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

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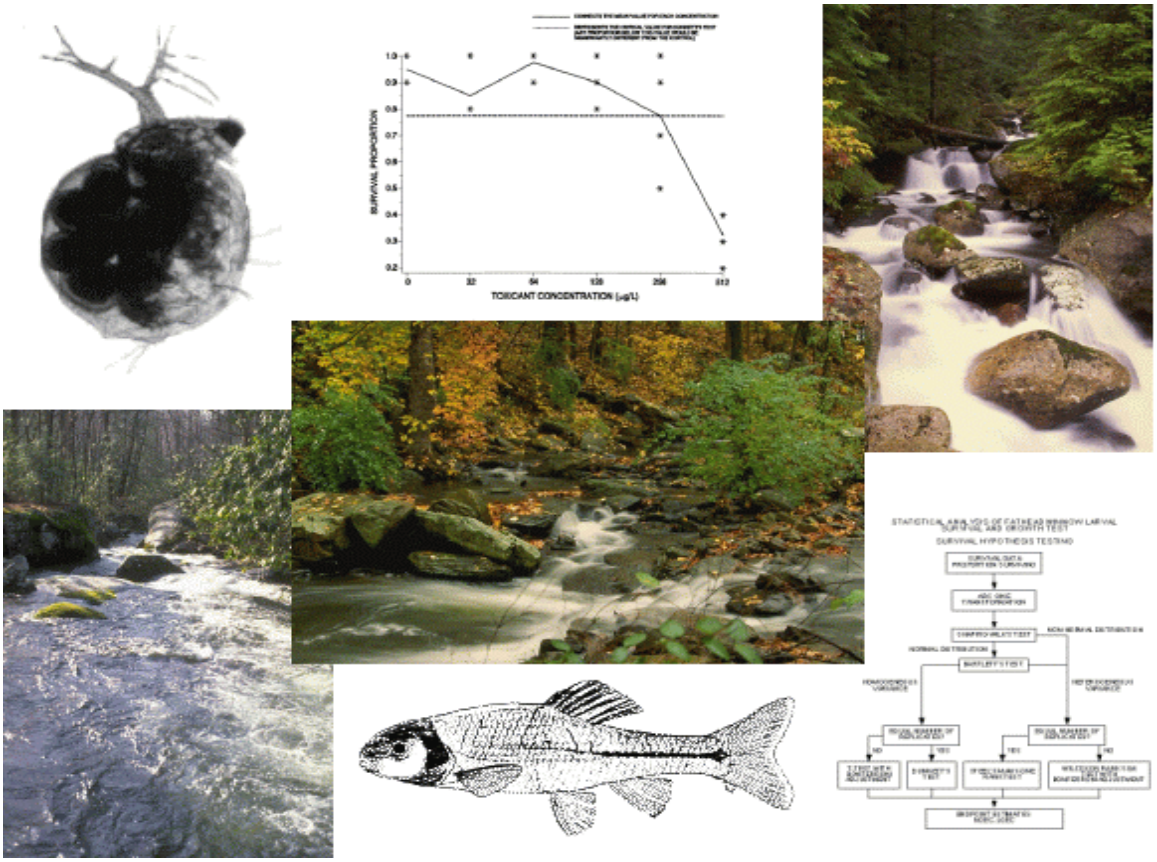

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SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50, NOEC, IC50 or IC25 (see Section 9, Chronic Toxicity Endpoints and Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; and USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 2002a), the short-term chronic toxicity test methods for marine and estuarine organisms (USEPA, 2002b), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991c). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1.6 These freshwater short-term toxicity tests are similar to those developed for marine and estuarine organisms to evaluate the toxicity of effluents discharged to marine and estuarine waters under the NPDES permit program. Methods are presented in this manual for three species of freshwater organisms from three phylogenetic groups. The methods are all static renewal type seven-day tests except the green alga, *Selenastrum capricornutum*, test which lasts four days.

1.7 The three species for which test methods are provided are the fathead minnow, *Pimephales promelas*; the daphnid, *Ceriodaphnia dubia*; and the green alga, *Selenastrum capricornutum*.

1.7.1 Two of the methods incorporate the chronic endpoint of growth in addition to lethality and one incorporates reproduction. The fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test incorporates teratogenic effects in addition to lethality. The green alga, *Selenastrum capricornutum*, growth test has the advantage of a relatively short exposure period (96 h).

1.8 The validity of the freshwater chronic methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1984; USEPA, 1985b; USEPA, 1985c; USEPA, 1985d; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d; Birge et al., 1989; and Eagleson et al., 1990).

1.9 The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of aquatic toxicity tests and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life-cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady-state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life-cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life-cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life-cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life-stages were the most sensitive stages. He proposed the use of partial life-cycle toxicity tests with the early life-stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life-stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life-cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry". Critical life-stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30 - 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In

1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect, and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, OH (USEPA, 1984), and at many other locations. Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 Mount and Norberg (1984) developed a seven-day cladoceran partial life-cycle test and experimented with a number of diets for use in culturing and testing the daphnid, *Ceriodaphnia reticulata* (Norberg and Mount, 1985). As different laboratories began to use this cladoceran test, it was discovered that apparently more than one species was involved in the tests conducted by the same laboratory. Berner (1986) studied the problem and determined that perhaps as many as three variant forms were involved and it was decided to recommend the use of the more common *Ceriodaphnia dubia* rather than the originally reported *Ceriodaphnia reticulata*. The method was adopted for use in the first edition of the freshwater short-term chronic methods (USEPA, 1985e).

2.1.15 The green alga, *Selenastrum capricornutum*, bottle test was developed, after extensive design, evaluation, and application, for the National Eutrophication Research Program (USEPA, 1971). The test was later modified for use in the assessment of receiving waters and the effects of wastes originating from industrial, municipal, and agricultural point and non-point sources (USEPA, 1978a).

2.1.16 The use of short-term toxicity tests including subchronic and chronic tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 21-day daphnid, *Daphnia magna*, life-cycle test.

2.2 **TYPES OF TESTS**

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the hatchability, gross morphological abnormalities, survival, growth, and/or reproduction within the prescribed period of time (four to seven days). The results of the tests are expressed in terms of the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which static type of test is to be conducted.

2.3 **STATIC TESTS**

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 **ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES**

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management. This program should include (1) a safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each of the laboratory staff, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel will determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks, and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone or other means to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers are to be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 **DISEASE PREVENTION**

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 **SAFETY MANUALS**

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e) and Walters and Jameson (1984).

3.6 **WASTE DISPOSAL**

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in performing the toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991a) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) prepare a quality assurance plan with stated data quality objectives (DQOs); (3) prepare a written description of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc.; and (4) provide an adequate, qualified technical staff for culturing and testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity test data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory evaluation related to toxicity testing, see FDA, (1978); USEPA, (1979d), USEPA (1980b), USEPA (1980c), and USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*. The fish and invertebrates should appear healthy, behave normally, feed well, and have low mortality in the cultures, during holding, and in test controls. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. For tests performed to meet NPDES objectives, synthetic, moderately hard water should be used.

The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section 5, Facilities, Equipment and Supplies. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 mg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 **EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING**

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 **TEST CONDITIONS**

4.6.1 Water temperature should be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one test vessel for the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH should be checked at the beginning of each test and daily throughout the test period.

4.7 **QUALITY OF TEST ORGANISMS**

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.15) and to document ongoing laboratory performance (Subsection 4.16).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.16). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicant and control conditions (see Section 6, Test Organisms).

4.7.4 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If routine reference toxicant tests fail to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 **FOOD QUALITY**

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts, and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 mg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 **ACCEPTABILITY OF SHORT-TERM CHRONIC TOXICITY TESTS**

4.9.1 For the tests to be acceptable, control survival in fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*, tests must be 80% or greater. At the end of the test, the average dry weight of surviving seven-day-old fathead minnows in control chambers must equal or exceed 0.25 mg. In *Ceriodaphnia dubia* controls, 60% or more of the surviving females must have produced their third brood in 7 ± 1 days, and the number of young per surviving female must be 15 or greater. In algal toxicity tests, the mean cell density in the controls after 96 h must equal or exceed 1×10^6 cells/mL and not vary more than 20% among replicates. If these criteria are not met, the test must be repeated.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from the test.

4.10 **ANALYTICAL METHODS**

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, and conductivity, must be calibrated and standardized according to instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity and total residual chlorine must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; and (5) the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 TEST PRECISION

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions and employing a known toxicant, such as a reference toxicant.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests with two species using the reference toxicants potassium chloride and copper sulfate are shown in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). The effluent sample was a municipal wastewater spiked with KCl, the receiving waster sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

TABLE 1. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING A REFERENCE TOXICANT[1]

Organism	Endpoint	No. Labs	% Effluent[2]	SD	CV(%)
Pimephales promelas	Survival, NOEC	146	NA	NA	NA
	Growth, IC25	124	4.67	1.87	40.0
	Growth, IC50	117	6.36	2.04	32.1
	Growth, NOEC	142	NA	NA	NA
Ceriodaphnia dubia	Survival, NOEC	162	NA	NA	NA
	Reproduction, IC25	155	2.69	1.96	72.9
	Reproduction, IC50	150	3.99	2.35	58.9
	Reproduction, NOEC	156	NA	NA	NA

[1] From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory- Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring.

[2] Expressed as % effluent; in reality it was a reference toxicant (KCl) but was not known by the persons conducting the tests.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1].

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Pimephales promelas	Growth, IC25	73	20.9
Ceriodaphnia dubia	Reproduction, IC25	34	35.0
Selenastrum capricornutum (with EDTA)	Growth, IC25	21	34.3
	Growth, IC50	22	32.2
Selenastrum capricornutum (without EDTA)	Growth, IC25	21	58.5
	Growth, IC50	22	58.5

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

2 Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

3 CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Control (see pp. 2-4, and 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis) precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, NOEC ± (NOEC minus LOEC).

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. As a result of the variability of different dilution factors, **USEPA recommends the use of the dilution factor of 0.5 or greater**. Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature

control; and feeding.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in the laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and the same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The chart should plot logarithm of concentration on the vertical axis against the date of the test or test number on the horizontal axis. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean ($\bar{X}$) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive test result. Endpoints from hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.16.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability. Note: Because NOECs can only be a fixed number of discrete values, the mean, standard deviation, and CV cannot be interpreted and applied in the same way that these descriptive statistics are interpreted and applied for continuous variables such as the IC25 or LC50.

4.16.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results

must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.16.5 If the toxicity value from a given test with a reference toxicant falls well outside the expected range for the other test organisms when using the standard dilution water and other test conditions, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of ± 2S will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls "well" outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.16.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

4.17 **REFERENCE TOXICANTS**

4.17.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release USEPA-certified solutions of cadmium and copper for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

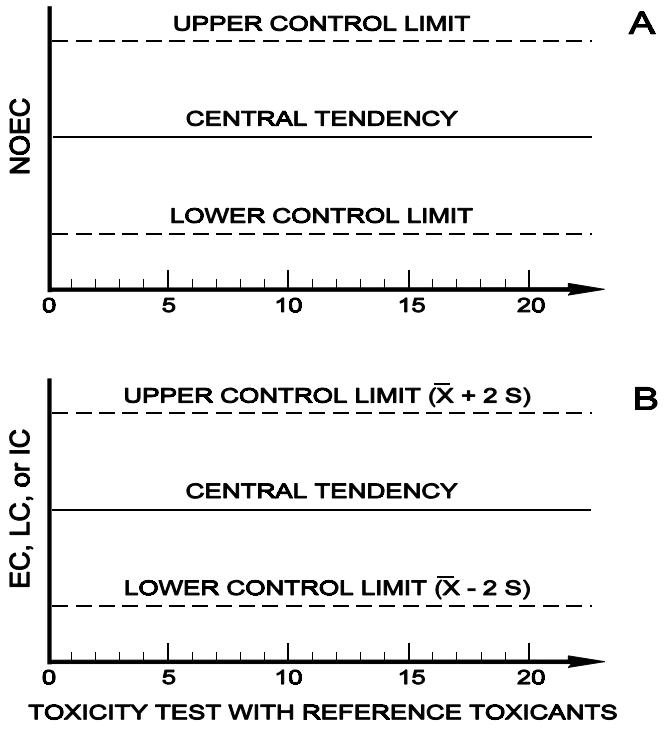

Figure 1. Control charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \frac{(\sum_{i=1}^{n} X_i)^2}{n}}{n-1}}$$

Where: X_i = Successive toxicity values from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean toxicity value.

S = Standard deviation.

4.18 **RECORD KEEPING**

4.18.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, receiving water, dechlorinated tap water, or reconstituted synthetic water. Dechlorination can be accomplished by carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce l.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters, and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free from fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample holding areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity test areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely- fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters, such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used for test chambers. The use of large ($\geq$ 20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment and Supplies, Subsection 5.3.2). Fiberglass and stainless steel, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 **TEST CHAMBERS**

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 **CLEANING TEST CHAMBERS AND LABORATORY APPARATUS**

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.3.2 All non-disposable sample containers, test vessels, tanks, and other equipment that have come in contact with effluent must be washed after use to remove contaminants as described below.

1. Soak 15 min in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 Special requirements for cleaning glassware used in the green alga, *Selenastrum capricornutum*, toxicity tests (Method 1003.0, Section 14). Prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal assays as follows:

1. Wash with non-phosphate detergent solution, preferably heated to $\geq$ 50°C. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher (under-counter type) is highly recommended.
2. Rinse with tap water.
3. Test flasks should be thoroughly rinsed with acetone and a 10% solution (by volume) of reagent grade hydrochloric acid (HCl). It may be advantageous to soak the flasks in 10% HCl for several days. Fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.
4. Rinse twice with MILLIPORE® MILLI-Q® OR QPAK™$_2$, or equivalent, water.
5. New test flasks, and all flasks which through use may become contaminated with toxic organic substances, must be rinsed with pesticide-grade acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, go to 7. If acetone is used, go to 6.
6. Rinse thoroughly with MILLIPORE® MILLI-Q® or QPAK™$_2$, or equivalent water, and dry in an 105°C oven. All glassware should be autoclaved before use and between uses.
7. Cover the mouth of each chamber with aluminum foil or other closure, as appropriate, before storing.

5.3.4 The use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

5.3.5 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.4 **APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS**

5.4.1 Apparatus and equipment requirements for culturing and testing are specified in each toxicity test method. Also, see USEPA, 2002a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

5.5 **REAGENTS AND CONSUMABLE MATERIALS**

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine shrimp, *Artemia* sp., cysts -- Many commercial sources of brine shrimp cysts are available.
2. Frozen adult brine shrimp, *Artemia* -- Available from most pet supply shops or other commercial sources.
3. Flake fish food -- TETRAMIN® and BIORIL® are available from most pet shops.
4. Trout chow -- Available from commercial sources.
5. Cereal leaves, CEROPHYLL® or equivalent -- Available from commercial sources.
6. Yeast -- Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store or commercial sources.
7. Alfalfa Rabbit Pellets -- Available from feed stores as Purina rabbit chow.
8. Algae - Available from commercial sources.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method section. Also, see Section 4, Quality Assurance.

5.6 **TEST ORGANISMS**

5.6.1 Test organisms should be obtained from inhouse cultures or from commercial suppliers (see specific test method; Section 4, Quality Assurance; and Section 6, Test Organisms).

5.7 **SUPPLIES**

5.7.1 See test methods (see Sections 11-14) for specific supplies.

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in the toxicity tests must be identified to species. If there is any doubt as to the identity of the test organism, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual also, see USEPA, 2002a.

6.1.3 The organisms used in the short-term chronic toxicity tests described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia* (Berner, 1986), and the green alga, *Selenastrum capricornutum*.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive, or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species in Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or the use of recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and the one or more recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥ 1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 The test organisms recommended in this manual can be cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections. The fathead minnow, *Pimephales promelas*, culture method is given in Section 11 and not repeated in Section 12. Also, see USEPA (2002a).

6.2.2 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms or starter cultures should be purchased from experienced commercial suppliers (see USEPA, 2002a).

6.2.3 Starter cultures of the green algae, *Selenastrum capricornutum, S. minutum,* and *Chlamydomonas reinhardti* are available from commercial suppliers.

6.2.4 Because the daphnid, *Ceriodaphnia dubia*, must be cultured individually in the laboratory for at least seven days before the test begins, it will be necessary to obtain a starter culture from a commercial source at least three weeks before the test is to begin if they are not being cultured inhouse.

6.2.5 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For detailed guidance on identification, see the individual test methods.

6.2.6 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.6.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be a logical approach. However, it is generally impractical and not recommended for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collecting permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. The fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*, are easily cultured in the laboratory or readily available commercially.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of the test organism be known to species level, it would be necessary to examine each organism caught in the wild to confirm its identity. This would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.6.2 Guidelines for collecting natural occurring organisms are provided in USEPA (1973), USEPA (1990) and USEPA (1993b).

6.2.7 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms can be obtained from commercial stock certified as "disease-free".

6.3 **LIFE STAGE**

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as larval fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 **LABORATORY CULTURING**

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in the respective test methods (also, see USEPA, 2002a).

6.5 **HOLDING AND HANDLING TEST ORGANISMS**

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature in any 12 h period or 2 units of pH in any 24-h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch a dry surface or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk batting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as PROPIPETTE®) should be used for transferring smaller organisms such as larval fish.

6.5.3 Holding tanks for fish are supplied with good quality water (see Section 5, Facilities, Equipment, and Supplies) with flow-through rate of at least two tank volumes per day. Otherwise use a recirculation system where water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding must be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The solution of oxygen depends on temperature and altitude. The DO must be maintained at a minimum of 4.0 mg/L. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.6 Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia*, or frozen adult brine shrimp, or dry food (frozen food should be completely thawed before use). Adult brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 **TRANSPORTATION TO THE TEST SITE**

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags or by use of an airstone supplied by a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, the organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is to be used as the dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially

to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms may be transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if the receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.7 **TEST ORGANISM DISPOSAL**

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study.

7.1.1.1 If the objective of the test is to estimate the absolute chronic toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness, alkalinity, and conductivity) as the receiving water. Seasonal variations in the quality of receiving waters may affect effluent toxicity. Therefore, the pH, alkalinity, hardness, and conductivity of receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 3 and 4). The source water for the deionizer can be ground water or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or

equivalent system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges (see Section 5, Facilities, Equipment, and Supplies).

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent) followed by a final bacteria filter. The QPAK™ 2 water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 3 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \cdot 2H_20$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia dubia* culturing and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 mg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 3.

TABLE 3. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

Water Type	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

1 Taken in part from Marking and Dawson (1973).
2 Add reagent grade chemicals to deionized water.
3 Approximate equilibrium pH after 24 h of aeration.
4 Expressed as mg $CaCO_3$/L.

7.2.3.2 If large volumes of synthetic reconstituted water will be needed, it may be advisable to mix 1 L portions of concentrated stock solutions of $NaHCO_3$, $MgSO_4$, and KCl for use in preparation of the reconstituted waters.

7.2.3.3 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using mineral water such as PERRIER® Water, or equivalent (Table 4), follow the instructions below.

1. Place 16 L of MILLI-Q® or equivalent water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness and alkalinity of the aerated water will be as indicated in Table 4.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

TABLE 4. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Approximate Final Water Quality pH[3]	Hardness[4]	Alka-linity[4]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	57-64
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[5]	---	---	---	---	---

[1] From Mount et al. (1987), and data provided by Philip Lewis, EMSL-Cincinnati, OH.
[2] Add mineral water to Milli-Q® water, or equivalent, to prepare Diluted Mineral Water (DMW).
[3] Approximate equilibrium pH after 24 h of aeration.
[4] Expressed as mg $CaCO_3$/L.
[5] Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

7.3 **USE OF RECEIVING WATER AS DILUTION WATER**

7.3.1 If the objectives of the test require the use of uncontaminated receiving water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water upstream of, or close to, but outside of the zone influenced by the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow through tests, the sample should be chilled to 0-6°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 Receiving water containing debris or indigenous organisms that may be confused with or attack the test organisms should be filtered through a sieve having 60 mm mesh openings prior to use.

7.3.4 Where toxicity-free dilution water is required in a test, the water is considered acceptable if test organisms show the required survival, growth, and reproduction in the controls during the test.

7.3.5 The regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated receiving water with a suitable hardness, or adjusting the hardness of an otherwise suitable receiving water by addition of reagents as indicated in Table 3.

7.4 **USE OF TAP WATER AS DILUTION WATER**

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and passed through a deionizer and carbon filter. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 **DILUTION WATER HOLDING**

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, 1988a). Conditions for exception would be: (l) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below (also see USEPA, 2002a).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 GRAB SAMPLES

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling and the probability of missing a spike is high.

8.2.1.2 COMPOSITE SAMPLES

Advantages:

1. A single effluent sample is collected over a 24-h period.

2. The sample is collected over a much longer period of time than a single grab sample and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 **EFFLUENT SAMPLING RECOMMENDATIONS**

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples, except for the green alga, *Selenastrum capricornutum*, test which is not renewed.

8.3.2 When tests are conducted off-site, a minimum of three samples are collected. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Day 2. The second sample would be used for test solution renewal on Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6, and 7.

8.3.3 Sufficient sample volume must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests.

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

8.3.4.1.1 If the facility discharge is continuous, a single 24-h composite sample is to be taken.

8.3.4.2 Intermittent discharges

8.3.4.2.1 If the facility discharge is intermittent, a composite sample is to be collected for the duration of the discharge but not more than 24 hours.

8.4 **RECEIVING WATER SAMPLING**

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, based on the requirements of the test, a single grab sample or daily grab sample of receiving water is collected for use in the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, samples should be collected from mid-stream and at mid-depth, if accessible. In lakes the samples are collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 **EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING**

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), they should be chilled and maintained at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e) should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h, 48 h, and/or 72 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. If shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the permitting authority may allow the continued use of the most recently used sample for test renewal. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (l-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 **SAMPLE RECEIVING**

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h unless a variance has been granted by the NPDES permitting authority.

8.7 **PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING**

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (> 36 h, but ≤ 72 h) is requested by a permittee, (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of samples must be obtained by comparing the results of multi-concentration chronic toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test should be held under the same conditions as during shipment and holding.

8.8 **PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS**

8.8.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® is used), or by using an appropriate discharge valve (spigot).

8.8.2 With the daphnid, *Ceriodaphnia dubia*, and fathead minnow, *Pimephales promelas*, tests, effluents and receiving waters should be filtered through a 60-µm plankton net to remove indigenous organisms that may attack or be confused with the test organisms (see the daphnid, *Ceriodaphnia dubia*, test method for details). Receiving waters used in green alga, *Selenastrum capricornutum*, toxicity tests must be filtered through a 0.45-µm pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. Because filtration may increase the dissolved oxygen (DO) in the effluent, the DO should be checked both before and after filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution:** filtration may remove some toxicity.

8.8.3 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature, samples should be aerated moderately (approximately 500 mL/min) for a few minutes using an airstone. If DO is below 4.0 mg/L after warming to test temperature, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range (≥4.0 mg/L). **Caution:** avoid excessive aeration.

8.8.4 The DO concentration in the samples should be near saturation prior to use. Aeration may be used to bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

8.8.4.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase their toxicity by altering pH. However, the DO in the test solutions should not be allowed to fall below 4.0 mg/L.

8.8.4.2 In static tests (renewal or non-renewal), low DOs may commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at a rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

8.8.5 At a minimum, pH, conductivity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

8.8.5.1 It is recommended that total alkalinity and total hardness also be measured in the undiluted effluent test water, receiving water, and the dilution water.

8.8.6 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by un-ionized ammonia (i.e., where total ammonia $\geq$ 5 mg/L). The concentration (mg/L) of un-ionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 5, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

8.8.7 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (APHA, 1992). Note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water (see Section 7, Dilution Water, Subsection 7.4.1). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

8.8.8 Mortality or impairment of growth or reproduction due to pH alone may occur if the pH of the sample falls outside the range of 6.0 - 9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0 by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

TABLE 5. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26°C AND pH 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1] Table provided by Teresa Norberg-King, ERL, Duluth, Minnesota. Also see Emerson et al. (1975), Thurston et al. (1974), and USEPA (1985a).

8.9 **PRELIMINARY TOXICITY RANGE-FINDING TESTS**

8.9.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, chronic, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

8.9.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as

100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (see Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

8.9.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up chronic definitive test because: (1) the definitive test is longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

8.10 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

8.10.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of an IC25, IC50, or LC50, or (2) a no-observed-effect-concentration (NOEC) defined in terms of mortality, growth, reproduction, and/or teratogenicity and obtained by hypothesis testing. The tests may be static renewal or static non-renewal.

8.10.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a $\geq$0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

8.10.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration. This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

8.10.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., pH, temperature, water hardness, salinity, etc.

8.11 RECEIVING WATER TESTS

8.11.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness of the control should be comparable to the receiving water.

8.11.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. Four replicates and 10 organisms per replicate are required for each treatment (see Summary of Test Conditions and Test Acceptability Criteria in the specific test method).

8.11.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a multi-concentration test is performed by preparing dilutions of the receiving water, using a $\geq$ 0.5 dilution series, with a suitable control water.

SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 ENDPOINTS

9.1.1 The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the responses observed in these tests are usually limited to hatchability, gross morphological abnormalities, survival, growth, and reproduction, and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these responses, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The terms used in this manual are as follows:

9.1.1.1 Safe Concentration - The highest concentration of toxicant that will permit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

9.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

9.1.1.3 Lowest-Observed-Effect-Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle (short-term) test, which causes adverse effects on the test organisms (i.e., where the values for the observed responses are statistically significantly different from the controls).

9.1.1.4 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect on a quantal, "all or nothing," response (such as death, immobilization, or serious incapacitation) in a given percent of the organisms, calculated by point estimation techniques. If the observable effect is death or immobility, the term, Lethal Concentration (LC), should be used (see Subsection 9.1.1.5). A certain EC or LC value might be judged from a biological standpoint to represent a threshold concentration, or lowest concentration that would cause an adverse effect on the observed response.

9.1.1.5 Lethal Concentration (LC) - The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observed adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

9.1.1.6 Inhibition Concentration (IC) - The toxicant concentration that would cause a given percent reduction in a non-quantal biological measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in mean young per female or in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction.

9.2 RELATIONSHIP BETWEEN ENDPOINTS DETERMINED BY HYPOTHESIS TESTING AND POINT ESTIMATION TECHNIQUES

9.2.1 If the objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe or no-effect concentration" of these substances, it is imperative to understand how the statistical endpoints of these tests are related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank

Sum Test with the Bonferroni adjustment), whereas LCs, ICs, and ECs are determined by point estimation techniques (Probit Analysis, Spearman-Karber Method, Trimmed Spearman-Karber Method, Graphical Method or Linear Interpolation Method). There are inherent differences between the use of a NOEC or LOEC derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, EC, IC, or other point estimates derived from curve fitting, interpolation, etc.

9.2.2 Most point estimates, such as the LC, IC, or EC, are derived from a mathematical model that assumes a continuous dose-response relationship. By definition, any LC, IC, or EC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe", in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

9.2.3 The use of NOECs and LOECs, on the other hand, assumes either (1) a continuous dose-response relationship, or (2) a non-continuous (threshold) model of the dose-response relationship.

9.2.3.1 In the case of a continuous dose-response relationship, it is also assumed that adverse effects that are not "statistically observable" are also not important from a biological standpoint, since they are not pronounced enough to test as statistically significant against some measure of the natural variability of the responses.

9.2.3.2 In the case of non-continuous dose-response relationships, it is assumed that there exists a true threshold, or concentration below which there is no adverse effect on aquatic life, and above which there is an adverse effect. The purpose of the statistical analysis in this case is to estimate as closely as possible where that threshold lies.

9.2.3.3 In either case, it is important to realize that the amount of adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design, such as the number of concentrations of toxicant, number of replicates per concentration, number of organisms per replicate, and use of randomization. Other factors that affect the sensitivity of the test include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration.

9.2.3.4 Where the assumption of a continuous dose-response relationship is made, by definition some amount of adverse effect might be present at the NOEC, but is not great enough to be detected by hypothesis testing.

9.2.3.5 Where the assumption of a non-continuous dose-response relationship is made, the NOEC would indeed be an estimate of a "safe" or "no-effect" concentration if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. If, however, the amount of adverse effect at the threshold were not great enough to test as statistically different, some amount of adverse effect might be present at the NOEC. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

9.2.4 In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a truly "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

9.2.5 A better understanding of the relationship between endpoints derived by hypothesis testing (NOECs) and point estimation techniques (LCs, ICs, and ECs) would be very helpful in choosing methods of data analysis. Norberg-King (1991) reported that the IC25s were comparable to the NOECs for 23 effluent and reference toxicant data sets analyzed. The data sets included short-term chronic toxicity tests for the fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*. Birge et al. (1985) reported that LC1s derived from Probit Analysis of data from short-term embryo-larval tests with reference toxicants were comparable to NOECs for several organisms. Similarly, USEPA (1988d) reported that the IC25s were comparable to the NOECs for a set of daphnid, *Ceriodaphnia dubia*, chronic tests with a single reference toxicant. However, the scope of these comparisons was very limited, and sufficient information is not yet available to establish an overall relationship between these two types of endpoints, especially when derived from effluent toxicity test data.

9.3 **PRECISION**

9.3.1 HYPOTHESIS TESTS

9.3.1.1 When hypothesis tests are used to analyze toxicity test data, it is not possible to express precision in terms of a commonly used statistic. The results of the test are given in terms of two endpoints, the No-Observed- Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test. The width of the NOEC-LOEC interval is a function of the dilution series, and differs greatly depending on whether a dilution factor of 0.3 or 0.5 is used in the test design. Therefore, **USEPA recommends the use of the ≥ 0.5 dilution factor** (see Section 4, Quality Assurance). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed with the same toxicant, toxicant concentrations, and test species, were analyzed with hypothesis tests, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that maximum precision would be attained if all tests yielded the same NOEC-LOEC interval. In practice, the precision of results of repetitive chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below a central tendency. Using these guidelines, the "normal" range of NOECs from toxicity tests using a 0.5 dilution factor (two-fold difference between adjacent concentrations), would be four-fold.

