SECTION 11

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

11.1 SCOPE AND APPLICATION

11.1.1 This method estimates the chronic toxicity of effluents and receiving water to the fathead minnow, *Pimephales promelas*, using newly hatched larvae in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

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

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

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

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

11.2 SUMMARY OF METHOD

11.2.1 Fathead minnow, *Pimephales promelas*, larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and weight of the larvae.

11.3. INTERFERENCES

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

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

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

11.3.4 Pathogenic and/or predatory organisms in effluent samples or receiving water that is used for dilution may affect test organism survival and confound test results. When pathogen interference is suggested by observation (11.3.4.1) and data evaluations (11.3.4.2) and confirmed by parallel testing (11.3.4.4), steps should be taken to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Pathogen control techniques that do not require modification of effluent samples, such as use of the modified test design described in Subsection 11.3.4.5, are recommended for controlling pathogen interference. Upon approval by the regulatory authority, analysts also may use additional pathogen control techniques that require sample modification (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence

of pathogen interference and demonstrates successful pathogen control (11.3.4.6).

11.3.4.1 A typical indication that pathogen interference has occurred in a WET test is when test organisms exhibit "sporadic mortality". This sporadic morality phenomenon is characterized by an unexpected concentration-response relationship (i.e., effects that do not increase with increasing effluent concentration) and organism survival that varies greatly among replicates and among effluent dilutions (USEPA, 2000a). The observed sporadic mortality among replicates may occur in receiving water controls, lower effluent concentrations, and occasionally in full-strength effluent on day 3 or day 4 of the chronic test. When sporadic mortality occurs, a fungal growth may appear directly on the fish, especially in the gill area. The fungus has not been definitively identified, but the fungal growth appears to be compatible with *Saprolegnea sp.* (Downey *et al.*, 2000). Microbiological evaluations on receiving waters, the fish, and the food indicated the ubiquitous nature of pathogenic organisms (e.g., *Flexibacter spp*., *Aeromonas hydrophila*), and eradicating them from the test through the decontamination of the fish and their food has not been practical (Geis *et al.*, 2000).

11.3.4.2 When pathogen interference is suspected, a series of data evaluations are required. The test data must be reviewed to determine a cause for any unexpected concentration-response pattern and subsequently to determine the validity of calculated results (USEPA, 2000a). USEPA (2000a) provides guidance on reviewing concentration-response relationships including specific response patterns that may indicate pathogen effects. Each treatment (including the control) should be evaluated for an unusually high mortality response and unevenness of mortalities among replicates. Within-treatment coefficient of variation (CVs) for survival of >40% in effluent or receiving water treatments but relatively small for control replicates in a standard reconstituted water may be an indication of pathogen interference. Receiving water controls from improper preparation or collection also should be evaluated.

11.3.4.3 Because of the ubiquitous nature of the pathogens or predatory organisms, all test equipment, glassware, and pipettes must be kept clean and dry when not in use. Use of separate glassware, pipettes, and siphons for each concentration is recommended to minimize cross contaminating replicates of all treatments. Care also should be taken to properly clean test chambers by removing excess food, dead fish larvae, and other debris prior to daily renewal (see Subsection 11.10.7). When proper laboratory hygiene and filtration through a 2-4 mm mesh opening (Subsection 8.8.2) do not eliminate the sporadic mortality, the analyst should determine the source and confirm pathogen interference using parallel testing (11.3.4.4).

11.3.4.4 Parallel tests should be conducted using reconstituted water and receiving water as diluents with the effluent to confirm that the test results are due to pathogen interference and to determine the source of pathogens in the test. This determination is an important step in controlling pathogen interference. When the dilution water exhibits the interference (i.e., pathogen interference is not observed in the test using reconstituted laboratory water for dilution), reconstituted laboratory water instead of receiving waters should be used to eliminate the interference. However, if receiving water is required, the analyst may modify the test design to control pathogen interference (Subsection 11.3.4.5) or treat the dilution water prior to testing to remove the interference (Subsection 11.3.4.6). If pathogen interference is due to pathogens in the effluent (i.e., pathogen interference is still observed in the test using reconstituted laboratory water for dilution), it is recommended that the analyst modify the test design to control pathogen interference (Subsection 11.3.4.5). Upon approval by the regulatory authority, analysts also may use various sample sterilization techniques to control pathogen interference (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence of pathogen interference and demonstrates successful pathogen control.

11.3.4.5 When data evaluation indicates that sporadic mortality has occurred as described in Subsections 11.3.4.1 - 11.3.4.2, the test design can be modified as described below to control pathogen interference. The use of 2 fish per 20 ml in each 1 ounce plastic cup test solution or 2 fish per 50 ml in each 4 ounce plastic cup can be used rather than 10 fish per test chamber. The total number of fish tested remains unchanged (i.e., 40 per treatment). At test initiation, for each test concentration and replicate, the test cups must be labeled to easily recombine the fish to the original replicate at the end of the test. For example, for replicate A, each of the five plastic test cups would be identified as subreplicate A1, A2, A3, A4, and A5 repeating the pattern for subsequent replicates (e.g., for replicate B, each cup would be identified as subreplicate B1, B2, B3, B4, and B5). At test termination, all test organisms

from the five A subreplicates are combined for a survival and weight determination. Document the recombination of replicates in records.

11.3.4.5.1 All test chambers must be randomized using a template for randomization or by using a table of random numbers. Test chambers are randomized once at the beginning of the test (see Subsection 11.10.2.3). When using templates, a number of different templates should be prepared, so that the same template is not used for every test. Randomization procedures must be documented with daily records.

11.3.4.5.2 When adding or transferring the larvae to test chambers, the amount of excess water added to the chambers should be kept to a minimum to avoid unnecessary dilution of the test concentrations. The fish in each test chamber should be fed 0.1 mL of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4 h intervals, or 0.15 mL should be fed twice daily at an interval of 6 h. (NOTE: to prevent low dissolved oxygen levels, the amount of food added to cups should be adjusted to account for the modified test design that uses smaller test chambers). Dead test organisms should be removed as soon as they are observed.

11.3.4.5.3 Fish are transferred to new or clean test chambers daily. At the time of the daily renewal of the test solutions, the fish are transferred to a new test chamber containing fresh test solution using a pipette which has at least a 5mm bore diameter. Separate pipettes should be used for each treatment. Water transfer is kept to a minimum by allowing the fish to swim out of the pipette into the new test chamber. Any potential injury to individual fish should be recorded on the test sheets.

11.3.4.5.4 At test termination, the surviving larvae in each chamber must be counted and all subreplicates within a replicate (e.g., A1, A2, A3, A4, and A5) combined. For example, all test cups (within a treatment) labeled A would be combined for a survival and dry weight determination.

11.3.4.6 When parallel testing has confirmed pathogen interference, the regulatory authority may allow modifications of the effluent samples or receiving water diluent to remove or inactivate the pathogens (Subsection 11.3.4.6.1 - 11.3.4.6.4). Techniques that control pathogen interference without modifying the effluent sample (11.3.4.5) are recommended, but they may not always be able to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Therefore, regulatory authorities may allow appropriate pathogen control techniques (including those that modify the effluent sample) on a case-by-case basis. TIE approaches (USEPA, 1991b; USEPA, 1992) and the following procedures (Subsection 11.3.4.6.1 - 11.3.4.6.4) can be used alone or in combination to ascertain the adverse influence on tests caused by pathogens. Prior to routine use of pathogen control techniques that modify the sample, the effects of pathogenic bacteria and the effectiveness of the selected pathogen control technique must be confirmed by parallel and simultaneous testing of the technique with altered and unaltered samples.

11.3.4.6.1 Use of ultra-violet light to irradiate the sample. The rate of pumping specified by the manufacturer of the apparatus should be used (provided that adequate disinfection is achieved), and the life of the UV light source must follow manufacturers' recommendations and be documented. For example, one liter of water can be irradiated for 20 min using an 8 watt UV light (Aquatic Ecosystems, Apopka, FL) prior to use each day of the test. Light sources have limited lifetimes and their effectiveness will decrease with age. The delivery pump and the light source should be on the same electrical circuit to ensure that when power is interrupted both terminate operation. QA/QC procedures should be put into place to assure that the light source is on at the beginning and at the end of the procedure. Treatment of the large volumes of water necessary for test dilution also may be impractical. Caution: Since the effluent or receiving water samples must be passed through the UV sterilizer and then test treatments prepared, there may be potential effects of UV light on the sample. UV exposure may increase or decrease toxicity from other pollutants in the sample. UV treatment is known to cause photoactivation of some organic compounds, which may increase toxicity. UV treatment also is known to cause the photochemical breakdown of certain organic compounds, which could decrease toxicity (if the parent compound is toxic) or increase toxicity (if reaction products are toxic). These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The effectiveness of UV for

sterilization may decrease with turbid or stained samples. Bacteria can escape exposure by being lodged in crevices of particulate matter in the sample. All toxicity tests using a sterilized sample must include a blank preparation consisting of similarly sterilized laboratory water.

