SECTION 12

MYSID, *Holmesimysis costata* SURVIVAL AND GROWTH TEST METHOD

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SECTION 12

MYSID, *HOLMESIMYSIS COSTATA* SURVIVAL AND GROWTH TEST

12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the mysid, *Holmesimysis costata,* using three-to-four day old juveniles in a seven-day, static-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and additive components which adversely affect the physiological and biochemical functions of the test organisms.

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

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

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

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

12.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

12.2 SUMMARY OF METHOD

12.2.1 This method provides step-by-step instructions for

performing a 7-day static-renewal toxicity test using growth and survival juvenile mysids to determine the toxicity of substances in marine waters. The test endpoints are survival and growth.

12.3 **INTERFERENCES**

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

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

12.3.3 The test results can be confounded by (1) the presence of pathogenic and/or predatory organisms in the dilution water, effluent, and receiving water, (2) the condition of the brood stock from which the test animals were taken, (3) the amount and type of natural food in the effluent, receiving water, or dilution water, (4) nutritional value of the brine shrimp, *Artemia* nauplii, fed during the test, and (5) the quality of the brine shrimp, *Artemia* nauplii, or other food added during the test, which may sequester metals and other toxic substances, and lower the DO.

12.4 **SAFETY**

12.4.1 See Section 3, Health and Safety.

12.5 **APPARATUS AND EQUIPMENT**

12.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult mysids, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

12.5.2 Air pump, air lines, and air stones -- for aerating water containing mysids for supplying air to test solutions with low dissolved oxygen.

12.5.3 Constant temperature chambers or water baths -- for maintaing test solution temperature and keeping dilution water

supply, juvenile mysids, and stock suspensions at test temperature (13 or 15EC) prior to the test.

12.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

12.5.5 Refractometer -- for determining salinity.

12.5.6 Hydrometer(s) -- for calibrating refractometer.

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

12.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

12.5.9 pH and DO meters -- for routine physical and chemical measurements.

12.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

12.5.11 Winkler bottles -- for dissolved oxygen determinations.

12.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g (for weighing reference toxicants).

12.5.13 Microbalance -- Analytical, capable of accurately weighing to 0.000001 g (for weighing mysids).

12.5.14 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

12.5.15 Glass stirring rods -- for mixing test solutions.

12.5.16 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions.

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

12.5.18 Pipets, automatic -- adjustable, to cover a range of

delivery volumes from 0.010 to 100 mL.

12.5.19 Pipet bulbs and fillers -- PROPIPET® or equivalent.

12.5.20 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

12.5.21 Wash bottles -- for dilution water.

12.5.22 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

12.5.23 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

12.5.24 Pipets, volumetric: 1, 10, 25, 50, and 100 mL -- for dilutions.

12.5.25 Plastic randomization cups (approximately 100 mL, one for each test chamber).

12.5.26 Brine shrimp, *Artemia*, culture unit -- see Subsection 12.6.24 and Section 4, Quality Assurance.

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

12.5.28 Mysid culture apparatus (see Section 12.6.25.5). This test requires 400 three- to four-day-old juvenile mysids.

12.5.29 Gear for collecting adult mysids, including a small boat, 0.5 mm-mesh hand nets, plastic buckets, and portable air supply (mysids may also be obtained from commercial suppliers;).

12.5.30 Pipet bulbs and glass tubes (4 mm diameter, with fire-polished edges) for handling adult mysids.

12.5.31 Siphon tubes (fire polished glass with attached silicone tubing) -- for test solution renewals.

12.5.32 Fire-polished wide-bore 10 mL pipet -- for handling juveniles.

12.5.33 Forceps with fine points -- for transferring juveniles to weighing pans.

12.5.34 Light box -- for examining organisms.

12.5.35 Drying oven, 50-105EC range -- for drying organisms.

12.5.36 Desiccator -- for holding dried organisms.

12.5.37 Clean NITEX® mesh sieves (# 150 µm, 500-1000µm) -- for concentrating organisms. (NITEX® is available from Sterling Marine Products, 18 Label Street, Montclair, NJ 07042; 201-783-9800).

12.5.38 60 µm NITEX® filter - for filtering receiving water.

12.6 **REAGENTS AND SUPPLIES**

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

12.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

12.6.3 Tape, colored -- for labelling test chambers and containers.

12.6.4 Markers, water-proof -- for marking containers, etc.

12.6.5 Parafilm -- to cover graduated cylinders and vessels.

12.6.6 Gloves, disposable -- for personal protection from contamination.

12.6.7 Pipets, serological -- l-l0 mL, graduated.

12.6.8 Pipet tips -- for automatic pipets.

12.6.9 Coverslips -- for microscope slides.

12.6.10 Lens paper -- for cleaning microscope optics.

12.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

12.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

12.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

12.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

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

12.6.16 Test chambers -- 1000 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid 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 a plastic sheet (6 mm thick).

12.6.17 Micro-weighing pans, aluminum -- to determine the dry weight of organisms. Weighting pan should be about 5 mg or less to minimize noise in measurement of the small mysids.

12.6.18 Fronds of kelp (*Macrocystis*) for habitat in culture.

12.6.19 Reference toxicant solutions (see Subsection 12.10.2.4 and see Section 4, Quality Assurance).

12.6.20 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 and Section 7, Dilution Water).

12.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 12.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

12.6.23 HYPERSALINE BRINES

12.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

12.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine 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 it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

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

12.6.23.4 Freeze Preparation of Brine

12.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

12.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

12.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

12.6.23.5 Heat Preparation of Brine

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

12.6.23.5.2 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 reagent water rinses.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

12.6.23.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

12.6.23.5.4 After the required salinity is attained, the HSB

should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

12.6.23.6 Artificial Sea Salts

12.6.23.6.1 No data from mysids using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

12.6.23.7 Dilution Water Preparation from Brine

12.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

12.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part, plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

12.6.23.8 Test Solution Salinity Adjustment

12.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

12.6.23.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide(see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

12.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

12.6.23.9 Preparing Test Solutions

12.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

12.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see

equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

12.6.23.10 Brine Controls

12.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 12.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

12.6.24 BRINE SHRIMP, *ARTEMIA SP*., NAUPLII -- for feeding cultures and test organisms.

12.6.24.1 Newly hatched *Artemia sp.* nauplii are used for food for the stock cultures and test organisms. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size. (One source that has been found to be acceptable is Aquarium Products, 180L Penrod Ct., Glen Burnie, Maryland 21061). For commercial sources of brine shrimp, *Artemia*, cysts, see Table 2 of Section 5, Facilities, Equipment, and Supplies); and Section 4, Quality Assurance.

12.6.24.2 Each new batch of *Artemia* cysts should be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be

a previously tested and acceptable batch of cysts, or may be obtained from the Quality Assurance Research Division, EMSL, Cincinnati, OH 45268, 513-569-7325. 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 0.15 ug/g wet weight or that the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight (For analytical methods see USEPA, 1982).

12.6.24.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or an aqueous unionized salt (NaCl) solution prepared with 35 g salt or artificial sea salts per liter, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27EC. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985a; USEPA, 1993a; ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes with a dark cloth or paper towel. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a funnel fitted with a #150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

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

12.6.24.4.1 The primary criteria for acceptability of each new supply of brine shrimp cysts is adequate survival, and growth of the mysids. The mysids used to evaluate the acceptability of the brine shrimp nauplii must be the same geographical origin and stage of development (3 to 4 days old) as those used routinely in the toxicity tests. Two 7-day chronic tests are performed side-by-side, each consisting of five replicate test vessels containing five juveniles (25 organisms per test, total of 50

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with deionized water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

organisms). The juveniles in one set of test chambers is fed reference (acceptable) nauplii and the other set is fed nauplii from the "new" source of *Artemia* cysts.

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

12.6.24.4.3 Results of the brine shrimp, *Artemia*, nauplii 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 or growth of the mysids fed the two sources of nauplii.

12.6.25 TEST ORGANISMS

12.6.25.1 The test organisms for this method are juveniles of the mysid crustacean, *Holmesimysis costata* (Holmes 1900; previously referred to as *Acanthomysis sculpta*). *H. costata* occurs in the surface canopy of the giant kelp *Macrocystis pyrifera* where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970; Turpen et al., 1994). *H. costata* is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). Mysids are called opossum shrimp because females brood their young in an abdominal pouch, the marsupium. *H. costata* eggs develop for about 20 days in the marsupium before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12EC), and may have multiple broods throughout their approximately 120-day life.