9.3.2 POINT ESTIMATION TECHNIQUES

9.3.2.1 Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse (inhibiting) effect, the precision of which can be quantitatively assessed (1) within tests by calculation of 95% confidence limits, and (2) across tests by calculating a standard deviation and coefficient of variation.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

9.4 **DATA ANALYSIS**

9.4.1 ROLE OF THE STATISTICIAN

9.4.1.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. If the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.4.1.2 The statistical methods recommended in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. Certainly there are other reasonable and defensible methods of statistical analysis for this kind of toxicity data. Among alternative hypothesis tests some, like Williams' Test, require additional assumptions, while others, like the bootstrap methods, require computer-intensive computations. Alternative point estimation approaches most probably would require the services of a statistician to determine the appropriateness of the model (goodness of fit), higher order linear or nonlinear models, confidence intervals for estimates generated by inverse regression, etc. In addition, point estimation or regression approaches would require the specification by biologists or toxicologists of some low level of adverse effect that would be deemed acceptable or safe. The statistical methods contained in this manual have been chosen because they are (1) applicable to most of the different toxicity test data sets for which they are recommended, (2) powerful statistical tests, (3) hopefully "easily" understood by nonstatisticians, and (4) amenable to use without a computer, if necessary.

9.4.2 PLOTTING THE DATA

9.4.2.1 The data should be plotted, both as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, and as an aid in interpretation of the results. Further discussion and plotted sets of data are included in the methods and the Appendices.

9.4.3 DATA TRANSFORMATIONS

9.4.3.1 Transformations of the data, (e.g., arc sine square root and logs), are used where necessary to meet assumptions of the proposed analyses, such as the requirement for normally distributed data.

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

9.4.4.1 Statistical independence among observations is a critical assumption in all statistical analysis of toxicity data. One of the best ways to insure independence is to properly follow rigorous randomization procedures. Randomization techniques should be employed at the start of the test, including the randomization of the placement of test organisms in the test chambers and randomization of the test chamber location within the array of chambers. Discussions of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

9.4.5 REPLICATION AND SENSITIVITY

9.4.5.1 The number of replicates employed for each toxicant concentration is an important factor in determining the sensitivity of chronic toxicity tests. Test sensitivity generally increases as the number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

9.4.5.2 In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration.

9.4.6 RECOMMENDED ALPHA LEVELS

9.4.6.1 The data analysis examples included in the manual specify an alpha level of 0.01 for testing the assumptions of hypothesis tests and an alpha level of 0.05 for the hypothesis tests themselves. These levels are

common and well accepted levels for this type of analysis and are presented as a recommended minimum significance level for toxicity test data analysis.

9.5 **CHOICE OF ANALYSIS**

9.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flowchart in Figure 2. An initial decision is made to use point estimation techniques (the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method, or Linear Interpolation Method) and/or to use hypothesis testing (Dunnett's Test, the t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank Sum Test with the Bonferroni adjustment). **NOTE: For the NPDES Permit Program, the point estimation techniques are the preferred statistical methods in calculating end points for effluent toxicity tests.** If hypothesis testing is chosen, subsequent decisions are made on the appropriate procedure for a given set of data, depending on the results of the tests of assumptions, as illustrated in the flowchart. A specific flow chart is included in the analysis section for each test.

9.5.2 Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and reproduction), the lowest estimate of a "no-observed-effect concentration" for any of the responses would be used as the "no-observed-effect concentration" for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed responses would not be subsequently tested for an effect on some other response. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent hypothesis test for effects on another parameter such as reproduction. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis. In performing the point estimation techniques recommended in this manual, an all-data approach is used. For example, data from concentrations above the NOEC for survival are included in determining ICp estimates using the Linear Interpolation Method.

9.5.3 ANALYSIS OF GROWTH AND REPRODUCTION DATA

9.5.3.1 Growth data from the fathead minnow, *Pimephales promelas*, larval survival and growth test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. The above mentioned growth data may also be analyzed by generating a point estimate with the Linear Interpolation Method. Data from effluent concentrations that have tested significantly different from the control for survival are excluded from further hypothesis tests concerning growth effects. Growth is defined as the dry weight per original number of test organisms when group weights are obtained. When analyzing the data using point estimation techniques, data from all concentrations are included in the analysis.

9.5.3.2 Reproduction data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. In hypothesis testing, data from effluent concentrations that have significantly lower survival than the control, as determined by Fisher's Exact test, are not included in the hypothesis tests for reproductive effects. Data from all concentrations are included when using point estimation techniques.

9.5.4 ANALYSIS OF ALGAL GROWTH RESPONSE DATA

9.5.4.1 The growth response data from the green alga, *Selenastrum capricornutum*, toxicity test, after an appropriate transformation, if necessary, to meet the assumptions of normality and homogeneity of variance, may be analyzed by hypothesis testing according to the flowchart in Figure 2. Point estimates, such as the IC25 and IC50, would also be appropriate in analyzing algal growth data.

9.5.5 ANALYSIS OF MORTALITY DATA

9.5.5.1 Mortality data are analyzed by Probit Analysis, if appropriate, or other point estimation techniques (i.e., the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method) (see Appendices I-L and the discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine square root transformation (see Appendix B-F), according to the flowchart in Figure 2.

9.5.5.2 Mortality data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed by Fisher's Exact Test (Appendix G) prior to the analysis of the reproduction data. The mortality data may also be analyzed by Probit Analysis, if appropriate or other methods (see Subsection 9.5.5.1).

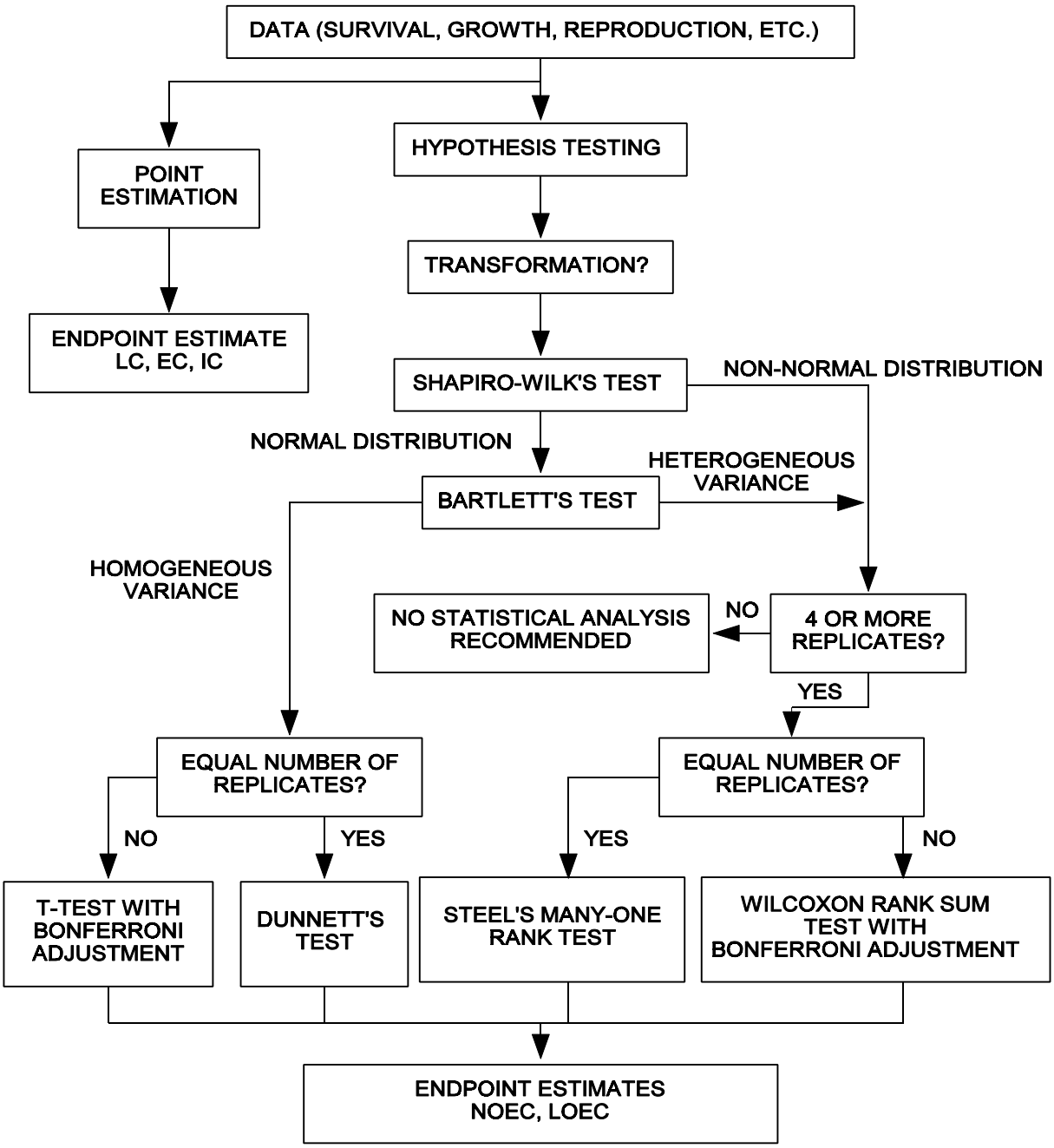

Figure 2. Flowchart for statistical analysis of test data

9.6 **HYPOTHESIS TESTS**

9.6.1 DUNNETT'S PROCEDURE

9.6.1.1 Dunnett's Procedure is used to determine the NOEC. The procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison procedure for comparing each of the treatment means with the control mean, in a series of paired tests (see Appendix C). Use of Dunnett's Procedure requires at least three replicates per treatment to check the assumptions of the test. In cases where the numbers of data points (replicates) for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons (see Appendix D), instead of using Dunnett's Procedure.

9.6.1.2 The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. Before analyzing the data, these assumptions must be tested using the procedures provided in Appendix B.

9.6.1.3 If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-one Rank Test should be used if there are four or more data points (replicates) per toxicant concentration. If the numbers of data points for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

9.6.1.4 Some indication of the sensitivity of the analysis should be provided by calculating (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

9.6.1.5 A step-by-step example of the use of Dunnett's Procedure is provided in Appendix C.

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

9.6.2.1 A t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus Dunnett's Procedure is a more powerful test.

9.6.2.2 The assumptions upon which the use of the t test with Bonferroni's adjustment is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. These assumptions must be tested using the procedures provided in Appendix B.

9.6.2.3 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of the use of the t test with Bonferroni's adjustment is provided in Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

9.6.3.1 Steel's Many-one Rank Test is a multiple comparison procedure for comparing several treatments with a control. This method is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption of normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-one Rank Test can be much more efficient (Hodges and Lehmann, 1956).

9.6.3.2 It is necessary to have at least four replicates per toxicant concentration to use Steel's test. Unlike Dunnett's procedure, the sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean that can be detected as statistically significant.

9.6.3.3 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of Steel's Many-one Rank Test is provided in Appendix E.

9.6.4 WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

9.6.4.1 The Wilcoxon Rank Sum Test with the Bonferroni Adjustment is a nonparametric test for comparing treatments with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i.e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

9.6.4.2 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test with Bonferroni Adjustment is provided in Appendix F.

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

9.6.5.1 If in the calculation of an NOEC by hypothesis testing, two tested concentrations cause statistically significant adverse effects, but an intermediate concentration did not cause statistically significant effects, the results should be used with extreme caution.

9.7 **POINT ESTIMATION TECHNIQUES**

9.7.1 PROBIT ANALYSIS

9.7.1.1 Probit Analysis is used to estimate the LC1, LC50, EC1, or EC50 and the associated 95% confidence interval. The analysis consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

9.7.1.2 The assumption upon which the use of Probit Analysis is contingent is a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit Analysis should not be used. It is important to check the results of Probit Analysis to determine if use of the analysis is appropriate. The chi-square test for heterogeneity provides one good test of appropriateness of the analysis. The computer program (see Appendix I) checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

9.7.1.3 A discussion of Probit Analysis, and examples of computer program input and output, are found in Appendix I.

9.7.1.4 In cases where Probit Analysis is not appropriate, the LC50 and associated confidence interval may be estimated by the Spearman-Karber Method (Appendix J) or the Trimmed Spearman-Karber Method (Appendix K). If the test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC50 may be estimated using the Graphical Method (Appendix L).

9.7.2 LINEAR INTERPOLATION METHOD

9.7.2.1 The Linear Interpolation Method (see Appendix M) is a procedure to calculate a point estimate of the effluent or other toxicant concentration [Inhibition Concentration, (IC)] that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms. The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests.

9.7.2.2 Use of the Linear Interpolation Method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. The assumption for piecewise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean.

9.7.2.3 The inability to test the monotonicity and piecewise linear response assumptions for this method makes it difficult to assess when the method is, or is not, producing reliable results. Therefore, the method should be used with caution when the results of a toxicity test approach an "all or nothing" response from one concentration to the next in the concentration series, and when it appears that there is a large deviation from monotonicity. See Appendix M for a more detailed discussion of the use of this method and a computer program available for performing calculations.

SECTION 10

REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address
6. Objective of test

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (at time of sampling)
   f. Sample temperature when received at the laboratory
   g Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source

b. Collection date(s) and time(s)
c. Pretreatment
d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test, test results, and current control chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated for sublethal endpoints determined by hypothesis testing in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc. (as required in the applicable NPDES permit)
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended

methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 When NPDES permits require sublethal hypothesis testing endpoints from Methods 1000.0,1002.0, or 1003.0 (e.g., growth or reproduction NOECs and LOECs), within-test variability must be reviewed and variability criteria must be applied as described in this section (10.2.8.2). When the methods are used for non-regulatory purposes, the variability criteria herein are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

10.2.8.2.1 To measure test variability, calculate the percent minimum significant difference (PMSD) achieved in the test. The PMSD is the smallest percentage decrease in growth or reproduction from the control that could be determined as statistically significant in the test. The PMSD is calculated as 100 times the minimum significant difference (MSD) divided by the control mean. The equation and examples of MSD calculations are shown in Appendix C. PMSD may be calculated legitimately as a descriptive statistic for within-test variability, even when the hypothesis test is conducted using a non-parametric method. The PMSD bounds were based on a representative set of tests, including tests for which a non-parametric method was required for determining the NOEC or LOEC. The conduct of hypothesis testing to determine test results should follow the statistical flow charts provided for each method. That is, when test data fail to meet assumptions of normality or heterogeneity of variance, a non-parametric method (determined following the statistical flowchart for the method) should be used to calculate test results, but the PMSD may be calculated as described above (using parametric methods) to provide a measure of test variability.

10.2.8.2.2 Compare the PMSD measured in the test with the upper PMSD bound variability criterion listed in Table 6. When the test PMSD exceeds the upper bound, the variability among replicates is unusually large for the test method. Such a test should be considered insufficiently sensitive to detect toxic effects on growth or reproduction of substantial magnitude. A finding of toxicity at a particular concentration may be regarded as trustworthy, but a finding of "no toxicity" or "no statistically significant toxicity" at a particular concentration should not be regarded as a reliable indication that there is no substantial toxic effect on growth or reproduction at that concentration.

10.2.8.2.3 If the PMSD measured for the test is less than or equal to the upper PMSD bound variability criterion in Table 6, then the test's variability measure lies within normal bounds and the effect concentration estimate (e.g., NOEC or LOEC) would normally be accepted unless other test review steps raise serious doubts about its validity.

10.2.8.2.4 If the PMSD measured for the test exceeds the upper PMSD bound variability criterion in Table 6, then one of the following two cases applies (10.2.8.2.4.1, 10.2.8.2.4.2).

10.2.8.2.4.1 If toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC or LOEC), then the test shall be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity.

10.2.8.2.4.2 If toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC or LOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample.

10.2.8.2.5 To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds shall also be applied when a hypothesis test result (e.g., NOEC or LOEC) is reported. Lower PMSD bounds, which are based on the 10^{th} percentiles of national PMSD data, are presented in Table 6. The 10^{th} percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a

regular basis and most do not achieve it even occasionally. In determining hypothesis test results (e.g., NOEC or LOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bounds in Table 6. See USEPA, 2000b for specific examples of implementing lower PMSD bounds.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability. The log of PMSD will provide an approximately normal variate useful for control charting.

TABLE 6. VARIABILITY CRITERIA (UPPER AND LOWER PMSD BOUNDS) FOR SUBLETHAL HYPOTHESIS TESTING ENDPOINTS SUBMITTED UNDER NPDES PERMITS.[1]

Test Method	Endpoint	Lower PMSD Bound	Upper PMSD Bound
Method 1000.0, Fathead Minnow Larval Survival and Growth Test	growth	12	30
Method 1002.0, *Ceriodaphnia dubia* Survival and Reproduction Test	reproduction	13	47
Method 1003.0, *Selenastrum capricornutum* Growth Test	growth	9.1	29

[1] Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

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^oC.

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:________________________

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

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

		Day						
Conc:	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 0-6°C until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (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 60°C, for 24 h or at 100°C 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).

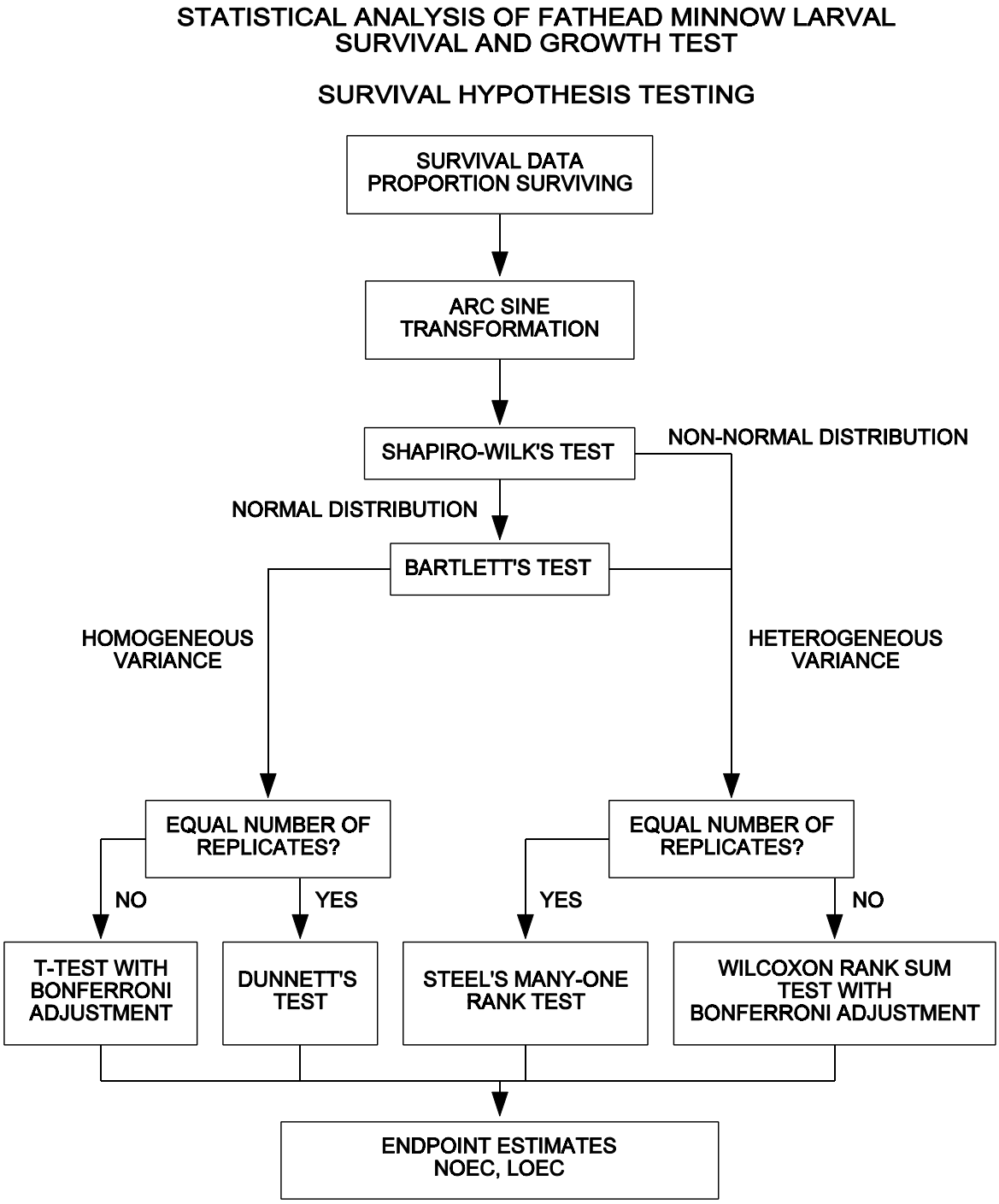

Figure 5. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas*, larval survival data by hypothesis testing.

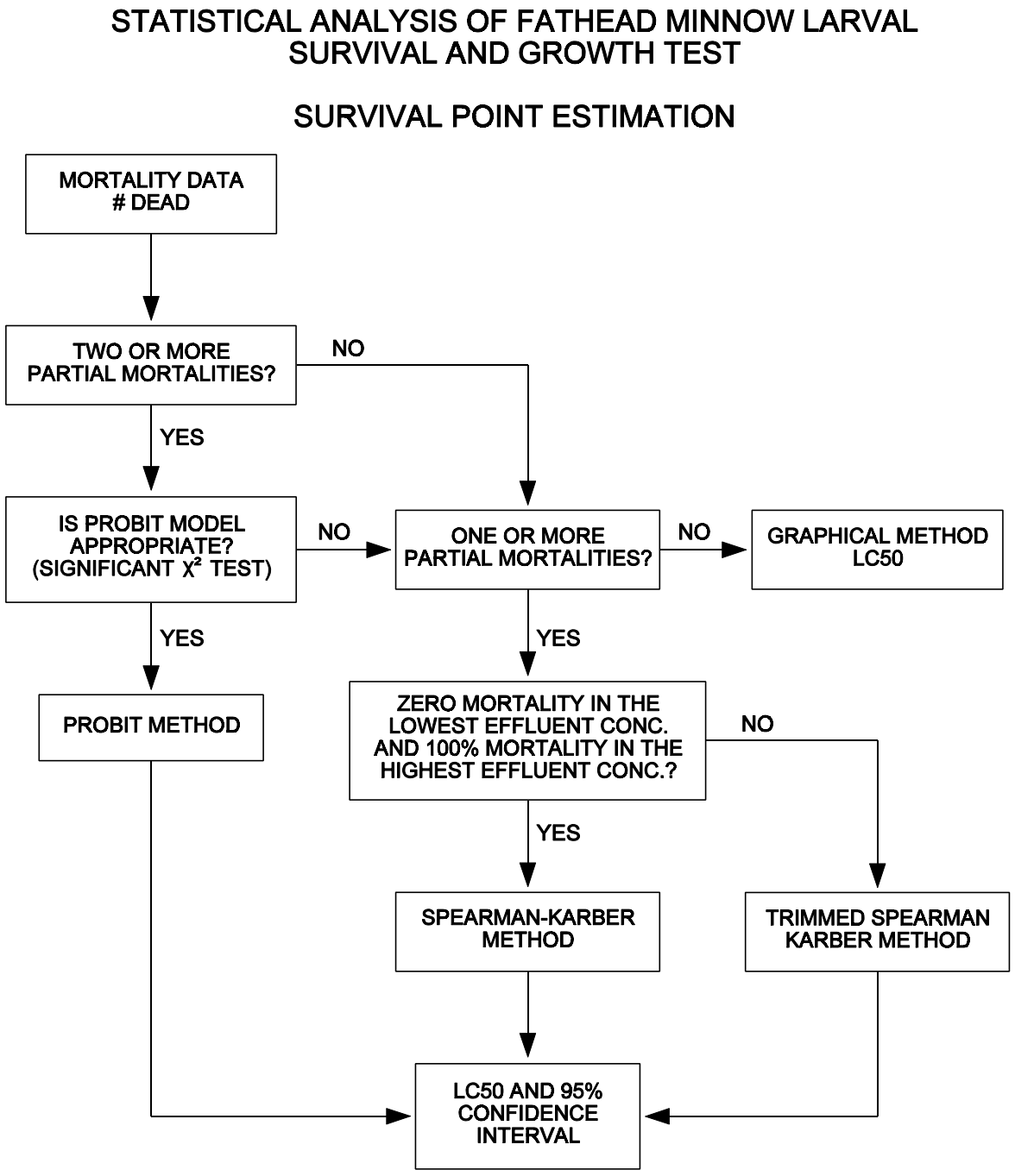

Figure 6. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas*, larval survival data by point estimation.

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

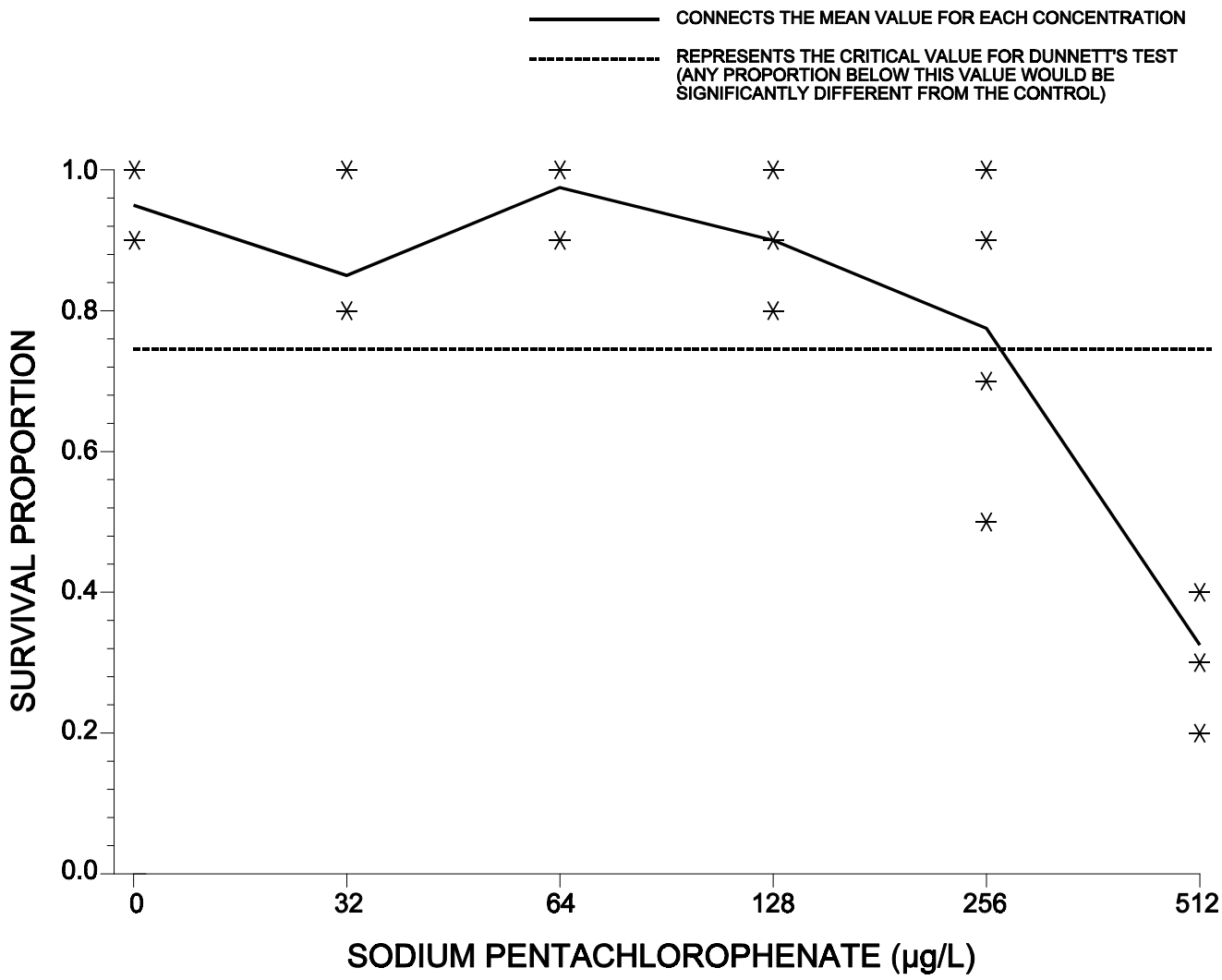

Figure 7. Plot of survival proportion data in Table 3.

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:

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

Where: V_i = degrees of freedom for each 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 $n_1 + n_2 ... + n_p$

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

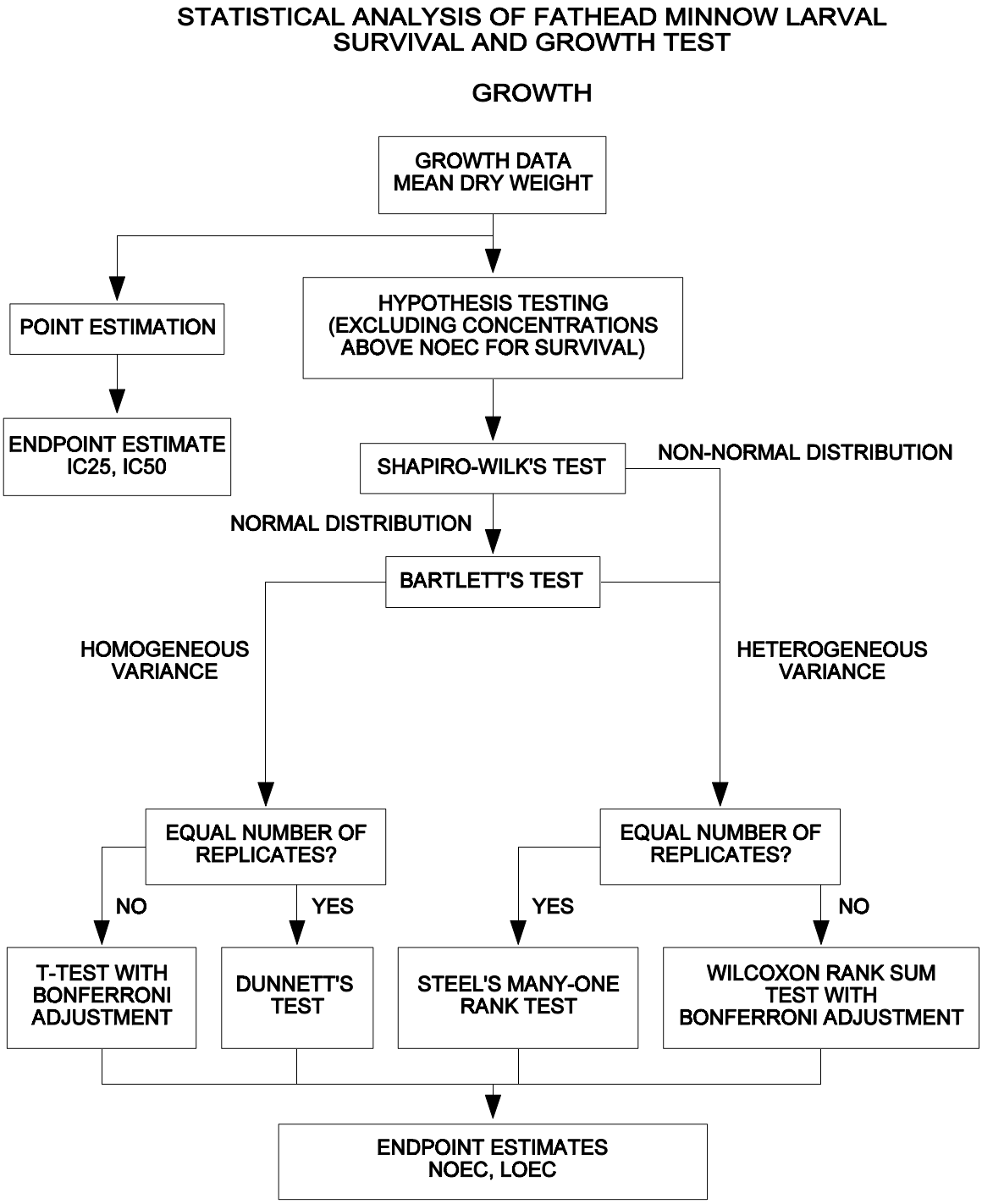

Figure 9. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas*, larval growth data.