11.3.4.6.2 Ultra-filtration through a 0.22 µm pore diameter filter (such as Gelman Suprocap®) may be conducted on sample aliquots before daily use. Samples may need to be filtered through a glass fiber filter prior to the 0.22 µm filter. This is time consuming and volume restricted. Treatment of the large volumes of water necessary for test dilution may be impractical. Caution: Since the effluent or receiving water samples must be passed through the filter, the effect of filtering must be evaluated. Filtration can remove toxicity if toxic components of the sample are bound to particles (USEPA, 1991b; 1992). The removal of suspended solids also may influence the bioavailability of chemical pollutants. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The removal of toxicity by filtration must be evaluated for each sample by testing samples before and after filtration. All toxicity tests using a sterilized sample also must include a blank preparation consisting of similarly sterilized reconstituted laboratory water.

11.3.4.6.3 Use of chlorination and dechlorination. In some cases, pathogens can survive the chlorination/ dechlorination process and the pathogenic effects may increase due to lack of competition from other organisms. Sufficient data must be collected and documented to determine the effective dosage required. Caution: Chlorination of effluent samples could cause unknown effects on the sample. Chlorination could increase or decrease sample toxicity by oxidizing organic compounds or forming chlorination by-products. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. Toxicity tests conducted with the addition of chlorine and subsequent dechlorination (USEPA, 1991b; 1992) to either effluent or receiving water samples also must include a blank preparation consisting of similarly treated laboratory water.

11.3.4.6.4 Use of antibiotics. The addition of wide spectrum antibiotics has been effective in removing the pathogen effect (Downey *et al.*, 2000). Antibacterial treatment such as those commonly used in aquaculture or home aquarium maintenance (e.g., oxytetracycline, chloramphenicol, and actinomycin) may be effective. Sufficient data must be collected to determine the effective dosage required. Caution: While antibiotics are effective, easy to use, inexpensive, and readily available, the antibiotic treatment may alter the sample in unknown or undesirable ways and may make the sample too cloudy. Large volumes of a sample may need to be treated. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. All toxicity tests using antibiotic treatments also must include treatment blanks of similarly prepared laboratory water.

11.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2.

The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

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

11.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 11.3.6.1.1).

11.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

11.4 SAFETY

11.4.1 See Section 3, Health and Safety.

11.5 APPARATUS AND EQUIPMENT

11.5.1 Fathead minnow and brine shrimp culture units -- see USEPA, 1985a and USEPA, 2002a. This test requires 240-360 larvae. It is preferable to obtain larvae from an in-house fathead minnow culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be shipped in well oxygenated water in insulated containers.

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

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

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

11.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

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

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

11.5.8 Test chambers -- four borosilicate glass or non-toxic disposable plastic test chambers are required for each concentration and control. Test chambers may be 1 L, 500 mL or 250 mL beakers, 500 mL plastic cups, or fabricated rectangular (0.3 cm thick) glass chambers, 15 cm x 7.5 cm x 7.5 cm. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

11.5.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions. 5.10

11.5.10 Volumetric pipets -- Class A, 1-100 mL.

11.5.11 Serological pipets -- 1-10 mL, graduated.

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

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

11.5.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

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

11.5.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

11.5.17 Thermometers, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calabrate laboratory themometers.

11.5.18 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

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

11.6 REAGENTS AND CONSUMABLE MATERIALS

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

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

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

11.6.4 Weighing boats, aluminum -- 24 per test.

11.6.5 Tape, colored -- for labeling test chambers.

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

11.6.7 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

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

11.6.9 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

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

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

11.6.12 Reference toxicant solutions (see Section 4, Quality Assurance).

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

11.6.14 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).

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

11.6.16 Brine Shrimp, *Artemia*, Nauplii -- for feeding cultures and test organisms

11.6.16.1 Newly-hatched *Artemia* nauplii are used as food (see USEPA, 2002a) for fathead minnow, *Pimephales promelas*, larvae in toxicity tests and frozen brine shrimp and flake food are used in the maintenance of continuous stock cultures. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

11.6.16.2 Each new batch of brine shrimp, *Artemia*, cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (see Leger et al., 1985; Leger et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A

sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982).

11.6.16.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L deionized water, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24-h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used) (see USEPA, 1991b; USEPA, 2002a and ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 min. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a beaker or funnel fitted with a ≤ 150 µm Nitex® or stainless steel screen, and rinse with deionized water, or equivalent, before use.

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

11.6.16.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the fathead minnow larvae (see Subsection 11.12). The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

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

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

11.6.17 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

11.6.17.1 Newly hatched fish less than 24 h old should be used for the test. If organisms must be shipped to the testing site, fish up to 48 h old may be used, all hatched within a 24-h window.

11.6.17.2 If the fish are kept in a holding tank or container, most of the water should be siphoned off to concentrate the fish. The fish are then transferred one at a time randomly to the test chambers until each chamber contains ten fish. Alternately, fish may be placed one or two at a time into small beakers or plastic containers until they each contain five fish. Three (minimum of two) of these beakers/plastic containers are then assigned to randomly-arranged control and exposure chambers.

11.6.17.2.1 The fish are transferred directly to the test vessels or intermediate beakers/plastic containers, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb, or a large volumetric pipet with tip removed and fitted with a safety type bulb filler. The glass or plastic containers should only contain a small volume of dilution water.

11.6.17.2.2 It is important to note that larvae should not be handled with a dip net. Dipping small fish with a net may result in damage to the fish and cause mortality.

11.6.17.3 The test is conducted with a minimum of four test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 40) fish are exposed at each test concentration.

11.6.17.4 Sources of organisms

11.6.17.4.1 Fathead minnows, *Pimephales promelas*, may be obtained from commercial biological supply houses. Fish obtained from outside sources for use as brood stock or in toxicity tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be of (1) the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of contemporary fish to reference toxicants.

11.6.17.5 Inhouse Sources of Fathead Minnows, *Pimephales promelas*

11.6.17.5.1 Problems in obtaining suitable fish from outside laboratories can be avoided by developing an inhouse laboratory culture facility. Fathead minnows, *Pimephales promelas*, can be easily cultured in the laboratory from eggs to adults in static, recirculating, or flow-through systems. The larvae, juveniles, and adult fish should be kept in 60 L (15 gal) or 76 L (20 gal) rearing tanks supplied with reconstituted water, dechlorinated tap water, or natural water. The water should be analyzed for toxic metals and organics quarterly (see Section 4, Quality Assurance).

11.6.17.5.1.1 If a static or recirculating system is used, it is necessary to equip each tank with an outside activated carbon filter system, similar to those sold for tropical fish hobbyists (or one large activated carbon filter system for a series of tanks) to prevent the accumulation of toxic metabolic wastes (principally nitrite and ammonia) in the water.

11.6.17.5.2 Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture tanks should be shielded from extraneous disturbances using opaque curtains, and should be isolated from toxicity testing activities to prevent contamination.

11.6.17.5.3 To avoid the possibility of inbreeding of the inhouse brood stock, fish from an outside source should be introduced yearly into the culture unit.

11.6.17.5.4 Dissolved oxygen -- The DO concentration in the culture tanks should be maintained near saturation, using gentle aeration with 15 cm air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all dissolved oxygen concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the re-aeration of flowing water for culture systems.

11.6.17.5.5 Culture Maintenance

11.6.17.5.5.1 Adequate procedures for culture maintenance must be followed to avoid poor water quality in the culture system. The spawning and brood stock culture tanks should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a glass siphon tube attached to a plastic hose leading to the floor drain. The tanks are more thoroughly cleaned as required. Algae, mostly diatoms and green algae, growing on the glass of the spawning tanks are left in place, except for the front of the tank, which is kept clean for observation. To avoid excessive build-up of algal growth, the walls of the tanks are periodically scraped. The larval culture tanks are cleaned once or twice a week to reduce the mass of fungus growing on the bottom of the tank.

11.6.17.5.5.2 Activated charcoal and floss in the tank filtration systems should be changed weekly, or more often if needed. Culture water may be maintained by preparation of reconstituted water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

11.6.17.5.5.3 Before new fish are placed in tanks, salt deposits are removed by scraping or with 5% acid solution, the tanks are washed with detergent, sterilized with a hypochlorite solution, and rinsed well with hot tap water and then with laboratory water.

11.6.17.5.6 Obtaining Embryos for Toxicity Tests

11.6.17.5.6.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory as described below.

11.6.17.5.6.2 For breeding tanks, it is convenient to use 60 L (15 gal) or 76 L (20 gal) aquaria. The spawning unit is designed to simulate conditions in nature conducive to spawning, such as water temperature and photoperiod. Spawning tanks must be held at a temperature of 25 ± 2°C. Each aquarium is equipped with a heater, if necessary, a continuous filtering unit, and spawning substrates. The photoperiod for the culture system should be maintained at 16 h light and 8 h darkness. For the spawning tanks, this photoperiod must be rigidly controlled. A convenient photoperiod is 5:00 AM to 9:00 PM. Fluorescent lights should be suspended about 60 cm above the surface of the water in the brood and larval tanks. Both DURATEST® and cool-white fluorescent lamps have been used, and produce similar results. An illumination level of 50 to 100 ft-c is adequate.

11.6.17.5.6.3 To simulate the natural spawning environment, it is necessary to provide substrates (nesting territories) upon which the eggs can be deposited and fertilized, and which are defended and cared for by the males. The recommended spawning substrates consist of inverted half-cylinders, 7.6 cm × 7.6 cm (3 in × 3 in) of Schedule 40 PVC pipe. The substrates should be placed equi-distant from each other on the bottom of the tanks.