12.6.25.2 *H. costata* has been used in previous toxicity studies with a variety of toxicants (Tatem and Portzer, 1985; Davidson et al., 1986; Machuzac and Mikel, 1987; Reish and Lemay, 1988; Asato, 1988; Martin et al., 1989; Singer et al., 1990; 1991; Hunt et al., In Press). Mysids are useful as toxicity test organisms because of their widespread availability, ecological importance, sensitivity to toxicants, and amenability to laboratory culture

(Nimmo et al., 1977; Mauchline, 1980; Gentile et al., 1982; Lussier et al., 1985).

12.6.25.3 Species Identification

12.6.25.3.1 Laboratories unfamiliar with the test organism should collect preliminary samples to verify species identification. Refer to Holmquist (1979) or send samples of mysids and any similar co-occurring organisms to a qualified taxonomist. Request certification of species identification from any organism suppliers. Records of verification should be maintained along with a few preserved specimens.

12.6.25.3.2 There have been recent revisions to the taxonomy of *H. costata.* Previous authors have referred to this species as *Acanthomysis sculpta.* However, Holmquist's (1979) review considers previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata*; we consider Holmquist's designation to be definitive.

12.6.25.4 Obtaining Broodstock

12.6.25.4.1 *H. costata* can be collected by sweeping a small-mesh (0.5 - 1 mm) hand net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants.

12.6.25.4.2 Mysids can be transported for a short time (< 3 hours) in tightly covered 20 liter plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation. Transport temperatures should remain within 3EC of the temperature at the collection site.

12.6.25.4.3 For longer transport times of up to 36 hours, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully: 1)

fill the plastic bag with one liter of dilution water seawater, 2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes, 3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag, 4) for adults add about 20 *Artemia* nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of ground Tetramin® flake food and 200 newly-hatched *Artemia* nauplii, 5) seal the bag securely, eliminating any airspace, then 6) place it within a second sealed bag in an ice chest. Do not overfeed mysids in transport, as this may deplete dissolved oxygen, causing stress or mortality in transported mysids. A well insulated ice chest should be cooled to approximately 15EC by adding one 1-liter blue ice block for every five 1-liter bags of mysids (a temperature range of 12 to 16EC is tolerable). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

12.6.25.5 Broodstock Culture and Handling

12.6.25.5.1 After collection, the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater or adequate aeration and filtration. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5EC per hour). The water temperature should be held at 15 ± 1EC. **Note:** Mysids collected north of Pt. Conception, California, should be held and tested at 13 ± 1EC.

12.6.25.5.2 Mysids can be cultured in tanks ranging from 4 to 1000 liters. Tanks should be equipped with gentle aeration and blades of *Macrocystis* to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per liter by culling out adult males or juveniles.

12.6.25.5.3 Adult mysids should be fed 100 *Artemia* nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Static chambers should be carefully monitored and rations adjusted to

prevent overfeeding and fouling of culture water. Refer to section 12.6.19 for details of *Artemia* culture and quality control.

12.6.25.6 Culture Materials

12.6.25.6.1 Refer to Section 5, Facilities and Equipment, for a discussion of suitable materials to be used in laboratory culture of mysids. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before re-use.

12.6.25.7 Test Organisms

12.6.25.7.1 Approximately 150 gravid female mysids should be isolated to provide approximately 400 juveniles for each set of toxicity tests (5 juveniles/chamber x 30 reference toxicant chambers and approximately 35 effluent chambers, plus additional mysids so that only healthy active juveniles are used in the test). Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. Marsupia appear distended and gray when females are ready to release young, due to presence of the juveniles.

12.6.25.7.2 Gravid females are easily isolated from other mysids using the following technique: (1) use a small dip net to capture about 100 mysids from the culture tank, (2)transfer the mysids to a screen-bottomed plastic tube (150 µm-mesh, 25-cm diam.) partly immersed in a water bath or bucket, (3)lift the screen-tube out of the water to immobilize mysids on the damp screen, (4)gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm bore), (5) collect the gravid females in a separate screen tube. Re-immerse the screen continuously during the isolation process; mysids should not be exposed to air for more than a few seconds at a time.

12.6.25.7.3 Four or five days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-liter aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid

inadvertently mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched *Artemia* nauplii (approximately 200 per mysid) to help stimulate juvenile release. *Artemia* can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000 *Artemia*. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of *Macrocystis* for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the aquarium. Outflows on flow-through aquaria should be screened (150-µm-mesh) to retain juveniles and allow some *Artemia* to escape.

12.6.25.7.4 Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a 150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juveniles that might mix with the next cohort.

12.6.25.7.5 After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles to conduct the necessary tests, they can be mixed with juveniles from one previous or subsequent release so that the test is initiated with three and/or four-day old juveniles. Initial experiments indicate that mysids 2-days-old and younger survive poorly in toxicity tests and that mysids older than four days may vary in their toxicant sensitivity or survival rate (Hunt et al., 1989; Martin et al., 1989).

12.6.25.7.6 Test juveniles should be transferred to additional screen-tubes (or to 4-liter static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-liter bucket so that dilution water seawater (0.5 liter/min) can flow into the tube, through the screen, and overflow from the bucket. Check water flow rates (< one liter/min) to make sure that juveniles or *Artemia* nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-liter beaker). Juveniles

should be fed 40 newly hatched *Artemia* nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. A blade of *Macrocystis* (well rinsed in seawater) should be added to each chamber. Chambers should be gently aerated and temperature controlled at 15 ± 1EC (or 13 ± 1EC if collected north of Pt. Conception). Half of the seawater in static chambers should be changed at least once between isolation and test initiation.

12.6.25.7.7 The day juveniles are isolated is designated day 0 (the morning after their nighttime release). The toxicity test should begin on day three or four. For example, if juveniles are isolated on Friday, the toxicity test should begin on the following Monday or Tuesday.

12.7 **EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE**

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

12.8 **CALIBRATION AND STANDARIZATION**

12.8.1 See Section 4, Quality Assurance.

12.9 **QUALITY CONTROL**

12.9.1 See Section 4, Quality Assurance.

12.10 **TEST PROCEDURES**

12.10.1 TEST DESIGN

12.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

12.10.1.2 Effluent concentrations are expressed as percent effluent.

12.10.2 TEST SOLUTIONS

12.10.2.1 Receiving waters

12.10.2.1.1 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 five replicates chambers per test, each containing 200 mL would require approximately 1 L or more of sample per test per renewal.

12.10.2.2 Effluents

12.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

12.10.2.2.2 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%).

12.10.2.2.3 The volume of effluent required for a 75% renewal of five replicates per concentration for five concentrations of effluent and two controls, each containing 200 mL of test solution, is approximately 370 mL.

12.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

12.10.2.3 Dilution Water

12.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

12.10.2.4 Reference Toxicant Test

12.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

12.10.2.4.2 The preferred reference toxicant for mysids is zinc sulfate ($ZnSO_4 \circ 7H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \circ 7H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

12.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 µg/L total zinc. Prepare one liter of each concentration by adding 0, 1.0, 1.8, 3.2, 5.6, and 10.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

12.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use juvenile originating from or released from the same pool of gravid females. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

12.10.3 START OF THE TEST

12.10.3.1 Prior to Beginning the Test

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

12.10.3.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (13 or 15 ± 1EC) and maintained at that temperature during the addition of dilution water.

12.10.3.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (13 or 15 ± 1EC).

12.10.3.1.4 Randomize the placement 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 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. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the mysids have been examined at the end of the test.

12.10.3.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

12.10.3.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

12.10.3.2 Randomized Assignment of Mysids to Test Chambers

12.10.3.2.1 The juvenile mysids must be randomized before placing them into the test chambers. Pool all of the test juveniles into a 1-liter beaker. Using a 10-mL wide-bore pipet or fire-polished glass tube (approximately 2-3 mm inside diameter), place one or two juveniles into as many plastic cups as there are test chambers (including reference toxicant chambers). These cups should contain enough clean dilution seawater to maintain water quality and temperature during the transfer process (approximately 50 mL per cup). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 5 animals.

