)+
(0.269)(0.731)(2.0000 - 1.3979)^2/4(39)

= 0.0005505

11.13.2.8.7 Calculate the 95% confidence interval for m: $m \pm 2.0 \sqrt{V(m)}$

11.13.2.8.7.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.755873 \pm 2 \sqrt{0.0005505} = (1.754772, 1.756974)$$

11.13.2.8.8 The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking $base_{10}$ antilogs of the above values.

11.13.2.8.8.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.755873) = 57.0%.

11.13.2.8.8.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.754772) = 56.9%

upper limit: antilog(1.756974) = 57.1%

11.13.3 EXAMPLE OF ANALYSIS OF SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 8. The response used in the statistical analysis is mean weight per original organism for each replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. The IC25 and IC50 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis testing consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the 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, Steels' 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 determined by the parametric test.

11.13.3.3 Additionally, 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. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 13. A plot of the mean weights for each treatment is provided in Figure 9. Since there is no survival in the 100% concentration, it is not considered in the growth analysis. Additionally, since there is significant mortality in the 50% effluent concentration, its effect on growth is not considered.

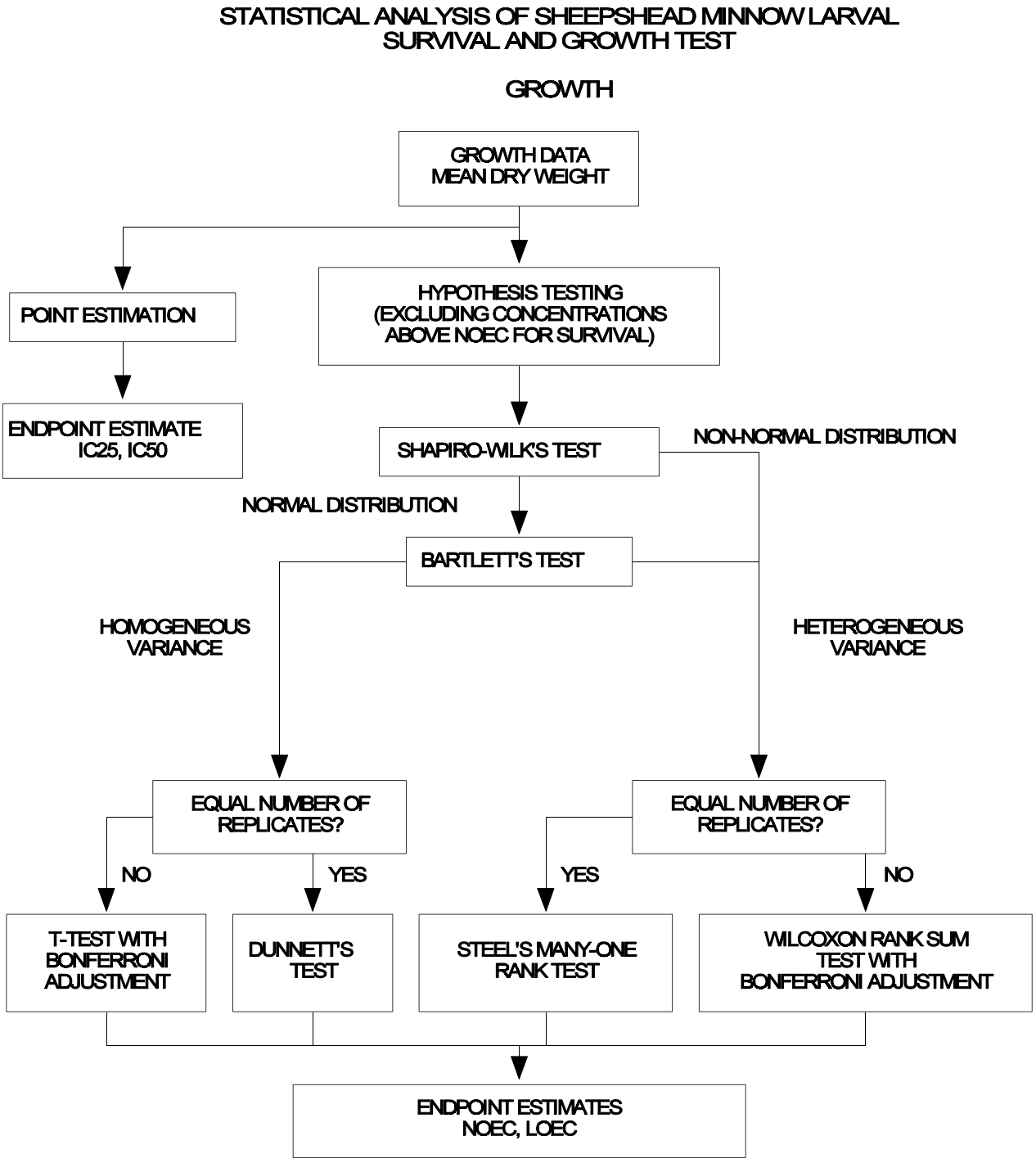

Figure 8. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval growth data.

TABLE 13. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, GROWTH DATA

		Effluent Concentration (%)				
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	1.29	1.27	1.32	1.29	-	-
B	1.32	1.00	1.37	1.33	-	-
C	1.59	0.97	1.35	1.20	-	-
D	1.27	0.97	1.34	0.94	-	-
Mean ($\bar{Y}_i$)	1.368	1.053	1.345	1.190	-	-
S^2_i	0.0224	0.0212	0.0004	0.0307	-	-
i	1	2	3	4	5	6

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 14.

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0
A	-0.078	0.217	-0.025	0.100
B	-0.048	-0.053	0.025	0.140
C	0.222	-0.083	0.005	0.010
D	-0.098	0.083	-0.005	-0.250

11.13.3.5.2 Calculate the denominator, D, of the test 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.

For this set of data, n = 16

$$\bar{X} = \frac{1}{16}(-0.004) = 0.00024 = 0.00$$

D = 0.2245

11.13.3.5.3 Order the centered observations from smallest to largest:

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

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.250	9	-0.005
2	-0.098	10	0.005
3	-0.083	11	0.010
4	-0.083	12	0.025
5	-0.078	13	0.100
6	-0.053	14	0.140
7	-0.048	15	0.217
8	-0.025	16	0.222