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

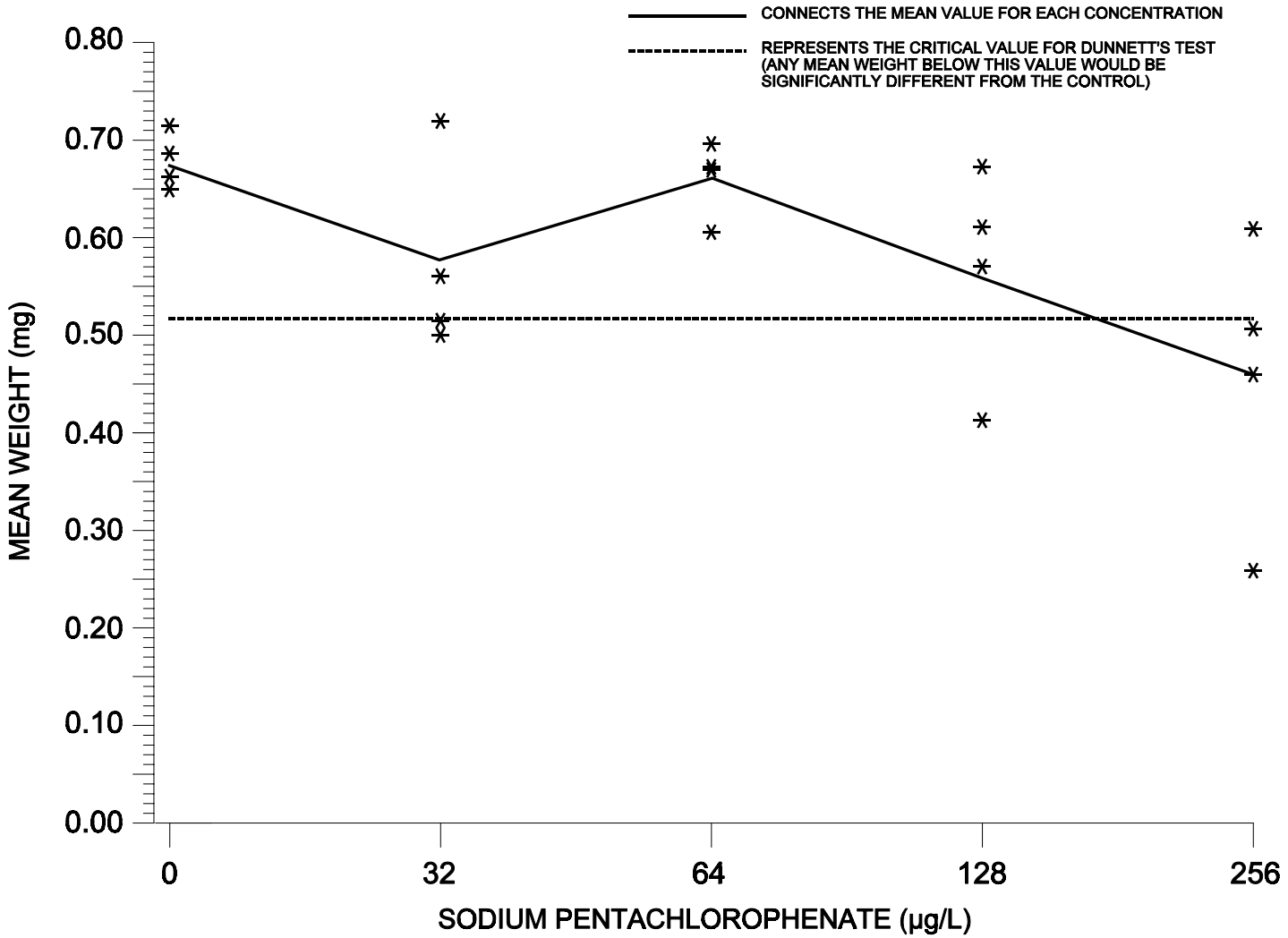

Figure 10. Plot of weight data from fathead minnow, *Pimephales promelas*, larval survival and growth test for point estimate testing.

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 overall 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, ..., 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:

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

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

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

$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$

$= T_4 \quad Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$

$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

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

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

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

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

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

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

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

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.

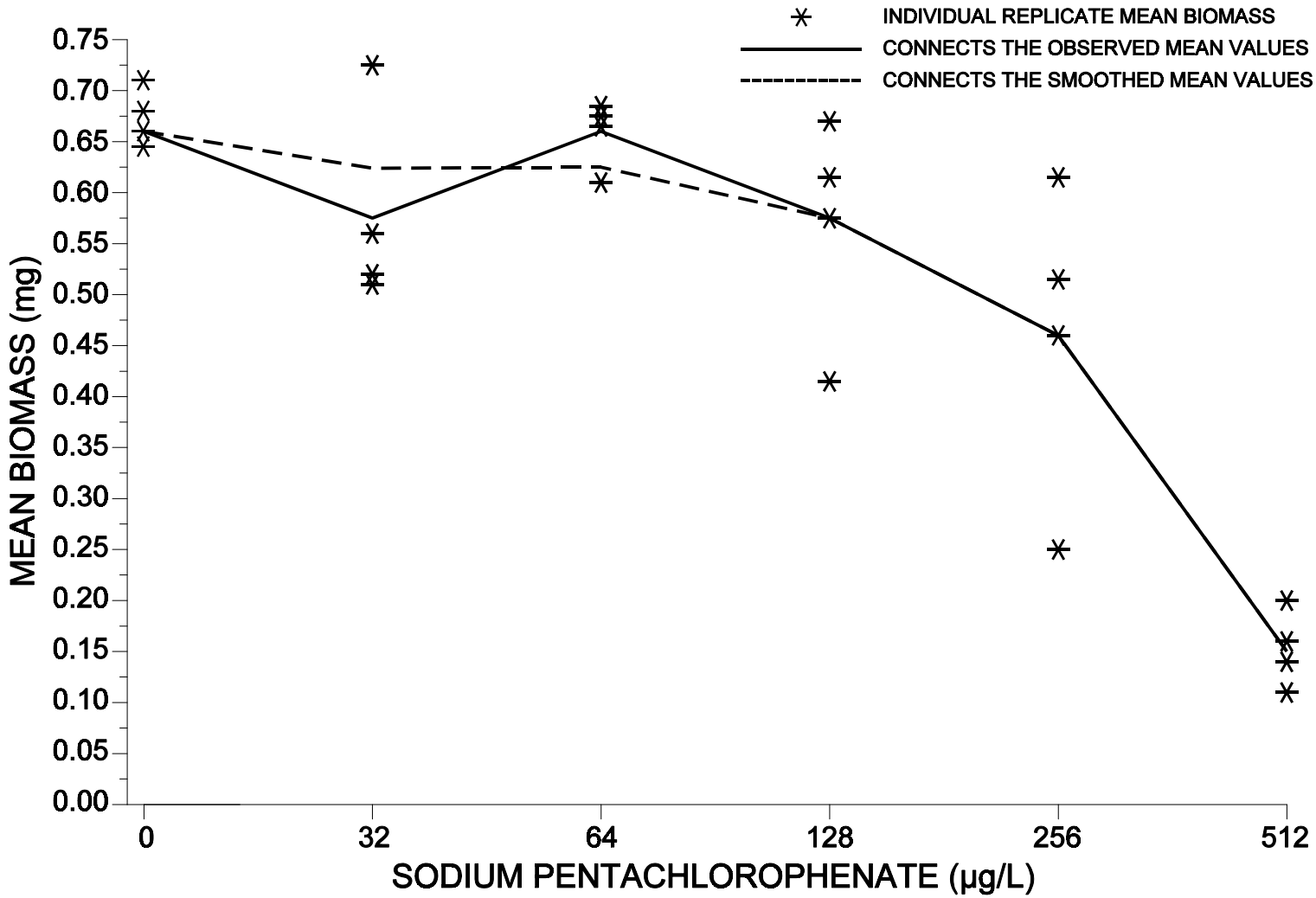

Figure 11. Plot of raw data, observed means, and smoothed means for the fathead minnow, *Pimephales promelas*, growth data in Tables 2 and 18.

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:

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

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

$$= 194 \text{ µg/L}$$

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

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

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

$$= 353 \text{ µg/L}$$

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.

SECTION 12

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1001.0

12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, *Pimephales promelas*, using embryos 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. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

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

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

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

12.1.5 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.

12.2 SUMMARY OF METHOD

12.2.1 Fathead minnow, *Pimephales promelas*, embryos are exposed in a static renewal system to different concentrations of effluent or to receiving water for seven days, starting shortly after fertilization of the eggs. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

12.3 INTERFERENCES

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

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

12.3.3 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).

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

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with

increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 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).

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

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.6.1.1).

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

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). 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.

12.4 **SAFETY**

12.4.1 See Section 3, Health and Safety.

12.5 **APPARATUS AND EQUIPMENT**

12.5.1 Fathead minnow and brine shrimp culture units -- See Section 11, Fathead Minnow, *Pimephales Promelas*, Larval Survival and Growth Test, and USEPA, 2002a. To test effluent toxicity on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from a laboratory fathead minnow culture unit. If necessary, embryos can be shipped in well oxygenated water in insulated containers. In cases where shipping is necessary, up to 48-h old embryos may be used for the test.

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

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

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

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

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

12.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of material to be weighed.

12.5.8 Test chambers -- four borosilicate glass or disposable, non-toxic plastic labware, per test solution, such as: 500-mL beakers; 100 mm x 15 mm or 100 mm x 20 mm glass or disposable polystyrene Petri dishes; or 12-cm OD, stackable "Carolina" culture dishes. The chambers should be covered with safety glass plates or sheet plastic during the test to avoid potential contamination from the air and excessive evaporation of the test solutions during the test.

12.5.9 Dissecting microscope, or long focal length magnifying lens, hand or stand supported -- for examining embryos and larvae in the test chambers.

12.5.10 Light box, microscope lamp, or flashlight -- for illuminating chambers during examination and observation of embryos and larvae.

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

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

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

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

12.5.15 Droppers, and glass tubing with fire polished edges, 2-mm ID -- for transferring embryos, and 4-mm ID -- for transferring larvae.

12.5.16 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

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

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

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

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

12.6 **REAGENTS AND CONSUMABLE MATERIALS**

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

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

12.6.3 Tape, colored -- for labelling test chambers.

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

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

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

12.6.7 Standard pH 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).

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

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

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

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

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

12.6.13 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

12.6.13.1 Fathead minnow embryos, less than 36-h old, are used for the test. The test is conducted with four (minimum of three) test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 30) embryos are exposed at each test concentration and 360 (minimum of 180) embryos would be needed for a test consisting of five effluent concentrations and a control.

12.6.13.2 Sources of Organisms

12.6.13.2.1 It is recommended that the embryos be obtained from inhouse cultures or other local sources if at all possible, because it is often difficult to ship the embryos so that they will be less than 36 h old for beginning the test. Receipt of embryos via Express Mail, air express, or other carrier, from a reliable outside source is an acceptable alternative, but they must not be over 48 h old when used to begin the test.

12.6.13.2.2 Culturing methods for fathead minnows, *Pimephales promelas*, are described in Section 6, Section 11 and in USEPA, 2002a.

12.6.13.2.3 Fish obtained from outside sources (see Section 5, Facilities, Equipment, and Supplies) such as commercial biological supply houses for use as brood stock should be guaranteed to be (1) of 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 the contemporary fish to reference toxicants.

12.6.13.3 Obtaining Embryos for Toxicity Tests from Inhouse Cultures.

12.6.13.3.1 Spawning substrates with the newly-spawned, fertilized embryos are removed from the spawning tanks or ponds, and the embryos are separated from the spawning substrate by using the index finger and rolling the embryos gently with a circular movement of the finger (see Gast and Brungs, 1973). The embryos are then combined and washed from the spawning substrate onto a 400 µm NITEX® screen, sprayed with a stream of deionized water to remove detritus and food particles, and back-washed with dilution water into a crystallizing dish for microscopic examination. Damaged and infertile eggs are discarded.

12.6.13.3.2 The embryos from three or more spawns are pooled in a single container to provide a sufficient number to conduct the tests. These embryos may be used immediately to start a test inhouse or may be transported for use at a remote location. When transportation is required, embryos should be taken from the substrates within 12 h of spawning. This permits off-site tests to be started with less than 36-h old embryos. Embryos should be transported or shipped in clean, opaque, insulated containers, in well aerated or oxygenated fresh culture or dilution water, and should be protected from extremes of temperature and any other stressful conditions during transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution water, should be less than 2°C. Sudden changes in pH, dissolved ions, osmotic strength, and DO should be avoided.

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

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

12.8 **CALIBRATION AND STANDARDIZATION**

12.8.1 See Section 4, Quality Assurance.

12.9 **QUALITY CONTROL**

12.9.1 See Section 4, Quality Assurance.

12.10 **TEST PROCEDURES**

12.10.1 TEST SOLUTIONS

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. 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 100 mL, and 400 mL for chemical analysis, would require approximately one liter, or more, of sample per test day.

12.10.1.2 Effluents

12.10.1.2.1 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%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

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

12.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 100 mL of test solution, is 1.5 L. Sufficient test solution (approximately 1000 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

12.10.1.2.4 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 the 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).

12.10.1.2.5 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.

12.10.1.2.6 The DO of the test solutions should be checked prior to 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 below 4.0 mg/L, all of the solutions and the control must be gently aerated.

12.10.1.3 Dilution Water

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

12.10.1.3.2 If the hardness of the test solutions (including the control) does not equal or exceed 25 mg/L as $CaCO_3$, it may be necessary to adjust the hardness by adding reagents for synthetic softwater as listed in Table 3, Section 7. In this case parallel tests should be conducted, one with the hardness adjusted and one unadjusted.

12.10.2 START OF THE TEST

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

12.10.2.2 Tests performed in laboratories that have inhouse fathead minnow breeding cultures must initiate tests with embryos less than 36 h old. When the embryos must be shipped to the test site from a remote location, it may be necessary to use embryos older than 36 h because of the difficulty of coordinating test organism shipments with field operations. However, in the latter case, the embryos must not be more than 48 h old at the start of the test and should all be within 24 h of the same age.

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

12.10.2.4 The test organisms should come from a pool of embryos consisting of at least three separate spawnings. Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are thoroughly mixed.

12.10.2.5 Using a small bore (2 mm ID) glass tube, the embryos are 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) embryos, for a total of 60 (minimum of 30) embryos for each concentration (see Appendix A). The amount of water added to the chambers when transferring the embryos to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with new undamaged embryos. Placing the test chambers on a light table may facilitate examining and counting the embryos.

12.10.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

12.10.3.1 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.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO 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 new 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 l-mL KIMAX® serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the embryos.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

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

12.10.6.1.2 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 temperature variation in the environmental chamber.

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

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

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

12.10.6.3 Routine Biological Observations

12.10.6.3.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine the embryos. Remove the dead embryos (milky colored and opaque) and record the number (Figure 2). If the rate of mortality (including those with fungal infection) exceeds 20% in the control chambers, or if excessive non-concentration-related mortality occurs, terminate the test and start a new test with new embryos.

12.10.6.3.2 At 25°C, hatching may begin on the fourth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed larvae, daily. Deformed larvae are those with gross morphological abnormalities such as lack of appendages, lack of fusiform shape (non-distinct mass), lack of mobility, a colored, beating heart in an opaque mass, or other characteristics that preclude survival. Count and remove dead embryos and larvae as previously discussed and record the numbers for all of the test observations (Figure 2). Upon hatching, deformed larvae are counted as dead.

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

12.10.7 DAILY CLEANING OF TEST CHAMBERS

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

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 Freshly prepared solutions are used to renew the tests daily. 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 Handling 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 0-6°C until used.

12.10.8.2 The test solutions are renewed immediately after removing dead embryos and/or larvae. During the daily renewal process, 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 should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the embryos or larvae.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:	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*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:	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*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations (CONTINUED)

Discharger:______________________________ Test Dates:____________________________

Location:________________________________ Analyst:______________________________

____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Control	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Comments:

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data (CONTINUED).

12.10.9 TERMINATION OF THE TEST

12.10.9.1 The test is terminated after seven days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each (Figure 2). The deformed larvae are treated as dead in the analysis of the data. Keep a separate record of the total number and percent of deformed larvae for use in reporting the teratogenicity of the test solution.

12.10.9.2 Prepare a summary of the data as illustrated in Figure 3.

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

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

12.12 **ACCEPTABILITY OF TEST RESULTS**

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

Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

Treatment	Control					
No. dead embryos and larvae						
No. terata						
Total mortality (dead and deformed)						
Total mortality (%)						
Terata (%)						
Hatch (%)						

Comments:

Figure 3. Summary data for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 μE/m^2/s or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	150 mL (recommended minimum)
7.	Test solution volume:	70 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 36-h old embryos (Maximum of 48-h if shipped) (required)
10.	No. embryos per test chamber:	15 (recommended) 10 (required minimum)
11.	No. replicate test chambers per concentration:	4 (recommended) 3 (required minimum)
12.	No. embryos per concentration:	60 (recommended) 30 (required minimum)
13.	Feeding regime:	Feeding not required
14.	Aeration:	None unless DO falls below 4.0 mg/L (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*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0) (CONTINUED)

15.	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). The hardness of the test solutions should equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching success (available options)
16.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
17.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None, or ≥ 0.5 (recommended)
18.	Test duration:	7 days (required)
19.	Endpoint:	Combined mortality (dead and deformed organisms) (required)
20.	Test acceptability criteria:	80% or greater survival in controls (required)
21.	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)
22.	Sample volume required:	1.5 to 2.5 L/day depending on the volume of test solutions used (recommended)

12.13 **DATA ANALYSIS**

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data (Figure 3).

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, and dead and deformed larvae. The EC1 is calculated using Probit Analysis (Finney, 1971; see Appendix I). Separate analyses are performed for the estimation of LOEC and NOEC endpoints and for the estimation of the EC1 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint. See the Appendices for examples of the manual computations and examples of data input and output for the computer programs.

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

12.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined on the flowchart in Figure 4. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% total mortality in all of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint.

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

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

12.13.2.4 Probit Analysis (Finney, 1971) 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.

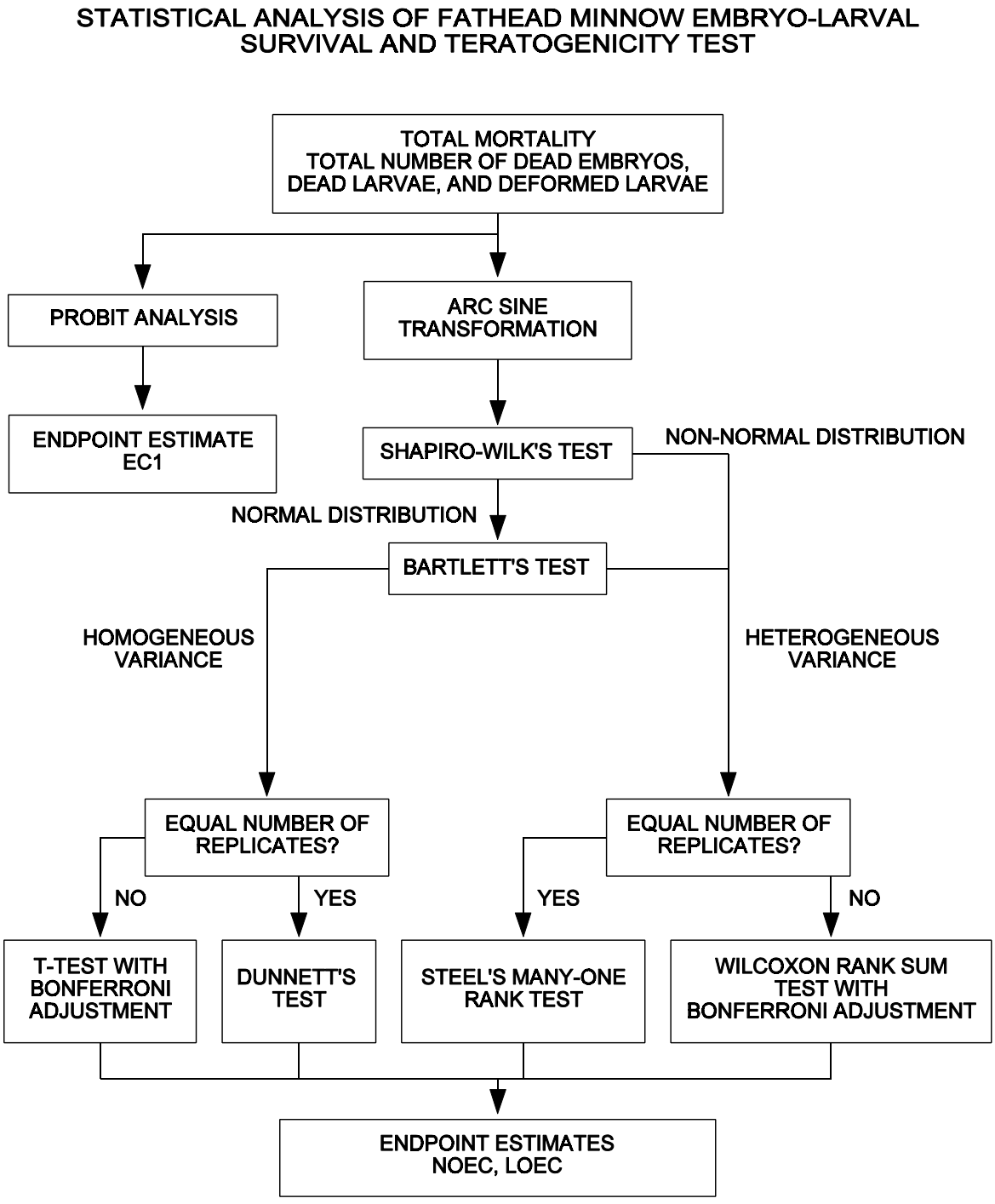

Figure 4. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas*, embryo-larval data.

12.13.2.5 The data for this example are listed in Table 2. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 3. A plot of the data is provided in Figure 5. Since there is 100% total mortality in replicates for the 50.0% concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

TABLE 2. DATA FROM FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL TOXICITY TEST WITH GROUND WATER EFFLUENT

Effluent Conc. (%)	No. Eggs at Start	Dead at Test Termination		Deformed at Test Termination		Dead + Deformed at Termination	
		No.	%	No.	%	No.	%
Control	10	0	0	0	0	0	0
	10	2	20	0	0	2	20
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
3.125	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
6.25	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
12.5	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
25.0	10	1	10	9	90	10	100
	10	2	20	8	80	10	100
	10	2	20	8	80	10	100
	10	1	10	4	40	5	50
50.0	10	4	40	6	60	10	100
	10	3	30	7	70	10	100
	10	5	50	5	50	10	100
	10	3	30	7	70	10	100

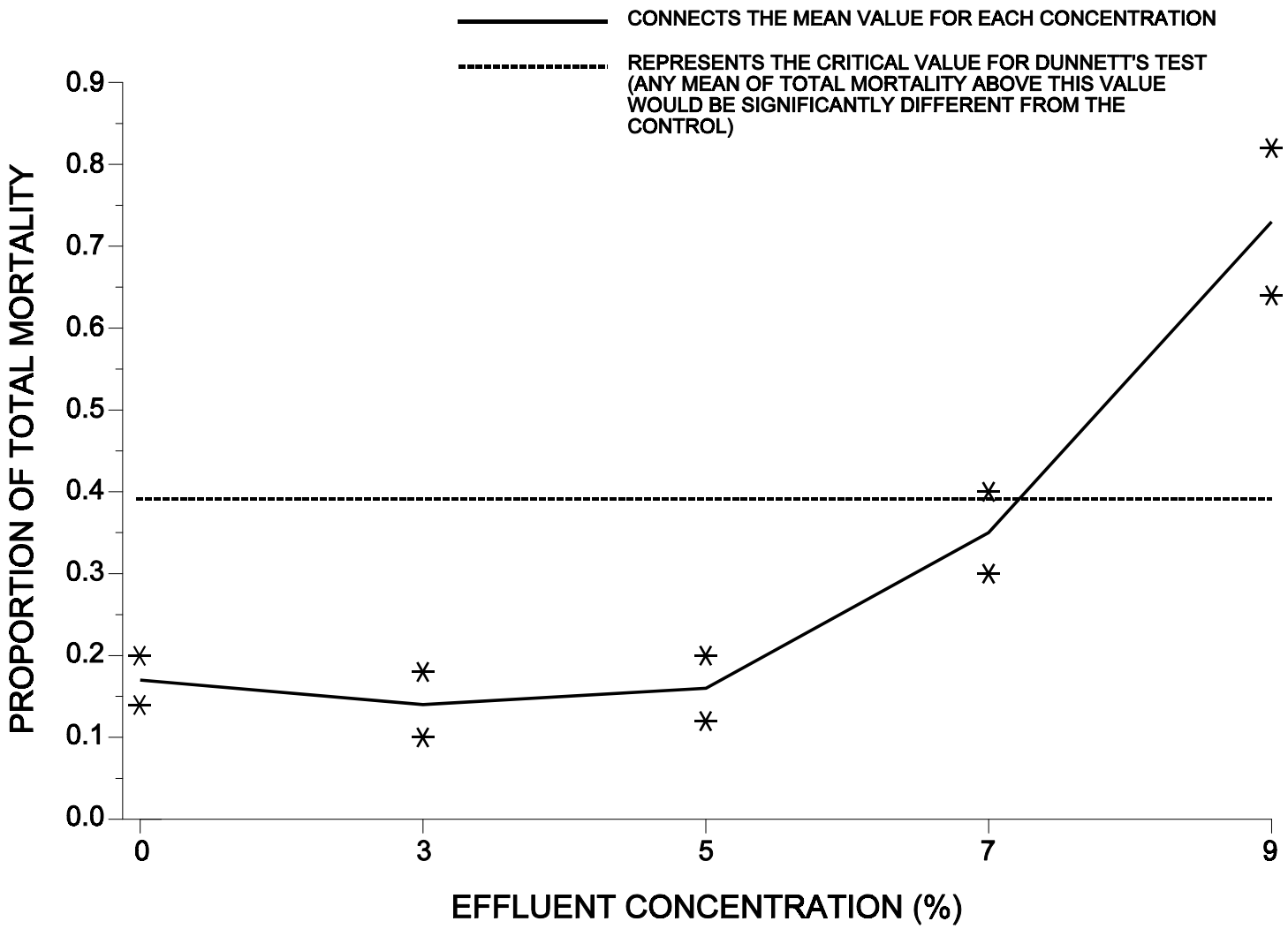

Figure 5. Plot of fathead minnow, *Pimephales promelas*, total mortality data from the embryo-larval test.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL TOTAL MORTALITY DATA

			Effluent Concentration (%)				
	Replicate	Control	3.125	6.25	12.5	25.0	50.0
RAW	A	0.00	0.00	0.00	0.00	1.00	1.00
	B	0.20	0.10	0.00	0.00	1.00	1.00
	C	0.00	0.00	0.00	0.00	1.00	1.00
	D	0.10	0.10	0.10	0.10	0.50	1.00
ARC SINE	A	0.159	0.159	0.159	0.159	1.412	-
TRANS-	B	0.464	0.322	0.159	0.159	1.412	-
FORMED	C	0.159	0.159	0.159	0.159	1.412	-
	D	0.322	0.322	0.322	0.322	0.785	-
Mean($\bar{Y}_i$)		0.276	0.241	0.200	0.200	1.255	
S_i^2		0.022	0.009	0.007	0.007	0.098	
i		1	2	3	4	5	

12.13.2.6 Test for Normality

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

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

		Effluent Concentration (%)				
Replicate	Control	3.125	6.25	12.5	25.0	50.0
A	-0.117	-0.082	-0.041	-0.041	0.157	-
B	0.188	0.081	-0.041	-0.041	0.157	-
C	-0.117	0.081	-0.041	-0.041	0.157	-
D	0.046	-0.082	0.122	0.122	-0.470	-

12.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

12.13.2.6.3 For this set of data, $n = 20$

$$\bar{X} = \frac{1}{20}(-0.003) = 0.000$$

$$D = 0.4261$$

12.13.2.6.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ 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	-0.470	11	-0.041
2	-0.117	12	0.046
3	-0.117	13	0.081
4	-0.082	14	0.081
5	-0.082	15	0.122
6	-0.041	16	0.122
7	-0.041	17	0.157
8	-0.041	18	0.157
9	-0.041	19	0.157
10	-0.041	20	0.188

12.13.2.6.5 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 6.