12.10.3.2.2 Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test mysids. This 5 mL volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test chambers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mLs of water are added to the test solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1EC of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant-temperature room.

12.10.3.2.3 Verify that all five animals are in the test chambers by counting the number in each chamber after transfer. This initial count is important because mysids unaccounted for at the end of the test are assumed to be dead.

12.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

12.10.4.1 The light quality and intensity should be at ambient laboratory conditions are generally adequate. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle. A 30 minute phase-in/out period is recommended.

12.10.4.2 The water temperature in the test chambers should be maintained at 13 or 15 ± 1EC. It is critical that the test water temperature be maintained at 13 ± 1EC (for mysids collected north of Pt. Conception, California) or 15 ± 1EC (for mysids collected south of Pt. Conception, California). If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

12.10.4.3 The test salinity should be in the range of 34 ± 2‰. 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.

12.10.4.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

12.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

12.10.6 FEEDING

12.10.6.1 *Artemia* nauplii are prepared as described above.

12.10.6.2 The feeding rates in the test beakers should be closely controlled to avoid overfeeding and fouling of test

solutions. Add 40 newly hatched *Artemia* nauplii per mysid per day. *Artemia* nauplii should be well rinsed with clean seawater and concentrated so that no more than one mL of seawater is added during feeding. (Use a 100-µm-mesh screen tube for rinsing and concentrating the nauplii; see Section 12.6.24.3). Test performance may be enhanced by feeding half the ration twice daily. If mysids die during the course of the experiment, the ration should be reduced proportionally. The mysids should not be fed on day 7.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Before the renewal of test solutions, uneaten and dead *Artemia*, dead mysids and other debris are removed from the bottom of the test chambers with a pipette. As much of the uneaten *Artemia* as possible should be removed from each chamber to ensure that the mysids eat primarily newly hatched nauplii. By placing the test chambers on a light box, inadvertent removal of live mysids can be greatly reduced because they can be more easily seen. If a mysid is lost during siphoning, note the test chamber from it came, and reduce the initial count from five to four for that chamber when calculating survival at the end of the test.

12.10.8 OBSERVATIONS DURING THE TEST

12.10.8.1 Routine Chemical and Physical Observations

12.10.8.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

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

12.10.8.1.3 Record all the measurements on the data sheet.

12.10.8.2 Routine Biological Observations

12.10.8.2.1 The number of live mysids are counted and recorded each day. Dead animals and excess food should be removed with a pipette before test solutions are renewed. This is necessary to avoid cannibalism and to prevent fouling of test solutions.

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

12.10.9 TEST SOLUTION RENEWAL

12.10.9.1 The test duration is 7 days. Because effluent toxicity may change over short time periods in test chambers, the test solutions must be renewed after 48 h and 96 h. Prepare renewal test solutions in the same way as initial test solutions. Remove three quarters of the original test solution from each chamber, taking care to avoid losing or damaging mysids. This can be done by siphoning with a small-bore (2 to 3 mm) fire-polished glass tube or pipet. Attach the glass tube to clear plastic tubing fitted with a pinch clamp so that the siphon flow can be stopped quickly if necessary to release entrained mysids. It is convenient to siphon old solutions into a small (500 mL) chamber in order to check to make sure that no mysids have been inadvertently removed during solution renewals. If a mysid is siphoned, return it to the test chamber and note it on the data sheet. Follow the chamber randomization sheet to siphon first from the controls, then work sequentially to the highest test concentration to avoid cross-contamination.

12.10.9.2 To minimize disturbance to the juvenile mysids, refill the chambers to the 200-mL mark by carefully siphoning new test solution into the test chambers using small diameter plastic tubing attached to a bent clean glass rod that directs incoming solution upward or to the side to slow the current and minimize turbulence.

12.10.9.3 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 4EC. Plastic chambers 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).

12.10.9.4 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 13 ± 1EC or 15 ± 1EC to prepare the test solutions. A sufficient quantity of effluent should be warmed to make the test solutions.

12.10.10 TERMINATION OF THE TEST

12.10.10.1 Ending the Test

12.10.10.1.1 Record the time the test is terminated.

12.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

12.10.10.1.3 On the last day of the test, examine each test chamber, and remove and record any dead mysids. Sum the cumulative total of all mortalities observed in each test chamber over the 7 days of the test, subtract this from the initial number of mysids (5), and verify the number of survivors. Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

12.10.10.2 Weighing

12.10.10.2.1 To prepare mysids for weighing at the end of the exposure period, remove any remaining dead mysids, then carefully pour the contents of the test chamber through a small mesh screen (<300µm). Count the mysids before screening, and take care to keep track of them on the screen. Make sure mortality counts have already been recorded. Briefly dip the screen containing the mysids in deionized water to rinse away the salt. Using fine point forceps, carefully transfer the mysids from the screen to a preweighed and labelled micro-weigh boat. Carefully fold the

foil weigh boats over the mysids to avoid loss while drying test organisms.

12.10.10.2.2 To prepare weigh boats prior to testing, write the test chamber number on each with a fine felt-tipped marker, dry the ink and weigh boat in a drying oven, allow the dry weigh boats to cool in a desiccator, weigh the weigh boats to the nearest 1 microgram (µg) on a microbalance, and record the weight and chamber number on the data sheet. Place the weighed weigh boats in a clean ziplock bag until ready to use for weighing mysids. The juvenile mysids are very small, and light (60 µg) relative to the weigh boats (4 mg). Take all precautions to make sure weigh boats remain clean and dry during weighing and subsequent storage, so that mysid weights may be accurately determined by subtraction.

12.10.10.2.3 When all mysids are loaded onto weigh boats, arrange them all in a dish, small tray or other small open chamber, and place them in a clean drying oven. Dry for at least 24 hours at 60EC or for at least 6 hours at 105EC. Remove the weigh boats with mysids from the drying oven and place them in a desiccator to cool for one hour. When cool, carefully weigh each weigh boat on a microbalance (accurate to 1 µg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

12.10.10.3 Endpoint

12.10.10.3.1 Growth is measured as dry weight of surviving mysids. All surviving mysids from a single replicate test chamber are pooled together and weighed, then this total weight is divided by the number of original mysids to obtain the mean dry weight per individual for each replicate, which is used for statistical analysis.

12.10.10.3.2 The percentage of surviving mysids in each chamber at the end of the test will be used for subsequent statistical analysis.

12.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

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

12.12 **ACCEPTABILITY OF TEST RESULTS**

12.12.1 Test results are acceptable only if all the following requirements are met:

(1) Control survival must be at least 75%.

(2) The average weight of control mysids must be at least 40 µg per mysid.

(3) Between replicate variability in the mortality data must be low enough that the minimum significant difference (%MSD) is less than 40% in the reference toxicant test.

(4) Between replicate variability in the weight data must be low enough that the %MSD is less than 50 µg in the reference toxicant test.

(5) Both the mortality NOEC and LC50 must be less than 100 µg/L zinc in the reference toxicant test.

12.13 **DATA ANALYSIS**

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of survival and growth data.

12.13.1.2 The endpoints of the mysid 7-day chronic test are based on the adverse effects on survival and growth. The LC50 and the IC25 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 and IC25. 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 and IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

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

12.13.2 EXAMPLE OF ANALYSIS OF MYSID, *HOLMESIMYSIS COSTATA*, SURVIVAL DATA

12.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 1 and 2. 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 LC, EC, and IC endpoints.