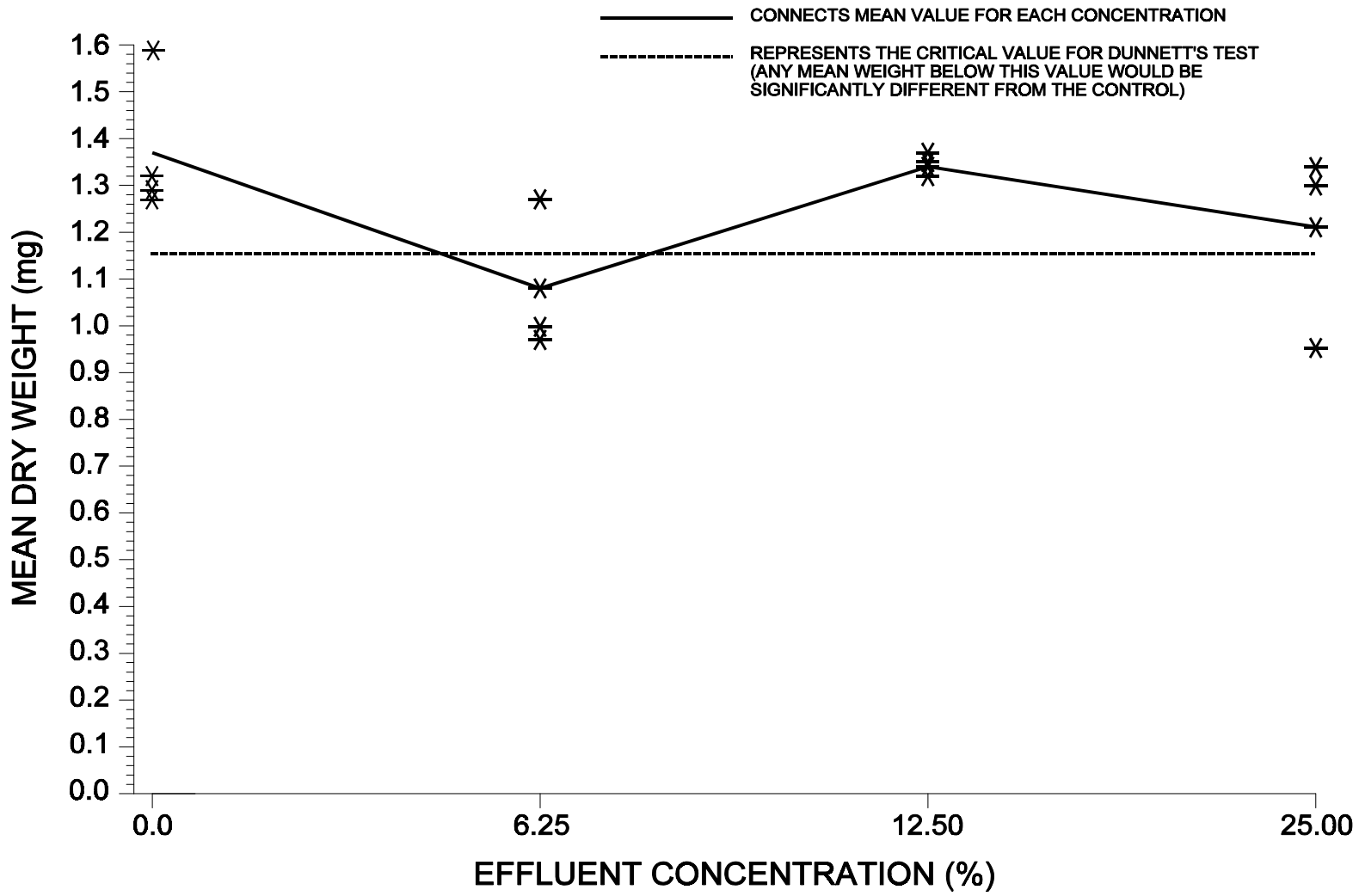

Figure 9. Plot of weight data from sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test.

11.13.3.5.4 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 = 16 and k = 8. The a_i values are listed in Table 16.

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5056	0.472	$X^{(16)} - X^{(1)}$
2	0.3290	0.315	$X^{(15)} - X^{(2)}$
3	0.2521	0.223	$X^{(14)} - X^{(3)}$
4	0.1939	0.183	$X^{(13)} - X^{(4)}$
5	0.1447	0.103	$X^{(12)} - X^{(5)}$
6	0.1005	0.063	$X^{(11)} - X^{(6)}$
7	0.0593	0.053	$X^{(10)} - X^{(7)}$
8	0.0196	0.020	$X^{(9)} - X^{(8)}$

11.13.3.5.5 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 16.

For this set of data:

$$W = \frac{1}{0.2245}(0.4588)^2 = 0.938$$

11.13.3.5.6 The decision rule for this test is to compare W with the 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 this example, the critical value at a significance level of 0.01 and 16 observations (n) is 0.844. Since W = 0.938 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i

p = number of levels of effluent concentration including the control

ln = $\log_e$

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

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

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

11.13.3.6.2 For the data in this example (see Table 14), all effluent concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(0.0187) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.139$$

$$= [12(-3.979) - 3(-18.876)]/1.139$$

$$= 8.882/1.139$$

$$= 7.798$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 11.345. Since B = 7.798 is less than the critical value of 11.345, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. 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)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 4$

