SECTION 13

TEST METHOD

INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH METHOD 1006.0

13.1 SCOPE AND APPLICATION

13.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the inland silverside, *Menidia beryllina*, using seven to 11-day old 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.

13.1.2 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).

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

13.1.4 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.

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

13.2 SUMMARY OF METHOD

13.2.1 Inland silverside, *Menidia beryllina*, seven to 11-day old larvae 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 growth of the larvae.

13.3 INTERFERENCES

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

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

13.3.3 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).

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

13.3.5 Food added during the test may sequester metals and other toxic substances and confound test results.

13.3.6 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 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. 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.

13.3.6.1 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 13.3.6.2. 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.

13.3.6.1.1 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).

13.3.6.1.2 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).

13.3.6.1.3 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 13.3.6.1.1).

13.3.6.1.4 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 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 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.

13.4 SAFETY

13.4.1 See Section 3, Health and Safety.

13.5 APPARATUS AND EQUIPMENT

13.5.1 Facilities for holding and acclimating test organisms.

13.5.2 Brine shrimp, *Artemia*, Culture Unit -- see Subsection 13.6.16 below and Section 4, Quality Assurance.

13.5.3 *Menidia Beryllina* Culture Unit -- see Subsection 13.6.17 below, Middaugh and Hemmer (1984), Middaugh et al. (1986), USEPA (1987g) and USEPA (2002a) for detailed culture methods. This test requires from 180-360 7 to 11 day-old larvae. 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 larvae can be obtained from other sources by shipping them in well oxygenated saline water in insulated containers.

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

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

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

13.5.7 Balance, analytical -- capable of accurately weighing to 0.00001 g.

13.5.8 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 weighing pans plus fish.

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

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

13.5.11 Air lines, plastic or pasteur pipettes, or air stones -- for gently aerating water containing the fragile larvae or for supplying air to test solution with low DO

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

13.5.13 Standard or micro-Winkler apparatus -- for calibrating DO (optional).

13.5.14 Desiccator -- for holding dried larvae.

13.5.15 Light box -- for counting and observing larvae.

13.5.16 Refractometer -- for determining salinity.

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

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

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

13.5.20 Test chambers -- four chambers per concentration. The chambers should be borosilicate glass or nontoxic disposable plastic labware. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or sheet plastic (6 mm thick).

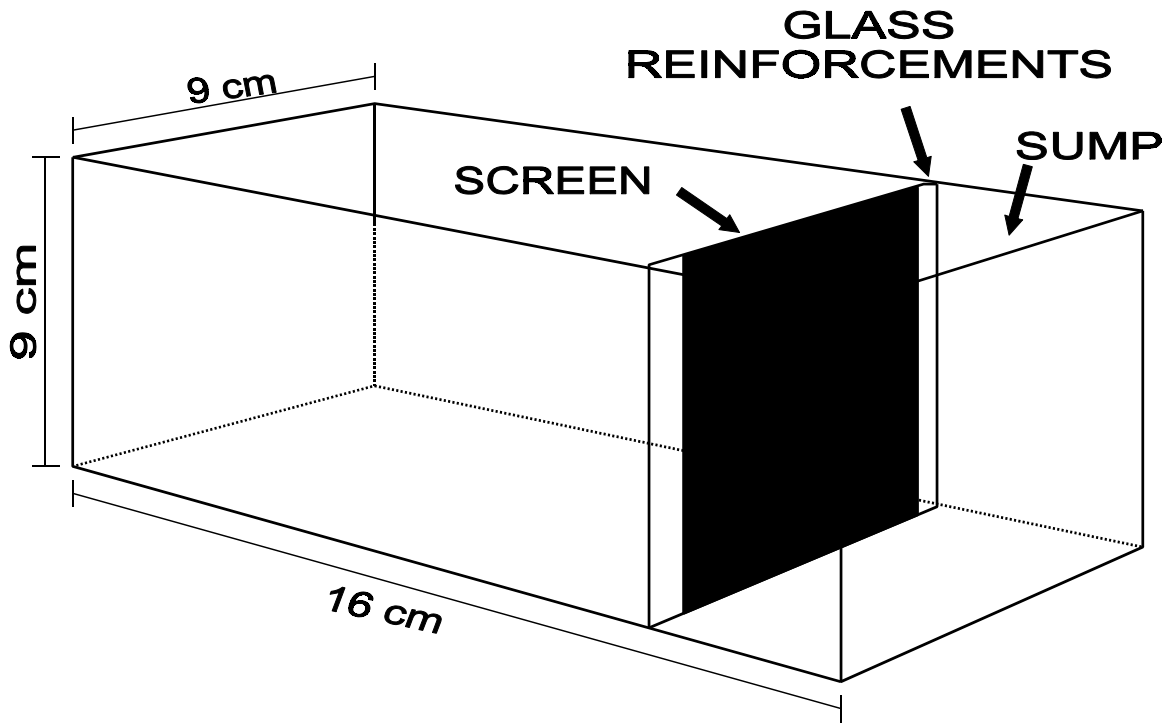

Figure 1. Glass chamber with sump area. Modified from Norberg and Mount (1985). From USEPA (1987c).

13.5.20.1 Each test chamber for the inland silverside should contain a minimum of 750 mL of test solution. A modified Norberg and Mount (1985) chamber (Figure 1), constructed of glass and silicone cement, has been used successfully for this test. This type of chamber holds an adequate column of test solution and incorporates a sump area from which test solutions can be siphoned and renewed without disturbing the fragile inland silverside larvae. Modifications for the chamber are as follows: 1) 200 µm mesh NITEX® screen instead of stainless steel screen; and 2) thin pieces of glass rods cemented with silicone to the NITEX® screen to reinforce the bottom and sides to

produce a sump area in one end of the chamber. Avoid excessive use of silicone, while still ensuring that the chambers do not leak and the larvae cannot get trapped or escape into the sump area. Once constructed, check the chambers for leaks and repair if necessary. Soak the chambers overnight in seawater (preferably in flowing water) to cure the silicone cement before use. Other types of glass test chambers, such as the 1000 mL beakers used in the short-term Sheepshead Minnow Larval Survival and Growth Test, may be used. It is recommended that each chamber contain a minimum of 50 mL per larvae and allow adequate depth of test solution (5.0 cm).

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

13.5.22 Mini-Winkler bottles -- for dissolved oxygen calibrations.

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

13.5.24 Crystallization dishes, beakers, culture dishes, or equivalent -- for incubating embryos.

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

13.5.26 Separatory funnels, 2 L -- Two - four for culturing *Artemia*.

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

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

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

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

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

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

13.5.33 Forceps -- for transferring dead larvae to weighing pans.

13.5.34 NITEX® Mesh Sieves (≤ 150 µm, 500 µm, 3-5 mm) -- for collecting *Artemia* nauplii and fish larvae.

13.6 REAGENTS AND CONSUMABLE MATERIALS

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

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

13.6.3 Tape, colored -- for labelling test chambers.

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

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

13.6.6 Weighing pans, aluminum -- 26/test (two extra).

13.6.7 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).

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

13.6.9 Laboratory quality assurance samples and standards -- for the above methods.

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

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

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

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

13.6.13.1 Saline test and dilution water -- the salinity of the test water must be in the range of 5 to 32‰. The salinity should vary 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.

13.6.13.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of *Menidia beryllina* 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. Artificial sea salts or hypersaline brine (100‰) derived from natural seawater may be used to adjust the salinities.

13.6.13.3 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 70% at 30‰ salinity and 80% at 20‰ salinity.

13.6.13.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a noncorrosive 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.

13.6.13.3.2 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.

13.6.13.3.3 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.

13.6.13.3.4 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 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.

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

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

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

13.6.13.3.8 Table 1 illustrates the composition of test solutions at 20‰ if they are made by combining effluent (0‰), deionized water and HSB at 100‰ salinity. The volume (mL) of brine required is determined by using the amount calculated above. In this case, 200 mL of brine is required for 1 L; therefore, 600 mL would be required for 3 L of solution. The volumes of HSB required are constant. The volumes of deionized water are determined by subtracting the volumes of effluent and brine from the total volume of solution: 3,000 mL - mL effluent - mL HSB = mL deionized water.

13.6.13.4 Artificial sea salts: A modified GP2 artificial seawater formulation (Table 2) has been successfully used to perform the inland silverside survival and growth test. The use of GP2 for holding and culturing of adults is not recommended at this time.

13.6.13.4.1 The GP2 artificial sea salts (Table 2) 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 gm $NaHCO_3$ in 500 mL deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

13.6.14 ROTIFER CULTURE --for feeding cultures and test organisms

13.6.14.1 At hatching *Menidia beryllina* larvae are too small to ingest *Artemia* nauplii and must be fed rotifers, *Brachionus plicatilis*. The rotifers can be maintained in continuous culture when fed algae (see Section 6 and USEPA, 1987g). Rotifers are cultured in 10-15 L Pyrex® carboys (with a drain spigot near the bottom) at 25-28°C and 25-35‰ salinity. Four 12 L culture carboys should be maintained simultaneously to optimize production. Clean carboys should be filled with autoclaved seawater. Alternatively, an immersion heater may be used to heat saline water in the carboy to 70-80°C for 1-h.

TABLE 1: PREPARATION OF 3 L SALINE WATER FROM DEIONIZED WATER AND A HYPERSALINE BRINE OF 100‰ NEEDED FOR TEST SOLUTIONS AT 20‰ SALINITY

Effluent Concentration	Volume of Effluent (0‰) (mL)	Volume of Deionized Water (mL)	Volume of Hypersaline Brine (mL)	Total Volume (mL)
80	2400	0	600	3000
40	1200	1200	600	3000
20	600	1800	600	3000
10	300	2100	600	3000
5	150	2250	600	3000
Control	0	2400	600	3000
Total	4,650	9,750	3,600	18,000

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, 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.