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

12.13.2.3 If equal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE MYSID, *HOLMESIMYSIS COSTATA*, GROWTH AND SURVIVAL TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	13 ± 1EC (mysids collected north of Pt. Conception) 15 ± 1EC (mysids collected south of Pt. Conception)
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (Ambient laboratory illumination)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber:	1000 mL
8. Test solution volume:	200 mL
9. Renewal of test solutions:	75% renewal at 48 and 96 hours
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	5
12. No. replicate chambers per concentration:	5
13. No. mysids per concentration:	25
14. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
15. Feeding regime:	Feed 40 nauplii per larvae daily (dividing into morning and evening feedings)

16. Cleaning:	Siphon during test solution renewal
17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups
18. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
19. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
20. Dilution factor:	Effluents: $0.5 series Receiving waters: None, or $0.5
21. Test duration:	7 days
22. Endpoints:	Survival and growth
23. Test acceptability criteria:	$75% survival, average dry weight $ 0.40 µg in the controls; survival MSD <40%; growth MSD <50 µg; and both survival and growth NOECs must be less than 100 µg/L with zinc
24. Sampling requirements:	For on-site tests, samples must be used within 24 h of the time they are removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
25. Sample volume required:	2 L per renewal

TABLE 4. DATA FOR HOLMESIMYSIS COSTATA 7-DAY SURVIVAL AND GROWTH TEST[1]

Treatment	Replicate Chamber	Total Mysids	No. Alive	Prop. Alive	Mean Weight
Control, Brine	1	5	5	1.00	0.051
	2	5	5	1.00	0.050
	3	5	5	1.00	0.040
	4	5	5	1.00	0.064
	5	5	5	1.00	0.039
Control, Dilution	1	5	5	1.00	0.048
	2	5	5	1.00	0.058
	3	5	5	1.00	0.047
	4	5	5	1.00	0.058
	5	5	5	1.00	0.051
1.80%		1	5	5	1.00
					0.055
	2	5	5	1.00	0.048
	3	5	5	1.00	0.042
	4	5	4	0.80	0.041
	5	5	5	1.00	0.052
3.20%		1	5	5	1.00
					0.057
	2	5	4	0.80	0.050
	3	5	5	1.00	0.046

t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

12.13.2.4 Probit Analysis (Finney, 1971; see Appendix G) 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 to estimate the LC50 (see Appendices H-K).

12.13.2.5 The proportion of survival in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 3.

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 6.

12.13.2.6.2 Calculate the denominator, D, of the test statistic:

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

Where: $\&_i$ = the ith centered observation

$\&$ = the overall mean of the centered observations

n = the total number of centered observations.

12.13.2.6.3 For this set of data, n = 25

$$\& = \frac{1}{25}(0.001) = 0.00$$

D = 0.227

12.13.2.6.4 Order the centered observations from smallest to largest:

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

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

TABLE 5. MYSID, HOLMESIMYSIS COSTATA, SURVIVAL DATA

	Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.00
	1	1.00	1.00	1.00	0.80	0.20
	2	1.00	1.00	0.80	1.00	0.00
RAW	3	1.00	1.00	1.00	1.00	0.00
	4	1.00	0.80	1.00	0.80	0.00
	5	1.00	1.00	0.80	0.80	0.00
ARC SINE	1	1.345	1.345	1.345	1.107	0.464
SQUARE	2	1.345	1.345	1.107	1.345	0.225
ROOT	3	1.345	1.345	1.345	1.345	0.225
TRANS-	4	1.345	1.107	1.345	1.107	0.225
FORMED	5	1.345	1.345	1.107	1.107	0.225
Mean($\bar{x}_i$)		1.345	1.297	1.250	1.202	0.273
S_i^2		0.000	0.011	0.017	0.017	0.011
i		1	2	3	4	5

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2,...., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

12.13.2.6.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8.

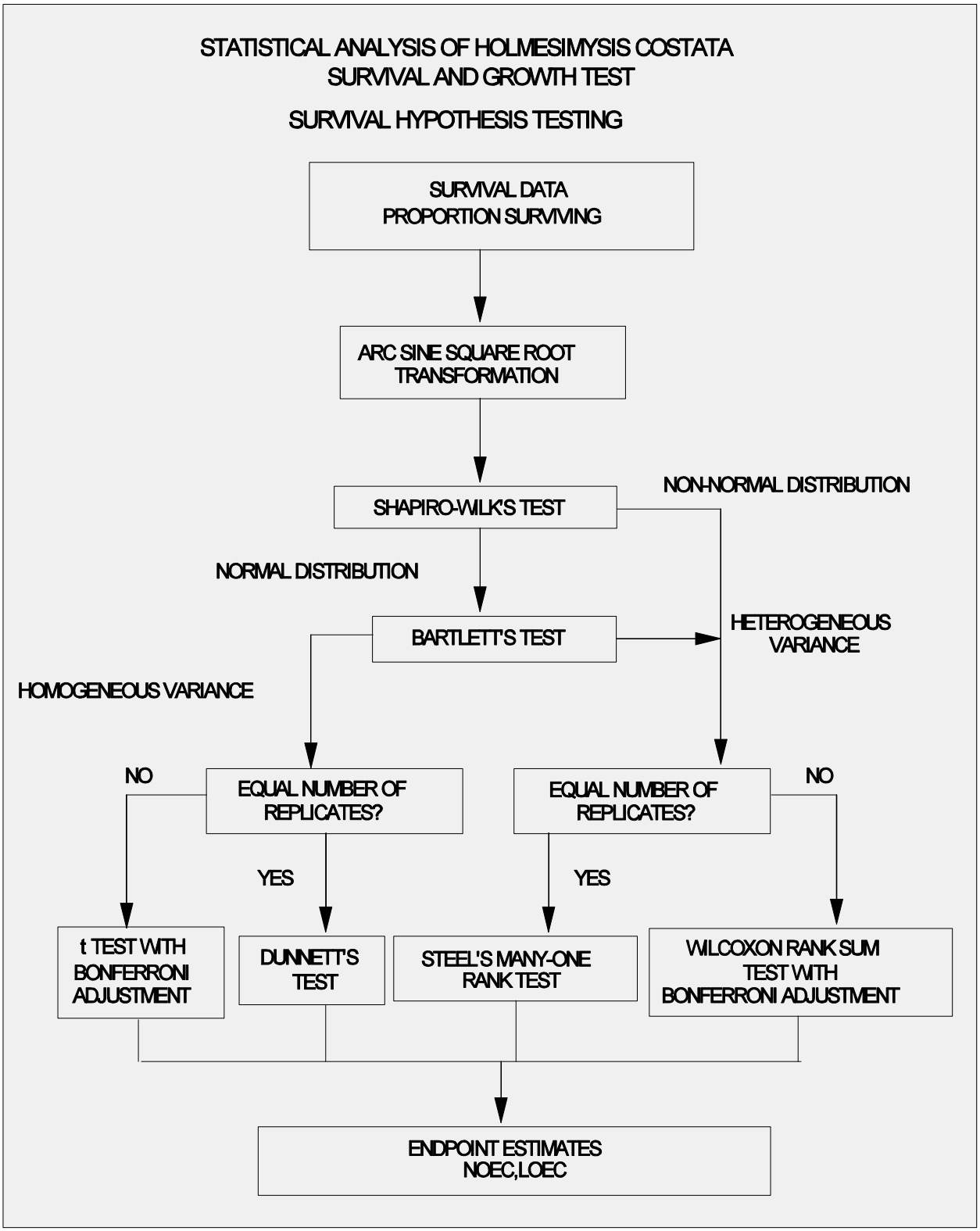

Figure 1. Flowchart for statistical analysis of mysid, *Holmesimysis costata*, survival data by hypothesis testing.