N = 16

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.47$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.21$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.38$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 4.76$

$G = T_1 + T_2 + T_3 + T_4 = 19.82$

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

$$= \frac{1}{4}(99.247) - \left(\frac{19.82}{16}\right)^2 = 0.260$$

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

$$= 25.036 - \left(\frac{19.82}{16}\right)^2 = 0.484$$

$$SSW = SST - SSB$$

$$= 0.484 - 0.260 = 0.224$$

$S_B^2 = SSB/(p-1) = 0.260/(4-1) = 0.087$

$S_W^2 = SSW/(N-p) = 0.224/(16-4) = 0.019$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	3	0.260	0.087
Within	12	0.224	0.019
Total	15	0.484	

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

$$t_i = \frac{(Y_1 - Y_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean dry weight for effluent concentration i

$\overline{Y}_1$ = mean dry weight 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.

11.13.3.7.5 Table 19 includes the calculated t values for each concentration and control combination. In this example, comparing the 6.25% concentration with the control, the calculation is as follows:

TABLE 19. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	3.228
12.5	3	0.236
25.0	4	1.824

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, 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, 12 degrees of freedom for error and three concentrations (excluding the control) the critical value is 2.29. The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_2 is greater than 2.29, the 6.25% concentration has significantly lower growth than the control. However, the 12.5% and 25% concentrations do not exhibit this effect. Hence the NOEC and the LOEC for growth cannot be calculated.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected 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 = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 2.29(0.10)\sqrt{(1/4) + (1/4)}$$

$$= 2.29\ (0.138)(0.707)$$

$$= 0.223$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.223 mg.

11.13.3.7.10 This represents a 16% reduction in mean weight from the control.

11.13.3.8 Calculation of the ICp

11.13.3.8.1 The growth data from Table 4 are utilized in this example. As seen from Table 4 and Figure 7, the observed means are not monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\overline{Y}_i$ and the smoothed

means by M_i.

11.13.3.8.2 Starting with the control mean, $\overline{Y}_1 = 1.368$ and $\overline{Y}_2 = 1.053$, we see that $\overline{Y}_1 > \overline{Y}_2$. Set $M_1 = \overline{Y}_1$. Comparing $\overline{Y}_2$ to $\overline{Y}_3$, $\overline{Y}_2 < \overline{Y}_3$.

11.13.3.8.3 Calculate the smoothed means:

$$M_2 = M_3 = (\overline{Y}_2 + \overline{Y}_3)/2 = 1.199$$

11.13.3.8.4 Since $\overline{Y}_6 = 0 < \overline{Y}_5 = 0.525 < \overline{Y}_4 = 1.190 < \overline{Y}_4 = 1.345$, set $M_3 = 1.199$, $M_4 = 1.190$, $M_5 = 0.525$, and set $M_6 = 0$.

11.13.3.8.5 Table 20 contains the response means and smoothed means and Figure 10 gives a plot of the smoothed response curve.

TABLE 20. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means (mg) Y_i	Smoothed Means (mg) M_i
Control	1	1.368	1.368
6.25	2	1.053	1.199
12.50	3	1.345	1.199
25.00	4	1.189	1.189
50.00	5	0.525	0.525
100.00	6	0.0	0.0