11.6.17.5.6.4 To establish a breeding unit, 15-20 pre-spawning adults six to eight months old are taken from a "holding" or culture tank and placed in a 76-L spawning tank. At this point, it is not possible to distinguish the sexes. However, after less than a week in the spawning tank, the breeding males will develop their distinct coloration and territorial behavior, and spawning will begin. As the breeding males are identified, all but two are removed, providing a final ratio of 5-6 females per male. The excess spawning substrates are used as shelter by the females.

11.6.17.5.6.5 Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained (Flickinger, 1966). Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

11.6.17.5.6.6 Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed from the spawning tanks because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

11.6.17.5.6.7 A breeding unit should remain in their spawning tank about four months. Thus, each brood tank or unit is stocked with new spawners about three times a year. However, the restocking process is rotated so that at any one time the spawning tanks contain different age groups of brood fish.

11.6.17.5.6.8 Fathead minnows spawn mostly in the early morning hours. They should not be disturbed except for a morning feeding (8:00 AM) and daily examination of substrates for eggs in late morning or early afternoon. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, for ease of handling, for purposes of recording embryo count and hatchability, and for the use of the newly hatched young fish for toxicity tests.

11.6.17.5.6.9 Daily, beginning six to eight hours after the lights are turned on (11:00 AM - 1:00 PM), the substrates in the spawning tanks are each lifted carefully and inspected for embryos. Substrates without embryos are immediately returned to the spawning tank. Those with embryos are immersed in clean water in a collecting tray, and replaced with a clean substrate. A daily record is maintained of each spawning site and the estimated number of embryos on the substrate.

11.6.17.5.6.10 Three different methods are described for embryo incubation.

1. Incubation of Embryos on the Substrates: Several (2-4) substrates are placed on end in a circular pattern (with the embryos on the innerside) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm airstone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25°C, 50% hatch occurs in five days. At 22°C embryos incubated on aerated tiles require 7 days for 50% hatch.

2. Incubation of Embryos in a Separatory Funnel: The embryos are removed from the substrates with a rolling action of the index finger ("rolled off") (Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor of approximately 430 embryos/mL. The embryos are incubated in about 1.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25°C. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days. Hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, unaerated container, a 50% hatch will occur in six days (instead of five) and a 100% hatch will occur in seven days. If the culture system is operated at 22°C, embryos incubated on aerated tiles require seven days for 50% hatch.

3. Incubation in Embryo Incubation Cups: The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation have been used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one only dead embryos are removed from the cups. During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier-illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungus should be done quickly, and the substrates should be returned to the incubation tanks as rapidly as possible so that the good eggs are not damaged by desiccation. Hatching takes four to five days at an optimal temperature of 25°C. Hatching can be delayed several (two to four) days by incubating at lower temperatures. A large plastic tank receiving recirculating water from a temperature control unit, can be used as a water bath for incubation of embryos.

11.6.17.5.6.11 Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Approximately 1500 newly hatched larvae are placed in a 60-L (15 gal) or 76-L (20 gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25°C, but sudden, extreme variations in temperature must be avoided.

11.6.17.5.7 Food and Feeding

11.6.17.5.7.1 The amount of food and feeding schedule affects both growth and egg production. The spawning fish and pre-spawners in holding tanks usually are fed all the adult frozen brine shrimp and tropical fish flake food or dry commercial fish food (No. l or No. 2 granules) that they can eat (*ad libitum*) at the beginning of the work day and in the late afternoon (8:00 AM and 4:00 PM). The fish are fed twice a day (twice a day with dry food and once a day with adult shrimp) during the week and once a day on weekends.

11.6.17.5.7.2 Fathead minnow larvae are fed freshly-hatched brine shrimp (*Artemia*) nauplii twice daily until they are four weeks old. Utilization of older (larger) brine shrimp nauplii may result in starvation of the young fish because they are unable to ingest the larger food organisms (see Subsection 11.6.16 or USEPA, 2002a for instructions on the preparation of brine shrimp nauplii).

11.6.17.5.7.3 Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#l and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate. (Starter, No. 1 and N. 2 granules, U.S. Fish and Wildlife Service Formulation Specification Diet SD9-30). Newly hatched brine shrimp nauplii, and frozen adult brine shrimp are fed to the fish cultures in volumes based on age, size, and number of fish in the tanks.

11.6.17.5.7.4 Fish in the larval tanks (from hatch to 30 days old) are fed commercial starter fish food at the beginning and end of the work day, and newly hatched brine shrimp nauplii (from the brine shrimp culture unit) once a day, usually mid-morning and mid-afternoon.

11.6.17.5.7.5 Attempts should be made to avoid introducing *Artemia* cysts and empty shells when the brine shrimp nauplii are fed to the fish larvae. Some of the mortality of the larval fish observed in cultures could be caused from the ingestion of these materials.

11.6.17.5.8 Disease Control

11.6.17.5.8.1 Fish are observed daily for abnormal appearance or behavior. Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

11.6.17.5.8.2 In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins et al., 1976). These changes could cause the death of the culture organisms.

11.6.17.5.8.3 Do not transfer equipment from one tank to another without first disinfecting tanks and nets. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite. Also to avoid the contamination of cultures or spread of disease, each time nets are used to remove live or dead fish from tanks, they are first sterilized with sodium hypochlorite or formalin, and rinsed in hot tap water. Before a new lot of fish is transferred to culture tanks, the tanks are cleaned and sterilized as described above.

11.6.17.5.8.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Newly hatched fathead minnow larvae less than 24 h old are used to monitor the chronic toxicity of the reference toxicant to the test fish produced by the culture unit (see Section 4, Quality Assurance).

11.6.17.5.9 Record Keeping

11.6.17.5.9.1 Records, kept in a bound notebook, include: (l) type of food and time of feeding for all fish tanks; (2) time of examination of the tiles for embryos, the estimated number of embryos on the tile, and the tile position number; (3) estimated number of dead embryos and embryos with fungus observed during the embryonic development stages; (4) source of all fish; (5) daily observation of the condition and behavior of the fish; and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

11.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance.

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance.

11.10 TEST PROCEDURES

11.10.1 TEST SOLUTIONS

11.10.1.1 Receiving Waters

11.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 250 mL, and 400 mL for chemical analyses, would require approximately 1.5 L or more of sample per test per day.

11.10.1.2 Effluents

11.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as the dilution factor is increased beyond 0.5, and declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

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

11.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 250 mL of test solution, is approximately 2.5 L. Sufficient test solution (approximately 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

11.10.1.2.4 Tests 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 for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

11.10.1.2.6 The DO of the test solutions should be checked prior to the test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

11.10.1.3 Dilution Water

11.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

11.10.2 START OF THE TEST

11.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have a minimum of four replicates.

11.10.2.2 Tests performed in laboratories that have in-house fathead minnow breeding cultures should use larvae less than 24 h old. When eggs or larvae must be shipped to the test site from a remote location, it may be necessary to use larvae older than 24 h because of the difficulty in coordinating test organism shipments with field operations. However, in the latter case, the larvae must not be more than 48 h old at the start of the test and must all be within 24 h of the same age.

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

11.10.2.4 The larvae are pooled and placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) larvae, for a total of 60 larvae (minimum of 40) for each concentration (see Appendix A). The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

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

11.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

11.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C.

11.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated.

The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.10.5 FEEDING

11.10.5.1 The fish in each test chamber are fed 0.1 g (approximately 700 to 1000) of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g are fed twice daily at an interval of 6 h. Equal amounts of nauplii must be added to each replicate chamber to reduce variability in larval weight. Sufficient numbers of nauplii should be provided to assure that some remain alive in the test chambers for several hours, but not in excessive amounts which will result in depletion of DO below acceptable levels (below 4.0 mg/L).

11.10.5.2 The feeding schedule will depend on when the test solutions are renewed. If the test is initiated after 12:00 PM, the larvae may be fed only once the first day. On following days, the larvae normally would be fed at the beginning of the work day, at least 2 h before test solution renewal, and at the end of the work day, after test solution renewal. However, if the test solutions are changed at the beginning of the work day, the first feeding would be after test solution renewal in the morning, and the remaining feeding(s) would be at the appropriate intervals. The larvae are not fed during the final 12 h of the test.

11.10.5.3 The nauplii should be rinsed with freshwater to remove salinity before use (see USEPA, 2002a). At feeding time pipette about 5 mL (5 g) of concentrated newly hatched brine shrimp nauplii into a 120 mesh nylon net or plastic cup with nylon mesh bottom. Slowly run freshwater through the net or rinse by immersing the cup in a container of fresh water several times. Resuspend the brine shrimp in 10 mL of fresh water in a 30 mL beaker or simply set the cup of washed brine shrimp in ¼ inch of fresh water so that the cup contains about 10 mL of water. Allow the container to set for a minute or two to allow dead nauplii and empty cysts to settle or float to the surface before collecting the brine shrimp from just below the surface in a pipette for feeding. Distribute 2 drops (0.1 g) of the brine shrimp to each test chamber. If the survival rate in any test chamber falls below 50%, reduce the feeding in that chamber to 1 drop of brine shrimp at each subsequent feeding.

11.10.6 OBSERVATIONS DURING THE TEST

11.10.6.1 Routine Chemical and Physical Determinations

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

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

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

11.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

11.10.6.1.5 Record all the measurements on the data sheet (Figure 1)

11.10.6.2 Routine Biological Observations

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

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

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations (CONTINUED).