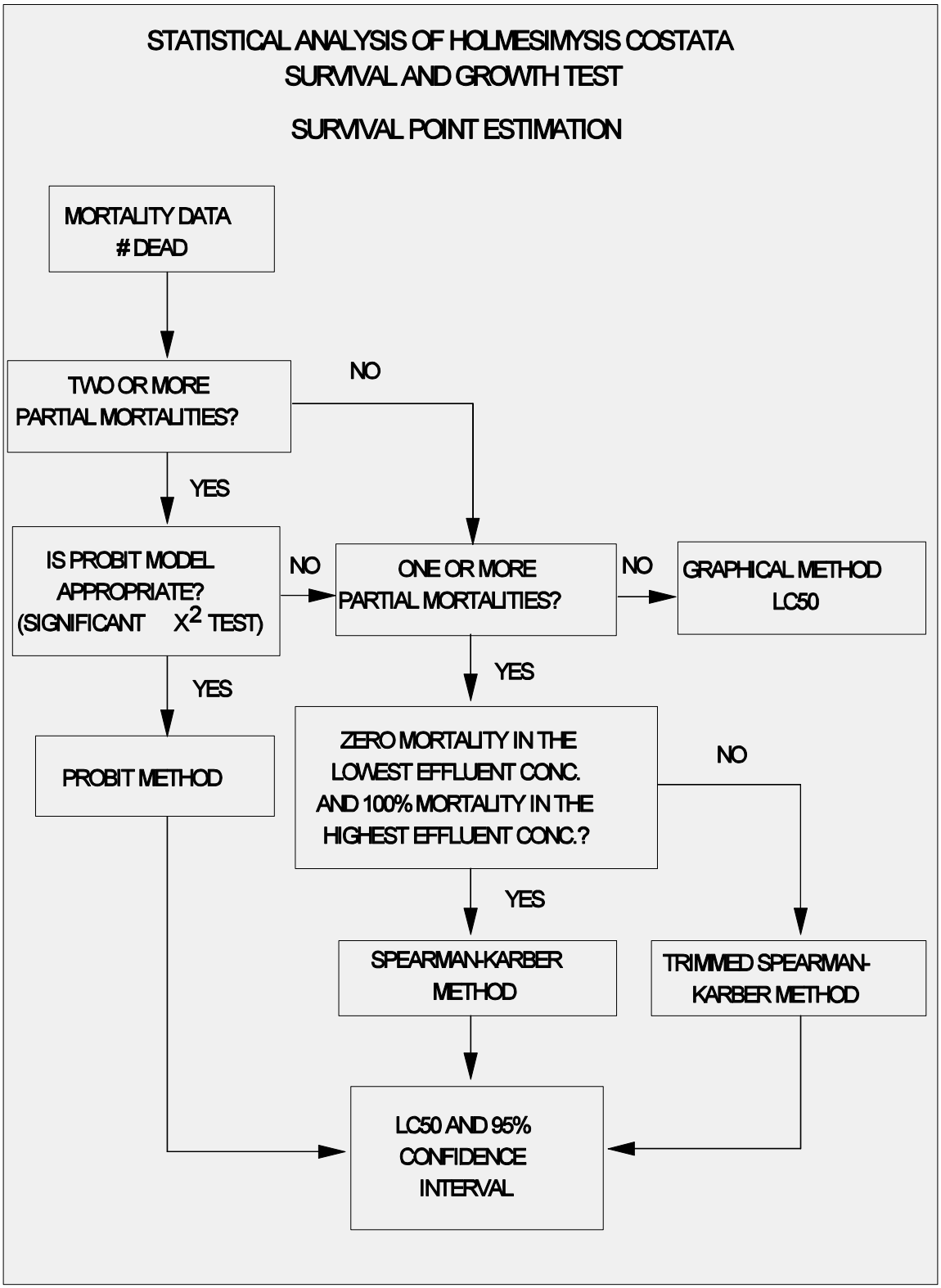

Figure 2.Flowchart for statistical analysis of mysid, *Holmesimysis costata*, survival data by point estimation.

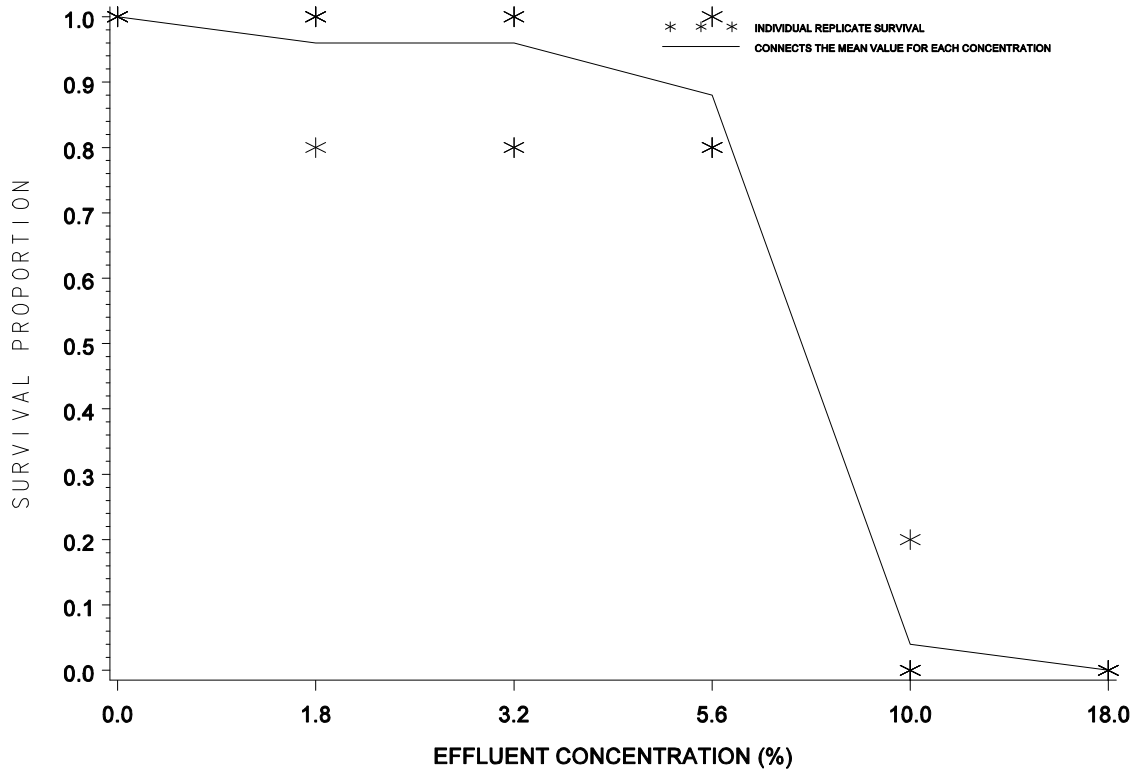

Figure 3. Plot of survival of mysids, *Holmesimysis costata*, at each treatment

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

Replicate	Control (Dilution)	Concentration 1.80	3.20	5.60	10.00
1	0.000	0.048	0.095	-0.095	0.191
2	0.000	0.048	-0.143	0.143	-0.048
3	0.000	0.048	0.095	0.143	-0.048
4	0.000	-0.190	0.095	-0.095	-0.048
5	0.000	0.048	-0.143	-0.095	-0.048

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.190	14	0.000
2	-0.143	15	0.000
3	-0.143	16	0.048
4	-0.095	17	0.048
5	-0.095	18	0.048
6	-0.095	19	0.048
7	-0.048	20	0.095
8	-0.048	21	0.095
9	-0.048	22	0.095
10	-0.048	23	0.143
11	0.000	24	0.143
12	0.000	25	0.191
13	0.000		

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.381	$X^{(25)} - X^{(1)}$
2	0.3069	0.286	$X^{(24)} - X^{(2)}$
3	0.2543	0.286	$X^{(23)} - X^{(3)}$
4	0.2148	0.190	$X^{(22)} - X^{(4)}$
5	0.1822	0.190	$X^{(21)} - X^{(5)}$
6	0.1539	0.190	$X^{(20)} - X^{(6)}$
7	0.1283	0.096	$X^{(19)} - X^{(7)}$
8	0.1046	0.096	$X^{(18)} - X^{(8)}$
9	0.0823	0.096	$X^{(17)} - X^{(9)}$
10	0.0610	0.096	$X^{(16)} - X^{(10)}$
11	0.0403	0.000	$X^{(15)} - X^{(11)}$
12	0.0200	0.000	$X^{(14)} - X^{(12)}$

For this data in this example:

$$W = \frac{1}{0.227} (0.4708)^2 = 0.976$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 6.6 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 set of data, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.976 is greater than the critical value, conclude that the data are normally distributed.

12.13.2.6.8 Since the variance of the control group is zero, Bartlett's test statistic can not be calculated. Therefore, the survival data variances are considered to be heterogeneous.

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

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 1.80% concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

12.13.2.7.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) is 17(See Table 5, Appendix E).

12.13.2.8.1 The data used to calculate the LC50 is summarized in Table 12. For this example, although there are two concentrations with partial mortalities, the chi-square test for heterogeneity was significant, indicating that Probit Analysis is inappropriate for this set of data. Inspection of the data reveals that the smoothed, adjusted proportion mortality for the lowest concentration will not be zero, indicating that the Trimmed Spearman-Karber Method is recommended to calculated the LC50 for this dataset.