13.6.14.2 When the water has cooled to 25-28°C, aerate and add a start-up sample of rotifers (50 rotifers/mL) and food (about 1 L of a dense algal culture). The carboys should be checked daily to ensure that adequate food is available and that the rotifer density is adequate. If the water appears clear, drain 1 L of culture water and replace it with algae. Excess water can be removed through the spigot drain and filtered through a ≤ 60 µm mesh screen. Rotifers collected on the screen should be returned to the culture. If a more precise measure of the rotifer population is needed, rotifers collected from a known volume of water can be resuspended in a smaller volume, killed with formalin and counted in a Sedgwick-Rafter cell. If the density exceeds 50 rotifers/mL, the amount of food per day should be increased to 2 L of algae suspension. The optimum density of approximately 300-400 rotifers/mL may be reached in seven to 10 days and is sustainable for two to three weeks. At these densities, the rotifers should be cropped daily. Keeping the carboys away from light will reduce the amount of algae attached to the carboy walls. When detritus accumulates, populations of ciliates, nematodes, or harpacticoid copepods that may have been inadvertently introduced can rapidly take over the culture. If this occurs, discard the cultures.

13.6.15 ALGAL CULTURES -- for feeding rotifer cultures

13.6.15.1 *Tetraselmus suecica* or *Chlorella* sp. (see USEPA, 1987a) can be cultured in 20 L polycarbonate carboys that are normally used for bottled drinking water. Filtered seawater is added to the carboys and then autoclaved (110°C for 30 minutes). After cooling to room temperature, the carboys are placed in a temperature chamber controlled at 18-20°C. One liter of *T. suecica* or *Chlorella* sp. starter culture and 100 mL of nutrients are added to each carboy.

13.6.15.2 Formula for algal culture nutrients.

13.6.15.2.1 Add 180 g $NaNO_3$, 12 g NaH_2PO_4, and 6.16 g EDTA to 12 L of deionized water. Mix with a magnetic stirrer until all salts are dissolved (at least 1-h).

13.6.15.2.2 Add 3.78 g $FeCl_3 \cdot 6\ H_2O$ and stir again. The solution should be bright yellow.

13.6.15.2.3 The algal culture is vigorously aerated via a pipette inserted through a foam stopper at the top of the carboy. A dense algal culture should develop in 7 to 10 days and should be used by Day 14. Thus, start-up of cultures should be made on a daily or every second day basis. Approximately 6 to 8 continuous cultures will meet the feeding requirements of four 12 L rotifer cultures. When emptied, carboys are washed with soap and water and rinsed thoroughly with deionized water before reuse.

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

13.6.16.1 Newly hatched *Artemia* nauplii are used as food for inland silverside larvae in toxicity tests. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are being used because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

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

13.6.16.2.1 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L of deionized water, to a 2 L separatory funnel or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985d; USEPA, 2002a; and ASTM, 1993.)
3. After 24-h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic and will concentrate at the bottom of the funnel if it is covered for 10-15 minutes to prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 minutes without aeration.
4. Drain the nauplii into a beaker or funnel fitted with ≤ 150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

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

13.6.16.3.1 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 inland silverside larvae (see Subsection 13.11). 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 chambers each containing a minimum of 15 larvae, for each type of food.

13.6.16.3.2 The feeding rate and frequency, test vessels and 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.

13.6.16.3.3 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.

13.6.16.4 Use of *Artemia* nauplii as food for inland silverside, *Menidia beryllina*, larvae.

13.6.16.4.1 *Menidia beryllina* larvae begin feeding on newly hatched *Artemia* nauplii about five days after hatching, and are fed *Artemia* nauplii daily throughout the 7-day larval survival and growth test. Survival of *Menidia beryllina* larvae seven to nine days old is improved by feeding newly hatched (< 24-h old) *Artemia* nauplii. Equal amounts of *Artemia* nauplii must be fed 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 below an acceptable level (below 4.0 mg/L). As much of the uneaten *Artemia* nauplii as possible should be siphoned from each chamber prior to test solution renewal to ensure that the larvae principally eat newly hatched nauplii.

13.6.17 TEST ORGANISMS, INLAND SILVERSIDE, *MENIDIA BERYLLINA*

13.6.17.1 The inland silverside, *Menidia beryllina*, is one of three species in the atherinid family that are amenable to laboratory culture; and one of four atherinid species used for chronic toxicity testing. Several atherinid species have been utilized successfully for early life stage toxicity tests using field collected (Goodman et al., 1985) and laboratory reared adults (Middaugh and Takita, 1983; Middaugh and Hemmer, 1984; and USEPA, 1987g). The inland silverside, *Menidia beryllina*, populates a variety of habitats from Cape Cod, Massachusetts, to Florida and west to Vera Cruz, Mexico (Johnson, 1975). It can tolerate a wide range of temperature, 2.9-32.5°C (Tagatz and Dudley, 1961; Smith, 1971) and salinity, of 0-58‰ (Simmons, 1957; Renfro, 1960), having been reported from the freshwaters of the Mississippi River drainage basin (Chernoff et al., 1981) to hypersaline lagoons (Simmons, 1957). Ecologically, *Menidia* spp. are important as major prey for many prominent commercial species (e.g., bluefish (*Pomatomus saltatrix*), mackerel (*Scomber scombrus*), and striped bass (*Morone saxatilis*) (Bigelow and Schroeder, 1953). The inland silverside, *Menidia beryllina*, is a serial spawner, and will spawn under controlled laboratory conditions. Spawning can be induced by diurnal interruption in the circulation of water in the culture tanks (Middaugh et al., 1986; USEPA, 1987a). The eggs are demersal, approximately 0.75 mm in diameter (Hildebrand and Schroeder, 1928), and adhere to vegetation in the wild, or to filter floss in laboratory culture tanks. The larvae hatch in six to seven days when incubated at 25°C and maintained in seawater ranging from 5-30‰ (USEPA, 1987a). Newly hatched larvae are 3.5-4.0 mm in total length (Hildebrand, 1922).

13.6.17.2 Inland silverside, *Menidia beryllina*, adults (see USEPA, 1987g and USEPA, 2002a for detailed culture methods) may be cultured in the laboratory or obtained from the Gulf of Mexico or Atlantic coast estuaries throughout the year (Figure 2). Gravid females can be collected from low salinity waters along the Atlantic coast during April to July, depending on the latitude. The most productive and protracted spawning stock can be obtained from adults brought into the laboratory. Broodstocks, collected from local estuaries twice each year (in April and October), will become sexually active after one to two months and will generally spawn for 4-6 months.

13.6.17.3 The fish can be collected easily with a beach seine (3-6 mm mesh), but the seine should not be completely landed onto the beach. Silversides are very sensitive to handling and should never be removed from the water by net -- only by beaker or bucket.

13.6.17.4 Samples may contain a mixture of inland silverside, *Menidia beryllina*, and Atlantic silverside, *Menidia menidia*, on the Atlantic coast or inland silverside and tidewater silverside, *Menidia peninsulae*, on the Gulf Coast (see USEPA, 1987g for additional information on morphological differences for identification). Johnson (1975) and Chernoff et al. (1981) have attempted to differentiate these species. In the northeastern United States, *M. beryllina* juveniles and adults are usually considerably smaller than *M. menidia* juveniles and adults (Bengtson, 1984), and can be separated easily in the field on that basis.

13.6.17.5 Record the water temperature and salinity at each collection site. Aerate (portable air pump, battery operated) the fish and transport to the laboratory as quickly as possible after collection. Upon arrival at the laboratory, the fish and the water in which they were collected are transferred to a tank at least 0.9 m in diameter. A filter system should be employed to maintain water quality (see USEPA, 1987g). Laboratory water is added to the tank slowly, and the fish are acclimated at the rate of 2°C per day, to a final temperature of 25°C, and about 5‰ salinity per day, to a final salinity in the range of 20-32‰. The seawater in each tank should be brought to a minimum volume of 150 L. A density of about 50 fish/tank is appropriate. Maintain a photoperiod of 16 h light/8 h dark. Feed the adult fish flake food or frozen brine shrimp twice daily and *Artemia* nauplii once daily. Siphon the detritus from the bottom of the tanks weekly.

13.6.17.6 Larvae for a toxicity test can be obtained from the broodstock by spawning onto polyester aquarium filter-fiber substrates, 15 cm long x 10 cm wide x 10 cm thick, which are suspended with a string 8-10 cm below the surface of the water and in contact with the side of the holding tanks for 24-48 h, 14 days prior to the beginning of a test. The floss should be gently aerated by placing it above an airstone, and weighted down with a heavy non-toxic object. The embryos, which are light yellow in color, can be seen on the floss, and are round and hard to the touch compared to the soft floss.

13.6.17.7 Remove as much floss as possible from the embryos. The floss should be stretched and teased to prevent the embryos from clumping. The embryos should be incubated at the test salinity and lightly aerated. At 25°C, the embryos will hatch in about six to eight days. Larvae are fed about 500 rotifer larvae/day from hatch through four days post-hatch. On Days 5 and 6, newly hatched (less than 12 h old) *Artemia* nauplii are mixed with the rotifers, to provide a transition period. After Day 7, only nauplii are fed, and the age range for the nauplii can be increased from 12 h old to 24 h old.