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

No. Surviving Organisms									
Conc:	Rep. No.	Day							
Control:		1	2	3	4	5	6	7	Remarks
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									

Comments:

Figure 2. Survival data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

11.10.7 DAILY CLEANING OF TEST CHAMBERS

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

11.10.8 TEST SOLUTION RENEWAL

11.10.8.1 Freshly prepared solutions are used to renew the tests daily immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Holding, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6ºC until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (250 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the larvae.

11.10.9 TERMINATION OF THE TEST

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

11.10.9.2 For immediate drying and weighing, place live larvae onto a 500 µm mesh screen in a large beaker to wash away debris that might contribute to the dry weight. Each group of larvae is rinsed with deionized water to remove food particles, transferred to a tared weighing boat that has been properly labeled, and dried at 60ºC, for 24 h or at 100ºC for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing boats are placed in a dessicator until weighed, to prevent the absorption of moisture from the air. All weights should be measured to the nearest 0.01 mg and recorded on data sheets (Figure 3). Subtract tare weight to determine the dry weight of the larvae in each replicate. For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight and record on the data sheet (Figure 3). For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (See Section 11.11). Average weights should be expressed to the nearest 0.001 mg.

11.10.9.3 Prepare a summary table as illustrated in Figure 4.

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

11.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

Discharge:_____ Test Date(s): _____ Drying Temperature (°C): _____
Location: _____ Weighing Date:_____ Drying Time (h): _____
Analyst: _____

Conc:	Rep. No.	A Wgt. of tare (mg)	B Dry wgt: tare and larvae (mg)	B-A Total dry wgt of larvae (mg)	C No. of original larvae (mg)	(B-A)/C Mean dry wgt of larvae (mg)	Remarks
Control							
Conc:							
Conc:							
Conc:							
Conc:							
Conc:							

Figure 3. Weight data for the fathead minnow, *Pimephales promelas*, larval survival and growth test. From USEPA (1989a).

Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

TREATMENT	CONTROL					
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WGT OF LARVAE (MG) ± SD						
TEMPERATURE RANGE (°C)						
DISSOLVED OXYGEN RANGE (MG/L)						
HARDNESS						
CONDUCTIVITY						

COMMENTS:

Figure 4. Summary data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 µE/m^2/s (50-100 ft-c)(ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h darkness (recommended)
6.	Test chamber size:	500 mL (recommended minimum)
7.	Test solution volume:	250 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Newly hatched larvae less than 24 h old. If shipped, not more than 48 h old, 24 h range in age (required)
10.	No. larvae per test chamber:	10 (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	No. larvae per concentration:	40 (required minimum)
13.	Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old) (required)
14.	Feeding regime:	On days 0-6, feed 0.1 g newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g twice daily at 6-h intervals (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. (recommended)

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

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0) (CONTINUED)

15.	Cleaning:	Siphon daily, immediately before test solution renewal (required)
16.	Aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/minimum (recommended)
17.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options)
18.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
19.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None or ≥ 0.5 (recommended)
20.	Test duration:	7 days (required)
21.	Endpoints:	Survival and growth (weight) (required)
22.	Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg (required)
23.	Sampling requirements:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device; For off-site tests, a minimum of three samples (e.g., collected on days one, three and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
24.	Sample volume required:	2.5 L/day (recommended)

11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 For the test results to be acceptable, survival in the controls must be at least 80%. The average dry weight per surviving control larvae at the end of the test must equal or exceed 0.25 mg.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is shown in Table 2.

TABLE 2. SUMMARY OF SURVIVAL AND GROWTH DATA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAE EXPOSED TO A REFERENCE TOXICANT FOR SEVEN DAYS[1]

NaPCP Conc. (µg/L)	Proportion of Survival in Replicate Chambers A	B	C	D	Mean Prop. Surv	Avg Dry Wgt (mg) In Replicate Chambers A	B	C	D	Mean Dry Wgt (mg)
0	1.0	1.0	0.9	0.9	0.95	0.711	0.662	0.646	0.690	0.677
32	0.8	0.8	1.0	0.8	0.85	0.517	0.501	0.723	0.560	0.575
64	0.9	1.0	1.0	1.0	0.975	0.602	0.669	0.694	0.676	0.660
128	0.9	0.9	0.8	1.0	0.90	0.566	0.612	0.410	0.672	0.565
256	0.7	0.9	1.0	0.5	0.775	0.455	0.502	0.606	0.254	0.454
512	0.4	0.3	0.4	0.2	0.325	0.143	0.163	0.195	0.099	0.150

[1] Four replicates of 10 larvae each.

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

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

11.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 5 and 6. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50, EC50, and IC endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoints.

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

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

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

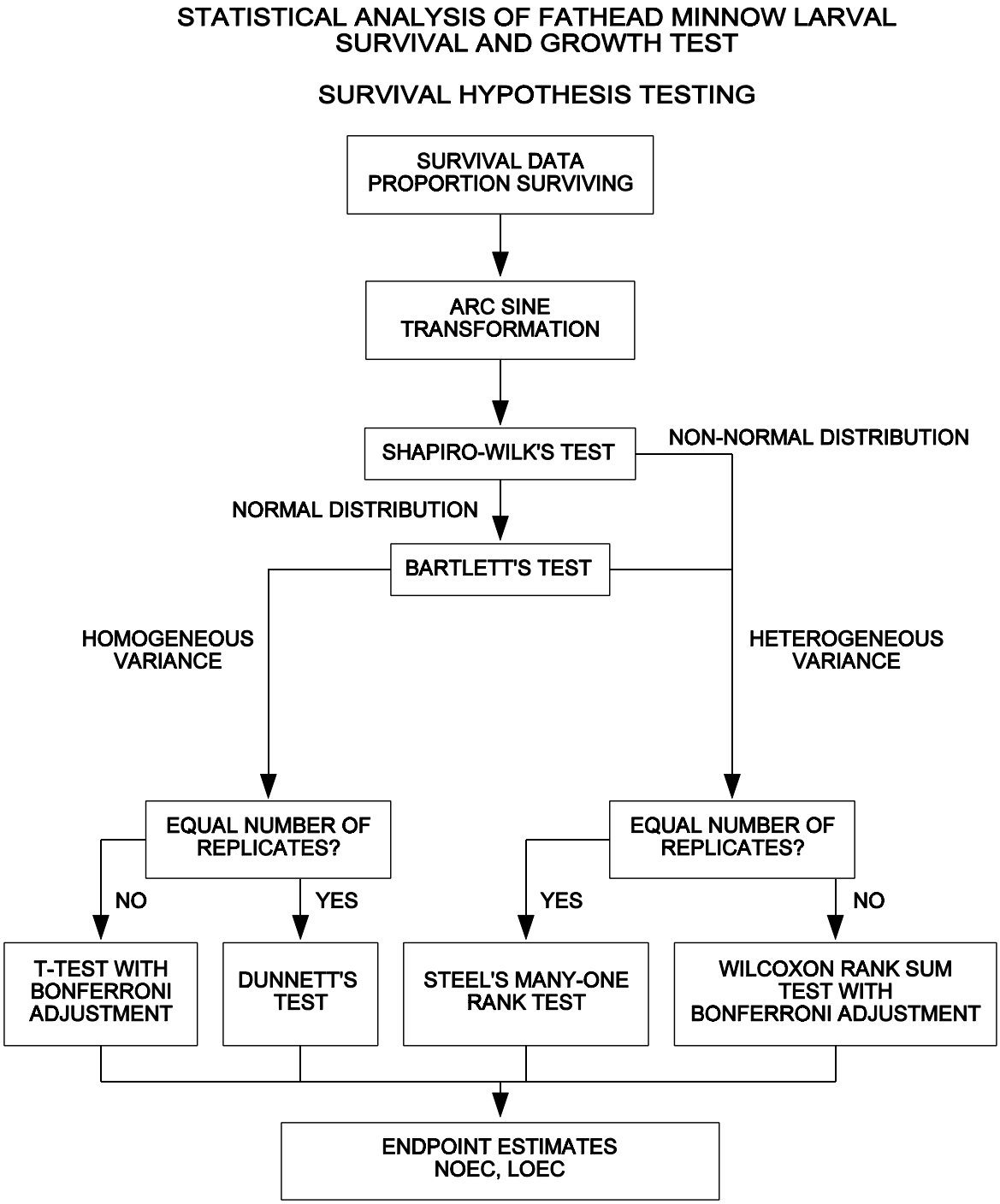

Figure 5. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas*, larval survival data by hypothesis testing.