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.658	$X^{(20)} - X^{(1)}$
2	0.3211	0.274	$X^{(19)} - X^{(2)}$
3	0.2565	0.274	$X^{(18)} - X^{(3)}$
4	0.2085	0.239	$X^{(17)} - X^{(4)}$
5	0.1686	0.204	$X^{(16)} - X^{(5)}$
6	0.1334	0.163	$X^{(15)} - X^{(6)}$
7	0.1013	0.122	$X^{(14)} - X^{(7)}$
8	0.0711	0.122	$X^{(13)} - X^{(8)}$
9	0.0422	0.087	$X^{(12)} - X^{(9)}$
10	0.0140	0.000	$X^{(11)} - X^{(10)}$

12.13.2.6.6 Compute the test statistic, W, as follows:

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

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

$$W = \frac{1}{0.4261}(0.6004)^2$$
$$= 0.846$$

12.13.2.6.7 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 = 20 observations is 0.868. Since W = 0.846 is less than the critical value, conclude that the data are not normally distributed.

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

12.13.2.7 Steel's Many-one Rank Test

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

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 3.125% effluent concentration is given in Table 7. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 8. The control group ranks are next summed for each effluent concentration pairing, as shown in Table 9.

TABLE 7. ASSIGNING RANKS TO THE CONTROL AND 3.125% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Mortality	Effluent Concentration (%)
2.5	0.159	Control
2.5	0.159	Control
2.5	0.159	3.125
2.5	0.159	3.125
6	0.322	Control
6	0.322	3.125
6	0.322	3.125
8	0.464	Control

TABLE 8. TABLE OF RANKS FOR STEEL'S MANY-ONE RANK TEST

			Effluent Concentration (%)							
Repl.	Control		3.125		6.25		12.5		25.0	
A	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
B	0.464	(8,8,8,4)	0.322	(6)	0.159	(3)	0.159	(3)	1.412	(7)
C	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
D	0.322	(6,6.5,6.5,3)	0.322	(6)	0.322	(3)	0.159	(3)	0.785	(5)

TABLE 9. RANK SUMS

Effluent Concentration (%)	Control Rank Sum
3.125	19
6.25	20.5
12.5	20.5
25.0	10

12.13.2.7.3 For this example, we want to determine if the total mortality in any of the effluent concentrations is significantly higher than the total mortality in the control. If this occurs, the rank sum of the control would be significantly less than the rank sum at that concentration. Thus we are only concerned with comparing the control rank sum for each pairing with the various effluent concentrations with some "minimum" or critical rank sum, at or below which the concentration total mortality would be considered significantly greater than the control. At a signficance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates per concentration is 10 (see Table 5, Appendix E).

12.13.2.7.4 Since the control rank sum for the 25.0% effluent concentration pairing is equal to the critical value, the total proportion mortality in the 25.0% concentration is considered significantly greater than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have signficantly higher total proportion mortality than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

12.13.2.8 Calculation of the LC50

12.13.2.8.1 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.

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

12.13.2.8.3 Figure 6 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

TABLE 10. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	3.125	6.25	12.5	25.0	50.0
Number Dead	3	1	0	1	6	15
Number Exposed	40	40	40	40	40	40

12.14 **PRECISION AND ACCURACY**

12.14.1 PRECISION

12.14.1.1 Single-laboratory Precision

12.14.1.1.1 Data shown in Tables 11 and 12 indicate that the precision of the embryo-larval survival and teratogenicity test, expressed as the relative standard deviation (or coefficient of variation, CV) of the LC1 values, was 62% for cadmium (Table 11) and 41% for Diquat (Table 12).

12.14.1.1.2 Precision data are also available from four embryo-larval survival and teratogenicity tests on trickling filter pilot plant effluent (Table 13). Although the data could not be analyzed by Probit Analysis, the NOECs and LOECs obtained using Dunnett's Procedure were the same for all four tests, 7% and 11% effluent, respectively, indicating maximum precision in terms of the test design.

12.14.1.2 Multilaboratory Precision

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

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

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

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

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity (tabular value) = 7.815

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

Estimated LC/EC Values and Confidence Limits

Point		Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC	1.00	1.346	0.453	1.922
LC/EC	50.00	3.018	2.268	3.672

Figure 6. Output for USEPA Probit Program, Version 1.5.

TABLE 11. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits	NOEC[4] (mg/L)
1	0.014	0.009 - 0.018	0.012
2	0.006	0.003 - 0.010	0.012
3	0.005	0.003 - 0.009	0.013
4	0.003	0.002 - 0.004	0.011
5	0.006	0.003 - 0.009	0.012
N	5		5
Mean	0.0068		NA
SD	0.0042		
CV(%)	62		NA

1 Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

2 Cadmium chloride was used as the reference toxicant. The nominal concentrations, expressed as cadmium (mg/L), were: 0.01, 0.032, 0.100, 0.320, and 1.000. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

3 Determined by Probit Analysis.

4 Highest no-observed-effect concentration determined by independent statistical analysis (2X2 Chi-square Fisher's Exact Test). NOEC range of 0.011 - 0.013 represents a difference of one exposure concentration.

TABLE 12. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits
1	0.58	0.32 - 0.86
2	2.31	--[4]
3	1.50	1.05 - 1.87
4	1.71	1.24 - 2.09
5	1.43	0.93 - 1.83
N	5	
Mean	1.51	
SD	0.62	
CV(%)	41.3	

[1] Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

[2] The Diquat concentrations were determined by chemical analysis. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

[3] Determined by Probit Analysis.

[4] Cannot be calculated.

TABLE 13. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[1,2,3]

Test No.	NOEC (% Effluent)	LOEC (% Effluent)
1	7	11
2	7	11
3	7	11
4	7	11

[1] Data provided by Timothy Neiheisel, Bioassessment and Ecotoxiology Branch, EMSL, USEPA, Cincinnati, OH.
[2] Effluent concentrations used: 3, 5, 7, 11 and 16%
[3] Maximum precision achieved in terms of NOEC-LOEC interval. For a discusssion of the precision of data from chronic toxicity tests (see Section 4, Quality Assurance).

SECTION 13

TEST METHOD

DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

13.1 SCOPE AND APPLICATION

13.1.1 This method measures the chronic toxicity of effluents and receiving water to the daphnid, *Ceriodaphnia dubia*, using less than 24 h old neonates during a three-brood (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.

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

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

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

13.1.5 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.

13.2 SUMMARY OF METHOD

13.2.1 *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent, or to receiving water, until 60% or more of surviving control females have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the surviving control organisms should produce 15 or more young in three broods. If these criteria are not met at the end of 8 days, the test must be repeated.

13.3 INTERFERENCES

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

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

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

13.3.4 The amount and type of natural food in the effluent or dilution water may confound test results.

13.3.5 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.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the initial pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 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).

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

13.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 13.3.6.1.1).

13.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). 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.

13.4 **SAFETY**

13.4.1 See Section 3, Health and Safety.

13.5 **APPARATUS AND EQUIPMENT**

13.5.1 *Ceriodaphnia* and algal culture units -- See *Ceriodaphnia* and algal culturing methods below and algal culturing methods in Section 14 and USEPA, 2002a.

13.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, capable of collecting a 24-h composite sample of 5 L or more.

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

13.5.4 Environmental chambers, incubators, or equivalent facilities with temperature control (25 ± 1°C).

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

13.5.6 Balance -- analytical, capable of accurately weighing 0.00001 g.

13.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the material to be weighed.

13.5.8 Test chambers -- 10 test chambers are required for each concentration and control. Test chambers such as 30-mL borosilicate glass beakers or disposable polystyrene cups are recommended because they will fit in the viewing field of most stereoscopic microscopes. The glass beakers and plastic cups are rinsed thoroughly with dilution water before use. To avoid potential contamination from the air and excessive evaporation of the test solutions during the test, the test vessels should be covered with safety glass plates or sheet plastic (6 mm thick).

13.5.9 Mechanical shaker or magnetic stir plates -- for algal cultures.

13.5.10 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

13.5.11 Fluorometer (optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

13.5.12 UV-VIS spectrophotometer (optional) -- capable of accommodating 1-5 cm cuvettes.

13.5.13 Cuvettes for spectrophotometer -- 1-5 cm light path.

13.5.14 Electronic particle counter (optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

13.5.15 Microscope with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope) -- for determining sex and verifying identification.

13.5.16 Dissecting microscope, stereoscopic, with zoom objective, magnification to 50X -- for examining and counting the neonates in the test vessels.

13.5.17 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

13.5.18 Centrifuge (optional) -- plankton, or with swing-out buckets having a capacity of 15-100 mL.

13.5.19 Centrifuge tubes -- 15-100 mL, screw-cap.

13.5.20 Filtering apparatus -- for membrane and/or glass fiber filters.

13.5.21 Racks (boards) -- to hold test chambers. It is convenient to use a piece of styrofoam insulation board, 50 cm x 30 cm x 2.5 cm (20 in x 12 in x 1 in), drilled to hold 60 test chambers, in six rows of 10 (see Figure 1).

13.5.22 Light box -- for illuminating organisms during examination.

13.5.23 Volumetric flasks and graduated cylinders -- class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for culture work and preparation of test solutions.

13.5.24 Pipettors, adjustable volume repeating dispensers -- for feeding. Pipettors such as the Gilson REPETMAN®, Eppendorf, Oxford, or equivalent, provide a rapid and accurate means of dispensing small volumes (0.1 mL) of food to large numbers of test chambers.

13.5.25 Volumetric pipets -- class A, 1-100 mL.

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

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

13.5.28 Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, $\geq$ 2mm ID -- for transferring organisms.

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

13.5.30 Thermometer, glass or electronic, laboratory grade, -- for measuring water temperatures.

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

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

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

13.6 **REAGENTS AND CONSUMABLE MATERIALS**

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

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

13.6.3 Vials, marked -- for preserving specimens for verification (optional).

13.6.4 Tape, colored -- for labeling test vessels.

13.6.5 Markers, waterproof -- for marking containers.

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

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

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

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

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

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

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

13.6.13 Effluent, surface 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.

13.6.14 Trout chow, yeast, and CEROPHYL® food (or substitute food) -- for feeding the cultures and test organisms.

13.6.14.1 Digested trout chow, or substitute flake food (TETRAMIN®, BIORIL®, or equivalent), is prepared as follows:

1. Preparation of trout chow or substitute flake food requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications.
2. Add 5.0 g of trout chow pellets or substitute flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

13.6.14.2 Yeast is prepared as follows:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® Yeast, Lake State Kosher Certified Yeast, or equivalent, to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

13.6.14.3 CEROPHYLL® is prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves, CEROPHYLL®, or equivalent are available from commercial sources. Dried, powdered, alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet shops.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

13.6.14.4 Combined yeast-cerophyl-trout chow (YCT) is mixed as follows:

1. Thoroughly mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks. Do not store frozen over three months.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7-1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

13.6.15 Algal food -- for feeding the cultures and test organisms.

13.6.15.1 Algal Culture Medium is prepared as follows:

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1. MACRONUTRIENTS			
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C.	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2. MICRONUTRIENTS			
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

13.6.15.2 Algal Cultures

13.6.15.2.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of *Selenastrum capricornutum, S. minutum,* and *Chlamydomonas reinhardti.*

13.6.15.2.2 Two types of algal cultures are maintained: "stock" cultures, and "food" cultures.

13.6.15.2.2.1 Establishing and Maintaining Stock Cultures of Algae:

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia dubia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia dubia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained at 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms (see Section 6, Test Organisms) every four to six months.

13.6.15.2.2.2 Establishing and Maintaining "Food" Cultures of Algae:

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia dubia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$ or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

13.6.15.2.3 Preparing Algal Concentrate for Use as *Ceriodaphnia dubia* Food:

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer (see Section 14, Green Alga, *Selenastrum capricornutum* Growth Test), and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia dubia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

13.6.15.3 Food Quality

13.6.15.3.1 USEPA recommends Fleishmann's® yeast, Cerophyll®, trout chow, and *Selenastrum capricornutum* as the preferred *Ceriodaphnia dubia* food combination. This recommendation is based on extensive data developed by many laboratories which indicated high *Ceriodaphnia dubia* survival and reproduction in culturing and testing. The use of substitute food(s) is acceptable only after side-by-side tests are conducted to determine that the quality of the substitute food(s) is equal to the USEPA recommended food combination based on survival and reproduction of *Ceriodaphnia dubia*.

13.6.15.3.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, algae, and/or any substitute food(s) should be determined in side-by-side comparisons of *Ceriodaphnia dubia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

13.6.16 TEST ORGANISMS, DAPHNIDS, *CERIODAPHNIA DUBIA*

13.6.16.1 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

13.6.16.2 Neonates used for toxicity tests must be obtained from individually cultured organisms. Mass cultures may be maintained, however, to serve as a reserve source of organisms for use in initiating individual cultures and in case of loss of individual cultures.

13.6.16.3 Starter animals may be obtained from commercial sources and may be shipped in polyethylene bottles. Approximately 40 animals and 3 mL of food are placed in a l-L bottle filled full with culture water for shipment. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

13.6.16.4 It is best to start the cultures with one animal, which is sacrificed after producing young, mounted on a microscope slide, and retained as a permanent slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by preparing slide mounts, regardless of the number of animals used to start the culture. The following procedure is recommended for making slide mounts of *Ceriodaphnia dubia* (modified from Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.

3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/10 Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-10 stained with enough acid fuchsin dye to color the mixture a light pink. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.
5. Using forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a 12 mm round cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing varnish around the edges of the coverslip.
9. Identify to species (see Pennak, 1978; Pennak, 1989; and Berner, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

13.6.16.5 Mass Culture

13.6.16.5.1 Mass cultures are used only as a "backup" reservoir of organisms.

13.6.16.5.2 One-liter or 2-L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

13.6.16.5.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal with new culture medium at least twice a week for two weeks. At each renewal, the adult survival is recorded, and the offspring and the old medium are discarded. After two weeks, the adults are also discarded, and the culture is re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia dubia* each week.

13.6.16.6 Individual Culture

13.6.16.6.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

13.6.16.6.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests (see Figure 1).

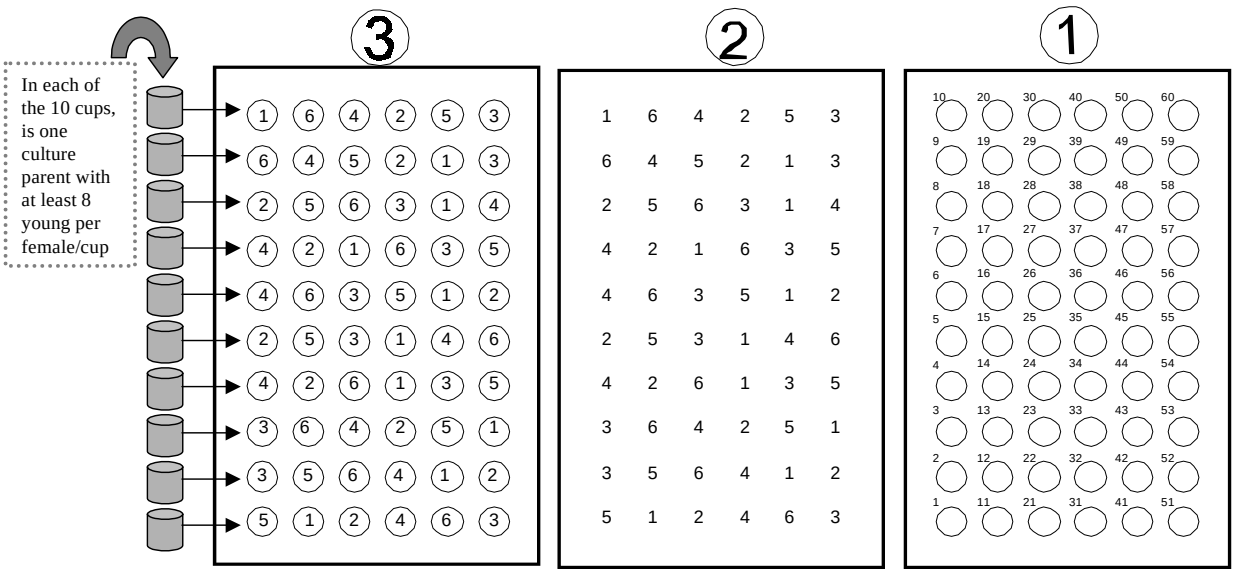

Figure 1. Examples of a test board and randomizing template: 1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets, 2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and 3) test board (1) placed on top of the randomizing template (2) for the purpose of assigning the position of test treatments (1-6) within each block (row on the test board). Following placement of test chambers, test organisms are allocated using blocking by known parentage. Test organisms from a single brood cup are distributed to each treatment within a given block (row on the test board).

13.6.16.6.3 Organisms are fed daily (see Subsection 13.6.16.9) and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

13.6.16.6.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

13.6.16.6.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

13.6.16.6.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults, or less than an average of 20 young per female would indicate problems, such as poor quality of culture media or food. Cultures that do not meet these criteria should not be used as a source of test organisms.

13.6.16.7 Culture Medium

13.6.16.7.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

13.6.16.8 Culture Conditions

13.6.16.8.1 The daphnid, *Ceriodaphnia dubia*, should be cultured at a temperature of 25 ± 1°C.

13.6.16.8.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A photoperiod of 16-h of light and 8-h of darkness is recommended. Light intensity should be 10-20 $\mu E/m^2/s$ or 50 to 100 ft-c.

13.6.16.8.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

13.6.16.8.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

13.6.16.9 Food and Feeding

13.6.16.9.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia dubia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

13.6.16.9.2 Other algal species (such as *S. minutum* or *Chlamydomonas reinhardti*), other substitute food combinations (such as Flake Fish Food), or different feeding rates may be acceptable as long as performance criteria are met and side-by-side comparison tests confirm acceptable quality (see Subsection 13.6.15.3).

13.6.16.9.3 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low

numbers of young, large numbers of males, and/or ephippial females. Also, their offspring may produce few young when used in toxicity tests.

13.6.16.9.4 Feed as follows:

1. If YCT is frozen, remove a bottle of food from the freezer 1h before feeding time, and allow to thaw.
2. YCT food mixture and algal concentrates should both be thoroughly mixed by shaking before dispensing.
3. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
4. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after two weeks.

13.6.16.10 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Daphnid, *Ceriodaphnia dubia*, neonates less than 24 h old, and all within 8 h of the same age are used to monitor the chronic toxicity of the reference toxicant to the *Ceriodaphnia dubia* produced by the culture unit (see Section 4, Quality Assurance).

13.6.16.11 Record Keeping

13.6.16.11.1 Records, kept in a bound notebook, include (1) source of organisms used to start the cultures, (2) type of food and feeding times, (3) dates culture were thinned and restarted, (4) rate of reproduction in individual cultures, (5) daily observations of the condition and behavior of the organisms in the cultures, and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

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

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

13.8 **CALIBRATION AND STANDARDIZATION**

13.8.1 See Section 4, Quality Assurance.

13.9 **QUALITY CONTROL**

13.9.1 See Section 4, Quality Assurance.

13.10 **TEST PROCEDURES**

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 The sampling point is determined by the objectives of the test. 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. For a test consisting of single receiving water and control, approximately 600 mL of sample would be required for each test, assuming 10 replicates of 15 mL, and sufficient additional sample for chemical analysis.

13.10.1.2 Effluents

13.10.1.2.1 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%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5, and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

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

13.10.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, with a dilution series of 0.5, is approximately 1 L/day. A volume of 15 mL of test solution is adequate for the organisms, and will provide a depth in which it is possible to count the animals under a stereomicroscope with a minimum of re-focusing. Ten test chambers are used for each effluent dilution and for the control. Sufficient test solution (approximately 550 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations.

13.10.1.2.4 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).

13.10.1.2.5 Just prior to test initiation (approximately one 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 preparation of the test solutions.

13.10.1.2.6 The DO of the test solutions should be checked prior to 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 the solutions and the control must be gently aerated.

13.10.1.3 Dilution Water

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

13.10.2 START OF THE TEST

13.10.2.1 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 ten replicates.

13.10.2.2 The test chambers must be randomly assigned to a board using a template (Figure 1) or by using random numbers (see Appendix A). Randomizing the position of test chambers as described in Figure 1 (or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4). A number of different templates should be prepared, and the template used for each test should be identified on the data sheet. The same template must not be used for every test.

13.10.2.3 Neonates less than 24 h old, and all within 8 h of the same age, are required to begin the test. The neonates must be obtained from individual cultures using brood boards, as described above in Subsection 13.6.16.6,

Individual Culture (also see Section 6, Test Organisms). Neonates must be taken only from adults in individual cultures that have eight or more young in their third or subsequent broods. These adults can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 mL YCT and 0.1 mL algal concentrate/15 mL of media). Record the age range of test organisms, source, and feeding of neonates on test data sheets.

13.10.2.4 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber in the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". The technique used to achieve blocking by known parentage should be recorded in the test data report. One effective technique is to block randomize the test board as described in Figure 1 and transfer one neonate from the first brood cup to each of the six test chambers in the first row on the test board. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 60 test chambers contains one neonate. The set of six test chambers (one for each test treatment) containing organisms derived from a single female parent is referred to as a block. When using the technique described in Figure 1, each row of the test board will represent a block.

13.10.2.4.1 The brood cups and test chambers may be placed on a light table to facilitate counting the neonates. However, care must be taken to avoid temperature increase due to heat from the light table.

13.10.2.4.2 Following the allocation of test organisms to the test board, additional neonates might remain in the ten brood cups that were selected for test setup. These additional neonates may be discarded, used as future culture organisms if needed, or used to start additional tests (provided that at least 6 neonates remain and these neonates continue to meet test organism age requirements).

13.10.2.5 Blocking by known parentage allows the performance of each test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. Also, by knowing the parentage of each test organism, blocks consisting largely of males can be omitted from all test treatments at the end of the test (see Subsection 13.13.1.4), decreasing variability among replicates.

13.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 $\mu E/m^2/s$, or 50 to 100 ft-c, with a photoperiod of 16 h of light and 8 h of darkness.

13.10.3.2 It is critical that the test water temperature be maintained at 25 ± 1°C to obtain three broods in seven days.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO 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 concentration should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). Aeration is generally not practical during the daphnid, *Ceriodaphnia dubia*, test. If the DO in the effluent and/or dilution water is low, aerate gently before preparing the test solutions. 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 organisms.

13.10.5 FEEDING

13.10.5.1 The organisms are fed when the test is initiated, and daily thereafter. Food is added to the fresh medium immediately before or immediately after the adults are transferred. Each feeding consists of 0.1 mL YCT and 0.1 mL *Selenastrum capricornutum* concentrate/15 mL test solution (0.1 mL of algal concentrate containing 3.0-3.5 X 10^7 cells/mL will provide 2-2.3 X 10^5 cells/mL in the test chamber).

13.10.5.2 The YCT and algal suspension can be added accurately to the test chambers by using automatic pipettors, such as Gilson, Eppendorf, Oxford, or equivalent.

13.10.6 OBSERVATIONS DURING THE TEST

13.10.6.1 Routine Chemical and Physical Determinations

13.10.6.1.1 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.

13.10.6.1.2 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 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 sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

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

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

13.10.6.1.5 Record the data on data sheet (Figure 2).

13.10.6.2 Routine Biological Observations

13.10.6.2.1 Three or four broods are usually obtained in the controls in a 7-day test conducted at 25 ± 1°C. A brood is a group of offspring released from the female over a short period of time when the carapace is discarded during molting. In the controls, the first brood of two-to-five young is usually released on the third or fourth day of the test. Successive broods are released every 30 to 36 h thereafter. The second and third broods usually consist of eight to 20 young each. The total number of young produced by a healthy control organism in three broods often exceeds 30 per female. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.6.2.2 The release of a brood may be inadvertently interrupted during the daily transfer of organisms to fresh test solutions, resulting in a split in the brood count between two successive days. For example, four neonates of a brood of five might be released on Day 3, just prior to test solution renewal, and the fifth released just after renewal, and counted on Day 4. Partial broods, released over a two-day period, should be counted as one brood.

13.10.6.2.3 Each day, the live adults are transferred to fresh test solutions, and the numbers of live young are recorded (see data form, Figure 3). The young can be counted with the aid of a stereomicroscope with substage lighting. Place the test chambers on a light box over a strip of black tape to aid in counting the neonates. The young are discarded after counting.

13.10.6.2.4 Some of the effects caused by toxic substances include, (1) a reduction in the number of young produced, (2) young may develop in the brood pouch of the adults, but may not be released during the exposure

period, and (3) partially or fully developed young may be released, but are all dead at the end of the 24-h period. Such effects should be noted on the data sheets (Figure 3).

13.10.6.2.5 Protect the daphnids, *Ceriodaphnia dubia*, from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and transfer of females carefully. Make sure the females remain immersed during the performance of these operations.

13.10.7 DAILY PREPARATION OF TEST CHAMBERS

13.10.7.1 The test is started (Day 0) with new disposable polystyrene cups or precleaned 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or precleaned glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are recleaned (see Section 5, Facilities, Equipment, and Supplies) for the following day, and previously used plastic cups are discarded.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		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 2. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		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 2. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Routine chemical and physical determinations (CONTINUED).

13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 Freshly prepared solutions are used to renew the test daily. 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 Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. No more than 36 h should elapse between collection of the sample and the first use in the test. Maintain the samples in the refrigerator at 0-6°C until used.

13.10.8.2 New test solutions are prepared daily, and the test organisms are transferred to the freshly prepared solutions using a small-bore (2 mm) glass or polyethylene dropper or pipet. The animals are released under the surface of the water so that air is not trapped under the carapace. Organisms that are dropped or injured are discarded.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 Tests should be terminated when 60% or more of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. Because of the rapid rate of development of *Ceriodaphnia dubia*, at test termination all observations on organism survival and numbers of offspring should be completed within two hours. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.9.2 Count the young, conduct required chemical measurements, and complete the data sheets (Figure 3).

13.10.9.3 Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In most cases, the animal will need to be placed on a microscope slide before examining (see Subsection 13.6.16.4).

13.10.9.3.1 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of the *Ceriodaphnia dubia* Survival and Reproduction Test, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress (Pennak, 1989), culture conditions should be examined whenever males are identified in a test.

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

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

13.12 **ACCEPTABILITY OF TEST RESULTS**

13.12.1 For the test results to be acceptable, at least 80% of all control organisms must survive, and 60% of surviving control females must produce at least three broods, with an average of 15 or more young per surviving female.

Discharger:______________________ Location: _______________________ Date Sample Collected: ____________

Analyst:______________________________ Test Start-Date/Time: __________________ Test Start-Date/time: ___________________

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data.

Discharger: ______________________
Location: ________________________
Date Sample Collected: ___________

Analyst: ______________________________
Test Start-Date/Time: _________________
Test Start-Date/time: __________________

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data (CONTINUED).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures should 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, or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	30 mL (recommended minimum)
7.	Test solution volume:	15 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 24 h; and all released within a 8-h period (required)
10.	No. neonates per test chamber:	1 Assigned using blocking by known parentage (Subsection 13.10.2.4) (required)
11.	No. replicate test chambers per concentration:	10 (required minimum)
12.	No. neonates per test concentration:	10 (required minimum)
13.	Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily (recommended)
14.	Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily (recommended)
15.	Aeration:	None (recommended)
16.	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)

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

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0) (CONTINUED)

17.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
18.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving Waters: None or ≥ 0.5 (recommended)
19.	Test duration:	Until 60% or more of surviving control females have three broods (maximum test duration 8 days) (required)
20.	Endpoints:	Survival and reproduction (required)
21.	Test acceptability criteria:	80% or greater survival of all control organisms and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control females must produce three broods (required)
22.	Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23.	Sample volume required:	1 L/day (recommended)

13.13 **DATA ANALYSIS**

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data. A sample set of survival and reproduction data is listed in Table 4.

TABLE 4. SUMMARY OF SURVIVAL AND REPRODUCTION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, EXPOSED TO AN EFFLUENT FOR SEVEN DAYS

Effluent Concentration (%)	No. of Young per Adult Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control	27	30	29	31	16	15	18	17	14	27	10
1.56	32	35	32	26	18	29	27	16	35	13	10
3.12	39	30	33	33	36	33	33	27	38	44	10
6.25	27	34	36	34	31	27	33	31	33	31	10
12.5	10	13	7	7	7	10	10	16	12	2	10
25.0	0	0	0	0	0	0	0	0	0	0	2

13.13.1.2 The endpoints of toxicity tests using the daphnid, *Ceriodaphnia dubia*, are based on the adverse effects on survival and reproduction. The LC50, the IC25, the IC50 and the EC50 are calculated using point estimation techniques, and LOEC and NOEC values for survival and reproduction are obtained using a hypothesis test approach such as Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962), Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, IC50 and EC50. 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 reproduction, but included in the estimation of the LC50, IC25, IC50, and EC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

13.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. 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.

13.13.1.4 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproduction endpoint (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of the survival endpoint (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of survival (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). Note that the exclusion of males from the analysis of reproduction may create unequal sample sizes among the concentrations, influencing the statistical methods chosen for analysis of reproduction (Figure 6). Determinations regarding test acceptability criteria for survival and reproduction (Subsection 13.12) must be made prior to exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the

control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample.

13.13.2 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined on the flowchart in Figure 4. The response used in the analysis is the number of animals surviving at each test concentration. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC50, LC50, IC25, or IC50 endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

13.13.2.2 Fisher's Exact Test is used to determine the NOEC and LOEC endpoints. It provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli (binomial) population. Additional information on Fisher's Exact Test is provided in Appendix G.