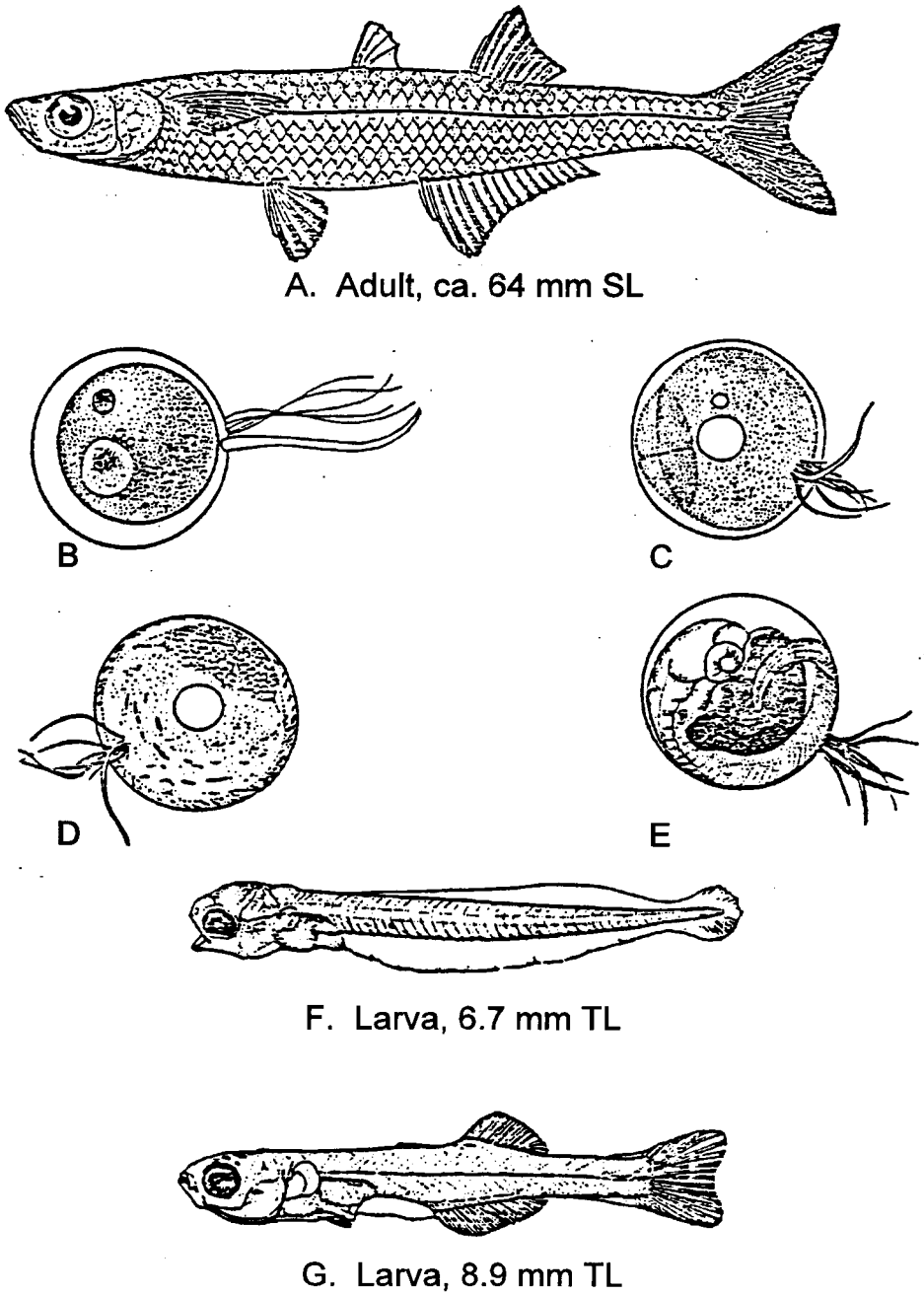

Figure 2. Inland silverside, *Menidia beryllina*: A. Adult, ca. 64 mm SL; B. Egg (diagrammatic), only bases of filaments shown; C. Egg, 2-cell stage; D. Egg, morula stage; E. Advanced embryo, two and one half days after fertilization. From Martin and Drewry (1978).

13.6.17.8 Silverside larvae are very sensitive to handling and shipping during the first week after hatching. For this reason, if organisms must be shipped to the test laboratory, it may be impractical to use larvae less than 11 days old because the sensitivity of younger organisms may result in excessive mortality during shipment. If organisms are to be shipped to a test site, they should be shipped only as (1) early embryos, so that they hatch after arrival, or (2) after they are known to be feeding well on *Artemia* nauplii (8-10 days of age). Larvae shipped at 8 - 10 days of age would be 9 to 11 days old when the test is started. Larvae that are hatched and reared in the test laboratory can be used at seven days of age.

13.6.17.9 If four replicates of 15 larvae are used at each effluent concentration and in the control, 360 larvae will be needed for each test.

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 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 day.

13.10.1.2 Effluents

13.10.1.2. 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%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥0.5 dilution factor.** If 100% salinity HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity, and 70% at 30‰ salinity.

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

13.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates per treatment for five concentrations of effluent and a control, each containing 750 mL of test solution, is approximately

5 L. Prepare enough test solution at each effluent concentration to provide 400 mL additional volume for chemical analyses.

13.10.1.2.4 Tests should begin as soon as possible after sample collection, preferably within 24 h. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity studies unless permission is granted by the permitting authority. In no case should the test be started more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4).

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

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

13.10.1.3 Dilution Water

13.10.1.3.1 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 3 in Section 7, Dilution Water). Other artificial sea salts may be used for culturing inland silverside minnows and for the larval survival and growth test if the control criteria for acceptability of test data are satisfied.

13.10.2 START OF THE TEST

13.10.2.1 Inland silverside larvae 7 to 11 days old can be used to start the survival and growth test. At this age, the inland silverside feed on newly-hatched *Artemia* nauplii. At 25°C, tests with inland silverside larvae can be performed at salinities ranging from 5 to 32‰. If the test salinity ranges from 16 to 32‰, the salinity for spawning, incubation, and culture of the embryos and larvae should be maintained within this salinity range. If the test salinity is in the range of 5 to 15‰, the embryos may be spawned at 30‰, but egg incubation and larval rearing should be at the test salinity. If the specific salinity required for the test differs from the rearing salinity, adjustments of 5‰ daily should be made over the three days prior to start of test.

13.10.2.2 One day Prior to Beginning of Test

13.10.2.2.1 Set up the *Artemia* culture so that newly hatched nauplii will be available on the day the test begins. (see Section 7).

13.10.2.2.2 Increase the temperature of water bath, room, or incubator to the required test temperature (25 ± 1°C).

13.10.2.2.3 Label the test chambers with a marking pen. Use of color coded tape to identify each concentration and replicate is helpful. A minimum of five effluent concentrations and a control should be selected for each test. Glass test chambers, such as crystallization dishes, beakers, or chambers with a sump area (Figure 1), with a capacity for 500-750 mL of test solution, should be used.

13.10.2.2.4 Randomize the position of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a table of random numbers or similar process (see Appendix A for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart.

13.10.2.2.5 Because inland silverside larvae are very sensitive to handling, it is advisable to distribute them to their respective test chambers which contain control water on the day before the test is to begin. Each test chamber

should contain a minimum of 10 larvae and it is required that there be four replicates minimum for each concentration and control.

13.10.2.2.6 Seven to 11 day old larvae are active and difficult to capture and are subject to handling mortality. Carefully remove larvae (two to three at a time) by concentrating them in a corner of the aquarium or culture vessel, and capture them with a wide-bore pipette, small petri dish, crystallization dish, 3-4 cm in diameter, or small pipette. They are active and will readily escape from a pipette. Randomly transfer the larvae (two to three at a time) into each test chamber until the desired number (15) is attained. See Appendix A for an example of randomization. After the larvae are dispensed, use a light table to verify the number in each chamber.

13.10.2.3 Before beginning the test remove and replace any dead larvae from each test chamber. The test is started by removing approximately 90% of the clean seawater from each test chamber and replacing with the appropriate test solution.

13.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50-100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1 °C. The test salinity should be in the range of 5-32‰, and the salinity should not vary by 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.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO The DO should 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 concentrations 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 to the fish.

13.10.5 FEEDING

13.10.5.1 *Artemia* nauplii are prepared as described above.

13.10.5.2 The test larvae are fed newly-hatched (less than 24 h old) *Artemia* nauplii once a day from Day 0 through Day 6; larvae are not fed on Day 7. Equal amounts of *Artemia* nauplii must be fed 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 of *Artemia* nauplii, should be provided to each replicate on a daily basis. Feeding excessive amounts of *Artemia* nauplii will result in a depletion in DO to below an acceptable level. Siphon as much of the uneaten *Artemia* nauplii as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

13.10.5.3 On Days 0-2, transfer 4 g wet weight or pipette 4 mL of concentrated, rinsed *Artemia* nauplii to seawater in a 100 mL beaker, and bring to a volume of 80 mL. Aerate or swirl the suspension to equally distribute the nauplii while withdrawing individual 2 mL portions of the *Artemia* nauplii suspension by pipette or adjustable syringe to transfer to each replicate test chamber. Because the nauplii will settle and concentrate at the tip of the pipette during the transfer, limit the volume of concentrate withdrawn each time to a 2 mL portion for one test chamber helps ensure an equal distribution to the replicate chambers. Equal distribution of food to the replicates is critical for successful tests.

13.10.5.4 On Days 3-6, transfer 6 g wet weight or 6 mL of the *Artemia* nauplii concentrate to seawater in a 100 mL beaker. Bring to a volume of 80 mL and dispense as described above.

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

13.10.6 DAILY CLEANING OF TEST CHAMBERS

13.10.6.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL), fitted with a safety pipet filler or rubber bulb, can be used. If the test chambers illustrated in Figure 1 are used, remove only as much of the test solution from the chamber as is necessary to clean, and siphon the remainder of the test solution from the sump area. Because of their small size during the first few days of the test, larvae are easily drawn into a siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of 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 up in the siphon can be retrieved, and returned by pipette to the appropriate test chamber and noted on data sheet. 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.