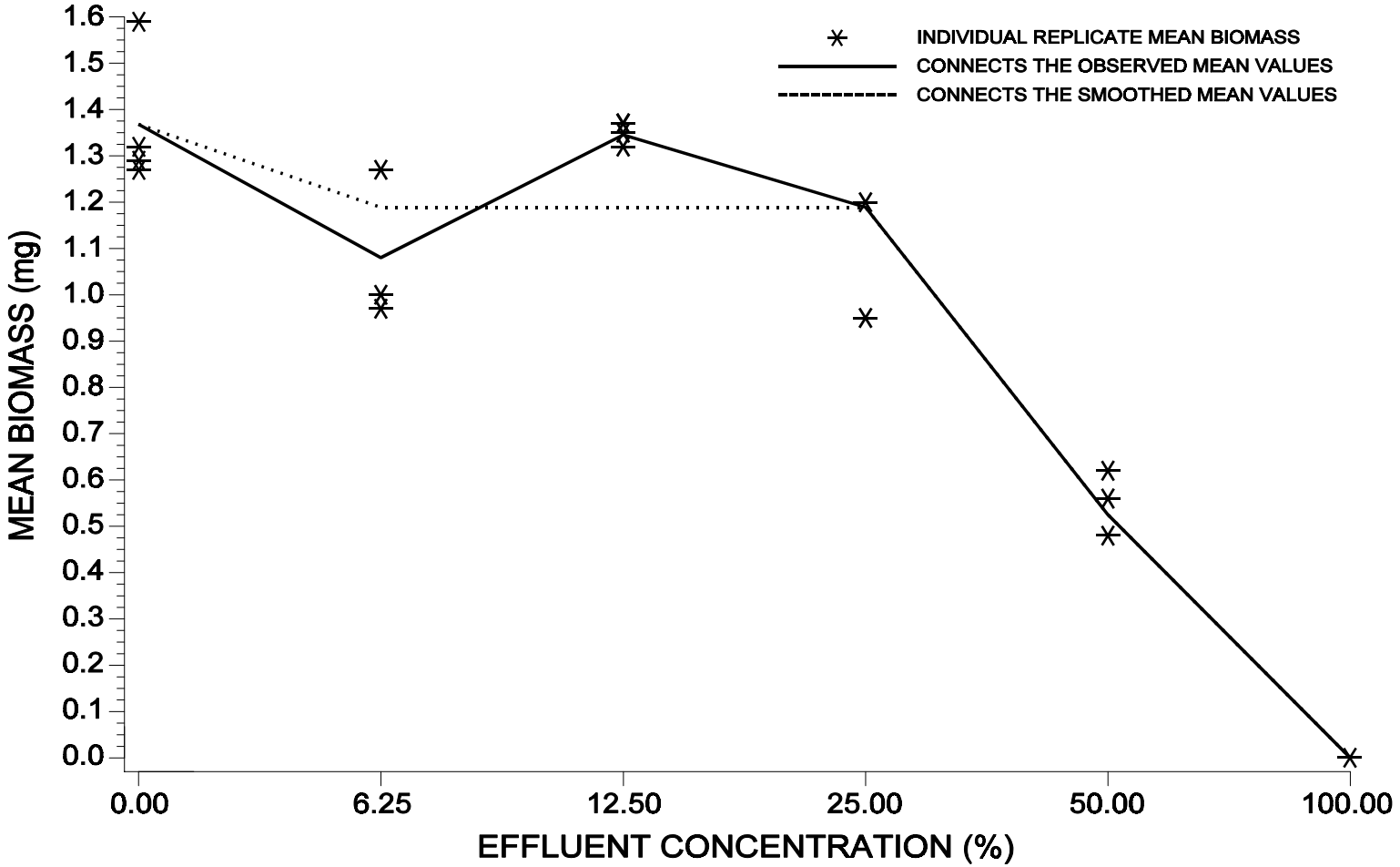

Figure 10. Plot of raw data, observed means, and smoothed means for the sheepshead minnow, *Cyprinodon varieagatus*, growth data from Tables 4 and 20.

11.13.3.8.6 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 1.026 mg, where $M_1(1\text{-}p/100) = 1.368(1\text{-}25/100)$. A 50% reduction in mean dry weight, compared to the controls, would result in a mean dry weight of 0.684 mg. Examining the smoothed means and their associated concentrations (Table 4), the response, 1.026 mg, is bracketed by $C_4 = 25.0\%$ effluent and $C_5 = 50.0\%$ effluent. The response (0.684 mg) is bracketed by $C_4 = 25.0\%$ effluent and $C_5 = 50\%$ effluent.