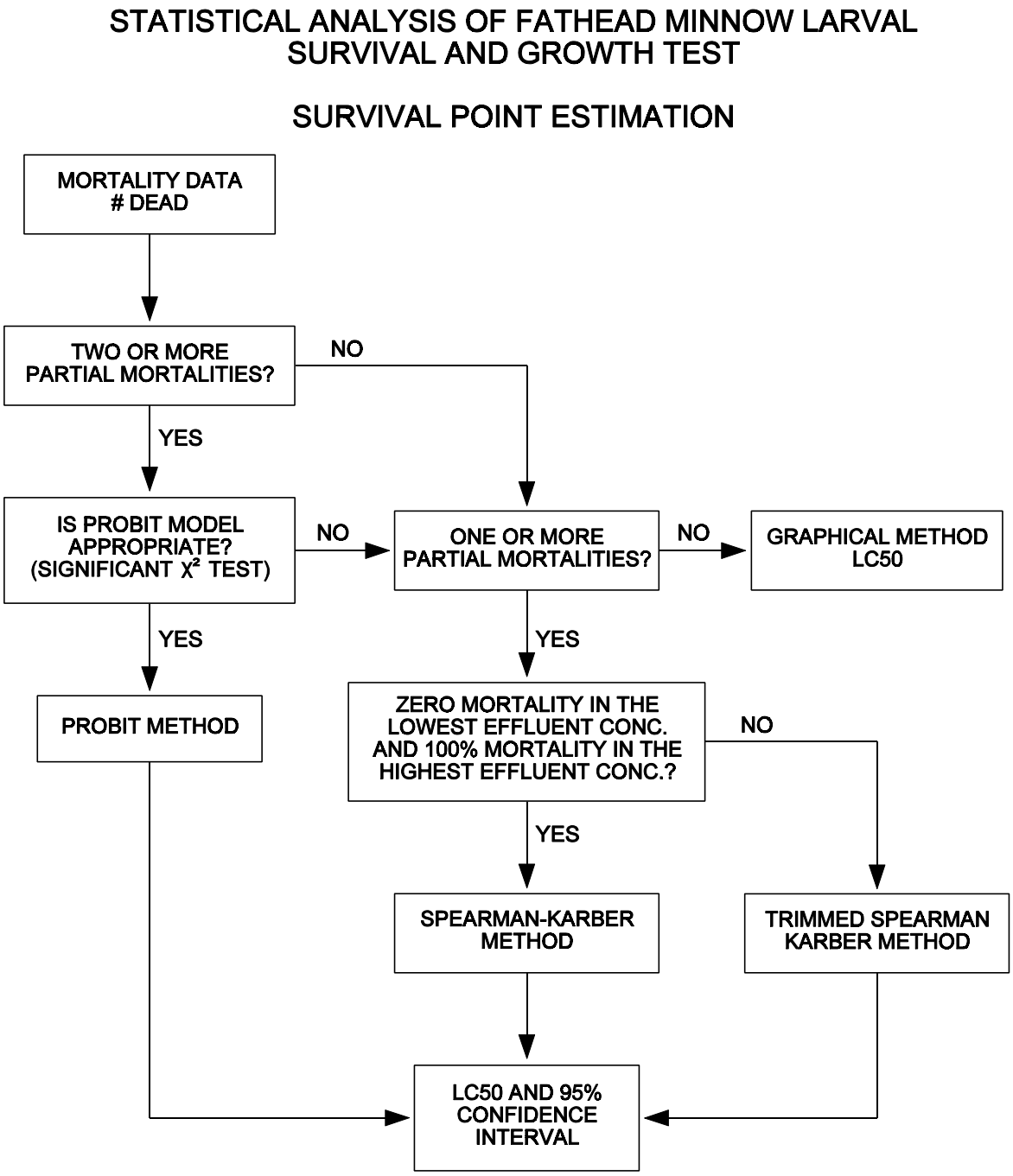

Figure 6. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas,* larval survival data by point estimation.

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Fathead Minnow Larval Survival and Growth Test (Table 2). The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each toxicant concentration and control are listed in Table 3. A plot of the survival proportions is provided in Figure 7.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

			NaPCP Concentration (µg/L)				
	Replicate	Control	32	64	128	256	512
RAW	A	1.0	0.8	0.9	0.9	0.7	0.4
	B	1.0	0.8	1.0	0.9	0.9	0.3
	C	0.9	1.0	1.0	0.8	1.0	0.4
	D	0.9	0.8	1.0	1.0	0.5	0.2
ARC SINE TRANS-FORMED	A	1.412	1.107	1.249	1.249	0.991	0.685
	B	1.412	1.107	1.412	1.249	1.249	0.580
	C	1.249	1.412	1.412	1.107	1.412	0.685
	D	1.249	1.107	1.412	1.412	0.785	0.464
Mean($\bar{Y}_i$)		1.330	1.183	1.371	1.254	1.109	0.604
S_i^2		0.0088	0.0232	0.0066	0.0155	0.0768	0.0111
i		1	2	3	4	5	6

11.13.2.6 Test for Normality

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

11.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

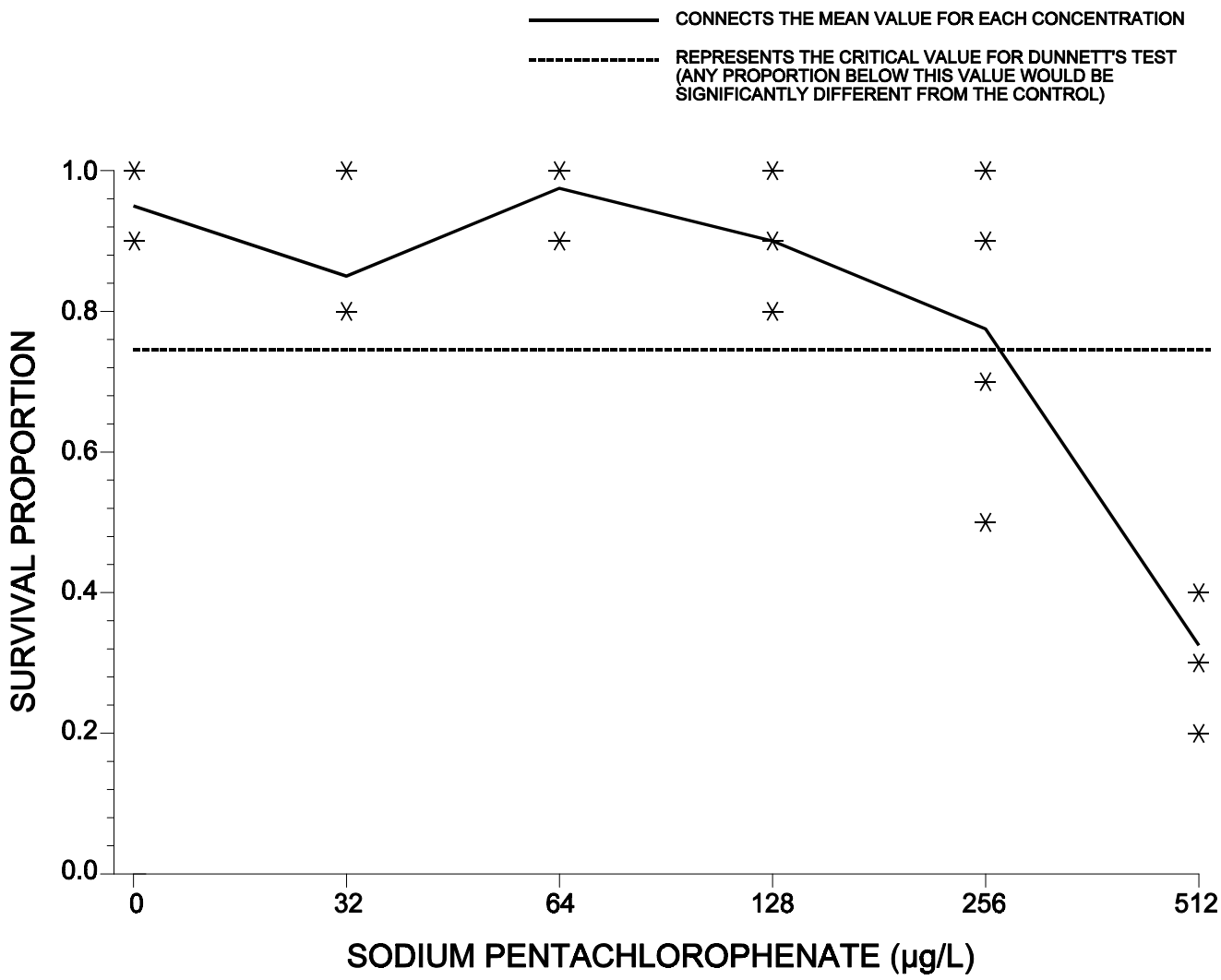

Figure 7. Plot of survival proportion data in Table 3.

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

Replicate	Control	NaPCP Concentration (µg/L) 32	64	128	256	512
A	0.082	-0.076	-0.122	-0.005	-0.118	0.081
B	0.082	-0.076	0.041	-0.005	0.140	-0.024
C	-0.081	0.229	0.041	-0.147	0.303	0.081
D	-0.081	-0.076	0.041	0.158	-0.324	-0.140

11.13.2.6.3 For this set of data: n = 24

$$\bar{X} = \frac{1}{24}(0.000) = 0.000$$

D = 0.4265

11.13.2.6.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.324	13	-0.005
2	-0.147	14	0.041
3	-0.140	15	0.041
4	-0.122	16	0.041
5	-0.118	17	0.081
6	-0.081	18	0.081
7	-0.081	19	0.082
8	-0.076	20	0.082
9	-0.076	21	0.140
10	-0.076	22	0.158
11	-0.024	23	0.229
12	-0.005	24	0.303

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 6.