13.10.7 OBSERVATIONS DURING THE TEST

13.10.7.1 Routine Chemical and Physical Determinations

13.10.7.l.l DO is measured at the beginning and end of each 24 h exposure period in one test chamber at all test concentrations and in the control.

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

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

13.10.7.1.4 Record all measurements on the data sheet (Figure 3)

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																																
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**			

TIME FED							

COMMENTS:

Figure 3. Data form for the inland silverside, *Menida beryllina,* larval survival and growth test. Daily record of larval survival and test conditions. (From USEPA, 1987c).

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																																
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**			

TIME FED							

COMMENTS:

Figure 3. Data form for inland silverside, *Menida beryllina*, larval survival and growth test. Daily record of larval survival and test conditions. (CONTINUED)(From USEPA, 1987c).

13.10.7.2 Routine Biological Observation

13.10.7.2.1 The number of live larvae in each test chamber are recorded daily (Figure 3), and the dead larvae are discarded.

13.10.7.2.2 Protect the larvae from unnecessary disturbances during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae. Make sure the larvae remain immersed at all times during the performance of the above operations.

13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 The test solutions are renewed daily using freshly prepared solutions, immediately after cleaning the test chambers. The water level in each chamber is lowered to a depth of 7-10 mm, leaving 10-15% of the test solution. New test solution is added slowly by refilling each chamber with the appropriate amount of test solution without excessively disturbing the larvae. If the modified chamber is used (Figure 1), renewals should be poured into the sump area using a narrow bore (approximately 9 mm ID) funnel.

13.10.8.2 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 0-6°C. Plastic containers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.10.8.3 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 25 ± 1°C to prepare the test solutions. A sufficient quantity of effluent should be warmed to make the daily test solutions.

13.10.8.3.1 An illustration of the quantities of effluent and seawater needed to prepare test solution at the appropriate salinity is provided in Table 2.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 The test is terminated after seven days 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 in 4% formalin or 70% ethanol. Preserved organisms are dried and weighed within seven days. For safety, formalin should be used under a hood.

13.10.9.2 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* to be rinsed away. Rinse the larvae with deionized water to remove salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of deionized water.

13.10.9. Small aluminum weighing pans can be used to dry and weigh larvae. An appropriate number of aluminum weigh pans (one per replicate) are marked for identification and weighed to 0.01 mg, and the weights are recorded (Figure 4) on the data sheets.

13.10.9.4 Immediately prior to drying, rinse the preserved larvae in distilled (or deionized) water. The rinsed larvae from each test chamber are transferred, using forceps, 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 to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing pans containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate. Record (Figure 4) the weights. Divide the dry weight by the number of original larvae per replicate to determine the average dry weight, and record (Figures 4 and 5) on the data sheets. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 13.11). Complete the summary data sheet (Figure 5) after calculating the average measurements and

statistically analyzing the dry weights and percent survival for the entire test. Average weights should be expressed to the nearest 0.001 mg.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

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

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 Test results are acceptable if (1) the average survival of control larvae is equal to or greater than 80%, and (2) where the test starts with seven-day old larvae, the average dry weight per surviving control larvae, when dried immediately after test termination, is equal to or greater than 0.50 mg, or the average dry weight of the control larvae preserved not more than seven days in 4% formalin or 70% ethanol equals or exceeds 0.43 mg.

13.13 DATA ANALYSIS

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data.

13.13.1.2 The endpoints of toxicity tests using the inland silverside 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 and examples of data input and program output.

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

Test Dates:_____ Species:_____

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

Figure 4. Data form for the inland silverside, *Menidia beryllina*, larval survival and growth test. Dry weights of larvae (from USEPA, 1987b).

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 5. Data form for the inland silverside, *Menidia beryllina*, larval survival and growth test. Summary of test results (from USEPA, 1987c).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1006.0)[1]

1.	Test type:	Static renewal (required)
2.	Salinity:	5‰ to 32‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory illumination (recommended)
5.	Light intensity:	l0-20 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 containers (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:	7-11 days post hatch; 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 (survival of 7-9 days old *Menidia beryllina* larvae improved by feeding 24 h old *Artemia*) (required)
15.	Feeding regime:	Feed 0.l0 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)

[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 INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1006.0) (CONTINUED)

17.	Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/minimum (recommended)
18.	Dilution water:	Uncontaminated source of natural sea water, artificial 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, 0.50 mg average dry weight of control larvae where test starts with 7-days old larvae and dried immediately after test termination, or 0.43 mg or greater average dry weight per surviving control larvae, preserved not more than 7 days in 4% formalin or 70% ethanol (required)
24.	Sampling requirement:	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)

13.13.2 EXAMPLE OF ANALYSIS OF INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 6 and 7. 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.

13.13.2.2 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 the 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.

13.13.2.3 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.

13.13.2.4 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 model, the Spearman-Karber method, the Trimmed Spearman-Karber method, or the Graphical method may be used (see Appendices H-K).

13.13.2.5 Example of Analysis of Survival Data

13.13.2.5.1 This example uses the survival data from the inland silverside larval survival and growth test. The proportion surviving in each replicate in this example must first be transformed by the arc sine 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 4. A plot of the data is provided in Figure 8. Since there is 100% mortality in all three replicates for the 50% and 100% concentrations, they are not included in this statistical analysis and are considered a qualitative mortality effect.

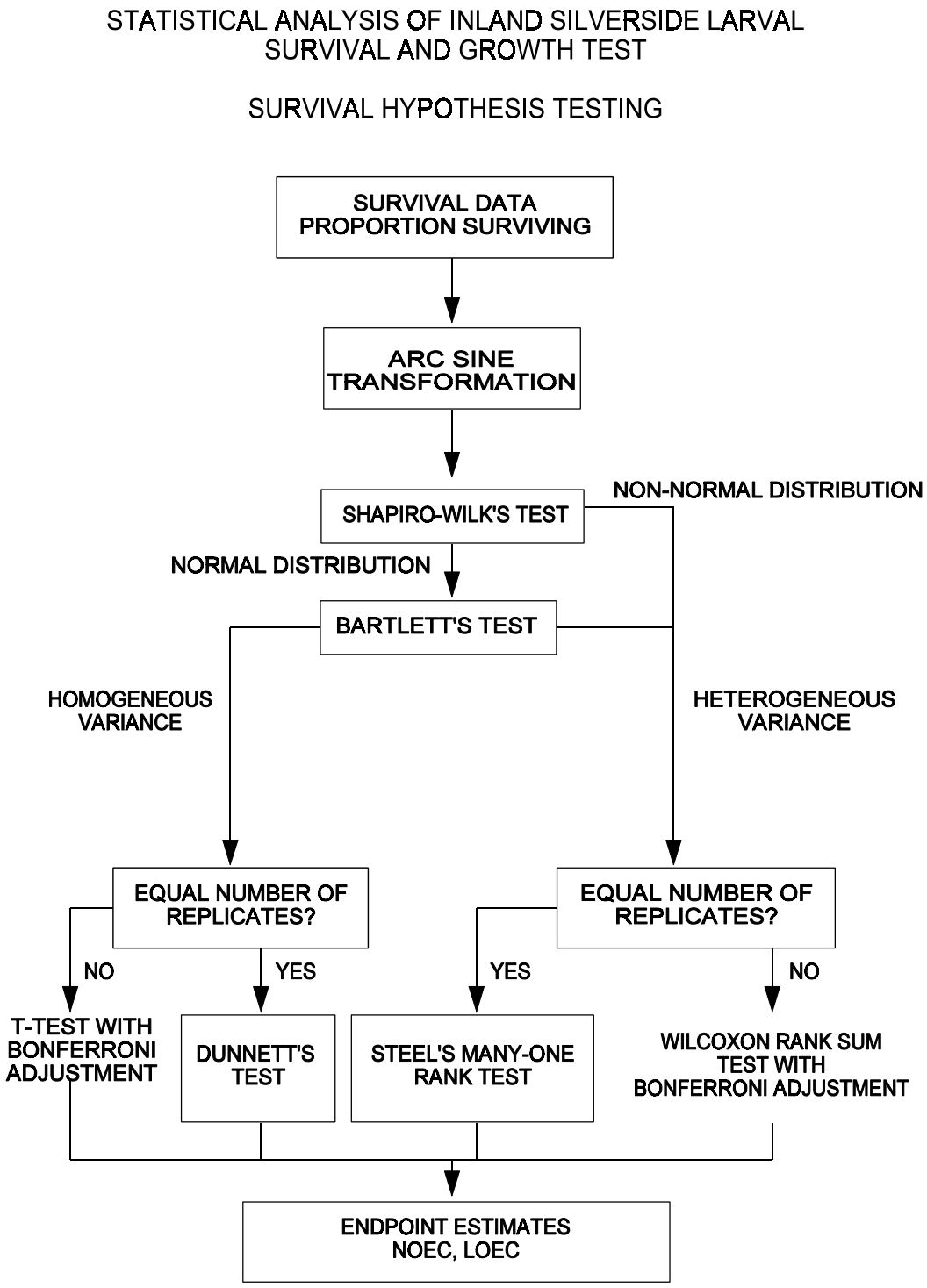

Figure 6. Flowchart for statistical analysis of the inland silverside, *Menidia beryllina*, survival data by hypothesis testing.