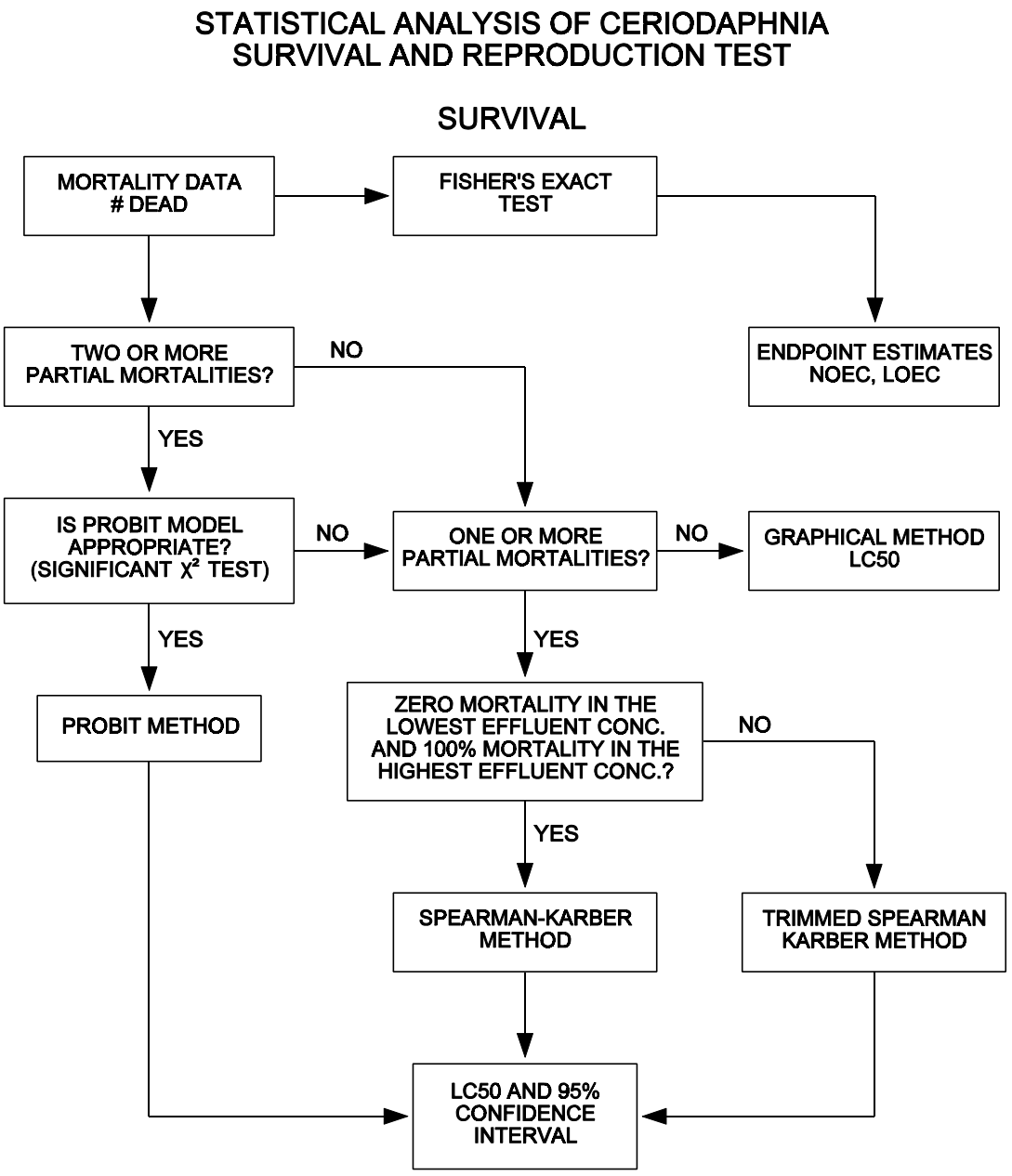

Figure 4. Flowchart for statistical analysis of the daphnid, *Ceriodaphnia dubia*, survival data.

13.13.2.3 Probit Analysis (Finney, 1971; Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total number dead at a given concentration is the response.

13.13.2.4 Example of Analysis of Survival Data

13.13.2.4.1 The data in Table 4 will be used to illustrate the analysis of survival data from the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test. As can be seen from the data in Table 4, there were no deaths in the 1.56%, 3.12%, 6.25%, and 12.5% concentrations. These concentrations are obviously not different from the control in terms of survival. This leaves only the 25% effluent concentration to be tested statistically for a difference in survival from the control.

13.13.2.5 Fisher's Exact Test

13.13.2.5.1 The basis for Fisher's Exact Test is a 2x2 contingency table. From the 2x2 table prepared by comparing the control and the effluent concentration, determine statistical significance by looking up a value in the table provided in Appendix G (Table G.5). However, to use this table the contingency table must be arranged in the format illustrated in Table 5.

TABLE 5. FORMAT OF THE 2x2 CONTINGENCY TABLE

	Number of		Number of
	Successes	Failures	Observations
Condition 1	a	A - a	A
Condition 2	b	B - b	B
Total	a + b	[(A+B) - a - b]	A + B

13.13.2.5.2 Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For these data, a success may be 'alive' or 'dead' whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is included in Appendix G, Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from that of the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

13.13.2.5.3 To compare the control and the effluent concentration of 25%, the appropriate contingency table for the test is given in Table 6.

13.13.2.5.4 Since $10/10 \geq 3/10$, the category 'alive' is regarded as a success. For A = 10, B = 10 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 6. Since the value of b (b = 3) from the contingency table (Table 6), is less than the value of b (b = 6) from Table G.5 in Appendix G, the test concludes that the proportion

surviving in the 25% effluent concentration is significantly different from the control. Thus the NOEC for survival is 12.5% and the LOEC is 25%.

TABLE 6. 2x2 CONTIGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		
	Alive	Dead	Number of Observations
Condition 1	10	0	10
Condition 2	3	7	10
Total	13	7	20

13.13.2.6 Calculation of the LC50

13.13.2.6.1 The data used for the Trimmed Spearman-Karber Method are summarized in Table 7. To perform the Trimmed Spearman-Karber Method, run the USEPA Trimmed Spearman-Karber Program. An example of the program input and output is supplied in Appendix J.

TABLE 7. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

	Effluent Concentration (%)					
	Control	1.56	3.12	6.25	12.5	25.0
Number Dead	0	0	0	0	0	8
Number Exposed	10	10	10	10	10	10

13.13.2.6.2 For this example, with only one partial mortality, Trimmed Spearman-Karber analysis appears appropriate for this data.

13.13.2.6.3 Figure 5 shows the output for the Trimmed Spearman-Karber Analysis of the data in Table 7 using the USEPA Program.

13.13.3 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION DATA

13.13.3.1 Formal statistical analysis of the reproduction data is outlined on the flowchart in Figure 6. The response used in the statistical analysis is the number of young produced per adult female, which is determined by taking the total number of young produced until either the time of death of the adult or the end of the experiment, whichever comes first. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test. An animal that dies before

producing young, if it has not been identified as a male, would be included in the analysis with zero entered as the number of young produced. The subsequent calculation of the mean number of live young produced per adult female for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction. An IC estimate can be calculated for the reproduction data using a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC for reproduction. Concentrations above the NOEC for survival are excluded from the hypothesis test for reproduction effects.

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

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

13.13.3.4 The data, mean, and variance of the observations at each concentration including the control are listed in Table 8. A plot of the number of young per adult female for each concentration is provided in Figure 7. Since there is significant mortality in the 25% effluent concentration, its effect on reproduction is not considered.

TABLE 8. THE DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2
Mean $\overline{Y}_I$	22.4	26.3	34.6	31.7	9.4
S_i^2	48.0	64.0	23.4	8.7	15.1
i	1	2	3	4	5

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: *Ceriodaphnia dubia*

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	10	0
1.25	10	0
3.12	10	0
6.25	10	0
12.5	10	0
25.0	10	8

SPEARMAN-KARBER TRIM: 20.00 %

SPEARMAN-KARBER ESTIMATES: LC50: 19.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure 5. Output for USEPA Trimmed Spearman-Karber program.

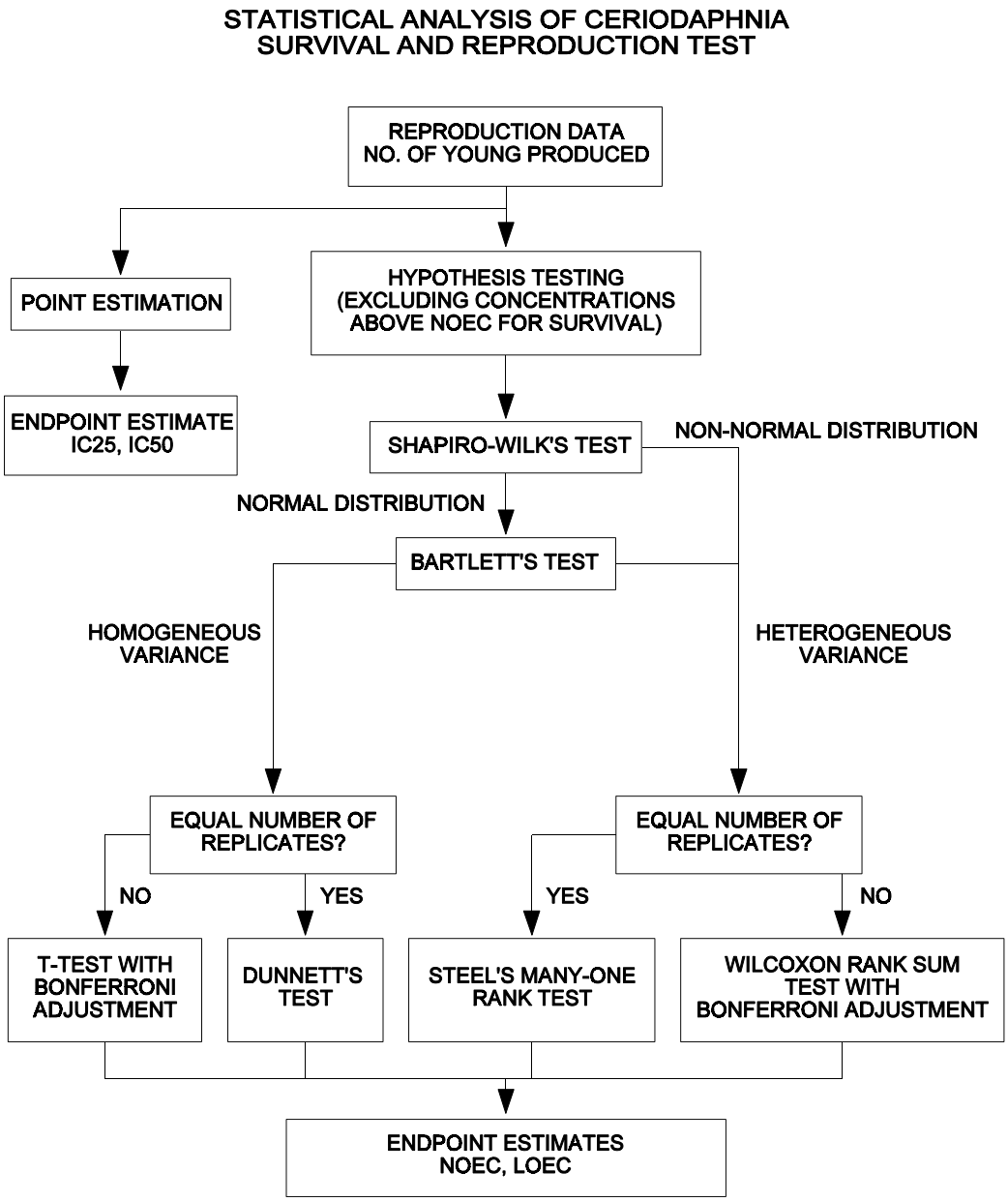

Figure 6. Flowchart for the statistical analysis of the daphnid, *Ceriodaphnia dubia*, reproduction data.

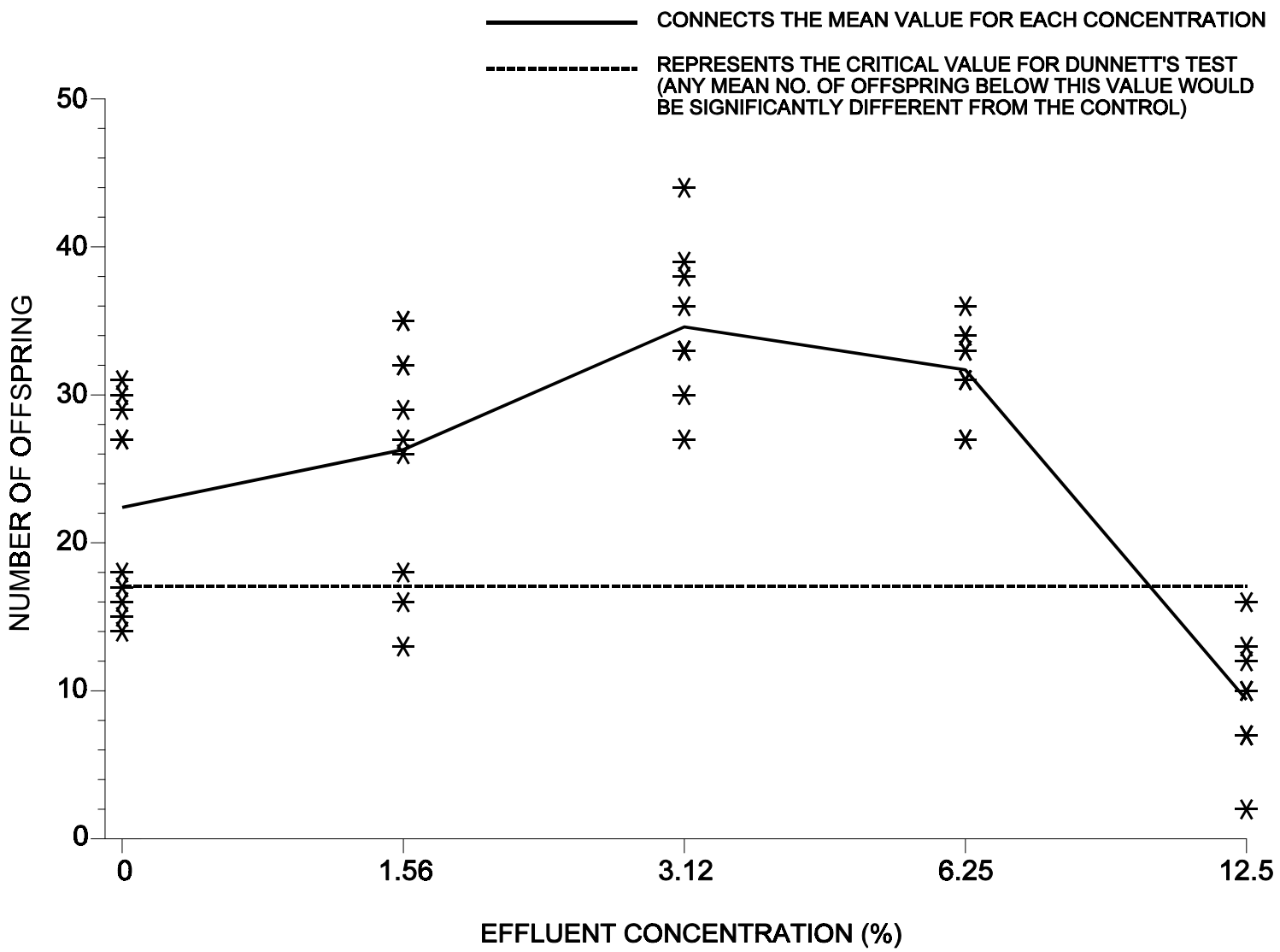

Figure 7. Plot of number of young per adult female from a daphnid, *Ceriodaphnia dubia*, survival and reproduction test.

13.13.3.5 Test for Normality

13.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 9.

TABLE 9. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	4.6	5.7	4.4	-4.7	0.6
2	7.6	8.7	-4.6	2.3	3.6
3	6.6	5.7	-1.6	4.3	-2.4
4	8.6	-0.3	-1.6	2.3	-2.4
5	-6.4	-8.3	1.4	-0.7	-2.4
6	-7.4	2.7	-1.6	-4.7	0.6
7	-4.4	0.7	-1.6	1.3	0.6
8	-5.4	-10.3	-7.6	-0.7	6.6
9	-8.4	8.7	3.4	1.3	2.6
10	4.6	-13.3	9.4	-0.7	-7.4

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

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations.

For this set of data,

$n = 50$

$\bar{X} = \frac{1}{50}(0.0) = 0.0$

$D = 1433.4$

13.13.3.5.3 Order the centered observations from smallest to largest

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

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 10.

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, ..., a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 50, k = 25. The a_i values are listed in Table 11.

13.13.3.5.5 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 11.

For this set of data:

$$W = \frac{1}{1433.4}(37.3)^2 = 0.97$$

TABLE 10. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-13.3	26	0.6
2	-10.3	27	0.6
3	-8.4	28	0.7
4	-8.3	29	1.3
5	-7.6	30	1.3
6	-7.4	31	1.4
7	-7.4	32	2.3
8	-6.4	33	2.3
9	-5.4	34	2.6
10	-4.7	35	2.7
11	-4.7	36	3.4
12	-4.6	37	3.6
13	-4.4	38	4.3
14	-2.4	39	4.4
15	-2.4	40	4.6
16	-2.4	41	4.6
17	-1.6	42	5.7
18	-1.6	43	5.7
19	-1.6	44	6.6
20	-1.6	45	6.6
21	-0.7	46	7.6
22	-0.7	47	8.6
23	-0.7	48	8.7
24	-0.3	49	8.7
25	0.6	50	9.4

TABLE 11. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3751	22.7	$X^{(50)} - X^{(1)}$
2	0.2574	19.0	$X^{(49)} - X^{(2)}$
3	0.2260	17.1	$X^{(48)} - X^{(3)}$
4	0.2032	16.9	$X^{(47)} - X^{(4)}$
5	0.1847	15.2	$X^{(46)} - X^{(5)}$
6	0.1691	14.0	$X^{(45)} - X^{(6)}$
7	0.1554	14.0	$X^{(44)} - X^{(7)}$
8	0.1430	12.1	$X^{(43)} - X^{(8)}$
9	0.1317	11.1	$X^{(42)} - X^{(9)}$
10	0.1212	9.3	$X^{(41)} - X^{(10)}$
11	0.1113	9.3	$X^{(40)} - X^{(11)}$
12	0.1020	9.0	$X^{(39)} - X^{(12)}$
13	0.0932	8.7	$X^{(38)} - X^{(13)}$
14	0.0846	6.0	$X^{(37)} - X^{(14)}$
15	0.0764	5.8	$X^{(36)} - X^{(15)}$
16	0.0685	5.1	$X^{(35)} - X^{(16)}$
17	0.0608	4.2	$X^{(34)} - X^{(17)}$
18	0.0532	3.9	$X^{(33)} - X^{(18)}$
19	0.0459	3.9	$X^{(32)} - X^{(19)}$
20	0.0386	3.0	$X^{(31)} - X^{(20)}$
21	0.0314	2.0	$X^{(30)} - X^{(21)}$
22	0.0244	2.0	$X^{(29)} - X^{(22)}$
23	0.0174	1.4	$X^{(28)} - X^{(23)}$
24	0.0104	0.9	$X^{(27)} - X^{(24)}$
25	0.0035	0.0	$X^{(26)} - X^{(25)}$

13.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 50 observations (n) is 0.930. Since W = 0.97 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 The test used to examine whether the variation in number of young produced is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

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

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

p = number of levels of effluent concentration and control

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

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

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

13.13.3.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 10 for all i). Thus, V_i = 9 for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(45)\ln(31.8) - 9\sum_{i=1}^{P} \ln(S_i^2)]/1.04$$

= [45(3.46) - 9(16.061)]/1.04

= 11.15/1.04

= 10.72

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = 10.7 is less than the critical value of 13.3, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 12.

TABLE 12. ANOVA TABLE

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

Where: p = number effluent concentrations including the control

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

n_i = number of observations in concentration i

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

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ 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 number of young produced by female j in effluent concentration i)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 10$

N = 50

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{110} = 224$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{210} = 263$

$T_3 = Y_{31} + Y_{32} + \ldots + Y_{310} = 346$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{410} = 317$
$T_5 = Y_{51} + Y_{52} + \ldots + Y_{510} = 94$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 1244$

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

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

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

$$= 36{,}272 - \frac{(1244)^2}{50} = 5321.28$$

$SSW = SST - SSB = 5321.28 - 3887.88 = 1433.40$

$S_B^2 = SSB/(p-1) = 3887.88/(5-1) = 971.97$

$S_W^2 = SSW/(N-p) = 1433.40/(50-5) = 31.85$

13.13.3.7.3 Summarize these calculations in an ANOVA table (Table 13).

TABLE 13. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	3887.88	971.97
Within	45	1433.40	31.85
Total	49	5321.28	

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

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

Where: $\bar{Y}_i$ = mean number of young produced for effluent concentration i

$\bar{Y}_1$ = mean number of young produced for the control

S_W = square root of within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decrease in reproduction from the control, the mean for concentration i is subtracted from the control mean in the t statistic above. However, if we were looking for an increased response over the control, the control mean would be subtracted from the mean at a concentration.

13.13.3.7.5 Table 14 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.56% concentration with the control the calculation is as follows:

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

TABLE 14. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
1.56	2	-1.55
3.12	3	-4.84
6.25	4	-3.69
12.5	5	5.16

13.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean reproduction, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. Since an entry for 45 degrees of freedom for error is not provided in the table, the entry for 40 degrees of freedom for error, an alpha level of 0.05 and four concentrations (excluding the control) will be used, 2.23. The mean reproduction for concentration "i" is considered significantly less than the mean reproduction for the control if t_i is greater than the critical value.

Since t_5 is greater than 2.23, the 12.5% concentration has significantly lower reproduction than the control. Hence the NOEC and the LOEC for reproduction are 6.25% and 12.5%, respectively.

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

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

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.

13.13.3.7.8 In this example:

$$MSD = 2.23(5.64)\sqrt{(\frac{1}{10}) + (\frac{1}{10})}$$

$$= 2.23\ (5.64)\ (0.447)$$

$$= 5.62$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 5.62.

13.13.3.7.10 This represents a 25% decrease in mean reproduction from the control.

13.13.3.8 Calculation of the IC

13.13.3.8.1 The reproduction data in Table 4 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

13.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1 = 22.4$, and the observed mean for the lowest effluent concentration, $\overline{Y}_2 = 26.3$, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

13.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 24.35$$

13.13.3.8.4 Since $\overline{Y}_3$= 34.6 is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + \overline{Y}_3)/3 = 27.7.$$

13.13.3.8.5 Additionally, $\overline{Y}_4$ = 31.7 is larger than M_3, and is pooled with the first three means. Thus:

$$(M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7 = M_1 = M_2 = M_3 = M_4$$

13.13.3.8.6 Since $M_4 > \overline{Y}_5$ = 9.4, set M_5 = 9.4. Likewise, $M_5 > \overline{Y}_6$ = 0, and M_6 becomes 0. Table 15 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

TABLE 15. DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Response Effluent Conc. (%)	i	Smoothed Means, Y_i (young/female)	Means, M_i (young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

13.13.3.8.7 Estimates of the IC25 and IC50 can be calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1 - p/100) = 28.75(1 - 25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1 - p/100) = 28.75(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 15), the two effluent concentrations bracketing 21.56 young per adult are C_4 = 6.25% effluent and C_5 = 12.5% effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also C_4 = 6.25% and C_5 = 12.5%.

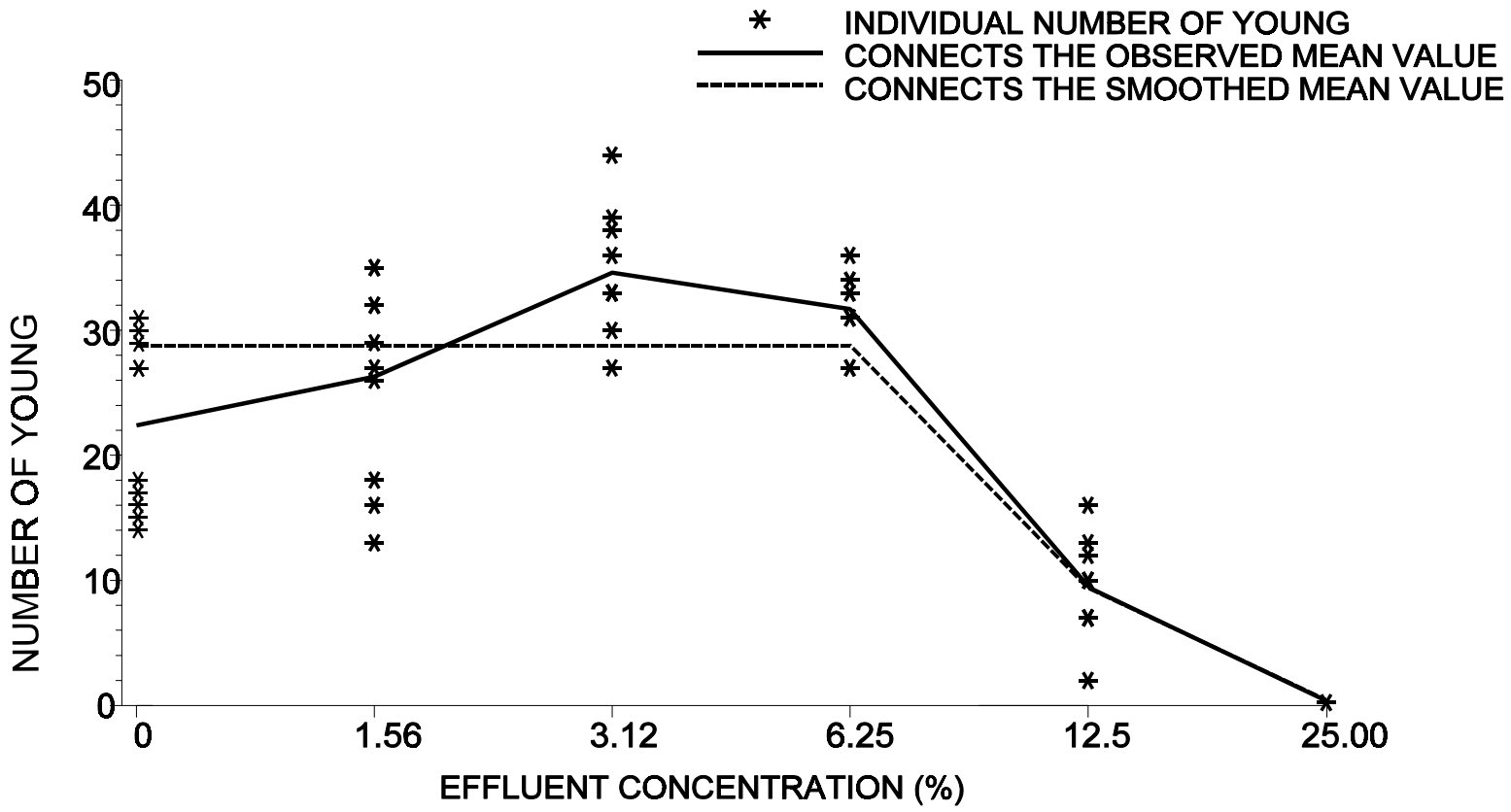

Figure 8. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

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

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

$$IC25 = 6.25 + [28.75(1 - \frac{25}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 8.57% effluent

13.13.3.8.9 The estimate of the IC50 is as follows:

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

$$IC50 = 6.25 + [28.75(1 - \frac{50}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 10.89% effluent

13.13.3.8.10 When the ICPIN program was used to analyze this data set for the IC25, requesting 80 resamples, the estimate of the IC25 was 8.5715% effluent. The empirical 95% confidence interval for the true mean was 8.3112% and 9.0418% effluent. The computer output for this data set is provided in Figure 9.

13.13.3.8.11 When the ICPIN program was used to analyze this data set for the IC50, requesting 80 resamples, the estimate of the IC50 was 10.8931% effluent. The empirical 95% confidence interval for the true mean was 10.4373% and 11.6269% effluent. The computer output for this data set is provided in Figure 10.

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0      1.56      3.12      6.25      12.5      25.0
-------------------------------------------------------------------------
Response  1         27        32        39        27        10         0
Response  2         30        35        30        34        13         0
Response  3         29        32        33        36         7         0
Response  4         31        26        33        34         7         0
Response  5         16        18        36        31         7         0
Response  6         15        29        33        27        10         0
Response  7         18        27        33        33        10         0
Response  8         17        16        27        31        16         0
Response  9         14        35        38        33        12         0
Response 10         27        13        44        31         2         0
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:             7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates               %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1         10             0.000         22.400      6.931     28.750
  2         10             1.560         26.300      8.001     28.750
  3         10             3.120         34.600      4.835     28.750
  4         10             6.250         31.700      2.946     28.750
  5         10            12.500          9.400      3.893      9.400
  6         10            25.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     8.5715   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   8.5891 Standard Deviation:     0.1831
Original Confidence Limits:   Lower:     8.3112 Upper:     9.0418
Resampling time in Seconds:     2.53  Random Seed: -641671986
```

Figure 9. Example of ICPIN program output for the IC25.

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0      1.56      3.12      6.25      12.5      25.0
-------------------------------------------------------------------------
Response  1         27        32        39        27        10         0
Response  2         30        35        30        34        13         0
Response  3         29        32        33        36         7         0
Response  4         31        26        33        34         7         0
Response  5         16        18        36        31         7         0
Response  6         15        29        33        27        10         0
Response  7         18        27        33        33        10         0
Response  8         17        16        27        31        16         0
Response  9         14        35        38        33        12         0
Response 10         27        13        44        31         2         0
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:             7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i50
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates               %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1         10             0.000         22.400      6.931     28.750
  2         10             1.560         26.300      8.001     28.750
  3         10             3.120         34.600      4.835     28.750
  4         10             6.250         31.700      2.946     28.750
  5         10            12.500          9.400      3.893      9.400
  6         10            25.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:    10.8931   Entered P Value: 50
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  10.9316 Standard Deviation:     0.3357
Original Confidence Limits:   Lower:    10.4373 Upper:    11.6269
Resampling time in Seconds:     2.58  Random Seed: 172869646
```

Figure 10. Example of ICPIN program output for the IC50.

13.14 **PRECISION AND ACCURACY**

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Information on the single-laboratory precision of the daphnid, *Ceriodaphnia dubia*, survival and reproduction test is based on the NOEC and LOEC values from nine tests with the reference toxicant sodium pentachlorophenate (NaPCP) is provided in Table 16. The NOECs and LOECs of all tests fell in the same concentration range, indicating maximum possible precision. Table 17 gives precision data for the IC25 and IC50 values for seven tests with the reference toxicant NaPCP. Coefficient of variation was 41% for the IC25 and 28% for the IC50.

13.14.1.1.2 Ten sets of data from six laboratories met the acceptability criteria, and were statistically analyzed using nonparametric procedures to determine NOECs and LOECs.

13.14.1.1.3 EPA evaluated within-laboratory precision of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test using a database of routine reference toxicant test results from 33 laboratories (USEPA, 2000b). The database consisted of 393 reference toxicant tests conducted in 33 laboratories using a variety of reference toxicants including: cadmium, copper, potassium chloride, sodium chloride, and sodium pentachlorophenate. Among the 33 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 reproduction endpoint. In 25% of laboratories, the within-laboratory CV was less than 17%; and in 75% of laboratories, the within-laboratory CV was less than 45%.