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.627	$X^{(24)} - X^{(1)}$
2	0.3098	0.376	$X^{(23)} - X^{(2)}$
3	0.2554	0.298	$X^{(22)} - X^{(3)}$
4	0.2145	0.262	$X^{(21)} - X^{(4)}$
5	0.1807	0.200	$X^{(20)} - X^{(5)}$
6	0.1512	0.163	$X^{(19)} - X^{(6)}$
7	0.1245	0.162	$X^{(18)} - X^{(7)}$
8	0.0997	0.157	$X^{(17)} - X^{(8)}$
9	0.0764	0.117	$X^{(16)} - X^{(9)}$
10	0.0539	0.117	$X^{(15)} - X^{(10)}$
11	0.0321	0.065	$X^{(14)} - X^{(11)}$
12	0.0107	0.000	$X^{(13)} - X^{(12)}$

1.13.2.6.6 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 6. For the data in this example,

$$W = \frac{1}{0.4265}(0.6444)^2 = 0.974$$

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.974 is greater than the critical value, conclude that the data are normally distributed.

11.13.2.7 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i

ln = $\log_e$

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

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

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

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

11.13.2.7.3 Bartlett's statistic is therefore:

$$B = [(18)\ln(0.0236) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.1296$$

$$= [18(-3.7465) - 3(-24.7516)]/1.1296$$

$$= 6.8178/1.1296$$

$$= 6.036$$

11.13.2.7.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test (from a table of chi-square distribution), at a significance level of 0.01 with five degrees of freedom, is 15.086. Since B = 6.036 is less than the critical value of 15.086, conclude that the variances are not different.

11.13.2.8 Dunnett's Procedure

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

TABLE 7. ANOVA TABLE

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

Where: p = number toxicant concentrations 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 proportion surviving for toxicant concentration i in test chamber j)

11.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

N = 24

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 27.408$

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

$$= \frac{1}{4}(131.495) - \frac{(27.408)^2}{24} = 1.574$$

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

$$= 33.300 - \frac{(27.408)^2}{24} = 2.000$$

$SSW = SST - SSB = 2.000 - 1.574 = 0.4260$

$S_B^2 = SSB/(p-1) = 1.574/(6-1) = 0.3150$

$S_W^2 = SSW/(N-p) = 0.426/(24-6) = 0.024$

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

TABLE 8. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	1.574	0.315
Within	18	0.426	0.024
Total	23	2.002	

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

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

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

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

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.330 - 1.183)}{[0.155\sqrt{(1/4) + (1/4)}]} = 1.341$$

TABLE 9. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

11.13.2.8.6 Since the purpose of this test is to detect a significant reduction in proportion surviving, 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, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. The mean proportion surviving for concentration i is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Since t_6 is greater than 2.41, the 512 µg/L concentration has significantly lower survival than the control. Hence the NOEC and the LOEC for survival are 256 µg/L and 512 µg/L, respectively.

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

11.13.2.8.8 In this example:

$$MSD = 2.41(0.155)\sqrt{(1/4) + (1/4)}$$

$$= 2.41\ (0.155)(0.707)$$

$$= 0.264$$

11.13.2.8.9 The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.330 - 0.264 = 1.066

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

   $[\text{Sine}\ (1.330)]^2 = 0.943$

   $[\text{Sine}\ (1.066)]^2 = 0.766$

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

   $MSD_u = 0.943 - 0.766 = 0.177$

11.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any toxicant concentration that can be detected as statistically significant is 0.177.

11.13.2.8.11 This represents a decrease in survival of 19% from the control.

11.13.2.9 Calculation of the LC50

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

TABLE 10. DATA FOR PROBIT ANALYSIS

		NaPCP Concentration (µg/L)				
	Control	32	64	128	256	512
Number Dead	2	6	1	4	9	27
Number Exposed	40	40	40	40	40	40

11.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant, thus Probit Analysis appears appropriate for this data.

11.13.2.9.3 Figure 8 shows the output data for the Probit Analysis of the data in Table 10 using the USEPA Probit Program.

11.13.3 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

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

11.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these 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.

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

Probit Analysis of Fathead Minnow Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	2	0.0500	0.0000
32.0000	40	6	0.1500	0.0779
64.0000	40	1	0.0250	-.0577
128.0000	40	4	0.1000	0.0237
256.0000	40	9	0.2250	0.1593
512.0000	40	27	0.6750	0.6474

Chi - Square for Heterogeneity (calculated) = 4.522
Chi - Square for Heterogeneity
(Tabular value at 0.05 level) = 7.815

Probit Analysis of Fathead Minnow Larval Survival Data

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	127.637	34.590	195.433
LC/EC 50.00	422.696	345.730	531.024

Figure 8. Output for USEPA Probit Analysis Program, Version 1.5

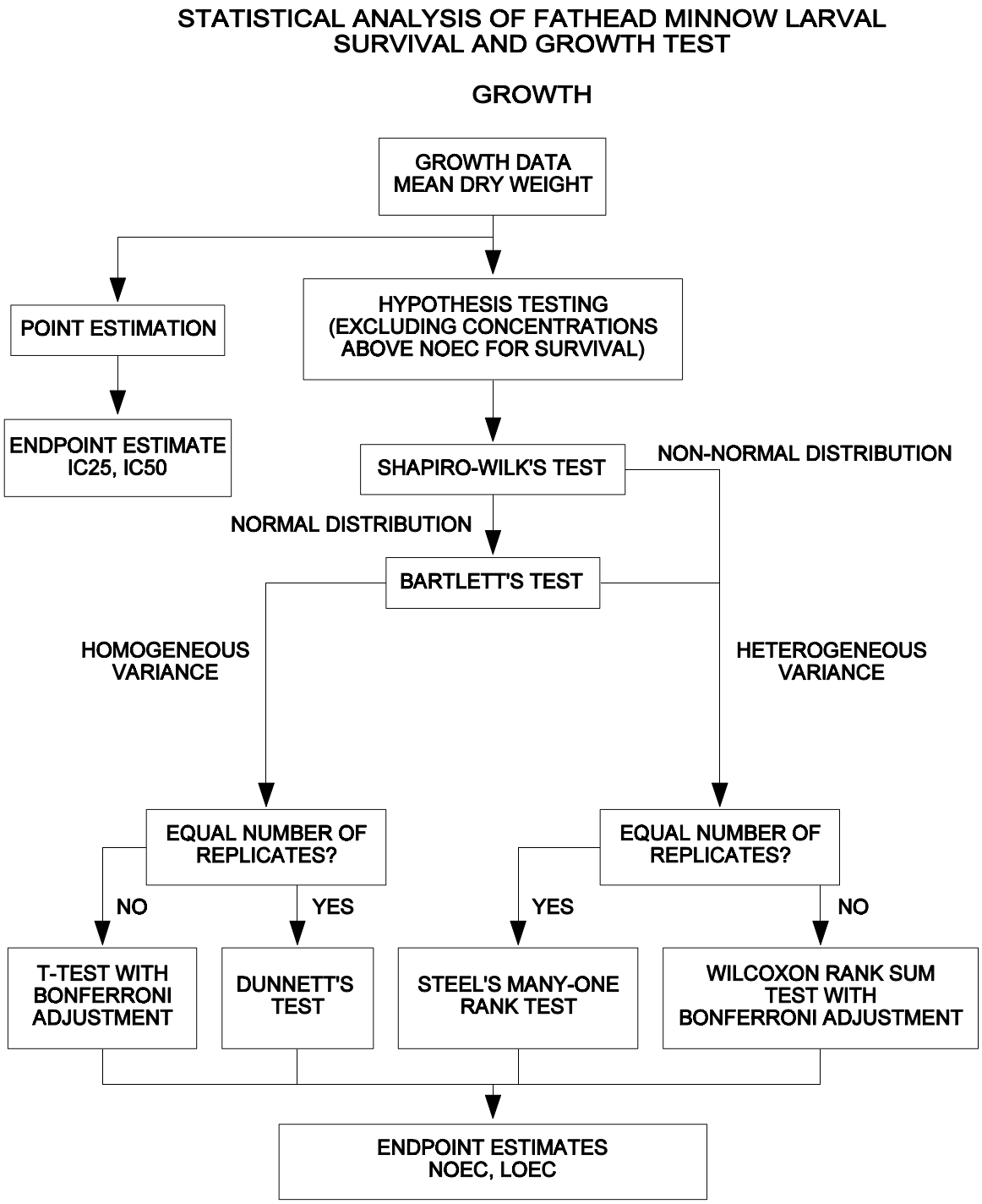

Figure 9. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas,* larval growth data.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 11. A plot of the weight data for each treatment is provided in Figure 10. Since there is significant mortality in the 512 µg/L concentration, its effect on growth is not considered.

TABLE 11. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

		NaPCP Concentration (µg/L)				
Replicate	Control	32	64	128	256	512
A	0.711	0.517	0.602	0.566	0.455	-
B	0.662	0.501	0.669	0.612	0.502	-
C	0.646	0.723	0.694	0.410	0.606	-
D	0.690	0.560	0.676	0.672	0.254	-
Mean($\bar{Y}_i$)	0.677	0.575	0.660	0.565	0.454	-
S_i^2	0.00084	0.01032	0.00162	0.01256	0.0218	-
i	1	2	3	4	5	6

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 12.