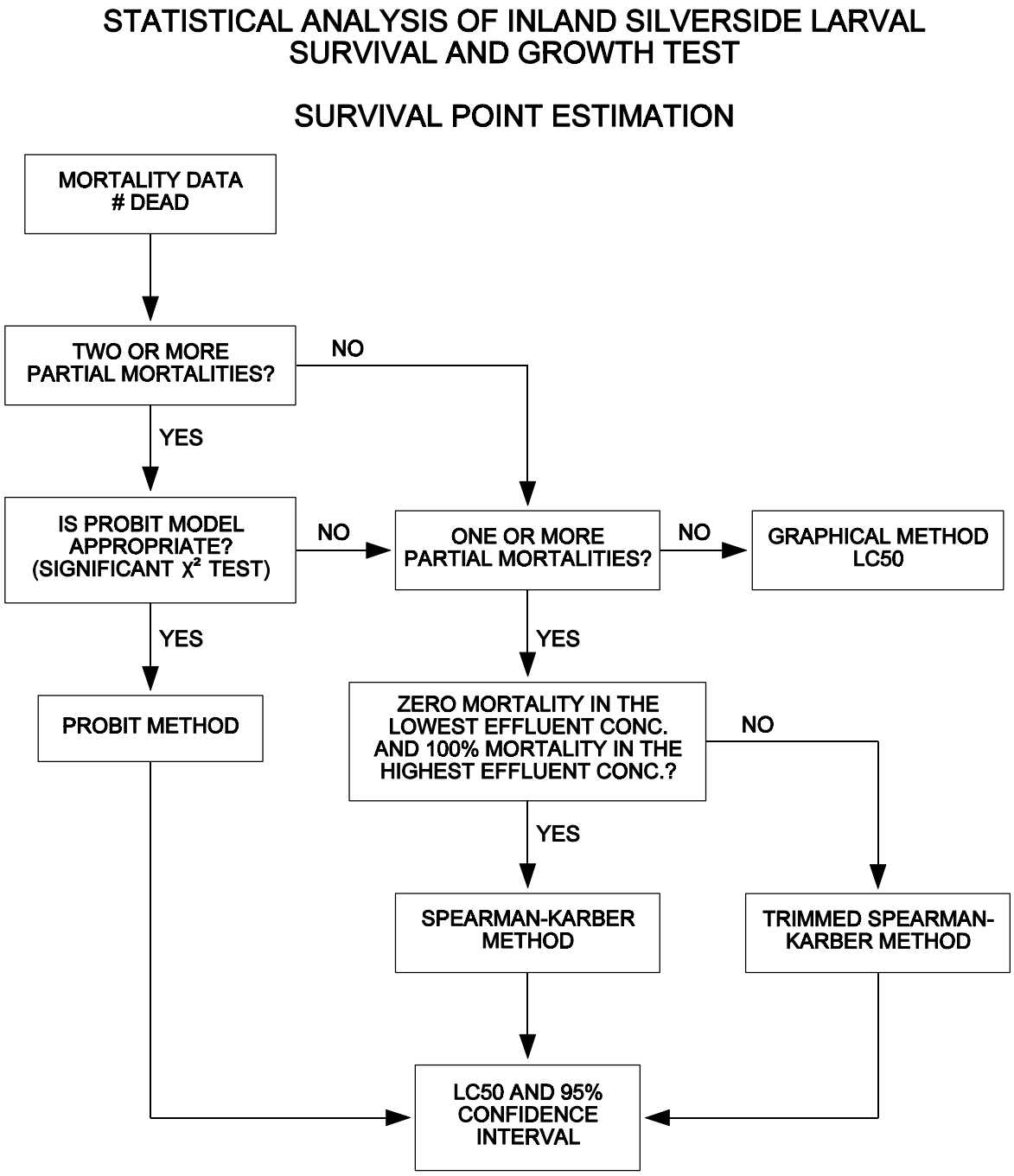

Figure 7. Flowchart for statistical analysis of the inland silverside, *Menidia beryllina*, survival data by point estimation.

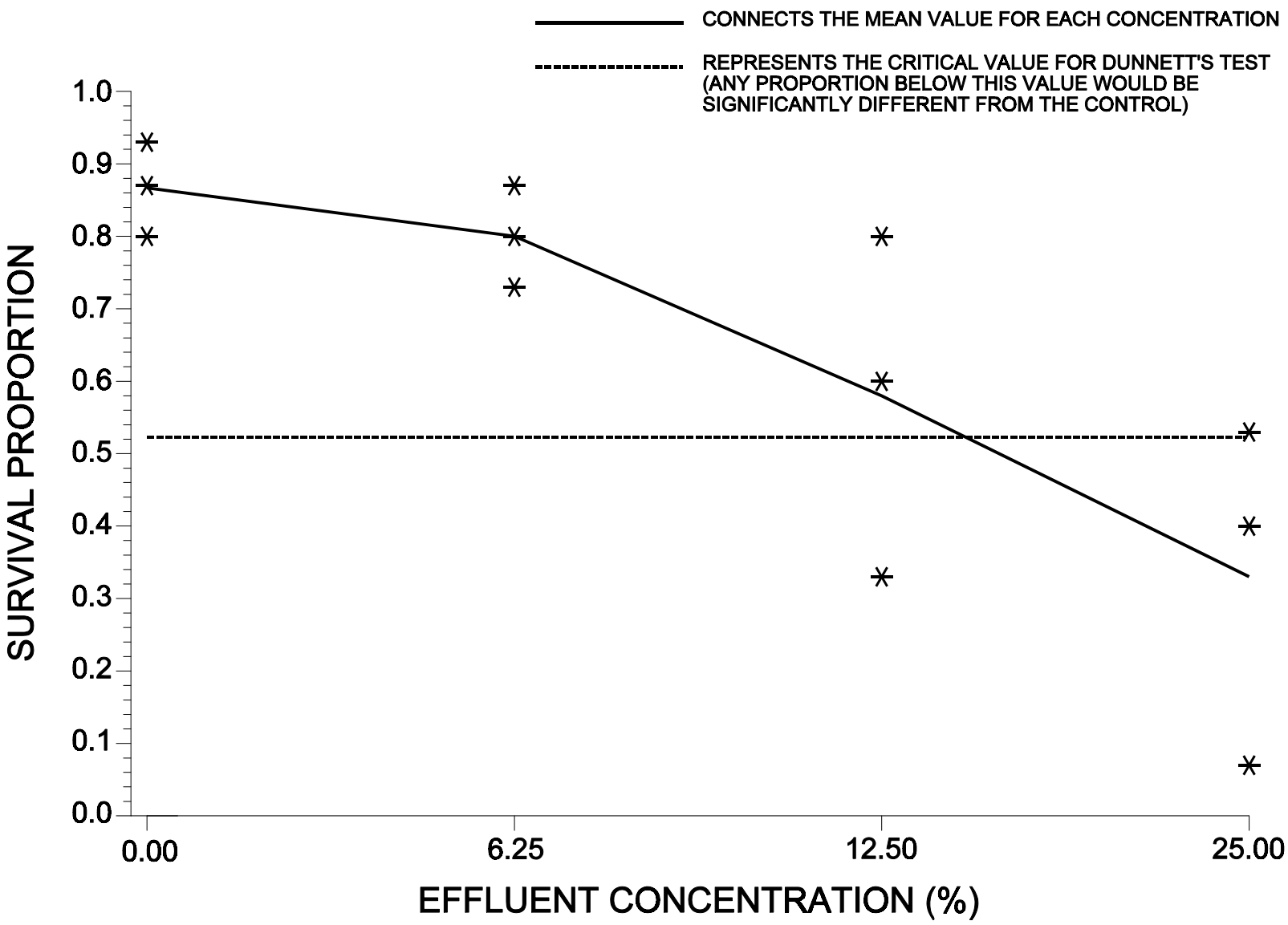

Figure 8. Plot of mean survival proportion of the inland silverside, *Menidia beryllina*, larvae.

TABLE 4. INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL DATA

			Concentration			
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	0.80	0.73	0.80	0.40	0.0	0.0
RAW B	0.87	0.80	0.33	0.53	0.0	0.0
C	0.93	0.87	0.60	0.07	0.0	0.0
ARC SINE A	1.107	1.024	1.107	0.685	-	-
TRANS- B	1.202	1.107	0.612	0.815	-	-
FORMED C	1.303	1.202	0.886	0.268	-	-
Mean($\bar{Y}_i$)	1.204	1.111	0.868	0.589		
S_i^2	0.010	0.008	0.061	0.082		
i	1	2	3	4		

13.13.2.6 Test for Normality

13.13.2.6.1 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 5.

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

		Effluent Concentration (%)		
Replicate	Control	6.25	12.5	25.0
A	-0.097	-0.087	0.239	0.096
B	-0.002	-0.004	-0.256	0.226
C	0.099	0.091	0.018	-0.321

13.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

13.13.2.6.3 For this set of data, n = 12

$$\bar{X} = \frac{1}{12}(0.002) = 0.0$$

$$D = 0.3214$$

13.13.2.6.4 Order the centered observations from smallest to largest:

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 6.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.321	7	0.018
2	-0.256	8	0.091
3	-0.097	9	0.096
4	-0.087	10	0.099
5	-0.004	11	0.226
6	-0.002	12	0.239

13.13.2.6.5 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 = 12 and k = 6. The a_i values are listed in Table 7.

13.13.2.6.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7. For the data in this example,

$$W = \frac{1}{0.3214}(0.5513)^2 = 0.945$$

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5475	0.560	$X^{(12)} - X^{(1)}$
2	0.3325	0.482	$X^{(11)} - X^{(2)}$
3	0.2347	0.196	$X^{(10)} - X^{(3)}$
4	0.1586	0.183	$X^{(9)} - X^{(4)}$
5	0.0922	0.095	$X^{(8)} - X^{(5)}$
6	0.0303	0.020	$X^{(7)} - X^{(6)}$

13.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 13.13.2.6.6 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 = 12 observations is 0.805. Since W = 0.945 is greater than the critical value, conclude that the data are normally distributed.