TABLE 16: SINGLE LABORATORY PRECISION OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[3]	0.25	0.50	0.35
2[4]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[5]	0.30	0.60	0.42
5	0.30	0.60	0.42
6	0.30	0.60	0.42
7	0.30	0.60	0.42
8	0.30	0.60	0.42
9	0.30	0.60	0.42

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

2 Data from Tests performed by Philip Lewis, Aquatic Biology Branch, EMSL-Cincinnati, OH. Tests were conduted in reconstituted hard water (hardness = 180 mg $CaC0_3$/L; pH - 8.1).

3 Concentrations used in Test 1 were: 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

4 Concentrations used in Tests 2 and 3 were: 0.007, 0.022, 0.067, 0.020, 0.60 mg NaPCP/L.

5 Concentrations used in Tests 4 through 9 were: 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

TABLE 17. THE DAPHNID, *CERIODAPHNIA DUBIA*, SEVEN-DAY SURVIVAL AND REPRODUCTION TEST PRECISION FOR A SINGLE LABORATORY USING NAPCP AS THE REFERENCE TOXICANT (USEPA, 1991a)

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
19	0.30	0.3754	0.4508
46A	0.20	0.0938	0.2608
46B	0.20	0.2213	0.2879
49	0.20	0.2303	0.2912
55	0.20	0.2306	0.3177
56	0.10	0.2241	0.2827
n	7	7	7
Mean	NA	0.2157	0.2953
CV(%)	NA	41.1	27.9

13.14.1.2 Multilaboratory Precision

13.14.1.2.1 A multilaboratory study was performed by the Aquatic Biology Branch, EMSL-Cincinnati in 1985e, involving a total of 11 analysts in 10 different laboratories (Neiheisel et. al., 1988; USEPA, 1988e). Each analyst performed one-to-three seven-day tests using aliquots of a copper-spiked effluent sample, for a total of 25 tests. The tests were performed on the same day in all participating laboratories, using a pre-publication draft of Method 1002.0. The NOECs and LOECs for these tests were within one concentration interval which, with a dilution factor of 0.5, is equivalent to a two-fold range in concentration (Table 18).

13.14.1.2.2 A second multilaboratory study of Method 1002.0 (using the first edition of this manual; USEPA, 1985c), was coordinated by Battelle, Columbus Division, and involved 11 participating laboratories (Table 19) (DeGraeve et al., 1989). All participants used 10% DMW (10% PERRIER® Water) as the culture and dilution water, and used their own formulation of food for culturing and testing the *Ceriodaphnia dubia*. Each laboratory was to conduct at least one test with each of eight blind samples. Each test consisted of 10 replicates of one organism each for five toxicant concentrations and a control. Of the 116 tests planned, 91 were successfully initiated, and 70 (77%) met the survival and reproduction criteria for acceptability of the results (80% survival and nine young per initial female). If the reproduction criteria of 15 young/female, used in this edition of the method, had been applied to the results of the interlaboratory study, 22 additional tests would have been unacceptable. The overall precision (CV) of the test was 27% for the survival data (7-day LC50s) and 37.5% and 39.0% for the reproduction data (IC50s and IC25s, respectively).

13.14.1.2.3 In 2000, EPA conducted an interlaboratory variability study of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 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 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 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 3.70%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 20 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 35.0% for IC25 results. Table 21 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant and effluent sample types and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 97.2%, 91.3%, 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, three concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 18. INTERLABORATORY PRECISION FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST WITH COPPER SPIKED EFFLUENT (USEPA, 1988e)

		Endpoints (% Effluent)			
		Reproduction		Survival	
Analyst	Test	NOEC	LOEC	NOEC	LOEC
3	1	12	25	25	50
4	1	6	12	12	25
4	2	6	12	25	50
5	1	6	12	12	25
5	2	12	25	12	25
6	1	12	25	25	50
6	2	6	12	25	50
10	1	6	12	12	25
10	2	6	12	12	25
11	1	12	25	25	50

TABLE 19. INTERLABORATORY PRECISION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, SUMMARIZED FOR EIGHT REFERENCE TOXICANTS AND EFFLUENTS (USEPA, 1991a)

Test Material	Mean IC50	CV%	Mean IC25	CV%
Sodium chloride	1.34	29.9	1.00	34.3
Industrial	3.6	83.3	3.2	78.1
Sodium chloride	0.96	57.4	0.09	44.4
Pulp and Paper	60.0	28.3	47.3	27.0
Potassium dichromate	35.8	30.8	23.4	32.7
Pulp and Paper	70.2	7.5	55.7	12.2
Potassium dichromate	53.2	25.9	29.3	46.8
Industrial	69.8	37.0	67.3	36.7
n		8		8
Mean		37.5		39.0
Standard Deviation		23.0		19.1

TABLE 20. 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	-	-	-
	Effluent	17.4	27.6	32.6
	Receiving water	-	-	37.4
	Average	17.4	27.6	35.0

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

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the reference toxicant sample type a majority of the results were outside of the test concentration range, so precision estimates were not calculated. For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

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

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

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

TABLE 21. 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	100%	97.2	0.00	2.78
	Effluent	25%	65.2	26.1	8.70
	Receiving water	25%	90.0	10.0	0.00
Growth NOEC	Reference toxicant	100%	72.2	11.1	16.7
	Effluent	12.5%	70.8	29.2	0.00
	Receiving water	25%	70.0	30.0	0.00

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

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

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

SECTION 14

TEST METHOD

GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TEST METHOD 1003.0

14.1 SCOPE AND APPLICATION

14.1.1 This method measures the chronic toxicity of effluents and receiving water to the freshwater green alga, *Selenastrum capricornutum*, in a four-day static 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.

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

14.1.3 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.

14.1.4 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.

14.1.5 This test is very versatile because it can also be used to identify wastewaters which are biostimulatory and may cause nuisance growths of algae, aquatic weeds, and other organisms at higher trophic levels.

14.2 SUMMARY OF METHOD

14.2.1 A green alga, *Selenastrum capricornutum*, population is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance.

14.3 INTERFERENCES

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

14.3.2 Adverse effects of high concentrations of suspended and/or dissolved solids, color, and extremes of pH may mask the presence of toxic substances.

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

14.3.4 Pathogenic organisms and/or planktivores in the dilution water and effluent may affect test organism survival and growth, and confound test results.

14.3.5 Nutrients in the effluent or dilution water may confound test results.

14.4 SAFETY

14.4.1 See Section 3, Safety and Health.

14.5 **APPARATUS AND EQUIPMENT**

14.5.1 Laboratory *Selenastrum capricornutum* culture unit -- see culturing methods below and USEPA, 2002a. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

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

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

14.5.4 Environmental chamber, incubator, or equivalent facility -- with "cool-white" fluorescent illumination (86 ± 8.6 $\mu E/m^2/s$, 400 ± 40 ft-c, or 4306 lux) and temperature control (25 ± 1°C).

14.5.5 Mechanical shaker -- capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

14.5.6 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

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

14.5.8 Balance -- analytical, capable of accurately weighing 0.00001 g.

14.5.9 Reference weights, class S -- for checking performance of balance.

14.5.10 Volumetric flasks and graduated cylinders -- class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

14.5.11 Volumetric pipets -- class A, 1-100 mL.

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

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

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

14.5.15 Test chambers -- four 125 or 250 mL borosilicate, Erlenmeyer flasks, with foam plugs or stainless steel or Shumadzu closures. For special glassware cleaning requirements (see Section 5, Facilities, Equipment, and Supplies).

14.5.16 Culture chambers -- 1-4 L borosilicate, Erlenmeyer flasks.

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

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

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

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

14.5.21 Tissue grinder -- for chlorophyll extraction.

14.5.22 Fluorometer (Optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

14.5.23 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

14.5.24 Cuvettes for spectrophotometer -- 1-5 cm light path.

14.5.25 Electronic particle counter (Optional) -- Coulter Counter, Model ZBI, or equivalent, with mean cell (particle) volume determination.

14.5.26 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condenser, and light source (inverted or conventional microscope).

14.5.27 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

14.5.28 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

14.5.29 Centrifuge tubes -- 15-100 mL, screw-cap.

14.5.30 Filtering apparatus -- for membrane and/or glass fiber filters.

14.6 **REAGENTS AND CONSUMABLE MATERIALS**

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

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

14.6.3 Tape, colored -- for labeling test chambers.

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

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

14.6.6 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).

14.6.7 Specific conductivity standards (see USEPA Method 120.1, USEPA, 1979b).

14.6.8 Standard particles -- such as chicken or turkey fibroblasts or polymer microspheres, 5.0 ± 0.03 µm diameter, 65.4 μm^3 volume, for calibration of electronic particle counters.

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

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

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

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

14.6.13 Effluent or 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 Testing.

14.6.14 Acetone -- pesticide-grade or equivalent.

14.6.15 Dilute (10%) hydrochloric acid -- carefully add 10 mL of concentrated HCl to 90 mL of MILLI-Q® water.

14.6.16 TEST ORGANISMS, GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*

14.6.16.1 *Selenastrum capricornutum*, a unicellular coccoid green alga, is the test organism. The genus and species name of this organism was formally changed to *Pseudokirchneriella subcapitata* (Hindak, 1990), however, the method manual will continue to refer to *Selenastrum capricornutum* to maintain consistency with previous versions of the method.

14.6.16.2 Algal Culture Medium is prepared as follows:

14.6.16.2.1 Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.

14.6.16.2.2 Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1N NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

14.6.16.2.3 Immediately filter the pH-adjusted medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.

14.6.16.2.4 If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels. If a 0.22 µg filter is used no sterilization is needed.

14.6.16.2.5 Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

14.6.16.2.6 When prepared according to Table 1, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). EPA requires the addition of EDTA to nutrient stock solutions when conducting the *Selenastrum capricornutum* Growth Test and submitting data under NPDES permits. The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC25 values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a). While the addition of EDTA improves test performance, EPA also cautions that the addition of EDTA may cause the *Selenastrum capricornutum* Growth Test to underestimate the toxicity of metals. Regulatory authorities should consider this possibility when selecting test methods for monitoring effluents that are suspected to contain metals. As recommended in EPA's Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a), the most sensitive of at least three test species from different phyla should be used for monitoring the toxicity of effluents.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1.	MACRONUTRIENTS		
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2.	MICRONUTRIENTS		
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

14.6.16.3 Stock Algal Cultures

14.6.16.3.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of the green alga, *Selenastrum capricornutum*.

14.6.16.3.2 Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125 mL flask to 2 L in a 4-L flask. The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.

14.6.16.3.3 Maintain the stock cultures at 25 ± 1°C, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c). Shake continuously at 100 cpm or twice daily by hand.

14.6.16.3.4 Transfer 1 to 2 mL of stock culture weekly to 50 - 100 mL of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination. Examine the stock cultures with a microscope for contaminating microorganisms at each transfer.

14.6.16.3.5 Viable unialgal culture material may be maintained for long periods of time if placed in a refrigerator at 4°C.

14.6.16.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Algal cells four to seven days old are used to monitor the chronic toxicity (growth) of the reference toxicant to the algal stock produced by the culture unit (see Section 4, Quality Assurance, Subsection 4.17).

14.6.16.5 Record Keeping

14.6.16.5.1 Records, kept in a bound notebook, include (1) dates culture media was prepared, (2) source of "starter" cultures, (3) date stock cultures were started, (4) cell density in stock cultures, and (5) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 TEST PROCEDURES

14.10.1 TEST SOLUTIONS

14.10.1.1 Receiving Waters

14.10.1.1.1 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 100 mL and 400 mL for chemical analyses, would require approximately 1 L or more of sample for the test.

14.10.1.2 Effluents

14.10.1.2.1 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 five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends using a ≥ 0.5 dilution factor**.

14.10.1.2.2 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 the effluent concentrations.

14.10.1.2.3 The volume of effluent required for the test is 1 to 2 L. Sufficient test solution (approximately 900 or 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. There is no daily renewal of test solution.

14.10.1.2.4 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 usedfor 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).

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

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

14.10.1.2.7 Effluents may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at a minimum the concentration of nutrients in the stock culture medium.

14.10.1.2.8 If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

14.10.1.3 Dilution Water

14.10.1.3.1 Dilution water may be stock culture medium, any uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other natural water (see Section 7, Dilution Water). However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution should be added per liter of dilution water. Natural waters used as dilution water must be filtered through a prewashed filter, such as a GF/A, GF/C, or equivalent filter, that provides 0.45 µm particle size retention.

14.10.1.3.2 If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the effluent and dilution water must be filtered through a GF/A or GF/C filter, or other filter providing 0.45 µm particle

size retention, and checked for "background" particle count before it is used in the test. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging.

14.10.1.4 Preparation of Inoculum

14.10.1.4.1 The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to-seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of approximately 10,000 cells/mL (± 10%) in the test flasks. Assuming the use of 250 mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL.

14.10.1.4.2 Estimate the volume of stock culture required to prepare the inoculum. As an example, if the four-to-seven-day-old stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 24 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 24,000,000 cells or 15 mL of stock solution (24,000,000/2,000,000) to provide sufficient inoculum. It is advisable to prepare a volume 20% to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

14.10.1.4.3 Prepare the inoculum as follows:

1. Centrifuge 15 mL of stock culture at 1000 x g for 5 min. This volume will provide a 50% excess in the number of cells.
2. Decant the supernatant and resuspend the cells in 10 mL of control medium.
3. Repeat the centrifugation and decantation step, and resuspend the cells in 10 mL control medium.
4. Mix well and determine the cell density in the algal concentrate. Some cells will be lost in the concentration process.
5. Determine the density of cells (cells/mL) in the stock culture (for this example, assume 2,000,000 per mL).
6. Calculate the required volume of stock culture as follows:

$$\text{Volume (mL) of Stock Culture Required} = \frac{\text{Number test flasks to be used} \times \text{Volume of test Solutions/flask} \times \text{10,000 cells/mL}}{\text{Cell density (cells/mL) in the stock culture}}$$

$$= \frac{24 \text{ flasks} \times 100 \text{ mL/flask} \times 10{,}000 \text{ cells/mL}}{2{,}000{,}000 \text{ cells/mL}}$$

$$= 12.0 \text{ mL Stock Culture}$$

7. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.
8. The volume of the algal inoculum should be considered in calculating the dilution of toxicant in the test flasks.

14.10.2 START OF THE TEST

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

14.10.2.2 Randomize the position of the test flasks at the beginning of the test (see Appendix A). Preparation of a position chart may be helpful.

14.10.2.3 The test begins when the algae are added to the test flasks. Mix the inoculum well, and add 1 mL to the test solution in each randomly arranged flask. Make a final check of the cell density in three of the test solutions at time "zero" (within 2 h of the inoculation).

14.10.2.3.1 Alkalinity, hardness, and conductivity are measured at the beginning of the test in the high, medium, and low effluent concentrations and control before they are dispensed to the test chambers and the data recorded on the data sheet (Figure 1).

Discharger:_____ Test Dates: _____
Location:_____ Analyst: _____

	Effluent Concentration								
Parameter	Control								Remarks
Temperature									
pH									
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the green alga, *Selenastrum capricornutum*, growth test. Routine chemical and physical determinations.

14.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

14.10.3.1 Test flasks are incubated under continuous illumination at 86 ± 8.6 µE/m^2/s (400 ± 40 ft-c), at 25 ± 1°C, and should be shaken continuously at 100 cpm on a mechanical shaker or twice daily by hand. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. If it can be verified that test specifications are met at all positions, this need not be done.

14.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.4.1 Because of the continuous illumination of the test flasks, DO concentration should never be a problem during the test and no aeration will be required.

14.10.5 OBSERVATIONS DURING THE TEST

14.10.5.1 Routine Chemical and Physical Determinations

14.10.5.1.1 Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be checked in a sufficient number of test vessels at least at the end of the test to determine variability in the environmental chamber.

14.10.5.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test flask at each concentration and in the control.

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

14.10.5.2 Biological Observations

14.10.5.2.1 Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one or two days in the test. It may be desirable, therefore, to determine the algal growth response daily. Otherwise, biological observations are not required until the test is terminated and the test solutions are not renewed during the test period.

14.10.6 TERMINATION OF THE TEST

14.10.6.1 The test is terminated 96 h after initiation. The algal growth in each flask is measured by one of the following methods: (a) cell counts, (b) chlorophyll content, or (c) turbidity (light absorbance).

14.10.6.2 Cell counts

14.10.6.2.1 Automatic Particle Counters

14.10.6.2.1.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in μm^3/cell. The Coulter Counter is widely used and is discussed in detail in USEPA (1978b).

14.10.6.2.1.2 If biomass data are desired for algal growth potential measurements, a Model ZM Coulter Counter is used. However, the instrument must be calibrated with a reference sample of particles of known volume.

14.10.6.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a l% sodium chloride electrolyte solution, such as ISOTON®, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-µm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.
3. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON®).
4. Determine the cell density (and MCV, if desired).

14.10.6.2.2 Manual microscope counting method

14.10.6.2.2.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA (1992) and USEPA (1973). Whenever feasible, 400 cells per replicate are counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

14.10.6.3 Chlorophyll Content

14.10.6.3.1 Chlorophyll may be estimated in-vivo fluorometrically, or in-vitro either fluorometrically or spectrophotometrically. In-vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al., 1982).

14.10.6.3.2 The in-vivo chlorophyll measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45 µm particle retention filter.
2. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 1 mL of culture from the flask with a sterile pipet.
3. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min).
4. Discard the sample.

14.10.6.3.3 For additional information on chlorophyll measurement methods, (see APHA, 1992).

14.10.6.4 Turbidity (Absorbance)

14.10.6.4.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the turbidity, or absorbance, of the cultures at a wavelength of 750 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it would be useful to construct a calibration curve to establish the relationship between absorbance and cell density.

14.10.6.4.2 The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The culture is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the test flask with a sterile pipet and transferred to a 1- to 5-cm cuvette.
4. The absorbance is read at 750 nm and divided by the light path length of the cuvette, to obtain an “absorbance-per-centimeter” value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

14.10.6.5 Record the data as indicated in Figure 2.

14.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

14.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 For the test results to be acceptable, the mean algal cell density in the control flasks must exceed 1×10^6 cells/mL at the end of the test, and the coefficient of variation (CV, calculated as standard deviation X 100 / mean) for algal cell density among the control replicates must not exceed 20%.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

Concentration	Cell Density Measurement Replicate				Treatment Mean	Comments
	1	2	3	4		
Control						
Conc:						
Conc:						
Conc:						
Conc:						
Conc:						

Comments:

Figure 2. Data form for the green alga, *Selenastrum capricornutum*, growth test, cell density determinations.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0)[1]

1.	Test type:	Static non-renewal (required)
2.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	"Cool white" fluorescent lighting (recommended)
4.	Light intensity:	86 ± 8.6 µE/m^2/s (400 ± 40 ft-c or 4306 lux) (recommended)
5.	Photoperiod:	Continuous illumination (required)
6.	Test chamber size:	125 mL or 250 mL (recommended)
7.	Test solution volume:	50 mL or 100 mL[2] (recommended)
8.	Renewal of test solutions:	None (required)
9.	Age of test organisms:	4 to 7 days (required)
10.	Initial cell density in test chambers:	10,000 cells/mL (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	Shaking rate:	100 cpm continuous, or twice daily by hand (recommended)
13.	Aeration:	None (recommended)
14.	Dilution water:	Algal stock culture medium, enriched 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)

[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.

[2] For tests not continuously shaken use 25 mL in 125 mL flasks and 50 mL in 250 mL flasks.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0) (CONTINUED)

15.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
16.	Test dilution factor:	Effluents: $\geq$ 0.5 (recommended) Receiving Waters: None or $\geq$ 0.5 (recommended)
17.	Test duration:	96 h (required)
18.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, or biomass) (required)
19.	Test acceptability criteria:[3]	Mean cell density of at least 1 X 10^6 cells/mL in the controls; and variability (CV%) among control replicates less than or equal to 20% (required)
20.	Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 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)
21.	Sample volume required:	1 or 2 L depending on test volume (recommended)

[3] If the test is conducted under non-NPDES applications (i.e., data are not submitted under NPDES permits) and used without EDTA in the nutrient stock solution, the test acceptability criteria are a mean cell density of at least 2 X 10^5 cells/mL in the controls, and variability (CV%) among control replicates less than or equal to 20%.

14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. A sample set of algal growth response data is shown in Table 4.

TABLE 4. GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH RESPONSE DATA

			Toxicant Concentration (µg Cd/L)				
	Replicate	Control	5	10	20	40	80
	A	1209	1212	826	493	127	49.3
	B	1180	1186	628	416	147	40.0
	C	1340	1204	816	413	147	44.0
Log_{10}	A	3.082	3.084	2.917	2.693	2.104	1.693
Trans-	B	3.072	3.074	2.798	2.619	2.167	1.602
formed	C	3.127	3.081	2.912	2.616	2.167	1.643
	Mean($\bar{Y}_i$)	3.094	3.080	2.876	2.643	2.146	1.646

14.13.1.2 The endpoints of toxicity tests using the green alga, *Selenastrum capricornutum*, are based on the adverse effects on cell growth (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). The EC50, the IC25, and the IC50 are calculated using the point estimation techniques, and LOEC and NOEC values for 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). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the EC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.1.3 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.

14.13.2 EXAMPLE OF ANALYSIS OF ALGAL GROWTH DATA

14.13.2.1 Formal statistical analysis of the growth data is outlined on the flowchart in Figure 3. The response used in the statistical analysis is the number of cells per milliliter per replicate. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints.

14.13.2.2 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 Tests, 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.

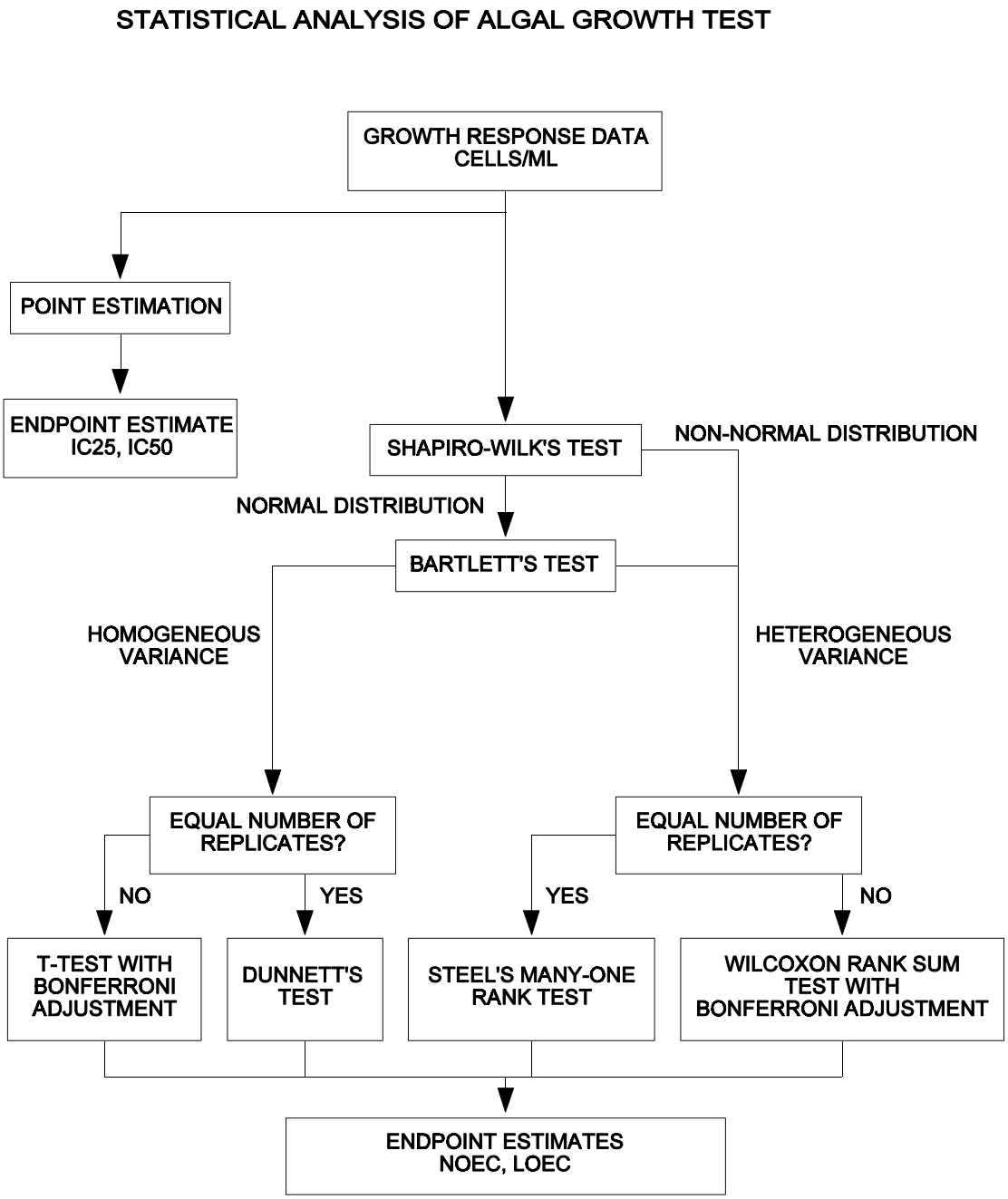

Figure 3. Flowchart for statistical analysis of the green alga, *Selenastrum capricornutum*, growth response data.

14.13.2.3 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).

14.13.2.4 Data from an algal growth test with cadmium chloride will be used to illustrate the statistical analysis. The cell counts were $\log_{10}$ transformed in an effort to stabilize the variance for the ANOVA analysis. The raw data, $\log_{10}$ transformed data, mean and standard deviation of the observations at each concentration including the control are listed in Table 4. A plot of the $\log_{10}$ transformed cell counts for each treatment is provided in Figure 4.

14.13.2.5 Test for Normality

14.13.2.5.1 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 5.

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

	Toxicant Concentration (µg Cd/L)					
Replicate	Control	5	10	20	40	80
A	-0.012	0.004	0.041	0.050	-0.042	0.047
B	-0.022	-0.006	-0.078	-0.024	0.021	-0.044
C	0.033	0.001	0.036	-0.027	0.021	-0.003

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

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations.

For this set of data, n = 18

$$\overline{X} = \frac{1}{18}(0.000) = 0.000$$

D = 0.0214

14.13.2.5.3 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 6.

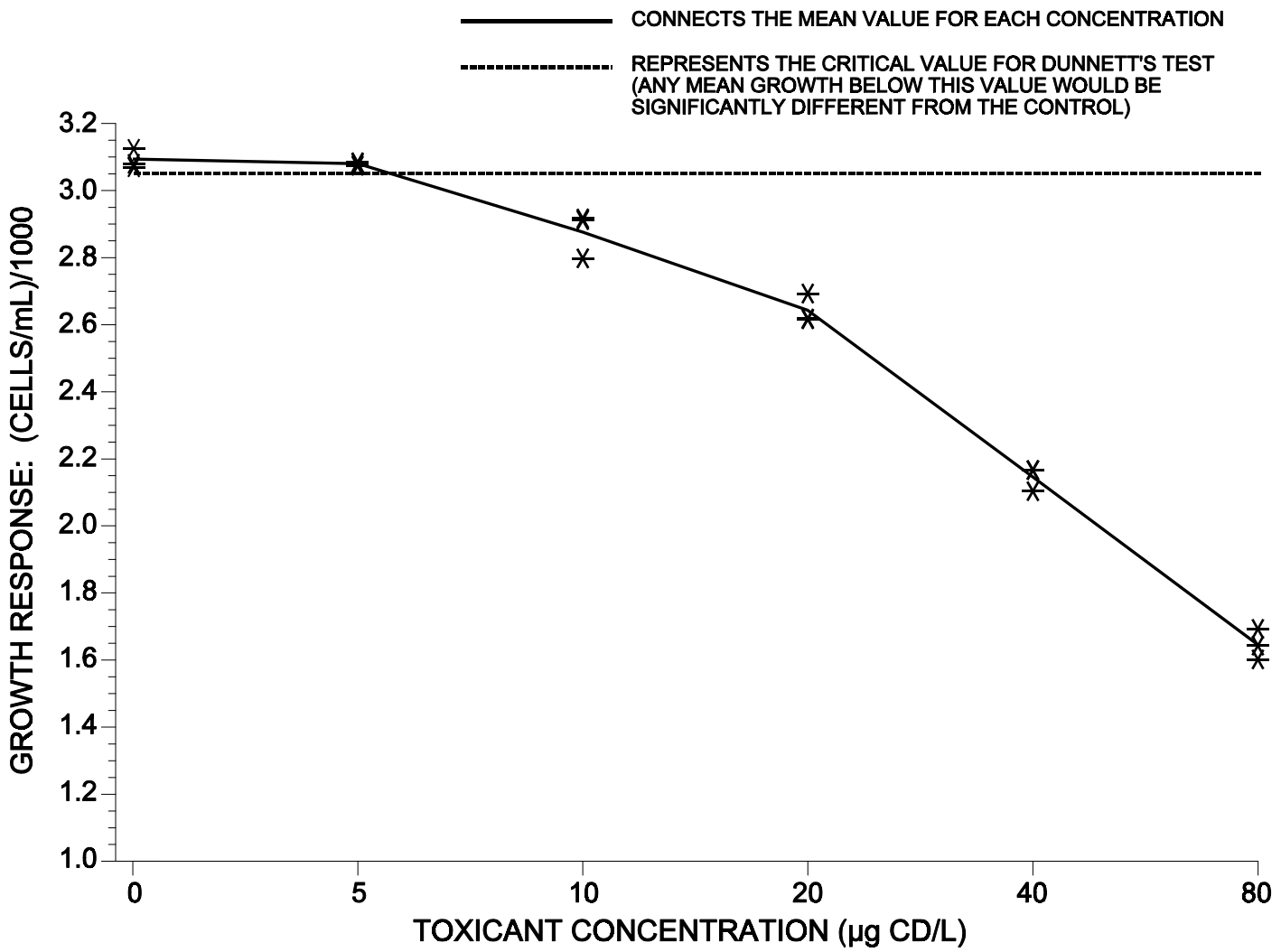

Figure 4. Plot of the $\log_{10}$ transformed cell count data from the green alga, *Selenastrum capricornutum*, growth response test in Table 4.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.078	10	0.001
2	-0.044	11	0.004
3	-0.042	12	0.021
4	-0.027	13	0.021
5	-0.024	14	0.033
6	-0.022	15	0.036
7	-0.012	16	0.041
8	-0.006	17	0.047
9	-0.003	18	0.050

14.13.2.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1 , a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 , k = 9. The a_i values are listed in Table 7.