11.13.3.8.7 Using the equation from Section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 25.0 + [1.368(1 - 25/100) - 1.189]\ \frac{(50.00 - 25.00)}{(0.525 - 1.189)}$$

$$= 31.2\%$$

11.13.3.8.8 Using the equation from Section 4.2 of Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 50.0 + [1.368(1\text{-}50/100) - 0525]\ \frac{(100.00\text{-}50.00)}{(0.0 - 0.525)}$$

$$= 44.0\%$$

11.13.3.8.9 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 31.1512%. The empirical 95% confidence interval for the true mean was 22.0420% and 36.3613%. The computer program output for the IC25 for this data set is shown in Figure 11.

11.13.3.8.10 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 44.0230%. The empirical 95% confidence interval for the true mean was 39.1011% and 49.0679%. The computer program output is shown in Figure 12.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	1.29	1.27	1.32	1.29	.62	0
Response 2	1.32	1	1.37	1.33	.560	0
Response 3	1.59	.972	1.35	1.2	.46	0
Response 4	1.27	.97	1.34	.936	.46	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Cyprinodon variegatus
Test Duration: 7-d
DATA FILE: sheep.icp
OUTPUT FILE: sheep.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	1.368	0.150	1.368
2	4	6.250	1.053	0.145	1.199
3	4	12.500	1.345	0.021	1.199
4	4	25.000	1.189	0.177	1.189
5	4	50.000	0.525	0.079	0.525
6	4	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 31.1512 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 30.6175 Standard Deviation: 2.9490
Original Confidence Limits: Lower: 25.4579 Upper: 34.4075
Expanded Confidence Limits: Lower: 22.0420 Upper: 36.3613
Resampling time in Seconds: 1.70 Random Seed: -2137496326

Figure 11. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	1.29	1.27	1.32	1.29	.62	0
Response 2	1.32	1	1.37	1.33	.560	0
Response 3	1.59	.972	1.35	1.2	.46	0
Response 4	1.27	.97	1.34	.936	.46	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Cyprinodon variegatus
Test Duration: 7-d
DATA FILE: sheep.icp
OUTPUT FILE: sheep.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	1.368	0.150	1.368
2	4	6.250	1.053	0.145	1.199
3	4	12.500	1.345	0.021	1.199
4	4	25.000	1.189	0.177	1.189
5	4	50.000	0.525	0.079	0.525
6	4	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 44.0230 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 44.3444 Standard Deviation: 1.7372
Original Confidence Limits: Lower: 40.9468 Upper: 47.1760
Expanded Confidence Limits: Lower: 39.1011 Upper: 49.0679
Resampling time in Seconds: 1.70 Random Seed: -156164614

Figure 12. ICPIN program output for the IC50.

11.14 PRECISION AND ACCURACY

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Data on the single-laboratory precision of the Sheepshead Minnow Larval Survival and Growth Test using FORTY FATHOMS® artificial seawater, natural seawater, and GP2 with copper sulfate, sodium dodecyl sulfate, and hexavalent chromium, as reference toxicants, are given in Tables 21-26. The IC25, IC50, or LC50 data (coefficient of variation), indicating acceptable precision for the reference toxicants (copper, sodium dodecyl sulfate, and hexavalent chromium), are also listed in these Tables.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test using a database of routine reference toxicant test results from five laboratories (USEPA, 2000b). The database consisted of 57 reference toxicant tests conducted in 5 laboratories using reference toxicants including: cadmium and potassium chloride. Among the 5 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 13% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 9%; and in 75% of laboratories, the within-laboratory CV was less than 14%.

11.14.1.2 Multilaboratory Precision

11.14.1.2.1 Data from a study of multilaboratory test precision, involving a total of seven tests by four participating laboratories, are listed in Table 27. The laboratories reported very similar results, indicating good interlaboratory precision. The coefficient of variation (IC25) was 44.2% and (IC50) was 56.9%, indicating acceptable precision.