12.13.2.8.2 For the Trimmed Spearman-Karber analysis, run the USEPA Trimmed Spearman-Karber program, TSK. An example of the program output is provided in Figure 4.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 1.80% CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion of Total Mortality	Concentration
1	1.107	1.80%
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%

TABLE 10. TABLE OF RANKS[1]

Replicate	Control	Concentration (%)			
		1.80	3.20	5.60	10.0
1	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.107(2)	0.464(5)
2	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.345(7)	0.225(2.5)
3	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.345(7)	0.225(2.5)
4	1.345(6,6.5,7,8)	1.107(1)	1.345(6.5)	1.107(2)	0.225(2.5)
5	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.107(2)	0.225(2.5)

[1]*Control ranks are given in the order of the concentration with which they were ranked.*

TABLE 11. RANK SUMS

Concentration	Rank Sum
1.80	25.0
3.20	22.5
5.60	20.0
10.00	15.0

TABLE 12. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

		Concentration (%)				
	Control	*1.80*	*3.20*	*5.60*	*10.0*	*18.0*
No Dead	*0*	*1*	*2*	*3*	*24*	*25*
No Exposed	*25*	*25*	*25*	*25*	*25*	*25*

12.13.3 EXAMPLE OF ANALYSIS OF MYSID, ***HOLMESIMYSIS COSTATA*** GROWTH DATA

12.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean weight per surviving organism per replicate. The IC25 can be calculated for the growth data via a point

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: TEST NUMBER: 1 DURATION: 7 days

TOXICANT : Effluent
SPECIES: Holmesimysis costata

RAW DATA: Concentration (%)	*Number Exposed*	*Mortalities*
.00	*25*	*0*
1.80	*25*	*1*
3.20	*25*	*2*
5.60	*25*	*3*
10.00	*25*	*24*
18.00	*25*	*25*

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: LC50: 6.95
95% LOWER CONFIDENCE: 6.22
95% UPPER CONFIDENCE: 7.76

Figure 4. Output for USEPA Trimmed Spearman-Karber Program, version 1.5.

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.

12.13.3.2 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

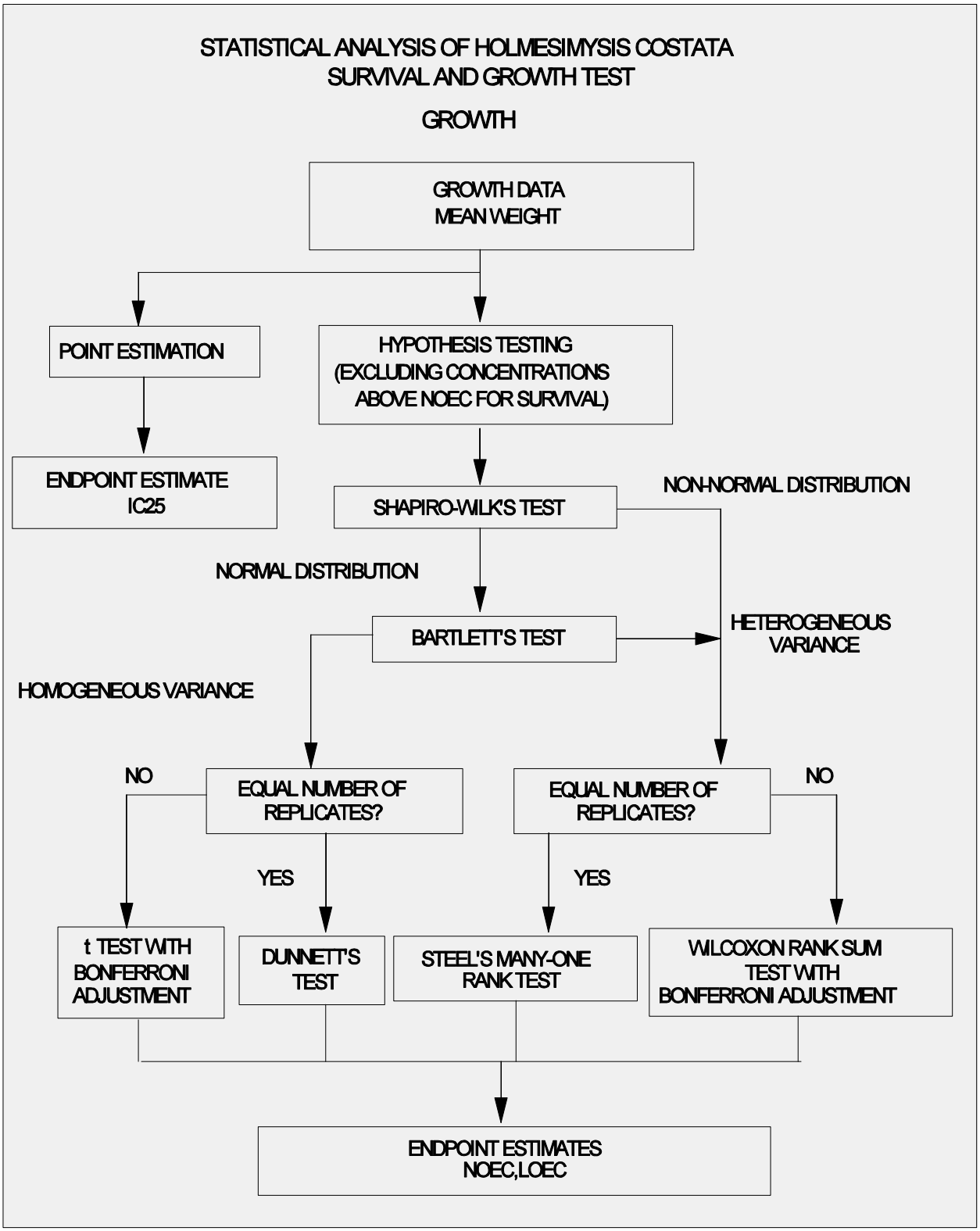

Figure 5. Flowchart for statistical analysis of mysid, *Holmesimysis costata*, growth data.

assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, 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.

12.13.3.3 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.

12.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6. Since there is significant mortality in the 10.0% concentration, its effect on growth is not considered.

12.13.3.5 Test for Normality

12.13.3.5.1 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 listed in Table 14.

12.13.3.5.2 Calculate the denominator, D, of the statistic:

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

TABLE 13. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.0
1	0.048	0.055	0.057	0.041	0.033
2	0.058	0.048	0.050	0.040	0.000
3	0.047	0.042	0.046	0.041	0.000
4	0.058	0.041	0.043	0.043	0.000
5	0.051	0.052	0.045	0.040	0.000
$Mean(\bar{Y}_i)$	0.052	0.048	0.048	0.041	0.007
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015	0.000218
i	1	2	3	4	5

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

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

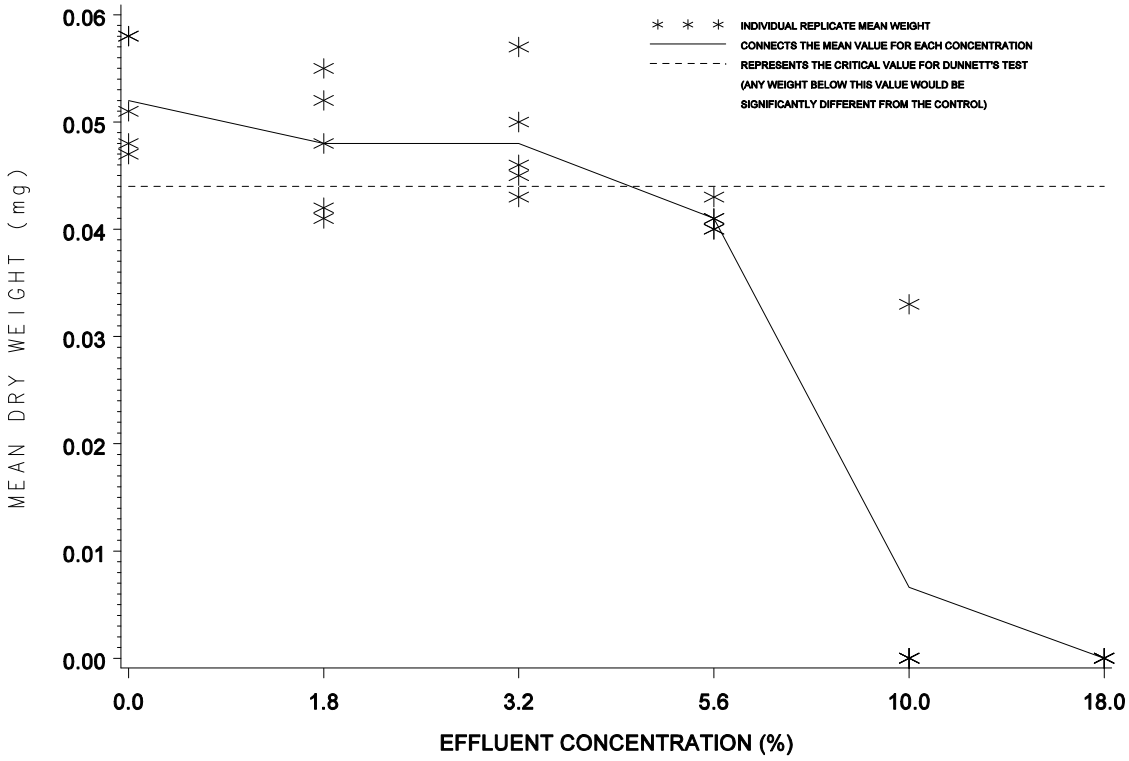

Figure 6. Plot of growth data for mysid, *Holmesimysis costata*, test.