TABLE 12. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.034	-0.058	-0.058	0.001	0.001
B	-0.015	-0.074	0.009	0.047	0.048
C	-0.031	0.148	0.034	-0.155	0.152
D	0.013	-0.015	0.016	0.107	-0.200

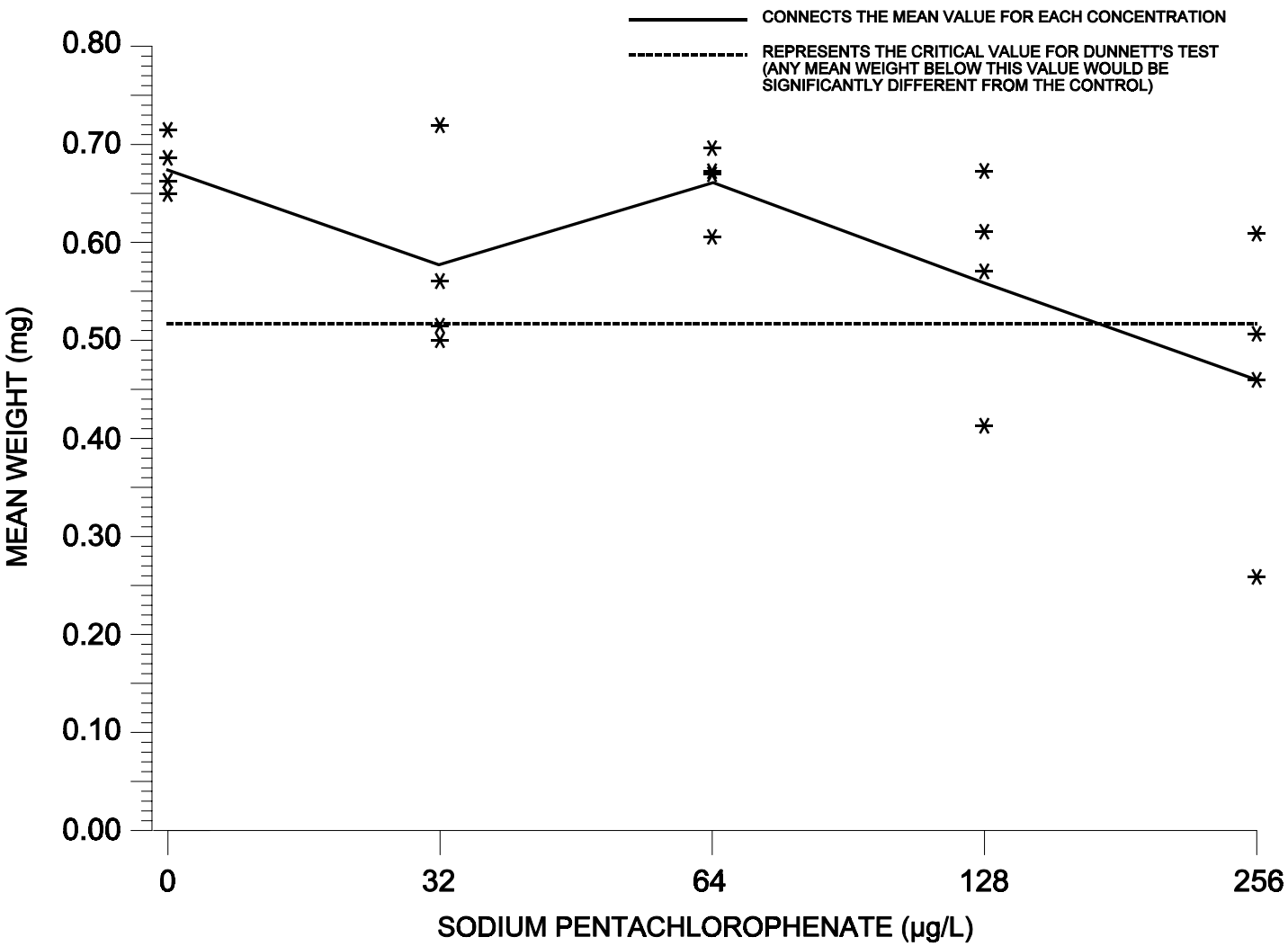

Figure 10. Plot of weight data from fathead minnow, *Pimephales promelas*, larval survival and growth test for point estimate testing.

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

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

Where: X_i = the ith centered observation

$\bar{X}$ = the <u>overall</u> mean of the centered observations

n = the total number of centered observations

For this set of data, n = 20

$\bar{X} = \frac{1}{20}(0.004) = 0.000$

D = 0.1414

11.13.3.5.3 Order the centered observations from smallest to largest

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

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 13.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.200	11	0.009
2	-0.155	12	0.013
3	-0.074	13	0.016
4	-0.058	14	0.034
5	-0.058	15	0.034
6	-0.031	16	0.047
7	-0.015	17	0.048
8	-0.015	18	0.107
9	0.001	19	0.148
10	0.001	20	0.152

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 14.

TABLE 14. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.352	$X^{(20)} - X^{(1)}$
2	0.3211	0.303	$X^{(19)} - X^{(2)}$
3	0.2565	0.181	$X^{(18)} - X^{(3)}$
4	0.2085	0.106	$X^{(17)} - X^{(4)}$
5	0.1686	0.105	$X^{(16)} - X^{(5)}$
6	0.1334	0.065	$X^{(15)} - X^{(6)}$
7	0.1013	0.049	$X^{(14)} - X^{(7)}$
8	0.0711	0.031	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.008	$X^{(11)} - X^{(10)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

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

the differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 14. For this set of data:

$$W = \frac{1}{0.1414}(0.3666)^2 = 0.9505$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 20 observations (n) is 0.868. Since W = 0.9505 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i.

ln = $\log_e$

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

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

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

11.13.3.6.2 For the data in this example, (see Table 11) all toxicant concentrations including the control have the same number of replicates (n_i = 4 for all i). Thus, V_i = 3 for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(15)\ln(0.00947) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.133$$

= [15(-5.9145) - 3(-26.2842]/1.133

= 8.8911/1.133

= 7.847

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.277. Since B = 7.847 is less than the critical value of 13.277, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 15.

TABLE 15. ANOVA TABLE

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

Where: p = number toxicant concentrations including the control

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

n_i = number of observations in concentration i

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

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

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

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

T_i = the total of the replicate measurements for concentration i

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

11.13.3.7.2 For the data in this example:

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

N = 20

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$

$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

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

$$= \frac{1}{4}(28.017) - \frac{(11.728)^2}{20} = 0.1270$$

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

$$= 7.146 - \frac{(11.728)^2}{20} = 0.2687$$

$$SSW = SST - SSB = 0.2687 - 0.1270 = 0.1417$$

$S_B^2 = SSB/(p-1) = 0.1270/(5-1) = 0.0318$

$S_W^2 = SSW/(N-p) = 0.041/(20-5) = 0.0094$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 16).

TABLE 16. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	0.1270	0.0318
Within	15	0.1417	0.0094
Total	19	0.2687	

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

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

Where: $\bar{Y}_i$ = mean dry weight for toxicant concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 17 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.677 - 0.575)}{[0.097\sqrt{(1/4) + (1/4)}]} = 1.487$$

TABLE 17. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.632
256	5	3.251

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 15 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.36. The mean weight for concentration "*i*" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_5 is greater than 2.36, the 256 µg/L concentration had significantly lower growth than the control. Hence the NOEC and the LOEC for growth are 128 µg/L and 256 µg/L, respectively.

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 2.36(0.052)\sqrt{(1/4) + (1/4)}$$

$$= 2.36\ (0.097)\ (0.707)$$

$$= 0.162$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.162 mg.

11.13.3.7.10 This represents a 24% reduction in mean weight from the control.

11.13.3.8 Calculation of the IC

11.13.3.8.1 The growth data in Table 2 modified to be mean weights per original number of fish are utilized in this example. As seen in Table 2 and Figure 11, the observed means are not monotonically non-increasing with respect to concentration (the mean response for each higher concentration is not less than or equal to the mean response for the previous concentration, and the responses between concentrations do not follow a linear trend). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

11.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.677$, we see that $\bar{Y}_1 > \bar{Y}_2$. Set $M_1 = \bar{Y}_1$ Comparing $\bar{Y}_2$ to $\bar{Y}_3$, $\bar{Y}_2 < \bar{Y}_3$.

11.13.3.8.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{Y}_2 + \bar{Y}_3)/2 = 0.618$$

11.13.3.8.4 For the remaining observed means, $M_3 > \bar{Y}_4 > \bar{Y}_5 > \bar{Y}_6$. Thus, M_4 becomes $\bar{Y}_4$, M_5 becomes $\bar{Y}_5$ etc., for the remaining concentrations. Table 18 contains the smoothed means, and Figure 11 provides a plot of the smoothed concentration response curve.