14.13.2.5.5 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7.

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	0.128	$X^{(18)} - X^{(1)}$
2	0.3253	0.091	$X^{(17)} - X^{(2)}$
3	0.2553	0.083	$X^{(16)} - X^{(3)}$
4	0.2027	0.063	$X^{(15)} - X^{(4)}$
5	0.1587	0.057	$X^{(14)} - X^{(5)}$
6	0.1197	0.043	$X^{(13)} - X^{(6)}$
7	0.0837	0.033	$X^{(12)} - X^{(7)}$
8	0.0496	0.010	$X^{(11)} - X^{(8)}$
9	0.0163	0.004	$X^{(10)} - X^{(9)}$

For this set of data:

$$W = \frac{1}{0.0214}(0.1436)^2 = 0.964$$

14.13.2.5.6 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 18 observations (n) is 0.858. Since W = 0.964 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

14.13.2.6 Test for Homogeneity of Variance

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

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

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

p = number of levels of toxicant concentration including the control

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

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

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

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

14.13.2.6.3 Bartlett's statistic is therefore:

$$B = \frac{[(12)\ln(0.0018) - 2\sum_{i=1}^{P} \ln(S_i^2)]}{1.194}$$

$$= [12(-6.3200) - 2(-41.9082)]/1.194$$

$$= 7.9764/1.194$$

$$= 6.6804$$

14.13.2.6.4 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 five degrees of freedom, is 15.09. Since B = 6.6804 is less than the critical value of 15.09, conclude that the variances are not different.

14.13.2.7 Dunnett's Procedure

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

TABLE 8. ANOVA TABLE

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

Where: p = number of toxicant concentrations including the control

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

n_i = number of observations in concentration i

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

$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$ 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 cell count for toxicant concentration i in test chamber j)

14.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$

$N = 18$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 9.281$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 9.239$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 8.627$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 7.928$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 6.438$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 4.938$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 46.451$

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

$$= \frac{1}{3}(374.606) - \frac{(46.451)^2}{18} = 4.997$$

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

$$= 124.890 - \frac{(46.451)^2}{18} = 5.018$$

$SSW = SST - SSB = 5.018 - 4.997 = 0.0210$

$S_B^2 = SSB/(p-1) = 4.996/(6-1) = 0.9990$

$S_W^2 = SSW/(N-p) = 0.021/(18-6) = 0.0018$

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

TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	5	4.997	0.999
Within	12	0.021	0.0018
Total	17	5.017	

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

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

Where: $\bar{Y}_i$ = mean cell count for toxicant concentration i

$\bar{Y}_1$ = mean cell count 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.

14.13.2.7.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 5 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(3.094 - 3.080)}{[0.0424\sqrt{(1/3)+(1/3)}]} = 0.405$$

TABLE 10. CALCULATED T VALUES

Toxicant Concentration (µg Cd/L)	i	t_i
5	2	0.405
10	3	6.300
20	4	13.035
40	5	27.399
80	6	41.850

14.13.2.7.6 Since the purpose of this test is to detect a significant reduction in mean cell count, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean count for concentration i is considered significantly less than the mean count for the control if t_i is greater than the critical value. Since t_3, t_4, t_5 and t_6 are greater than 2.50, the 10, 20, 40 and 80 µg/L concentrations have significantly lower mean cell counts than the control. Hence the NOEC and the LOEC for the test are 5 µg/L and 10 µg/L, respectively.

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

14.13.2.7.8 In this example:

$$MSD = 2.50(0.0424)\sqrt{(1/3)+(1/3)}$$

$$= 2.50\ (0.0424)(0.8165)$$

$$= 0.086$$

14.13.2.7.9 The MSD (0.086) is in transformed units. An approximate MSD in terms of cell count per 100 mL may be calculated via the following conversion.

1. Subtract the MSD from the transformed control mean.

   3.094 - 0.086 = 3.008

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

   $10^{(3.094)} = 1241.6$

   $10^{(3.008)} = 1018.6$

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

   $MSU_u = 1241.6 - 1018.6 = 223$

14.13.2.7.10 Therefore, for this set of data, the minimum difference in mean cell count between the control and any toxicant concentration that can be detected as statistically significant is 223.

14.13.2.7.11 This represents a decrease in growth of 18% from the control.

14.13.2.8 Calculation of the ICp

14.13.2.8.1 The growth data in Table 4 are utilized in this example. Table 11 contains the means for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp. See Figure 5 for a plot of the response curve.

TABLE 11. ALGAL MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Concentration (µg Cd/L)	i	Response means, $\bar{Y}_i$ (cells/mL)	Smoothed mean, M_i (cells/mL)
Control	1	1243	1243
5	2	1201	1201
10	3	757	757
20	4	441	441
40	5	140	140
80	6	44	44

14.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method (Appendix M). A 25% reduction in cell count, compared to the controls, would result in a mean count of 932 cells, where $M_1(1\text{-}p/100)$ = 1243(1-25/100). A 50% reduction in cell count, compared to the controls, would result in a mean count of 622 cells. Examining the means and their associated concentrations (Table 11), the response, 932 cells, is bracketed by C_2 = 5 µg Cd/L and C_3 = 10 µg Cd/L. The response, 622 cells, is bracketed by C_3 = 10 µg Cd/L and C_4 = 20 µg Cd/L.

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

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

$$IC25 = 5 + [1243(1 - 25/100) - 1201]\frac{(10 - 5)}{(757 - 1201)}$$

$$= 8 \text{µg Cd/L.}$$

14.13.2.8.4 The IC50 estimate is 14 µg Cd/L:

$$IC25 = 6.25 + [28.75(1 - 25/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$IC50 = 10 + [1243(1 - 50/100) - 757]\frac{(20 - 10)}{(441 - 757)}$$

$$= 14 \text{ µg Cd/L.}$$

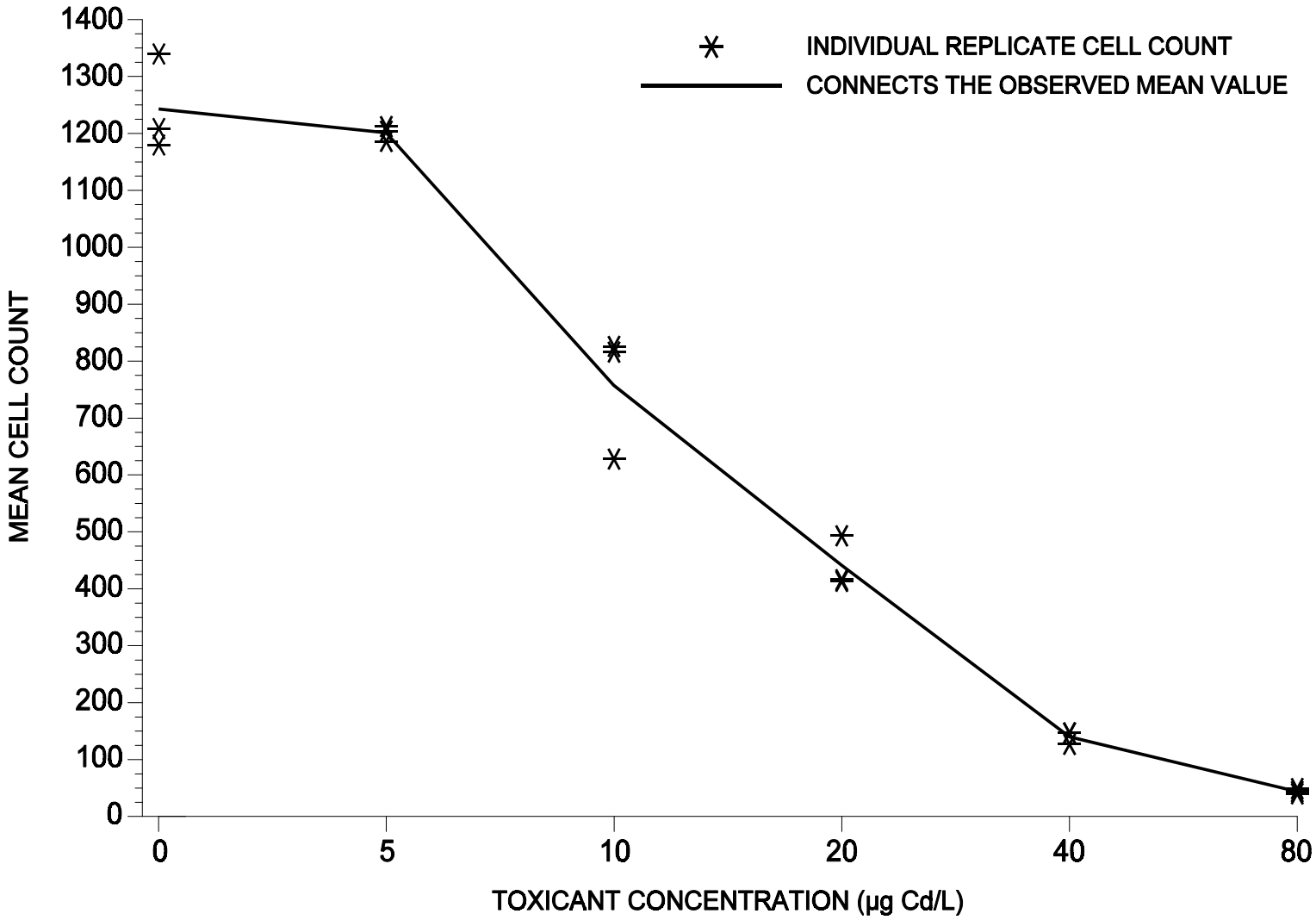

Figure 5. Plot of raw data and observed means for the green alga, *Selenastrum capricornutum*, growth data.

14.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.0227µg Cd/L. The empirical 95% confidence interval for the true mean was 6.4087 µg Cd/L and 10.0313 µg Cd/L. The ICPIN computer program output for the IC25 for this data set is shown in Figure 6.

14.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 14.2774 µg Cd/L. The empirical 95% confidence interval for the true mean was 9.7456 µg Cd/L and 18.5413 µg Cd/L. The computer program output for the IC50 for this data set is shown in Figure 7.

14.13.3 BIOSTIMULATION

14.13.3.1 Where the growth response in effluent (or surface water) exceeds growth in the control flasks, the percent stimulation, S(%), is calculated as shown below. Values which are significantly greater than the control indicate a possible degrading enrichment effect on the receiving water (Walsh et al., 1980):

$$S\ (\%)\ =\ \frac{T-C}{C}\ x\ 100$$

Where: T = Mean effluent or surface water response

C = Mean control response

14.14 **PRECISION AND ACCURACY**

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). 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.

14.14.1.1 Single-Laboratory Precison

14.14.1.1.1 Data from repetitive 96-h toxicity tests conducted with cadmium chloride as the reference toxicant, using medium containing EDTA, are shown in Table 12. The precision (CV) of the 10 EC50s was 10.2%.

14.14.1.1.2 EPA evaluated within-laboratory precision of the green alga, *Selenastrum capricornutum*, Growth Test using a database of routine reference toxicant test results from nine laboratories (USEPA, 2000b). The database consisted of 85 reference toxicant tests conducted in 9 laboratories using a variety of reference toxicants including: copper, sodium chloride, and zinc. Among the 9 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 25%; and in 75% of laboratories, the within-laboratory CV was less than 39%.

14.14.1.2 Multilaboratory Precision

14.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the green alga, *Selenastrum capricornutum*, Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of 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. Each sample was tested with and without the addition of EDTA. Of the 44 *Selenastrum capricornutum* Growth tests conducted with EDTA, 63.6% were successfully completed and met the required test acceptability criteria. Of the 44 tests conducted without EDTA, 65.9% were successfully completed and met the required test acceptability criteria. Of five tests that were conducted on blank samples with the addition of EDTA, none showed false positive results for the growth endpoint. Of 6 tests that were conducted on blank samples without the addition of EDTA, 2 showed false positive results for the growth endpoint, yielding a false positive rate of 33.3%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 13 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 34.3% and 58.5% for IC25 results in tests with EDTA and without EDTA, respectively. Table 14 shows the precision of growth NOEC endpoints for each sample type. NOEC values for tests with EDTA spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. NOEC values for tests without EDTA, spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median for tests conducted with EDTA was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. The percentage of values within one concentration of the median for tests conducted without EDTA was 40.0%, 50.0%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	1209	1212	826	493	127	49.3
Response 2	1180	1186	628	416	147	40.0
Response 3	1340	1204	816	413	147	44.0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration: 96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	1243.000	85.247	1243.000
2	3	5.000	1200.667	13.317	1200.667
3	3	10.000	756.667	111.541	756.667
4	3	20.000	440.667	45.347	440.667
5	3	40.000	140.333	11.547	140.333
6	3	80.000	44.433	4.665	44.433

The Linear Interpolation Estimate: 8.0227 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.1627 Standard Deviation: 0.4733
Original Confidence Limits: Lower: 7.2541 Upper: 8.9792
Expanded Confidence Limits: Lower: 6.4087 Upper: 10.0313
Resampling time in Seconds: 1.65 Random Seed: -1575623987

Figure 6. ICPIN program output for the IC25.

```
Conc. ID              1         2         3         4         5         6
-------------------------------------------------------------------------

Conc. Tested          0         5        10        20        40        80
-------------------------------------------------------------------------

Response  1        1209      1212       826       493       127      49.3
Response  2        1180      1186       628       416       147      40.0
Response  3        1340      1204       816       413       147      44.0

-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example   Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration:             96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i50

-------------------------------------------------------------------------

Conc.      Number     Concentration      Response       Std.       Pooled
 ID     Replicates             µg/l        Means       Dev.   Response Means
-------------------------------------------------------------------------

  1          3              0.000       1243.000     85.247   1243.000
  2          3              5.000       1200.667     13.317   1200.667
  3          3             10.000        756.667    111.541    756.667
  4          3             20.000        440.667     45.347    440.667
  5          3             40.000        140.333     11.547    140.333
  6          3             80.000         44.433      4.665     44.433

-------------------------------------------------------------------------

The Linear Interpolation Estimate:    14.2774   Entered P Value: 50

-------------------------------------------------------------------------

Number of Resamplings:   80
The Bootstrap Estimates Mean:  14.2057 Standard Deviation:     1.1926
Original Confidence Limits:   Lower:    12.1194 Upper:    16.3078
Expanded Confidence Limits:   Lower:     9.7456 Upper:    18.5413
Resampling time in Seconds:     1.65  Random Seed: -1751550803
```

Figure 7. ICPIN program output for the IC50.

TABLE 12. SINGLE LABORATORY PRECISION OF THE GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, 96-H TOXICITY TESTS, USING THE REFERENCE TOXICANT CADMIUM CHLORIDE (USEPA, 1991a)

Test Number	EC_{50} (mg/L)
1	2.3
2	2.4
3	2.3
4	2.8
5	2.6
6	2.1
7	2.1
8	2.1
9	2.6
10	2.4
n	10.0
Mean	2.37
CV (%)	10.2

TABLE 13. 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	10.9	20.8	23.5
(with EDTA)	Effluent	39.5	8.48	40.4
	Receiving water	-	-	38.9
	Average	25.2	14.6	34.3
IC25	Reference toxicant	25.6	83.6	87.5
(without EDTA)	Effluent	21.0	60.3	63.9
	Receiving water	-	-	24.1
	Average	23.3	72.0	58.5

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

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

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

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

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

TABLE 14. 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]
Growth NOEC	Reference toxicant	25%	57.1	28.6	14.3
(with EDTA)	Effluent	6.25%	42.9	57.1	0.00
	Receiving water	12.5%	28.6	57.1	14.3
Growth NOEC	Reference toxicant	18.8%	-[4]	40.0	60.0
(without EDTA)	Effluent	18.8%	-[4]	50.0	50.0
	Receiving water	6.25%	75.0	25.0	0.00

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

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

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

[4] The median NOEC fell between test concentrations, so no test results fell precisely on the median.

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APPENDICES

APPENDICES (CONTINUED)

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APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help ensure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the Fathead Minnow Larval Survival and Growth test. For the purpose of the example, the test design is as follows: five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are four replicate chambers per treatment. Each replicate chamber contains ten fish.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber four double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber
01, 25, 49, 73	Control, replicate chamber 1
02, 26, 50, 74	Control, replicate chamber 2
03, 27, 51, 75	Control, replicate chamber 3
04, 28, 52, 76	Control, replicate chamber 4
05, 29, 53, 77	6.25% effluent, replicate chamber 1
06, 30, 54, 78	6.25% effluent, replicate chamber 2
07, 31, 55, 79	6.25% effluent, replicate chamber 3
08, 32, 56, 80	6.25% effluent, replicate chamber 4
09, 33, 57, 81	12.5% effluent, replicate chamber 1
10, 34, 58, 82	12.5% effluent, replicate chamber 2
11, 35, 59, 83	12.5% effluent, replicate chamber 3
12, 36, 60, 84	12.5% effluent, replicate chamber 4
13, 37, 61, 85	25.0% effluent, replicate chamber 1
14, 38, 62, 86	25.0% effluent, replicate chamber 2
15, 39, 63, 87	25.0% effluent, replicate chamber 3
16, 40, 64, 88	25.0% effluent, replicate chamber 4
17, 41, 65, 89	50.0% effluent, replicate chamber 1
18, 42, 66, 90	50.0% effluent, replicate chamber 2
19, 43, 67, 91	50.0% effluent, replicate chamber 3
20, 44, 68, 92	50.0% effluent, replicate chamber 4
21, 45, 69, 93	100.0% effluent, replicate chamber 1
22, 46, 70, 94	100.0% effluent, replicate chamber 2
23, 47, 71, 95	100.0% effluent, replicate chamber 3
24, 48, 72, 96	100.0% effluent, replicate chamber 4

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 1 of the 25.0% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 1 of the 25.0% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10	09	73	25	33	76	52	01	35	86	34	67	35	43	76	80	95	90	91	17	39	29	27	49	45
37	54	20	48	05	64	89	47	42	96	24	80	52	40	37	20	63	61	04	02	00	82	29	16	65
08	42	26	89	53	19	64	50	93	03	23	20	90	25	60	15	95	33	47	64	35	08	03	36	06
99	01	90	25	29	09	37	67	07	15	38	31	13	11	65	88	67	67	43	97	04	43	62	76	59
12	80	79	99	70	80	15	73	61	47	64	03	23	66	53	98	95	11	68	77	12	27	17	68	33
66	06	57	47	17	34	07	27	68	50	36	69	73	61	70	65	81	33	98	85	11	19	92	91	70
31	06	01	08	05	45	57	18	24	06	35	30	34	26	14	86	79	90	74	39	23	40	30	97	32
85	26	97	76	02	02	05	16	56	92	68	66	57	48	18	73	05	38	52	47	18	62	38	85	79
63	57	33	21	35	05	32	54	70	48	90	55	35	75	48	28	46	82	87	09	83	49	12	56	24
73	79	64	57	53	03	52	96	47	78	35	80	83	42	82	60	93	52	03	44	35	27	38	84	35
98	52	01	77	67	14	90	56	86	07	22	10	94	05	58	60	97	09	34	33	50	50	07	39	98
11	80	50	54	31	39	80	82	77	32	50	72	56	82	48	29	40	52	42	01	52	77	56	78	51
83	45	29	96	34	06	28	89	80	83	13	74	67	00	78	18	47	54	06	10	68	71	17	78	17
88	68	54	02	00	86	50	75	84	01	36	76	66	79	51	90	36	47	64	93	29	60	91	10	62
99	59	46	73	48	87	51	76	49	69	91	82	60	89	28	93	78	56	13	68	23	47	83	41	13
65	48	11	76	74	17	46	85	09	50	58	04	77	69	74	73	03	95	71	86	40	21	81	65	44
80	12	43	56	35	17	72	70	80	15	45	31	82	23	74	21	11	57	82	53	14	38	55	37	63
74	35	09	98	17	77	40	27	72	14	43	23	60	02	10	45	52	16	42	37	96	28	60	26	55
69	91	62	68	03	66	25	22	91	48	36	93	68	72	03	76	62	11	39	90	94	40	05	64	18
09	89	32	05	05	14	22	56	85	14	46	42	75	67	88	96	29	77	88	22	54	38	21	45	98
91	49	91	45	23	68	47	92	76	86	46	16	28	35	54	94	75	08	99	23	37	08	92	00	48
80	33	69	45	98	26	94	03	68	58	70	29	73	41	35	53	14	03	33	40	42	05	08	23	41
44	10	48	19	49	85	15	74	79	54	32	97	92	65	75	57	60	04	08	81	22	22	20	64	13
12	55	07	37	42	11	10	00	20	40	12	86	07	46	97	96	64	48	94	39	28	70	72	58	15
63	60	64	93	29	16	50	53	44	84	40	21	95	25	63	43	65	17	70	82	07	20	73	17	90
61	19	69	04	46	26	45	74	77	74	51	92	43	37	29	65	39	45	95	93	42	58	26	05	27
15	47	44	52	66	95	27	07	99	53	59	36	78	38	48	82	39	61	01	18	33	21	15	94	66
94	55	72	85	73	67	89	75	43	87	54	62	24	44	31	91	19	04	25	92	92	92	74	59	73
42	48	11	62	13	97	34	40	87	21	16	86	84	87	67	03	07	11	20	59	25	70	14	66	70
23	52	37	83	17	73	20	88	98	37	68	93	59	14	16	26	25	22	96	63	05	52	28	25	62
04	49	35	24	94	75	24	63	38	24	45	86	25	10	25	61	96	27	93	35	65	33	71	24	72
00	54	99	76	54	64	05	18	81	59	96	11	96	38	96	54	69	28	23	91	23	28	72	95	29
35	96	31	53	07	26	89	80	93	45	33	35	13	54	62	77	97	45	00	24	90	10	33	93	33
59	80	80	83	91	45	42	72	68	42	83	60	94	97	00	13	02	12	48	92	78	56	52	01	06
46	05	88	52	36	01	39	09	22	86	77	28	14	40	77	93	91	08	36	47	70	61	74	29	41
32	17	90	05	97	87	37	92	52	41	05	56	70	70	07	86	74	31	71	57	85	39	41	18	38
69	23	46	14	06	20	11	74	52	04	15	95	66	00	00	18	74	39	24	23	97	11	89	63	38
19	56	54	14	30	01	75	87	53	79	40	41	92	15	85	66	67	43	68	06	84	96	28	52	07
45	15	51	49	38	19	47	60	72	46	43	66	79	45	43	59	04	79	00	33	20	82	66	95	41
94	86	43	19	94	36	16	81	08	51	34	88	88	15	53	01	54	03	54	56	05	01	45	11	76
98	08	62	48	26	45	24	02	84	04	44	99	90	88	96	39	09	47	34	07	35	44	13	18	80
33	18	51	62	32	41	94	15	09	49	89	43	54	85	81	88	69	54	19	94	37	54	87	30	43
80	95	10	04	06	96	38	27	07	74	20	15	12	33	87	25	01	62	52	98	94	62	46	11	71
79	75	24	91	40	71	96	12	82	96	69	86	10	25	91	74	85	22	05	39	00	38	75	95	79
18	63	33	25	37	98	14	50	65	71	31	01	02	46	74	05	45	56	14	27	77	93	89	19	36
74	02	94	39	02	77	55	73	22	70	97	79	01	71	19	52	52	75	80	21	80	81	45	17	48
54	17	84	56	11	80	99	33	71	43	05	33	51	29	69	56	12	71	92	55	36	04	09	03	24
11	66	44	98	83	52	07	98	48	27	59	38	17	15	39	09	97	33	34	40	88	46	12	33	56
48	32	47	79	28	31	24	96	47	10	02	29	53	68	70	32	30	75	75	46	15	02	00	99	94
69	07	49	41	38	87	63	79	19	76	35	58	40	44	01	10	51	82	16	15	01	84	87	69	38

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random assignments of fish to replicate chambers for the example are summarized in Table A.3.

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish		Assignment	
First	fish taken from tank	25.0% effluent,	replicate chamber 1
Second	fish taken from tank	6.25% effluent,	replicate chamber 2
Third	fish taken from tank	50.0% effluent,	replicate chamber 4
Fourth	fish taken from tank	100.0% effluent,	replicate chamber 4
Fifth	fish taken from tank	6.25% effluent,	replicate chamber 1
Sixth	fish taken from tank	25.0% effluent,	replicate chamber 4
Seventh	fish taken from tank	50.0% effluent,	replicate chamber 1
Eighth	fish taken from tank	100.0% effluent,	replicate chamber 3
Ninth	fish taken from tank	50.0% effluent,	replicate chamber 2
Tenth	fish taken from tank	100.0% effluent,	replicate chamber 4

2.3.5 Four double-digit numbers were assigned to each replicate chamber (instead of one, two, or three double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 24 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a four row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout.

TABLE A.4 RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

2.4.2 The second step is to assign each of the 24 positions four double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.5. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION

Assigned Numbers	Position
01, 25, 49, 73	1
02, 26, 50, 74	2
03, 27, 51, 75	3
04, 28, 52, 76	4
05, 29, 53, 77	5
06, 30, 54, 78	6
07, 31, 55, 79	7
08, 32, 56, 80	8
09, 33, 57, 81	9
10, 34, 58, 82	10
11, 35, 59, 83	11
12, 36, 60, 84	12
13, 37, 61, 85	13
14, 38, 62, 86	14
15, 39, 63, 87	15
16, 40, 64, 88	16
17, 41, 65, 89	17
18, 42, 66, 90	18
19, 43, 67, 91	19
20, 44, 68, 92	20
21, 45, 69, 93	21
22, 46, 70, 94	22
23, 47, 71, 95	23
24, 48, 72, 96	24

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 1. Thus, the first replicate chamber for the control will be placed in position 1.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 7. Thus, the second replicate chamber for the control will be placed in position 7.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first four numbers read will identify the positions for the control replicate chambers, the second four numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of

replicate chambers to positions for the example is displayed in Table A.6.

TABLE A.6. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNMENT OF ALL 24 POSITIONS

Control	100.0%	6.25%	6.25%	6.25%	12.5%
Control	12.5%	Control	25.0%	12.5%	25.0%
100.0%	50.0%	100.0%	Control	100.0%	25.0%
50.0%	50.0%	25.0%	50.0%	12.5%	6.25%

2.4.6 Four double-digit numbers were assigned to each position (instead of one, two, or three) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgment call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Fathead Minnow Larval Survival and Growth Test. The same data are used in the discussion of the homogeneity of variance determination in Paragraph 3 and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. FATHEAD LARVAL, *PIMEPHALES PROMELAS*, LARVAL GROWTH DATA (WEIGHT IN MG) FOR THE SHAPIRO-WILK'S TEST

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
i	1	2	3	4	5

2.3 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 listed in Table B.2.

TABLE B.2. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	-0.003	-0.028	-0.008	0.005	0.070
B	-0.052	-0.048	-0.008	0.056	-0.022
C	0.004	0.049	0.017	-0.111	0.026
D	0.053	0.026	-0.001	0.048	-0.072

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

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

Where: X_i = the centered observations and $\overline{X}$ is the overall mean of the centered observations. For this set of data, $\overline{X} = 0$, and D = 0.0412.

2.5 Order the centered observations from smallest to largest.

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations are listed in Table B.3.

TABLE B.3. EXAMPLE OF THE SHAPIRO-WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.111	11	0.004
2	-0.072	12	0.005
3	-0.052	13	0.017
4	-0.048	14	0.026
5	-0.028	15	0.026
6	-0.022	16	0.048
7	-0.008	17	0.049
8	-0.008	18	0.053
9	-0.003	19	0.056
10	-0.001	20	0.070

2.6 From Table B.4, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k, where k is n/2 if n is even, and (n-1)/2 if n is odd. For the data in this example, n = 20, k = 10. The a_i values are listed in Table B.5.

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

i \ n	2	3	4	5	6	7	8	9	10
	Number of Observations								
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

i \ n	11	12	13	14	15	16	17	18	19	20
	Number of Observations									
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3209	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

i \ n	21	22	23	24	25	26	27	28	29	30
	Number of Observations									
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0923	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONTINUED)

i \ n	Number of Observations									
	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1663	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

i \ n	Number of Observations									
	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	9,1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	9.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

2.7 Compute the test statistic, W, as follows:

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

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table B.5.

2.8 The decision rule for this test is to compare the critical value from Table B.6 to the computed W. If the computed value 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. The calculated value, 0.959, is not less than the critical value. Therefore, conclude that the data are normally distributed.

TABLE B.5. EXAMPLE OF THE SHAPIRO-WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i

	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.181	$X^{(20)} - X^{(1)}$
2	0.3211	0.128	$X^{(19)} - X^{(2)}$
3	0.2565	0.105	$X^{(18)} - X^{(3)}$
4	0.2085	0.097	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.048	$X^{(15)} - X^{(6)}$
7	0.1013	0.034	$X^{(14)} - X^{(7)}$
8	0.0711	0.025	$X^{(13)} - X^{(8)}$
9	0.0422	0.008	$X^{(12)} - X^{(9)}$
10	0.0140	0.005	$X^{(11)} - X^{(10)}$

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro-Wilk's Test for normality.

2.10 KOLMOGOROV "D" TEST

2.10.1 A formal two-sided test for normality is the Kolmogorov "D" Test. The test statistic is calculated by obtaining the difference between the cumulative distribution function estimated from the data and the standard normal cumulative distribution function for each standardized observation. This test is recommended for a sample size greater than 50. If the sample size is less than or equal to 50, then the Shapiro Wilk's Test is recommended. An example of the Kolmogorov "D" test is provided below.

2.10.2 The example uses reproduction data from the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test. The observed data and the mean of the observations at each concentration, including the control, are listed in Table B.7.

2.10.3 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 for the example are listed in Table B.8.