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

12.13.3.5.3 For this set of data, n = 20

$$\& = \frac{1}{20}(0.001) = 0.000$$

D = 0.000393

12.13.3.5.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

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

i	$X^{(i)}$	*i*	$X^{(i)}$
1	*-0.007*	*11*	*0.000*
2	*-0.006*	*12*	*0.000*
3	*-0.005*	*13*	*0.000*
4	*-0.005*	*14*	*0.002*
5	*-0.004*	*15*	*0.002*
6	*-0.003*	*16*	*0.004*
7	*-0.002*	*17*	*0.006*
8	*-0.001*	*18*	*0.006*
9	*-0.001*	*19*	*0.007*
10	*-0.001*	*20*	*0.009*

12.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 16.

12.13.3.5.6 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 16. For this set of data:

$$W = \frac{1}{0.000393} (0.0194)^2 = 0.958$$

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	*0.4734*	*0.016*	$X^{(20)} - X^{(1)}$
2	*0.3211*	*0.013*	$X^{(19)} - X^{(2)}$
3	*0.2565*	*0.011*	$X^{(18)} - X^{(3)}$
4	*0.2085*	*0.011*	$X^{(17)} - X^{(4)}$
5	*0.1686*	*0.008*	$X^{(16)} - X^{(5)}$
6	*0.1334*	*0.005*	$X^{(15)} - X^{(6)}$
7	*0.1013*	*0.004*	$X^{(14)} - X^{(7)}$
8	*0.0711*	*0.001*	$X^{(13)} - X^{(8)}$
9	*0.0422*	*0.001*	$X^{(12)} - X^{(9)}$
10	*0.0140*	*0.001*	$X^{(11)} - X^{(10)}$

12.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 12.13.3.5.6 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 this set of data, the critical value at a significance level of

0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

12.13.3.6 Test for Homogeneity of Variance

12.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

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

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

p = number of concentration levels including the control

ln = $\log_e$

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

n_i = the number of replicates for concentration i.

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

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

12.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

12.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p} \ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

12.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 9.210. Since B = 7.254 is less than the critical value of 9.210, conclude that the variances are not different.

12.13.3.7 Dunnett's Procedure

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

TABLE 17. ANOVA TABLE

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

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

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

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

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{p} T_i$$

T_i = the total of the replicate measurements for concentration i

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

12.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 5$

$N = 20$

$T_1 = Y_{11} + Y_{12} + ... + Y_{15} = 0.262$
$T_2 = Y_{21} + Y_{22} + ... + Y_{25} = 0.238$
$T_3 = Y_{31} + Y_{32} + ... + Y_{35} = 0.241$
$T_4 = Y_{41} + Y_{42} + ... + Y_{45} = 0.205$
$G = T_1 + T_2 + T_3 + T_4 = 0.946$

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

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

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

$$= 0.0455 - \frac{(0.946)^2}{} = 0.000754$$

$SSW = SST - SSB$

$= 0.000754 - 0.000254 = 0.000500$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

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

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	*df*	*Sum of Squares (SS)*	*Mean Square (MS) (SS/df)*
Between	*3*	*0.000254*	*0.0000847*
Within	*16*	*0.000500*	*0.0000313*
Total	*19*	*0.000754*	

12.13.3.7.4 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: $\overline{Y}_i$ = mean weight for concentration i

$\overline{Y}_1$ = mean 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

12.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 1.80% concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.052 - 0.048)}{[0.00559\sqrt{(1/5)+(1/5)}]}$$

$$= 1.131$$

TABLE 19. CALCULATED t VALUES

Concentration (ppb)	*i*	t_i
1.80	*2*	*1.131*
3.20	*3*	*1.131*
5.60	*4*	*3.111*

12.13.3.7.6 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, 16 degrees of freedom for error and three concentrations (excluding the control) the approximate critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 5.60% concentration has significantly lower mean weight than the control. Hence the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

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

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

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.

12.13.3.7.8 In this example:

$$MSD = 2.23(0.00559)\sqrt{(1/5)+(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

12.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.00788 mg.

12.13.3.7.10 This represents a 15.2% reduction in mean weight from the control.

12.13.3.8 Calculation of the ICp

12.13.3.8.1 The growth data from Table 13 are utilized in this example. As seen in the table, the observed means are monotonically non-increasing with respect to concentration. Therefore, the smoothed means will be simply the corresponding observed mean. The observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i. Table 20 contains the smoothed means and Figure 7 gives a plot of the smoothed response curve.

12.13.3.8.2 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean weight of 0.039 mg, where $M_1(1-p/100) = 0.052(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.039 mg, is bracketed by C_4 = 5.60% and C_5 = 10.0%.

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

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

$$IC25 = 5.60 + [0.052(1 - 25/100) - 0.041] \frac{(10.0 - 5.60)}{(0.0066 - 0.041)}$$

$$= 5.86\%.$$

12.13.3.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.86%. The empirical 95.0% confidence interval for the true mean was 4.9440% to 6.2553%. The computer program output for the IC25 for this data set is shown in Figure 8.

TABLE 20. MYSID, HOLMESIMYSIS COSTATA, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.0066	0.0066
18.00	6	0.000	0.000

12.14 **PRECISION AND ACCURACY**

12.12.1 PRECISION

12.12.1.1 Single-Laboratory Precision

12.12.1.1.1 Data on the single laboratory precision of the *Holmesimysis costata* growth and survival test with zinc sulfate

are shown in Table 21. NOECs for mysid survival were either 32 or 56 µg/L Zn. There was also good agreement among LC50s, with a coefficient of variation of 14%. Mysids did not exhibit a growth response at zinc concentrations below those causing significant mortality; NOEC values for growth were always greater than or equal to the highest zinc concentration. IC50 values for growth could not be calculated.

12.12.1.2 Multi-laboratory Precision

12.12.1.2.1 The multi-laboratory data indicate a similar level of test precision (Table 22). The four multi-laboratory tests were conducted over a two year period, and each used split effluent samples tested at two laboratories. Survival NOEC values were the same for both laboratories in three of the four tests, with the NOECs varying by one concentration in the fourth test. The mean coefficient of variation between LC50 values from different laboratories was 21%. The two available comparisons of growth NOEC values indicate similar responses at both laboratories. Growth was the more sensitive indicator of toxicity in three of the four effluent tests.