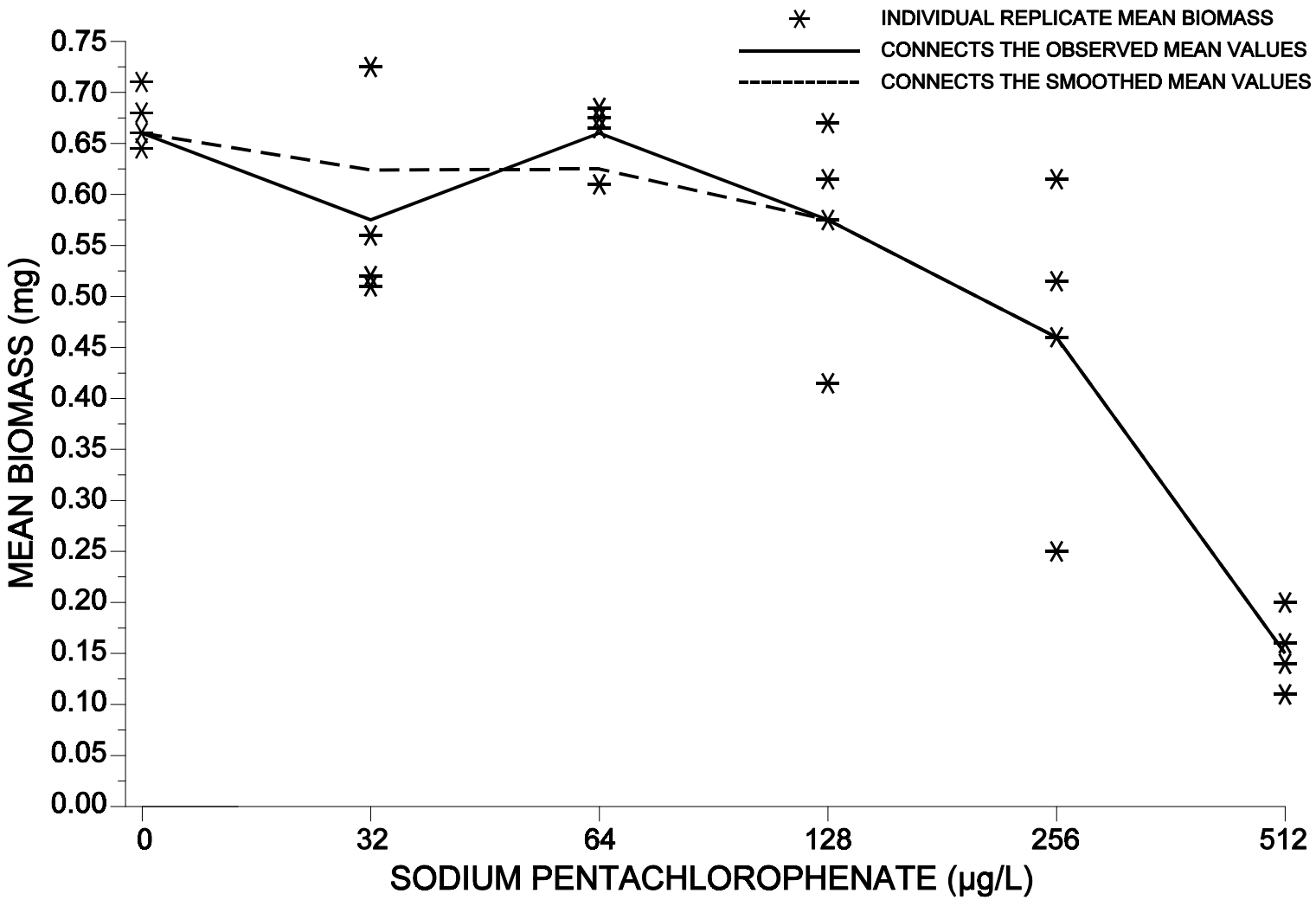

Figure 11. Plot of raw data, observed means, and smoothed means for the fathead minnow, *Pimephales promelas*, growth data in Tables 2 and 18.

TABLE 18. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

NaPCP Conc (µg/L)	i	Response means, $\bar{Y}_i$ (mg)	Smoothed means, M_i (mg)
Control	1	0.677	0.677
32	2	0.575	0.618
64	3	0.660	0.618
128	4	0.565	0.565
256	5	0.454	0.454
512	6	0.150	0.150

11.13.3.8.5 An IC25 and an IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.508 mg, where $M_1(1 - p/100) = 0.677(1 - 25/100)$. A 50% reduction in weight, compared to the controls, would result in a mean weight of 0.339 mg, where $M_1(1 - p/100) = 0.677(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 18), the response 0.508 mg is bracketed by C_4 = 128 µg/L and C_5 = 256 µg/L. For the 50% reduction (0.339 mg), the response (0.339 µg) is bracketed by C_5 = 256 µg/L and C_6 = 512 µg/L.

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

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

$$IC25 = 128 + [0.677(1 - 25/100) - 0.565]\frac{(256 - 128)}{(0.454 - 0.565)}$$

= 194 µg/L

11.13.3.8.7 Using the equation in Section 4.2 of Appendix M the estimate of the IC50 is calculated as follows:

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

$$IC50 = 256 + [0.677(1 - 50/100) - 0.454]\frac{(512 - 256)}{(0.150 - 0.454)}$$

= 353 µg/L

11.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 193.9503 µg/L. The empirical 95% confidence interval for the true mean was (54.9278 µg/L, 340.6617 µg/L). The computer program output for the IC25 for this data set is shown in Figure 12.

11.13.3.8.9 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 353.2884 µg/L. The empirical 95% confidence interval for the true mean was 208.4723 µg/L and 418.5276 µg/L. The computer program output is shown in Figure 13.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 193.9503 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 186.4935 Standard Deviation: 52.6094
Original Confidence Limits: Lower: 107.0613 Upper: 285.6449
Expanded Confidence Limits: Lower: 54.9278 Upper: 340.6617
Resampling time in Seconds: 1.81 Random Seed: 1272173518

Figure 12. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 353.2884 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 345.1108 Standard Deviation: 37.0938
Original Confidence Limits: Lower: 262.7783 Upper: 394.0629
Expanded Confidence Limits: Lower: 208.4723 Upper: 418.5276
Resampling time in Seconds: 1.87 Random Seed: 1126354766

Figure 13. ICPIN program output for the IC50.

11.14 PRECISION AND ACCURACY

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

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Information on the single-laboratory precision of the fathead minnow larval survival and growth test is presented in Table 19. The range of NOECs was only two concentration intervals, indicating good precision.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 19 laboratories (USEPA, 2000b). The database consisted of 205 reference toxicant tests conducted in 19 laboratories using a variety of reference toxicants including: cadmium, chromium, copper, potassium chloride, sodium chloride, sodium pentachlorophenate, and sodium dodecyl sulfate. Among the 19 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 21%; and in 75% of laboratories, the within-laboratory CV was less than 38%.

TABLE 19. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (µg/L)	LOEC (µg/L)	Chronic Value (µg/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181
n:	5	5	5
Mean:	NA	NA	253.4

[1] From Pickering, 1988.
[2] For a discussion of the precision of data from chronic toxicity tests, (see Section 4, Quality Assurance).

11.14.1.2 Multilaboratory Precision

11.14.1.2.1 A multilaboratory study of Method 1000.0 described in the first edition of this manual (USEPA, 1985e), was performed using seven blind samples over an eight month period (DeGraeve et. al., 1988). In this study, each of the 10 participating laboratories was to conduct two tests simultaneous with each sample, each test having two replicates of 10 larvae for each of five concentrations and the control. Of the 140 tests planned, 135 were completed. Only nine of the 135 tests failed to meet the acceptance criterion of 80% survival in the controls. Of the 126 acceptable survival NOECs reported, an average of 41% were median values, and 89% were within one concentration interval of the median (Table 20). For the growth (weight) NOECs, an average of 32% were at the median, and 84% were within one concentration interval of the median (Table 21). Using point estimate techniques, the precision (CV) of the IC50 was 19.5% for the survival data and 19.8% for the growth data. If the mean weight acceptance criterion of 0.25 mg for the surviving control larvae, which is included in this revised edition of the method, had applied to the test results of the interlaboratory study, one third of the 135 tests would have failed to meet the test criteria (Norberg-King, personal communication and 1989 memorandum; DeGraeve et al., 1991).

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 27 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 101 Fathead Minnow Larval Survival and Growth tests conducted in this study, 98.0% were successfully completed and met the required test acceptability criteria. Of 24 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 4.35%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 22 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 20.9% for IC25 results. Table 23 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 97.2%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type and four concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 86.1%, 91.7%, and 76.9% for the reference toxicant, effluent, and receiving water sample types, respectively.

11.14.2 ACCURACY

11.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 20. COMBINED FREQUENCY DISTRIBUTION FOR SURVIVAL NOECs FOR ALL LABORATORIES[1]

Sample	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	35	53	12	57	29	14
2. Sodium Pentachlorophenate (B)	42	42	16	56	44	0
3. Potassium Dichromate (A)	47	47	6	75	25	0
4. Potassium Dichromate (B)	41	41	18	50	50	0
5. Refinery Effluent 301	26	68	6	78	22	0
6. Refinery Effluent 401	37	53	10	56	44	0
7. Utility Waste 501	56	33	11	56	33	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

TABLE 21. COMBINED FREQUENCY DISTRIBUTION FOR WEIGHT NOECs FOR ALL LABORATORIES[1]

	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
Sample	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	59	41	0	57	43	0
2. Sodium Pentachlorophenate (B)	37	63	0	22	45	33
3. Potassium Dichromate (A)	35	47	18	88	0	12
4. Potassium Dichromate (B)	12	47	41	63	25	12
5. Refinery Effluent 301	35	53	12	75	25	0
6. Refinery Effluent 401	37	47	16	33	56	11
7. Utility Waste 501	11	61	28	33	56	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

TABLE 22. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.0	17.2	19.9
	Effluent	19.1	12.9	23.1
	Receiving water	-	-	19.8
	Average	14.6	15.0	20.9

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

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

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

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

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

TABLE 23. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	50%	75.0	22.2	2.78
	Effluent	12.5%	76.9	23.1	0.00
	Receiving water	25%	69.2	30.8	0.00
Growth NOEC	Reference toxicant	50%	58.3	27.8	13.9
	Effluent	12.5%	66.7	25.0	8.33
	Receiving water	12.5%	30.8	46.1	23.1

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.