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 7 participant laboratories tested 4 blind test samples that included blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a natural seawater spiked with KCl, and the reference toxicant sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater spiked with KCl. Of the 28 Sheepshead Minnow Larval Survival and Growth Tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of 7 tests that were conducted on blank samples, none showed false positive results for the survival endpoint or the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 28 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 10.5% for IC25 results. Table 29 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned two concentrations for the reference toxicant sample type and one concentration for the effluent and

receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types. For the growth endpoint, NOEC values spanned one concentration for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types.

11.14.2 ACCURACY

11.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 21. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING COPPER (CU) SULFATE AS A REFERENCE TOXICANT[1,2,3,4,5]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[6]
1	50	113.3	152.3	S
2	<50[7]	54.3	97.5	G
3	<50[7]	41.8	71.4	G
4	50	63.2	90.8	S
5	<50[7]	57.7	99.8	S
6	50	48.3	132.5	G
7	50	79.6	159.7	G
8	50	123.5	236.4	G
n:	5	8	8	
Mean:	NA	72.7	130.0	
CV(%):	NA	41.82	40.87	

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

2 Tests performed by Donald J. Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

3 All tests were performed using FORTY FATHOMS® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each copper concentration. Copper concentrations used in Tests 1-6 were: 50, 100, 200, 400, and 800 mg/L. Copper concentrations in Tests 7-8 were: 25, 50, 100, 200 and 400 mg/L.

4 Adults collected in the field.

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

6 Endpoints: G=growth; S=survival.

7 Lowest concentration tested was 50 µg/L (NOEC Range: < 50* - 50 µg/L).

TABLE 22. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[7]
1	1.0	1.2799	1.5598	S
2	1.0	1.4087	1.8835	S
3	1.0	2.3051	2.8367	S
4	0.5	1.9855	2.6237	G
5	1.0	1.1901	1.4267	S
6	0.5	1.1041	1.4264	G
n:	6	6	6	
Mean:	NA	1.5456	1.9595	
CV(%):	NA	31.44	31.82	

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

2 Tests performed by Donald J. Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

3 All tests were performed using FORTY FATHOMS® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each SDS concentration. SDS concentrations in Tests 1-2 were: 1.0, 1.9, 3.9, 7.7, and 15.5 mg/L. SDS concentrations in Tests 3-6 were: 0.2, 0.5, 1.0, 1.9, and 3.9 mg/L.

4 Adults collected in the field.

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

6 NOEC Range: 0.5 -1.0 mg/L (this represents a difference of one exposure concentration).

7 Endpoints: G=growth; S=survival

TABLE 23. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, USING COPPER (CU) SULFATE AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[7]
1	125	320.3	437.5	S
2	31	182.3	323.0	G
3	125	333.4	484.4	S
4	125	228.4	343.8	S
5	125	437.5	NC[8]	S
n:	5	5	4	
Mean:	NA	300.4	396.9	
CV(%):	NA	33.0	19.2	

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

2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.

3 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each copper concentration. Copper concentrations were: 31, 63, 125, 250, and 500 µg/L.

4 NOEC Range: 31 - 125 µg/L (this represents a difference of two exposure concentrations).

5 Adults collected in the field.

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

7 Endpoints: G=growth; S=survival.

8 NC = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 24. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, USING SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[7]
1	2.5	2.9	3.6	S
2	1.3	NC1[8]	NC2[9]	G
3	1.3	1.9	2.4	S
4	1.3	2.4	NC2	G
5	1.3	1.5	1.8	S
n:	5	4	3	
Mean:	NA	2.2	2.6	
CV(%):	NA	27.6	35.3	

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

2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.

3 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each SDS concentration. SDS concentrations were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L.