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

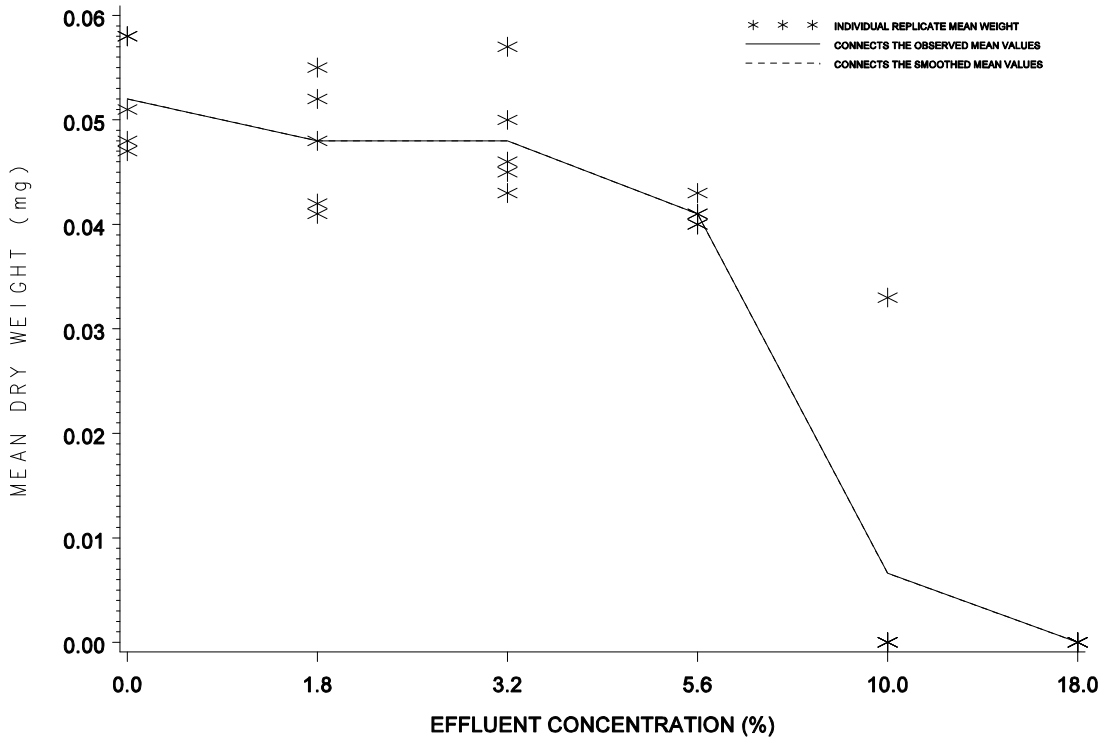

Figure 7. Plot of raw data, observed means, and smoothed means for the mysid, *Holmesimysis costata*, Growth data from tables 13 and 20

```
Conc. ID            1         2         3         4         5         6
------------------------------------------------------------------------
Conc. Tested        0      1.80      3.20      5.60      10.0      18.0
------------------------------------------------------------------------
Response  1      .048      .055      .057      .041      .033         0
Response  2      .058      .048      .050      .040         0         0
Response  3      .047      .042      .046      .041         0         0
Response  4      .058      .041      .043      .043         0         0
Response  5      .051      .052      .045      .040         0         0
------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration:          7 days
DATA FILE: mysid.icp
OUTPUT FILE:  mysid.i25
------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates                %       Means       Dev.   Response Means
------------------------------------------------------------------------
  1          5             0.000          0.052      0.005      0.052
  2          5             1.800          0.048      0.006      0.048
  3          5             3.200          0.048      0.006      0.048
  4          5             5.600          0.041      0.001      0.041
  5          5            10.000          0.007      0.015      0.007
  6          5            18.000          0.000      0.000      0.000
------------------------------------------------------------------------
The Linear Interpolation Estimate:     5.8174   Entered P Value: 25
------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   5.8205 Standard Deviation:     0.2673
Original Confidence Limits:   Lower:     4.9440 Upper:     6.2553
Expanded Confidence Limits:   Lower:     4.5073 Upper:     6.4743
Resampling time in Seconds:     0.22  Random_Seed: 526805435
```

Figure 8. Output for USEPA Linear Interpolation Program for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION DATA FOR THE MYSID, *HOLMESIMYSIS COSTATA* GROWTH AND SURVIVAL TEST WITH ZINC (ZN µG/L) SULFATE AS THE REFERENCE TOXICANT

Test	Survival		Growth
	NOEC	LC50	NOEC
1	32	47	>32
2	32	59	>32
3	56	62	>56
4	56	65	>56
N	4	4	4
Mean	44	58	>44
SD		7.9	
CV (%)		14	

No growth effect was observed in zinc concentrations below those causing significant mortality (10, 18, 32, 56 and 100 µg/L).

All tests were conducted at MPSL.

TABLE 22. MULTI-LABORATORY PRECISION DATA FOR THE MYSID, *HOLMESIMYSIS COSTATA* GROWTH AND SURVIVAL TEST WITH SPLIT EFFLUENT (%) ON THE SAME DATE.

Test	Effluent Type	Lab	Survival NOEC	Survival LC50	Growth NOEC
1	BKME	OSU	1.0	1.8	0.5[L]
1	BKME	MPSL	1.0	1.3	0.5[L]
			CV=26%		
2	POTW	ATL	3.2	4.1	>3.2[L]
2	POTW	MPSL	3.2	5.1	>3.2[L]
			CV=14%		
3	POTW	SRH	10.0	12.8	na
3	POTW	MPSL	10.0	11.7	3.2[W]
			CV=6%		
4	POTW	SRH	10.0	15.8	5.6[W]
4	POTW	MPSL	5.6	9.1	3.2[W]
			CV=38%		

Mean Interlaboratory CV= 21%

[L] Length was measured as the growth endpoint in tests 1 and 2,
[W] Weight was measured in test 3 and 4.
na Data was not available.

OSU is the Oregon State University Laboratory at the Hatfield Marine Science Center in Newport Oregon.

ATL is Aquatic Testing Laboratory in Ventura, California.

SRH is S.R. Hansen and Associates in Concord, California.

MPSL is the Marine Pollution Studies Laboratory near Monterey, California.

APPENDIX I. MYSID TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \circ 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 13 or 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Four to five days prior to the beginning of the toxicity test, isolate approximately 150 gravid female mysids in a screened (2-mm-mesh) compartment within an aerated 80-liter aquarium (15EC). Add a surplus of *Artemia* nauplii (200 per mysid, static; 500 per mysid, flow-through) to stimulate overnight release of juveniles. Add blades of kelp as habitat.

B. Isolate the newly released juveniles by slowly siphoning into a screen-tube (150-µm-mesh, 25 cm diam.) immersed in a bucket of clean seawater. Transfer juveniles into additional screen-tubes or static 4-liter beakers at a density of approximately 50 juveniles per liter. Juveniles should be fed five to ten newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Maintain the juveniles for three days at 13 to 15EC, changing the water at least once in static chambers.

C. After three days, begin randomized introduction of juveniles into the test chambers. Place one or two mysids at a time into as many plastic cups as there are test chambers. Repeat the process until each cup has exactly five juvenile mysids.

D. Eliminate excess water from the cups (no more than 5 mL should remain) and pipet the mysids into the test chambers using a wide bore glass tube or pipet (approximately 3 mm ID). Make sure no mysids are left in the randomization cups. Count the number of juveniles in each test chamber to verify that each has five.

E. Remove all dead mysids daily, and add 40 newly hatched *Artemia* nauplii/mysid/day, adjusting feeding to account for mysid mortality.

F. At 48 and 96 hours, renew 75% of the test solution in each chamber.

G. After 7 days, count and record the number of live and dead mysids in each chamber. After counting, use the randomization sheet to assign the correct test concentration to each chamber. Remove all dead mysids.

H. Carefully pour the contents of each test chamber through a small mesh screen (<300µm). Count the mysids and record before screening. Briefly dip the screen containing the mysids in fresh water to rinse away the salt. Carefully transfer the mysids from the screen to a prenumbered, preweighed micro-weigh boat using fine-tipped forceps. Dry for 24 hours at 60EC. Weigh each weigh boat on a microbalance (accurate to 1 µg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

I. Analyze the data.

J. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Juvenile Holmesimysis Toxicity Test

Test Start Date: Start Time: Mysid Source
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Mysid Age at Start:
Sample Source:

Test Cont. #	Toxic Conc.	Number Alive							Total Number Alive	Total Number at Start	Notes and Initials
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
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27											
28											
29											
30											
31											
32											
33											
34											
35											

Computer Data Storage
Disk:
File:

Note: See juvenile growth data on separate sheet.

Data Sheet for Weighing Juvenile Mysids

Test Start Date:

Start Time:

Mysid Source :

Test End Date:

End Time:

Collection/Arrival Date:

Reference Toxicant:

Mysid Age at Start:

Sample Source:

Sample Type:

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (µg)	Total Weight (µg)	Mysid Wt (Total - Foil) (mg)	Number of Mysids	Weight per Mysid (µg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
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29							
30							
31							
32							
33							
34							
35							

Computer Data Storage

Disk:

File:

Note: See mysid mortality data on separate sheet.