13.13.2.7 Test for Homogeneity of Variance

13.13.2.7.1 The test used to examine whether the variation in survival 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, $V_i = (n_i - 1)$

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

n_i = the number of replicates for concentration i.

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

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

13.13.2.7.2 For the data in this example (See Table 4), all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

13.13.2.7.3 Bartlett's statistic is therefore:

$$B = [(8)\ln(0.0402) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.2083$$

$$= [8(-3.21391) - 2(-14.731)]/1.2083$$

$$= 3.7508/1.2083$$

$$= 3.104$$

13.13.2.7.4 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 = 3.104 is less than the critical value of 11.345, conclude that the variances are not different.

13.13.2.8 Dunnett's Procedure

13.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 8.

TABLE 8. 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 SDS concentration levels including the control

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

n_i = number of observations in concentration i

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

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

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

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 proportion surviving for toxicant concentration i in test chamber j)

13.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 3$

$N = 12$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 3.612$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.333$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 2.605$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 1.768$

$G = T_1 + T_2 + T_3 + T_4 = 11.318$

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

$$= \frac{1}{3}(34.067) - \frac{(11.318)^2}{12} = 0.681$$

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

$$= 11.677 - \frac{(11.318)^2}{12} = 1.002$$

$SSW = SST - SSB = 1.002 - 0.681 = 0.321$

S_B^2 = SSB/(p-1) = 0.681/(4-1) = 0.227

S_W^2 = SSW/(N-p) = 0.321/(12-4) = 0.040

13.13.2.8.3 Summarize these calculations in the ANOVA table (Table 9).

TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	3	0.681	0.227
Within	8	0.321	0.040
Total	11	1.002	

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

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

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

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

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

13.13.2.8.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.0% concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.204 - 1.111)}{[0.020\sqrt{(1/3) + (1/3)}]} = 0.570$$

TABLE 10. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	0.570
12.5	3	2.058
25.0	4	3.766

13.13.2.8.6 Since the purpose of this test is to detect a significant reduction in survival, 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, eight degrees of freedom for error and three concentrations (excluding the control) the critical value is 2.42. The mean proportion surviving for concentration i is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Therefore, only the 25.0% concentration has a significantly lower mean proportion surviving than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

13.13.2.8.8 In this example:

$$MSD = 2.42\,(0.20)\sqrt{(1/3) + (1/3)}$$

$$= 2.42\ (0.20)\ (0.817)$$

$$= 0.395$$

13.13.2.8.9 The MSD (0.395) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.204 - 0.395 = 0.809

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

   [Sine (1.204)]^2 = 0.871

   [Sine (0.809)]^2 = 0.524

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

   MSD_u = 0.871 - 0.524 = 0.347

13.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any effluent concentration that can be detected as statistically significant is 0.347.

13.13.2.8.11 This represents a 40% decrease in survival from the control.

13.13.2.9 Calculation of the LC50

13.13.2.9.1 The data used for the Probit Analysis is summarized in Table 11. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix H.

TABLE 11. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	6.25	12.5	25.0	50.0	100.0
Number Dead	6	9	19	45	45	45
Number Exposed	45	45	45	45	45	45

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

13.13.2.9.3 Figure 9 shows the output data for the Probit Analysis of the data from Table 11 using the USEPA Probit Program.

13.13.3 ANALYSIS OF INLAND SILVERSIDE, *MENIDIA BERYLLINA,* GROWTH DATA

13.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 10. 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.

13.13.3.2 The statistical analysis using hypothesis tests 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 test 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 determined by the parametric test.

13.13.3.3 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.

Probit Analysis of Inland Silverside Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	45	6	0.1333	0.0000
6.2500	45	9	0.2000	0.0488
12.5000	45	19	0.4222	0.3130
25.0000	45	30	0.6667	0.6037
50.0000	45	45	1.0000	1.0000
100.0000	45	45	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 4.149
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Inland Silverside Larval Survival Data

Estimated LC/EC Values and Confidence Limits

	Point	Exposure Conc.	95% Confidence Limits Lower	Upper
LC/EC	1.00	4.980	2.023	7.789
LC/EC	50.00	18.302	13.886	22.175

Figure 9. Output for USEPA Probit Analysis Program, Version 1.5.

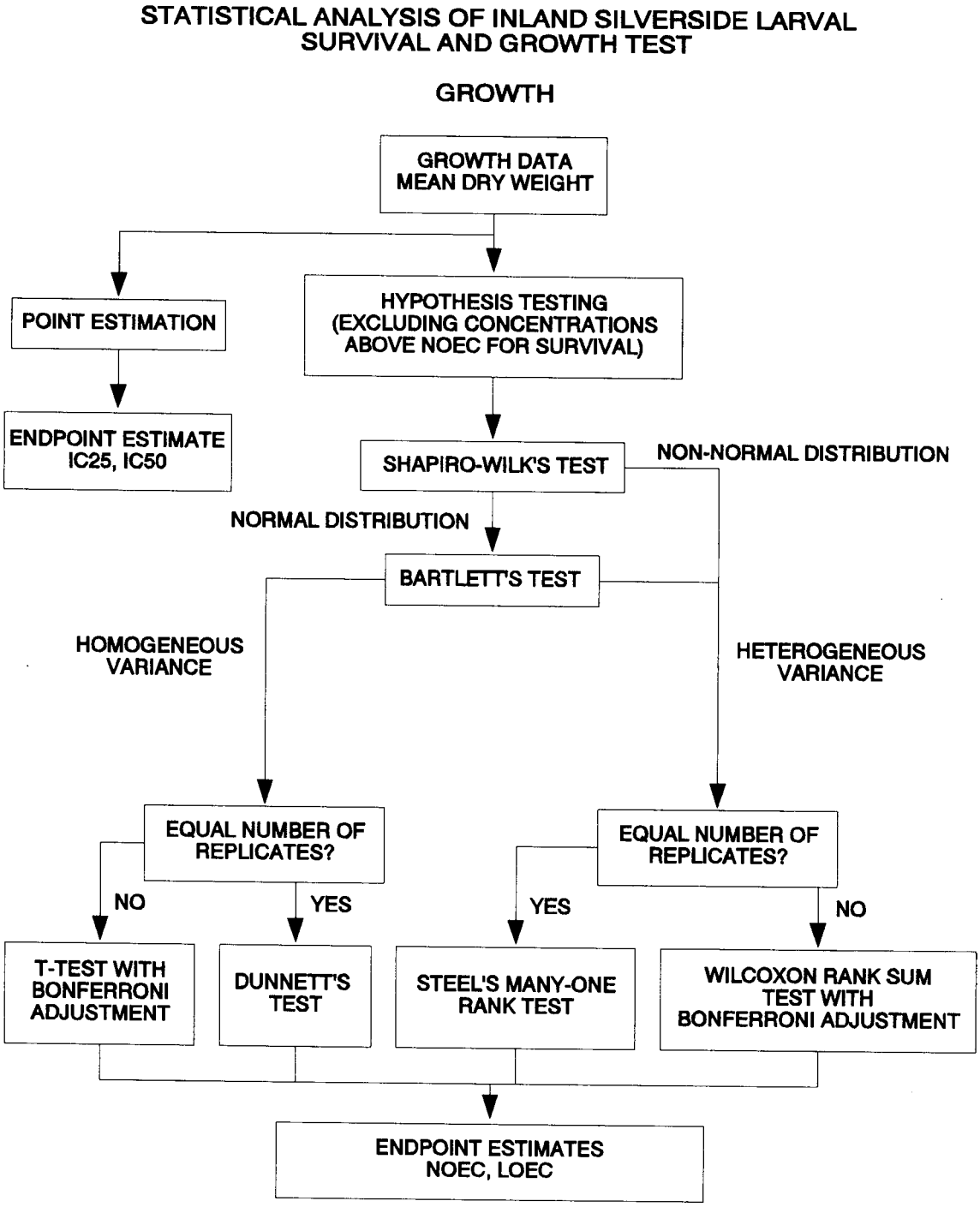

Figure 10. Flowchart for statistical analysis of the inland silverside, *Menida beryllina*, growth data.

13.13.3.4 The data, mean and variance of the growth observations at each concentration including the control are listed in Table 12. A plot of the data is provided in Figure 11. Since there was no survival in the 50% and 100% concentrations, these are not considered in the growth analysis. Additionally, since there is significant mortality in the 25% effluent concentration, its effect on growth is not considered.

TABLE 12. INLAND SILVERSIDE, *MENIDIA BERYLLINA*, GROWTH DATA

		Effluent Concentration %				
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	0.751	0.737	0.722	0.196	-	-
B	0.849	0.922	0.285	0.312	-	-
C	0.907	0.927	0.718	0.079	-	-
Mean($\bar{Y}_i$)	0.836	0.862	0.575	0.196	-	-
S_i^2	0.0062	0.0117	0.0631	0.0136	-	-
i	1	2	3	4	5	6

13.13.3.5 Test for Normality

13.13.3.5.1 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 13.