4 NOEC Range: 1.3 - 2.5 mg/L (this represents a difference of one exposure concentration).

5 Adults collected in the field.

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

7 Endpoints: G=growth; S=survival.

8 NC1 = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 75 percent of the control response mean.

9 NC2 = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 25. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, AND HEXAVALENT CHROMIUM AS THE REFERENCE TOXICANT[1,2,3,4,5]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[6]
1	2.0	5.8	11.4	G
2	1.0	2.9	9.9	G
3	4.0	6.9	11.5	G
4	2.0	2.4	9.2	G
5	1.0	3.1	10.8	G
n:	5	5	5	
Mean:	NA	4.2	10.6	
CV(%):	NA	47.6	9.7	

1 Tests performed by Donald Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

2 All tests were performed using Forty Fathoms® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each hexavalent chromium concentration. Hexavalent chromium concentrations used in Tests 1-5 were: 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mg/L.

3 NOEC Range: 1.0 - 4.0 mg/L (this represents a difference of four exposure concentrations)

4 Adults collected in the field.

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

6 Endpoints: G=growth; S=survival.

TABLE 26 COMPARISON OF LARVAL SURVIVAL (LC50) AND GROWTH (IC50) VALUES FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EXPOSED TO SODIUM DODECYL SULFATE (SDS) AND COPPER (CU) SULFATE IN GP2 ARTIFICIAL SEAWATER MEDIUM OR NATURAL SEAWATER[1,2,3,4]

	Survival		Growth	
SDS (mg/L)	GP2	NSW	GP2	NSW
	7.49	8.13	7.39	8.41
	8.70	8.87	8.63	8.51
	8.38	8.85	8.48	9.33
Mean	8.19	8.62	8.17	8.75
CV (%)	7.7	4.9	8.3	5.8
Copper(µg/L)	GP2	NSW	GP2	NSW
	455	412	341	333
	467	485	496	529
	390	528	467	776
Mean	437	475	435	546
CV (%)	9.4	12.3	18.9	40.7

1 Tests performed by George Morrison and Glen Modica, ERL-N, USEPA, Narragansett, RI.

2 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each SDS concentration. SDS concentrations were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L.

3 Adults collected in the field.

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

TABLE 27. DATA FROM INTERLABORATORY STUDY OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST USING AN INDUSTRIAL EFFLUENT AS A REFERENCE TOXICANT[1,2,3]

		Most Sensitive Endpoint[4]		
	Test Number	NOEC (%)	IC25 (%)	IC50 (%)
Laboratory A	1	3.2 (S,G)	7.4 (S)	7.4 (G)
	2	3.2 (S,G)	7.6 (S)	14.3 (G)
Laboratory B	1	3.2 (S,G)	5.7 (G)	9.7 (G)
	2	3.2 (S,G)	5.7 (G)	8.8 (G)
Laboratory C	1	1.0 (S)	4.7 (S)	7.2 (S)
Laboratory D	1	3.2 (S,G)	7.4 (G)	24.7 (G)
	2	1.0 (G)	5.2 (S)	7.2 (S)
n:		7	7	7
Mean:		NA	5.5	11.3
CV(%):		NA	44.2	56.9

[1] Data from USEPA (1987b), USEPA (1988a), and USEPA (1991a).

[2] Effluent concentrations were: 0.32, 1.0, 3.2, 10.0, and 32.0%.

[3] NOEC Range: 1.0 - 3.2% (this represents a difference of one exposure concentration).

[4] Endpoints: G=growth; S=survival.

TABLE 28. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type		CV (%)[2]
IC25	Reference toxicant		18.4
	Effluent		6.12
	Receiving water		7.15
		Average	10.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the total interlaboratory variability (including both within-laboratory and between-laboratory components of variability). Individual within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

TABLE 29. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	25%	57.1	42.9	0.00
	Effluent	25%	100	0.00	0.00
	Receiving water	25%	100	0.00	0.00
Growth NOEC	Reference toxicant	25%	100	0.00	0.00
	Effluent	12.5%	57.1	42.9	0.00
	Receiving water	12.5%	71.4	28.6	0.00

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

3 Percent of values two or more concentration intervals above or below the median.