TABLE 13. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)		
Replicate	Control	6.25	12.5
A	-0.085	-0.125	0.147
B	0.013	0.060	-0.290
C	0.071	0.065	0.143

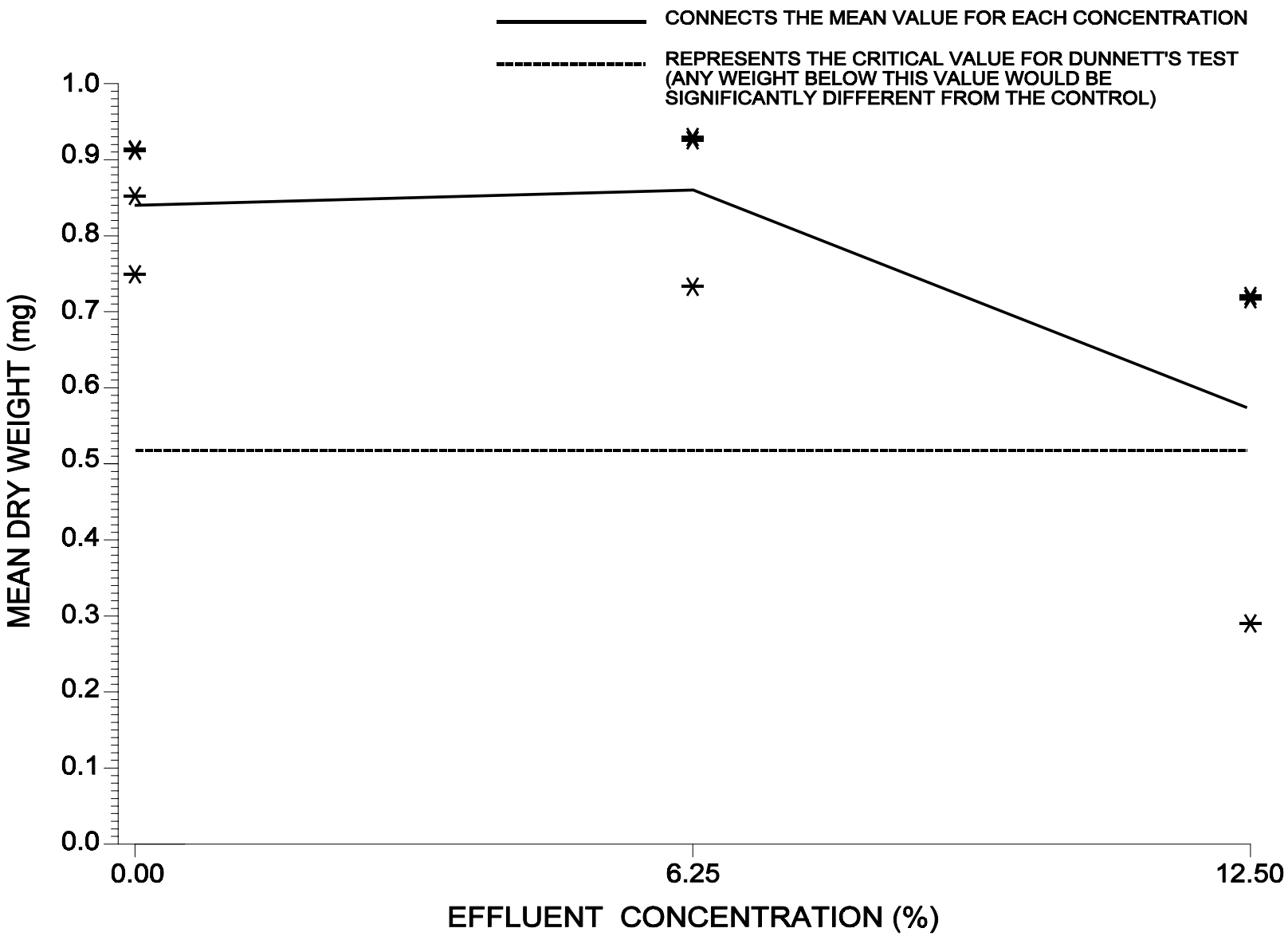

Figure 11. Plot of mean weights of inland silverside, *Menidia beryllina*, larval survival and growth test.

13.13.3.5.2 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

X = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, $n = 9$

$$\bar{X} = \frac{1}{9}(-0.002) = 0.000$$

$D = 0.162$

13.13.3.5.3 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 14.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.290	6	0.065
2	-0.125	7	0.071
3	-0.085	8	0.143
4	0.013	9	0.147
5	0.060		

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 9 and k = 4. The a_i values are listed in Table 15.

13.13.3.5.5 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 15. For this set of data:

$$W = \frac{1}{0.162}(0.3800)^2 = 0.89$$

TABLE 15. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5888	0.437	$X^{(9)} - X^{(1)}$
2	0.3244	0.268	$X^{(8)} - X^{(2)}$
3	0.1976	0.156	$X^{(7)} - X^{(3)}$
4	0.0947	0.052	$X^{(6)} - X^{(4)}$

13.13.3.5.6 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 nine observations (n) is 0.764. Since W = 0.964 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 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, $V_i = (n_i - 1)$

p = number of levels of effluent concentration including the control

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

ln = $\log_e$

n_i = number of replicates for concentration i

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

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

13.13.3.6.2 For the data in this example, (See Table 13) all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(6)\ln(0.027) - 2\sum_{i=1}^{p} \ln(S_i^2)/1.222$$

$$= [6(-3.612)-2(-12.290)]/1.222$$

$$= 2.909/1.222$$

$$= 2.38$$

13.13.3.6.4 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 2 degrees of freedom, is 9.210. Since B = 2.38 is less than the critical value of 9.210, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 16.

TABLE 16. ANOVA TABLE

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

Where: p = number of effluent concentrations 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

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

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

G = the grand total of all sample observations,

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 fish for toxicant concentration i in test chamber j)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = 3$

N = 9

$T_1 = Y_{11} + Y_{12} + Y_{13} = 0.751 + 0.849 + 0.907 = 2.507$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 0.727 + 0.922 + 0.927 = 2.576$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 0.722 + 0.285 + 0.718 = 1.725$

$G = T_1 + T_2 + T_3 = 6.808$

$$= \frac{1}{3}(15.896) - \frac{(6.808)^2}{9} = 0.1488$$

$$= 5.463 - \frac{(6.808)^2}{9} = 0.3131$$

$$= 0.3131 - 0.1488 = 0.1643$$

$$= SSB/(p-1) = 0.1488/(3-1) = 0.0744$$

$$= SSW/(N-p) = 0.1643/(9-3) = 0.0274$$

13.13.3.7.3 Summarize these calculations in the ANOVA table (Table 17).

TABLE 17. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	2	0.1488	0.0744
Within	6	0.1643	0.0274
Total	8	0.3131	

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

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

Where: Y_i = mean dry weight for effluent concentration i

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.

13.13.3.7.5 Table 18 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:

$$t_2 = \frac{(0.836 - 0.859)}{[0.1655\sqrt{(1/3) + (1/3)}]} = -0.120$$

TABLE 18. CALCULATED T VALUES

Effluent Concentration (ppb)	i	t_i
6.25	2	-0.120
12.5	3	1.931

13.13.3.7.6 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, six degrees of freedom for error and two concentrations (excluding the control) the critical value is 2.34. The mean weight for concentration i is considered significantly less than mean weight for the control if t_i is greater than the critical value. Therefore, all effluent concentrations in this example do not have significantly lower mean weights than the control. Hence the NOEC and the LOEC for growth cannot be calculated.

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

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

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.

13.13.3.7.8 In this example:

$$MSD = 2.34(0.1655)\sqrt{(1/3) + (1/3)}$$

$$= 2.34\ (0.1655)(0.8165)$$

$$= 0.316$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.316 mg.

13.13.3.7.10 This represents a 37.8% reduction in mean weight from the control.

13.13.3.8 Calculation of the ICp

13.13.3.8.1 The growth data from Tables 4 and 12 are utilized in this example. As seen in Table 19 and Figure 11, the observed means are not monotonically non-increasing with respect to concentration (the mean response for each higher concentration is not less than or equal to the mean response for the previous concentration, and the reponses between concentrations do not follow a linear trend). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

13.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.836$ and $\bar{Y}_2 = 0.859$, we see that $\bar{Y}_1 < \bar{Y}_2$. Set $M_i = Y_i$.

13.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1 + \bar{Y}_2)/2 = 0.847$$

13.13.3.8.4 Since $\bar{Y}_5 = 0 < \bar{Y}_4 = 0.196 < \bar{Y}_3 = 0.575 < M_2$, set $M_3 = 0.575$, $M_4 = 0.196$, and $M_5 = 0$.

13.13.3.8.5 Table 19 contains the response means and the smoothed means and Figure 12 gives a plot of the smoothed response curve.

TABLE 19. INLAND SILVERSIDE MEAN GROWTH RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means, Y_i (mg)	Smoothed Means, M_i (mg)
Control	1	0.836	0.847
6.25	2	0.859	0.847
12.50	3	0.575	0.575
25.00	4	0.196	0.196
50.00	5	0.00	0.0

13.13.3.8.6 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 0.627 mg, where $M_1(1\text{-}p/100) = 1.847(1\text{-}25/100)$. A 50% reduction in mean dry weight, compared to the controls, would result in a mean weight of 0.418 mg. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.627 mg, is bracketed by C_2 = 6.25% effluent and C_3 = 25.0% effluent. The response (0.418) is bracketed by C_3 = 12.5% and by C_4 = 25% effluent.

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

$$ICp = C_j + [m_1(1 - p/100) - M_j]\frac{(C_{j+1} - C_j)}{(m_{j+1} - M_j)}$$

$$IC25 = 6.25 + [0.847(1 - 25/100) - 0.847]\ \frac{(12.50 - 6.25)}{(0.575 - 0.847)}$$

$$= 11.1\%.$$

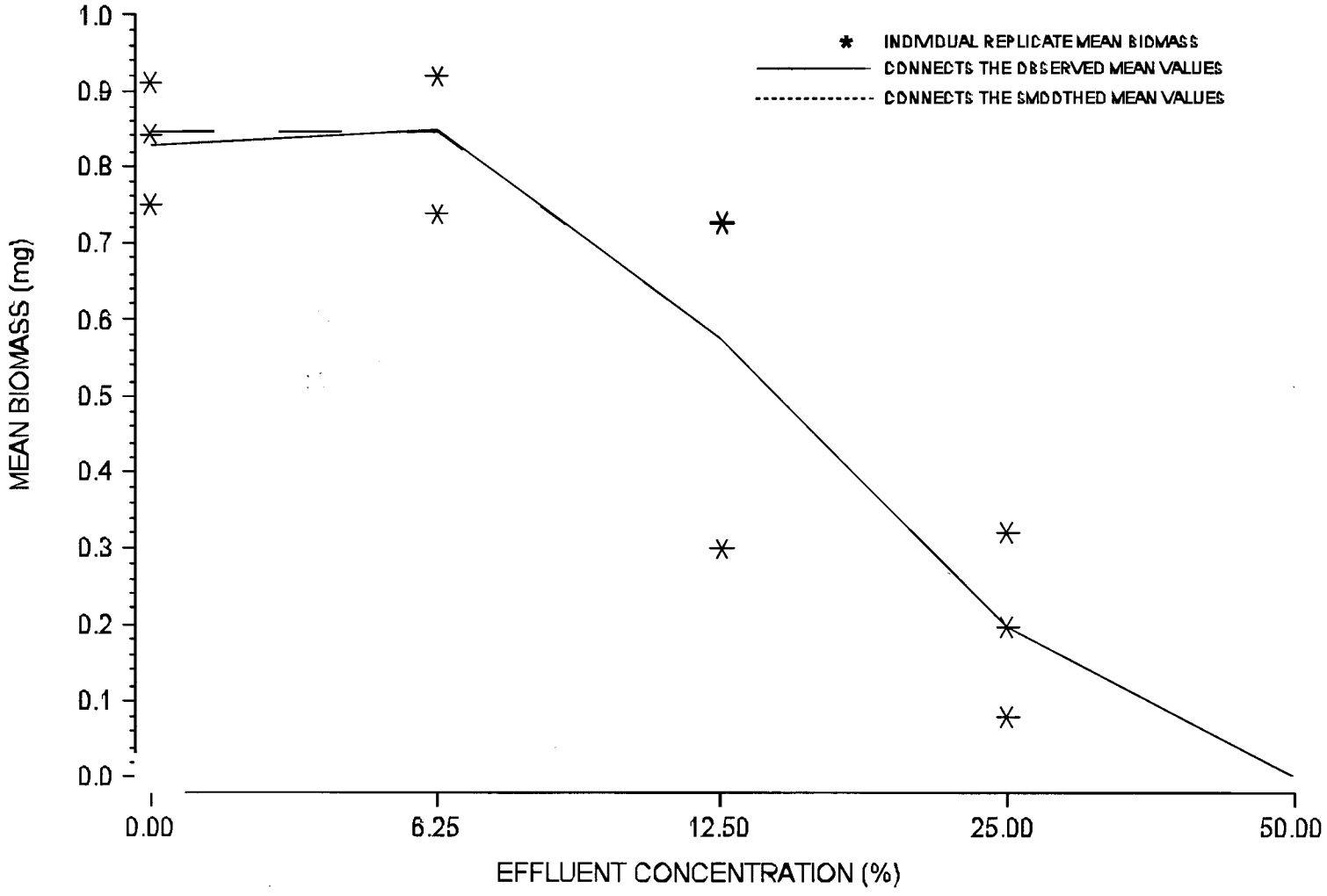

Figure 12. Plot of the raw data, observed means, and smoothed means from Tables 12 and 19.

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

$$ICp = C_j + [m_1(1 - p/100) - M_j]\frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

$$IC50 = 6.25 + [0.847(1 - 50/100) - 0.847] \frac{(12.50 - 6.25)}{(0.575 - 0.847)}$$

$$= 17.5\%.$$

13.13.3.8.9 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 11.1136%. The empirical 95% confidence interval for the true mean was 5.7119% to 19.2112%. The computer program output for the IC25 for this data set is shown in Figure 13.

13.13.3.8.10 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 17.4896%. The empirical 95% confidence interval for the true mean was 6.4891% to 22.4754% effluent. The computer program output is shown in Figure 14.

13.14 **PRECISION AND ACCURACY**

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). 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.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Data on the single-laboratory precision of the inland silverside larval survival and growth test using copper (CU) sulfate and sodium dodecyl sulfate (SDS) as reference toxicants, in natural seawater and GP2 are provided in Tables 20-22. In Tables 20-21, the coefficient of variation for copper based on the IC25 is 43.2% and for SDS is 43.2% indicating acceptable precision. In the five tests with each reference toxicant, the NOEC's varied by only one concentration interval, indicating good precision. The coefficient of variation for all reference toxicants based on the IC50 in two types of seawater (GP2 and natural) ranges from l.8% to 50.7% indicating acceptable precision. Data in Table 22 show no detectable differences between tests conducted in natural and artificial seawaters.

13.14.1.1.2 EPA evaluated within-laboratory precision of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 16 laboratories (USEPA, 2000b). The database consisted of 193 reference toxicant tests conducted in 16 laboratories using a variety of reference toxicants including: chromium, copper, potassium chloride, and sodium dodecyl sulfate. Among the 16 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 18%; and in 75% of laboratories, the within-laboratory CV was less than 43%.

13.14.1.2 Multilaboratory Precision

13.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 10 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater, the effluent sample was an industrial wastewater spiked with $CuSO_4$, the receiving water sample was a natural seawater spiked with $CuSO_4$, and the reference toxicant sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater spiked with $CuSO_4$. Of the 40 *Menidia beryllina* Larval Survival and Growth tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of seven tests that were conducted on blank samples, none showed false positive results for survival endpoints or for the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 23 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 43.8% for IC25 results. Table 24 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned five concentrations for the effluent, four concentrations for the reference toxicant sample type, and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 84.6%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned four concentrations for the reference toxicant and effluent sample types and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 91.7%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	.751	.727	.722	.196	0	0
Response 2	.849	.922	.285	.312	0	0
Response 3	.907	.927	.718	.079	0	0

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

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	0.836	0.079	0.847
2	3	6.250	0.859	0.114	0.847
3	3	12.500	0.575	0.251	0.575
4	3	25.000	0.196	0.117	0.196
5	3	50.000	0.000	0.000	0.000
6	3	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 11.1136 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 11.5341 Standard Deviation: 2.1155
Original Confidence Limits: Lower: 8.5413 Upper: 14.9696
Expanded Confidence Limits: Lower: 5.7119 Upper: 19.2112
Resampling time in Seconds: 1.43 Random Seed: -1912403737

Figure 13. 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	.751	.727	.722	.196	0	0
Response 2	.849	.922	.285	.312	0	0
Response 3	.907	.927	.718	.079	0	0

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

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	0.836	0.079	0.847
2	3	6.250	0.859	0.114	0.847
3	3	12.500	0.575	0.251	0.575
4	3	25.000	0.196	0.117	0.196
5	3	50.000	0.000	0.000	0.000
6	3	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 17.4896 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 16.9032 Standard Deviation: 2.49.73
Original Confidence Limits: Lower: 12.2513 Upper: 19.8638
Expanded Confidence Limits: Lower: 6.4891 Upper: 22.4754
Resampling time in Seconds: 1.43 Random Seed: -1440337465

Figure 14. ICPIN program output for the IC50.

TABLE 20. SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, AND COPPER (CU) AS A REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[6]
1	63	96.2	148.6	S
2	125	207.2	NC[8]	S
3	63	218.9	493.4	G
4	125	177.5	241.4	S
5	31	350.1	479.8	G
n:	5	5	4	
Mean:	NA	209.9	340.8	
CV(%):	NA	43.7	50.7	

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 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 Adults collected in the field.

5 S = Survival effects. G = Growth data at these toxicant concentrations were disregarded because there was a significant reduction in survival.

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

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

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

TABLE 21. SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint
1	1.3	0.3	1.7	S
2	1.3	1.6	1.9	S
3	1.3	1.5	1.9	S
4	1.3	1.5	1.9	S
5	1.3	1.6	2.2	S
n:	5	5	5	
Mean:	NA	1.3	1.9	
CV(%):	NA	43.2	9.4	

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 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 Adults collected in the field.
5 S = Survival Effects. Growth data at these toxicant concentrations were disregarded because there was a significant reduction in survival.
6 NOEC Range: 1.3 mg/L.
7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 22. COMPARISON OF THE SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL (LC50) AND GROWTH (IC50) VALUES EXPOSED TO SODIUM DODECYL SULFATE (SDS) OR COPPER (CU) SULFATE IN GP2 ARTIFICIAL SEAWATER MEDIUM OR NATURAL SEAWATER (NSW)[1,2,3,4]

	Survival		Growth	
SDS (mg/L)	GP2	NSW	GP2	NSW
	3.59	3.69	3.60	3.55
	4.87	4.29	5.54	5.27
	5.95	8.05	6.70	8.53
Mean	4.81	5.34	5.28	5.79
CV (%)	24.6	44.2	29.6	43.8
Copper (µg/L)	GP2	NSW	GP2	NSW
	247	256	260	277
	215	211	236	223
	268	240	NC[5]	238
Mean	243	236	248	246
CV (%)	10.9	9.8	6.9	11.2

1 Tests performed by George Morrison and Glen Modica, ERL-N, USEPA, Narragansett, RI.
2 Three replicate exposure chambers with 10-15 larvae per treatment.
3 Adults collected in the field.
4 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.
5 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 23. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	22.0	29.1	36.4
	Effluent	7.24	55.5	56.0
	Receiving water	-	-	39.1
	Average	14.6	42.3	43.8

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

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, 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.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 24. 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	12.5%	72.7	18.2	9.09
	Effluent	25%	38.5	46.1	15.4
	Receiving water	25%	57.1	28.6	14.3
Growth NOEC	Reference toxicant	12.5%	72.7	18.2	9.09
	Effluent	25%	41.7	50.0	8.33
	Receiving water	25%	57.1	28.6	14.3

[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.