TABLE B.6. QUANTILES OF THE SHAPIRO-WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

TABLE B.7. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FOR THE KOLMOGOROV "D" TEST

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	19	10
2	30	35	30	34	25	13
3	29	32	33	36	26	7
4	31	26	33	34	17	7
5	16	18	36	31	16	7
6	15	29	33	27	21	10
7	18	27	33	33	23	10
8	17	16	27	31	15	16
9	14	35	38	33	18	12
10	27	13	44	31	10	2
Mean	22.4	26.3	34.6	31.7	19.0	9.4

TABLE B.8. CENTERED OBSERVATIONS FOR KOLMOGOROV “D” EXAMPLE

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	4.6	5.7	4.4	-4.7	0.0	0.6
2	7.6	8.7	-4.6	2.3	6.0	3.6
3	6.6	5.7	-1.6	4.3	7.0	-2.4
4	8.6	-0.3	-1.6	2.3	-2.0	-2.4
5	-6.4	-8.3	1.4	-0.7	-3.0	-2.4
6	-7.4	2.7	-1.6	-4.7	2.0	0.6
7	-4.4	0.7	-1.6	1.3	4.0	0.6
8	-5.4	-10.3	-7.6	-0.7	-4.0	6.6
9	-8.4	8.7	3.4	1.3	-1.0	2.6
10	4.6	-13.3	9.4	-0.7	-9.0	-7.4

2.10.4 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, and n denotes the total number of centered observations. The ordered observations for the example are listed in Table B.9.

TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
1	-13.3	-2.51	0.0060	0.0107	0.0060
2	-10.3	-1.94	0.0262	0.0071	0.0095
3	-9.0	-1.70	0.0446	0.0054	0.0113
4	-8.4	-1.58	0.0571	0.0096	0.0071
5	-8.3	-1.57	0.0582	0.0251	-0.0085
6	-7.6	-1.43	0.0764	0.0236	-0.0069
7	-7.4	-1.40	0.0808	0.0359	-0.0192
8	-7.4	-1.40	0.0808	0.0525	-0.0359
9	-6.4	-1.21	0.1131	0.0369	-0.0202
10	-5.4	-1.02	0.1539	0.0128	0.0039
11	-4.7	-0.89	0.1867	-0.0034	0.0200
12	-4.7	-0.89	0.1867	0.0133	0.0034
13	-4.6	-0.87	0.1922	0.0245	-0.0078
14	-4.4	-0.83	0.2033	0.0300	-0.0134
15	-4.0	-0.75	0.2266	0.0234	-0.0067
16	-3.0	-0.57	0.2843	-0.0176	0.0343
17	-2.4	-0.45	0.3264	-0.0431	0.0597
18	-2.4	-0.45	0.3264	-0.0264	0.0431
19	-2.4	-0.45	0.3264	-0.0097	0.0264
20	-2.0	-0.38	0.3520	-0.0187	0.0353
21	-1.6	-0.30	0.3821	-0.0321	0.0488
22	-1.6	-0.30	0.3821	-0.0154	0.0321
23	-1.6	-0.30	0.3821	0.0012	0.0154
24	-1.6	-0.30	0.3821	0.0179	-0.0012
25	-1.0	-0.19	0.4247	-0.0080	0.0247
26	-0.7	-0.13	0.4483	-0.0150	0.0316
27	-0.7	-0.13	0.4483	0.0017	0.0150
28	-0.7	-0.13	0.4483	0.0184	-0.0017
29	-0.3	-0.06	0.4761	0.0072	0.0094
30	0.0	0.00	0.5000	0.0000	0.0167
31	0.6	0.11	0.5438	-0.0271	0.0438
32	0.6	0.11	0.5438	-0.0105	0.0271
33	0.6	0.11	0.5438	0.0062	0.0105
34	0.7	0.13	0.5517	0.0150	0.0017
35	1.3	0.25	0.5987	-0.0154	0.0320
36	1.3	0.25	0.5987	0.0013	0.0154
37	1.4	0.26	0.6026	0.0141	0.0026
38	2.0	0.38	0.6480	-0.0147	0.0313
39	2.3	0.43	0.6664	-0.0164	0.0331
40	2.3	0.43	0.6664	0.0003	0.0164
41	2.6	0.49	0.6879	-0.0046	0.0212
42	2.7	0.51	0.6950	0.0050	0.0117
43	3.4	0.64	0.7389	-0.0222	0.0389
44	3.6	0.68	0.7517	-0.0184	0.0350
45	4.0	0.75	0.7734	-0.0234	0.0401
46	4.3	0.81	0.7910	-0.0243	0.0410
47	4.4	0.83	0.7967	-0.0134	0.0300

TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC (CONTINUED)

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
48	4.6	0.87	0.8078	-0.0078	0.0245
49	4.6	0.87	0.8078	0.0089	0.0078
50	5.7	1.08	0.8599	-0.0266	0.0432
51	5.7	1.08	0.8599	-0.0099	0.0266
52	6.0	1.13	0.8708	-0.0041	0.0208
53	6.6	1.25	0.8944	-0.0111	0.0277
54	6.6	1.25	0.8944	0.0056	0.0111
55	7.0	1.32	0.9066	0.0101	0.0066
56	7.6	1.43	0.9236	0.0097	0.0069
57	8.6	1.62	0.9474	0.0026	0.0141
58	8.7	1.64	0.9495	0.0172	-0.0005
59	8.7	1.64	0.9495	0.0338	-0.0172
60	9.4	1.77	0.9616	0.0384	-0.0217

2.10.5 The next step is to standardize the ordered observations. Let z_i denote the standardized value of the ith ordered observation. Then,

$$z_i = \frac{X^{(i)}}{s} \quad and \quad s^2 = \frac{\Sigma[X^{(i)}]^2}{(n-1)}$$

For the example, s = 5.3, and the standardized observations are listed in Table B.9.

2.10.6 From Table B.10, obtain the value of the standard normal cumulative distribution function (standard normal CDF) at z_i. Denote this value as p_i. Note that negative z are not listed in Table B.10. The value of the standard normal CDF at a negative number is one minus the value of the standard normal CDF at the absolute value of that number. For example, since the value of the standard normal CDF at 3.21 is 0.9993, the value of the standard normal CDF at -3.21 is 1 - 0.9993 = 0.0007. The p_i values for the example data are listed in Table B.9.

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z

z	p	z	p	z	p	z	p
0.00	0.5000	0.41	0.6591	0.82	0.7939	1.23	0.8907
0.01	0.5040	0.42	0.6628	0.83	0.7967	1.24	0.8925
0.02	0.5080	0.43	0.6664	0.84	0.7995	1.25	0.8944
0.03	0.5120	0.44	0.6700	0.85	0.8023	1.26	0.8962
0.04	0.5160	0.45	0.6736	0.86	0.8051	1.27	0.8980
0.05	0.5199	0.46	0.6772	0.87	0.8078	1.28	0.8997
0.06	0.5239	0.47	0.6808	0.88	0.8106	1.29	0.9015
0.07	0.5279	0.48	0.6844	0.89	0.8133	1.30	0.9032
0.08	0.5319	0.49	0.6879	0.90	0.8159	1.31	0.9049
0.09	0.5359	0.50	0.6915	0.91	0.8186	1.32	0.9066
0.10	0.5398	0.51	0.6950	0.92	0.8212	1.33	0.9082
0.11	0.5438	0.52	0.6985	0.93	0.8238	1.34	0.9099
0.12	0.5478	0.53	0.7019	0.94	0.8264	1.35	0.9115
0.13	0.5517	0.54	0.7054	0.95	0.8289	1.36	0.9131
0.14	0.5557	0.55	0.7088	0.96	0.8315	1.37	0.9147
0.15	0.5596	0.56	0.7123	0.97	0.8340	1.38	0.9162
0.16	0.5636	0.57	0.7157	0.98	0.8365	1.39	0.9177
0.17	0.5675	0.58	0.7190	0.99	0.8389	1.40	0.9192
0.18	0.5714	0.59	0.7224	1.00	0.8413	1.41	0.9207
0.19	0.5753	0.60	0.7257	1.01	0.8438	1.42	0.9222
0.20	0.5793	0.61	0.7291	1.02	0.8461	1.43	0.9236
0.21	0.5832	0.62	0.7324	1.03	0.8485	1.44	0.9251
0.22	0.5871	0.63	0.7357	1.04	0.8508	1.45	0.9265
0.23	0.5910	0.64	0.7389	1.05	0.8531	1.46	0.9279
0.24	0.5948	0.65	0.7422	1.06	0.8554	1.47	0.9292
0.25	0.5987	0.66	0.7454	1.07	0.8577	1.48	0.9306
0.26	0.6026	0.67	0.7486	1.08	0.8599	1.49	0.9319
0.27	0.6064	0.68	0.7517	1.09	0.8621	1.50	0.9332
0.28	0.6103	0.69	0.7549	1.10	0.8643	1.51	0.9345
0.29	0.6141	0.70	0.7580	1.11	0.8665	1.52	0.9357
0.30	0.6179	0.71	0.7611	1.12	0.8686	1.53	0.9370
0.31	0.6217	0.72	0.7642	1.13	0.8708	1.54	0.9382
0.32	0.6255	0.73	0.7673	1.14	0.8729	1.55	0.9394
0.33	0.6293	0.74	0.7704	1.15	0.8749	1.56	0.9406
0.34	0.6331	0.75	0.7734	1.16	0.8770	1.57	0.9418
0.35	0.6368	0.76	0.7764	1.17	0.8790	1.58	0.9429
0.36	0.6406	0.77	0.7794	1.18	0.8810	1.59	0.9441
0.37	0.6443	0.78	0.7823	1.19	0.8830	1.60	0.9452
0.38	0.6480	0.79	0.7852	1.20	0.8849	1.61	0.9463
0.39	0.6517	0.80	0.7881	1.21	0.8869	1.62	0.9474
0.40	0.6554	0.81	0.7910	1.22	0.8888	1.63	0.9484

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
1.64	0.9495	2.05	0.9798	2.46	0.9931	2.87	0.9979
1.65	0.9505	2.06	0.9803	2.47	0.9932	2.88	0.9980
1.66	0.9515	2.07	0.9808	2.48	0.9934	2.89	0.9981
1.67	0.9525	2.08	0.9812	2.49	0.9936	2.90	0.9981
1.68	0.9535	2.09	0.9817	2.50	0.9938	2.91	0.9982
1.69	0.9545	2.10	0.9821	2.51	0.9940	2.92	0.9982
1.70	0.9554	2.11	0.9826	2.52	0.9941	2.93	0.9983
1.71	0.9564	2.12	0.9830	2.53	0.9943	2.94	0.9984
1.72	0.9573	2.13	0.9834	2.54	0.9945	2.95	0.9984
1.73	0.9582	2.14	0.9838	2.55	0.9946	2.96	0.9985
1.74	0.9591	2.15	0.9842	2.56	0.9948	2.97	0.9985
1.75	0.9599	2.16	0.9846	2.57	0.9949	2.98	0.9986
1.76	0.9608	2.17	0.9850	2.58	0.9951	2.99	0.9986
1.77	0.9616	2.18	0.9854	2.59	0.9952	3.00	0.9987
1.78	0.9625	2.19	0.9857	2.60	0.9953	3.01	0.9987
1.79	0.9633	2.20	0.9861	2.61	0.9955	3.02	0.9987
1.80	0.9641	2.21	0.9864	2.62	0.9956	3.03	0.9988
1.81	0.9649	2.22	0.9868	2.63	0.9957	3.04	0.9988
1.82	0.9656	2.23	0.9871	2.64	0.9959	3.05	0.9989
1.83	0.9664	2.24	0.9875	2.65	0.9960	3.06	0.9989
1.84	0.9671	2.25	0.9878	2.66	0.9961	3.07	0.9989
1.85	0.9678	2.26	0.9881	2.67	0.9962	3.08	0.9990
1.86	0.9686	2.27	0.9884	2.68	0.9963	3.09	0.9990
1.87	0.9693	2.28	0.9887	2.69	0.9964	3.10	0.9990
1.88	0.9699	2.29	0.9890	2.70	0.9965	3.11	0.9991
1.89	0.9706	2.30	0.9893	2.71	0.9966	3.12	0.9991
1.90	0.9713	2.31	0.9896	2.72	0.9967	3.13	0.9991
1.91	0.9719	2.32	0.9898	2.73	0.9968	3.14	0.9992
1.92	0.9726	2.33	0.9901	2.74	0.9969	3.15	0.9992
1.93	0.9732	2.34	0.9904	2.75	0.9970	3.16	0.9992
1.94	0.9738	2.35	0.9906	2.76	0.9971	3.17	0.9992
1.95	0.9744	2.36	0.9909	2.77	0.9972	3.18	0.9993
1.96	0.9750	2.37	0.9911	2.78	0.9973	3.19	0.9993
1.97	0.9756	2.38	0.9913	2.79	0.9974	3.20	0.9993
1.98	0.9761	2.39	0.9916	2.80	0.9974	3.21	0.9993
1.99	0.9767	2.40	0.9918	2.81	0.9975	3.22	0.9994
2.00	0.9772	2.41	0.9920	2.82	0.9976	3.23	0.9994
2.01	0.9778	2.42	0.9922	2.83	0.9977	3.24	0.9994
2.02	0.9783	2.43	0.9925	2.84	0.9977	3.25	0.9994
2.03	0.9788	2.44	0.9927	2.85	0.9978	3.26	0.9994
2.04	0.9793	2.45	0.9929	2.86	0.9979	3.27	0.9995

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
3.28	0.9995	3.46	0.9997	3.64	0.9999	3.82	0.9999
3.29	0.9995	3.47	0.9997	3.65	0.9999	3.83	0.9999
3.30	0.9995	3.48	0.0997	3.66	0.9999	3.84	0.9999
3.31	0.9995	3.49	0.9998	3.67	0.9999	3.85	0.9999
3.32	0.9995	3.50	0.9998	3.68	0.9999	3.86	0.9999
3.33	0.9996	3.51	0.9998	3.69	0.9999	3.87	0.9999
3.34	0.9996	3.52	0.9998	3.70	0.9999	3.88	0.9999
3.35	0.9996	3.53	0.9998	3.71	0.9999	3.89	0.9999
3.36	0.9996	3.54	0.9998	3.72	0.9999	3.90	1.0000
3.37	0.9996	3.55	0.9998	3.73	0.9999	3.91	1.0000
3.38	0.9996	3.56	0.9998	3.74	0.9999	3.92	1.0000
3.39	0.9997	3.57	0.9998	3.75	0.9999	3.93	1.0000
3.40	0.9997	3.58	0.9998	3.76	0.9999	3.94	1.0000
3.41	0.9997	3.59	0.9998	3.77	0.9999	3.95	1.0000
3.42	0.9997	3.60	0.9998	3.78	0.9999	3.96	1.0000
3.43	0.9997	3.61	0.9998	3.79	0.9999	3.97	1.0000
3.44	0.9997	3.62	0.9999	3.80	0.9999	3.98	1.0000
3.45	0.9997	3.63	0.9999	3.81	0.9999	3.99	1.0000

2.10.7 Next, calculate the following differences for each ordered observation:

$$D_i+ = (i/n) - p_i$$

$$D_i- = p_i - [(i-1)/n]$$

The differences for the example are listed in Table B.9.

2.10.8 Obtain the maximum of the D_i+, and denote it as D+. Obtain the maximum of the D_i-, and denote it as D-. For the example, D+ = 0.0525, and D- = 0.0597.

2.10.9 Next, obtain the maximum of D+ and D-, and denote it as D. For the example, D = 0.0597.

2.10.10 The test statistic, D*, is calculated as follows:

$$D^* = D(\sqrt{n} - 0.01 + \frac{0.85}{\sqrt{n}})$$

For the example, D* = 0.4684.

2.10.11 The decision rule for the two tailed test is to compare the critical value from Table B.11 to the computed D*. If the computed value is greater 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 is 1.035. The calculated value, 0.4684, is not greater than the critical value. Thus, the conclusion of the test is that the data are normally distributed.

2.10.12 In general, if the data fail the test for normality, a transformation such as the log transformation may normalize the data. After transforming the data, repeat the Kolmogorov "D" test for normality.

TABLE B.11. CRITICAL VALUES FOR THE KOLMOGOROV "D" TEST

Alpha Level	Critical Value
0.010	1.035
0.025	0.955
0.050	0.895
0.100	0.819
0.150	0.775

3. **TEST FOR HOMOGENEITY OF VARIANCE**

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices C and D. These data are listed in Table B.12, together with the calculated variance for the control and each toxicant concentration.

TABLE B.12. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
I	1	2	3	4	5

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

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

Where: V_i = degrees of freedom for each toxicant concentration and control

p = number of levels of toxicant concentration including the control

ln = $\log_e$

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

n_i = the number of replicates for concentration i.

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

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

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

3.5 For the data in this example, $V_i = 3$, p = 5, $\bar{S}^2 = 0.0027$, and C = 1.133. The calculated B value is:

$$B = \frac{(15)[\ln(0.0027)] - 3\sum_{i=1}^{P} \ln(S_i^2)}{1.133}$$

$$= \frac{15(-5.9145) - 3(-32.4771)}{1.133}$$

$$= 7.691$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.277 for a significance level of 0.01. Since B = 7.691 is less than the critical value of 13.277, conclude that the variances are not different.

4. **TRANSFORMATIONS OF THE DATA**

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than by nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993)

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to $P_i (1 - P_i)$, where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the t test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is

close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine $\sqrt{P}$) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the proportion of dead (or affected) organisms is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion dead is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, where:

RP = (number of surviving or "unaffected" organisms)/(number exposed)

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = arc sine $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0:

Angle (in radians) = arc sine $\sqrt{1/4\,N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 1.0:

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control (see Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, a t test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices B and D. These data are listed in Table C.1.

TABLE C.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR DUNNETT'S PROCEDURE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
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
Total(T_i)	2.709	2.301	2.641	2.260	1.817

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including:

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

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

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

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

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 7.146 - (11.728)^2/20$

$= 0.2687$

$SSB = \sum_i T_i^2/n_i - G^2/N$

$= ¼ (28.017 - 11.728)^2/20$

$= 0.1270$

$SSW = SST - SSB$

$= 0.2687 - 0.1270$

$= 0.1417$

1.5 Summarize these data in the ANOVA table (Table C.2).

TABLE C.2. ANOVA TABLE FOR DUNNETT'S PROCEDURE

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	

1.6 Summarize data for ANOVA (Table C.3).

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.1270	0.0318
Within	20 - 5 = 15	0.1417	0.0094
Total	19	0.2687	

1.7 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 for concentration i

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

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.633
256	5	3.251

1.9 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.36), with an overall alpha level of 0.05, 15 degrees of freedom and four concentrations excluding the control is read from the table of Dunnett's "T" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). 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 has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

(One-tailed) d^{α}_{k}

k / ν	α = .05									α = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.56	2.74	2.81	2.87	2.92	2.90	3.20	3.51	3.67	3.79	3.18	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.86	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.59	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.40
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.30	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.40
24	1.71	2.01	3.17	2.28	2.36	2.43	2.48	2.53	2.57	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.32	2.92	2.99	3.06	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42	2.33	2.56	2.68	2.77	2.34	2.90	2.93	2.97	3.00

1.10 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

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

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

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.36(0.097)[3\sqrt{(1/4)+(1/4)}] = 2.36(0.097)(\sqrt{2/4})$$

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

$$= 0.162$$

1.11 For this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.087 mg. This represents a decrease in growth of 24% from the control.

1.11.1 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.2 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2.1 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D.

$MSD_u = control_u - D_u$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.2.2 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows:

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

0.677 - 0.162 = 0.515

Step 2. Obtain untransformed values for the control mean (0.677) and the difference (0.515) obtained in Step 1 above.

$[\text{ Sine } (0.677)]^2 = 0.392$
$[\text{ Sine } (0.515)]^2 = 0.243$

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

$MSD_u = 0.392 - 0.243 = 0.149$

In this case, the MSD would represent a 38.0% decrease in survival from the control [(0.149/0.392)(100)].

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a t test with Bonferroni's adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.6 DATA INPUT AND OUTPUT

2.6.1 Reproduction data from a daphnid, *Ceriodaphnia dubia*, survival and reproduction test (Table C.6) are used to illustrate the data input and output for this program.

TABLE C.6. SAMPLE DATA FOR DUNNETT'S PROGRAM *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be entered:

1. Response proportions, like survival or fertilization proportions.
2. Counts and measurements, like offspring counts, cystocarp counts or weights.

2.6.2.2 After the type of data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for counts and measurements, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration:
   - number of observations
   - data for each observation

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

```
                    EMSL Cincinnati Dunnett Software
                              Version 1.5

      1)    Create a data file
      2)    Edit a data file
      3)    Perform ANOVA on existing data
      4)    Stop

Your choice ? 1


Number of groups, including control ? 5

Number of observations for group  1 ? 10

Enter the data for group  1 one observation at a time.

NO.  1?  27

NO.  2?  30

NO.  3?  29

NO.  4?  31

NO.  5?  16

NO.  6?  15

NO.  7?  18

NO.  8?  17

NO.  9?  14

NO. 10?  27

Number of observations for group  2  ?  10


Do you wish to save the data on disk  ?y

Disk file for output  ?  cerio
```

Figure C.1. Sample Data Input for Dunnett's Program for Reproduction Data from Table C.6.

2.6.3 Program Output

2.6.3.1 When Option 3 (Perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure and the minimum detectable difference are included in the program output.

EMSL Cincinnati Dunnett Software
Version 1.5

1) Create a data file
2) Edit a data file
3) Perform analysis on existing data set
4) Stop

Your choice ? 3

File name ? cerio

Available Transformations

1) no transform
2) square root
3) log10

Your choice ? 1

Dunnett's test as implemented in this program is
a one-sided test. You must specify the direction
the test is to be run; that is, do you expect the
means for the test groups to be less than or
greater than the mean for the control group mean.

Direction for Dunnett's test : L=less than, G=greater than ? L

Figure C.2. Example of Choosing Option 3 from the Menu of the Dunnett Program.

Ceriodaphnia Reproduction Data from Table C.6

Summary Statistics and ANOVA

Transformation = None

Group	n	Mean	s.d.	CV%
1 = control	10	22.4000	6.9314	30.9
2	10	26.3000	8.0007	30.4
3	10	34.6000	4.8351	14.0
4	10	31.7000	2.9458	9.3
5*	10	9.4000	3.8930	41.4

*) the mean for this group is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -5.628560
This difference corresponds to -25.13 percent of control

Between concentrations
Sum of squares = 3887.880000 with 4 degrees of freedom.

Error mean square = 31.853333 with 45 degrees of freedom.

Bartlett's test p-value for equality of variances = .029

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Reproduction Data from Table C.6.

APPENDIX D

T TEST WITH BONFERRONI'S ADJUSTMENT

1. The t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The t test with Bonferroni's adjustment is based on the same assumptions of normality and homogeneity of variance as Dunnett's Procedure (see Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the t test with Bonferroni's adjustment is provided below. The data used in the example are the same as in Appendix C, except that the third replicate from the 256 µg/L concentration is presumed to have been lost. Thus, Dunnett's Procedure cannot be used. The weight data are presented in Table D.1.

TABLE D.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
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	(LOST)
D	0.690	0.560	0.676	0.672	0.254
Mean($\bar{Y}$)	0.677	0.575	0.660	0.565	0.404
Total(T_i)	2.709	2.301	2.641	2.260	1.211

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including the control

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$$SSB = \sum_i T_i^2/n_i - G^2/N$$ Between Sum of Squares

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

Where: 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

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 4$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.211$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.122$

$$SSB = \sum_i T_i^2/n_i - G^2/N$$

$= 6.668 - (11.122)^2/19$

$= 0.158$

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$

$= 6.779 - (11.122)^2/19$

$= 0.269$

$$SSW = SST - SSB$$

= 0.269 - 0.158

= 0.111

3.3 Summarize these data in the ANOVA table (Table D.2):

TABLE D.2. ANOVA TABLE FOR BONFERRONI'S ADJUSTMENT

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	

3.4 Summarize these data in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.158	0.0395
Within	19 - 5 = 14	0.111	0.0029
Total	18	0.269	

3.5 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 for each concentration

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

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.6 Table D.4 includes the calculated t values for each concentration and control combination.

TABLE D.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.623
64	3	0.220
128	4	1.782
256	5	4.022

3.7 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.510), with an overall alpha level of 0.05, fourteen degrees of freedom and four concentrations excluding the control, was obtained from Table D.5. 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 2.510, the 256 µg/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.301	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.730	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.950	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788
26	1.706	2.056	2.248	2.379	2.479	2.559	2.627	2.684	2.734	2.779
27	1.704	2.052	2.243	2.374	2.473	2.553	2.620	2.677	2.727	2.771
28	1.702	2.049	2.239	2.369	2.468	2.547	2.613	2.670	2.720	2.764

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (see Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration and within the control is then calculated. To determine if the response in a concentration is significantly different from the response in the control, the minimum rank sum for each concentration and control combination is compared to the significant values of rank sums given later in this section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs reproduction data from a *Ceriodaphnia dubia* 7-day, chronic test. The data are listed in Table E.1. Significant mortality was detected via Fisher's Exact Test in the 50% effluent concentration. The data for this concentration is not included in the reproduction analysis.

TABLE E.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Control	20	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	20	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign ranks (1, 2, 3,..., 16) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table E.2. This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each effluent concentration, as shown in Table E.4.

TABLE E.2. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: ASSIGNING RANKS TO THE CONTROL AND 3% EFFLUENT CONCENTRATION

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
6	20	Control
8	23	Control
8	23	Control
8	23	3
10.5	24	Control
10.5	24	Control
12	25	3
15	26	Control
15	26	Control
15	26	Control
15	26	3
15	26	3
19	27	Control
19	27	Control
19	27	3

TABLE E.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%)							
			3		6		12		25	
1	20	(6,4.5,3,11)	13	(2.5)	18	(3)	14	(1)	9	(5)
2	26	(15,17,17,17)	15	(5)	22	(7.5)	22	(6)	0	(1)
3	26	(15,17,17,17)	14	(4)	13	(1.5)	20	(3)	9	(5)
4	23	(8,11.5,8.5,12.5)	13	(2.5)	13	(1.5)	23	(8.5)	7	(3)
5	24	(10.5,14.5,12,14.5)	23	(8)	23	(11.5)	20	(3)	6	(2)
6	27	(19,19.5,19.5,19.5)	26	(15)	22	(7.5)	23	(8.5)	10	(7)
7	26	(15,17,17,17)	0	(1)	20	(4.5)	25	(14.5)	12	(8)
8	23	(8,11.5,8.5,12.5)	25	(12)	22	(7.5)	24	(12)	14	(10)
9	27	(19,19.5,19.5,19.5)	26	(15)	23	(11.5)	25	(14.5)	9	(5)
10	24	(10.5,14.5,12,14.5)	27	(19)	22	(7.5)	21	(5)	13	(9)

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

TABLE E.4. RANK SUMS

Effluent Concentration (%)	Rank Sum
3	84
6	64
12	76
25	55

6. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and ten replicates is 76 (see Table E.5 , for R=4).

7. Comparing the rank sums in Table E.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus the NOEC and LOEC for reproduction are 3% and 6%, respectively.

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	--	--	--
	--	--	--	--	--	--	--	--
5	18	17	17	16	16	16	16	15
	15	--	--	--	--	--	--	--
6	27	26	25	25	24	24	24	23
	23	22	21	21	--	--	--	--
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287
	282	278	275	273	272	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. An example of the use of the Wilcoxon Rank Sum Test is provided in Table F.1. The data used in the example are the same as in Appendix E, except that two males are presumed to have occurred, one in the control and one in the 12% effluent concentration. Thus, there is unequal replication for the reproduction analysis.

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

TABLE F.1. EXAMPLE OF WILCOXON'S RANK SUM TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Cont	M	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	M	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum for the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and nine replicates in the control is 72 for those concentrations with ten replicates, and 60 for those concentrations with nine replicates (see Table F.5, for K = 4).

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus, the NOEC and LOEC for reproduction are 3% and 6%, respectively.

TABLE F.2. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND EFFLUENT CONCENTRATIONS

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
7	23	Control
7	23	Control
7	23	3
9.5	24	Control
9.5	24	Control
11	25	3
14	26	Control
14	26	Control
14	26	Control
14	26	3
14	26	3
18	27	Control
18	27	Control
18	27	3

TABLE F.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%) 3		6		12		25	
1	M		13	(2.5)	18	(3)	14	(1)	9	(5)
2	26	(14,16,15,16)	15	(5)	22	(6.5)	22	(4)	0	(1)
3	26	(14,16,15,16)	14	(4)	13	(1.5)	20	(2)	9	(5)
4	23	(7,10.5,6.5,11.5)	13	(2.5)	13	(1.5)	23	(6.5)	7	(3)
5	24	(9.5,13.5,10,13.5)	23	(7)	23	(10.5)	M		6	(2)
6	27	(18,18.5,17.5,18.5)	26	(14)	22	(6.5)	23	(6.5)	10	(7)
7	26	(14,16,15,16)	0	(1)	20	(4)	25	(12.5)	12	(8)
8	23	(7,10.5,6.5,11.5)	25	(11)	22	(6.5)	24	(10)	14	(10)
9	27	(18,18.5,17.5,18.5)	26	(14)	23	(10.5)	25	(12.5)	9	(5)
10	24	(9.5,13.5,10,13.5)	27	(18)	22	(6.5)	21	(3)	13	(9)

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

TABLE F.4. RANK SUMS

Effluent Concentration	Rank Sum	No. of Replicates	Critical Rank Sum
3	79	10	72
6	57	10	72
12	58	9	60
25	55	10	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicate Per Effluent Concentration							
		3	4	5	6	7	8	9	10
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

FISHER'S EXACT TEST

1. Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test if the proportion of successes is the same in two Bernoulli (binomial) populations. When used with the *Ceriodaphnia dubia* data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli population. Additionally, since it is a conservative test, a pair-wise comparison error rate of 0.05 is suggested rather that an experiment-wise error rate.

2. The basis for Fisher's Exact Test is a 2×2 contingency table. However, in order to use this table the contingency table must be arranged in the format shown in Table G.1. From the 2×2 table, set up for the control and the concentration you wish to compare, you can determine statistical significance by looking up a value in the table provided later in this section.

TABLE G.1. FORMAT FOR CONTINGENCY TABLE

	Number of		
	Successes	Failures	Number of Observations
Row 1	a	A - a	A
Row 2	b	B - b	B
Total	a + b	[(A + B) - a - b]	A + B

3. Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For the *Ceriodaphnia dubia* survival data, a success may be 'alive' or 'dead', whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from the survival proportion for the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

4. To illustrate Fisher's Exact Test, a set of survival data (Table G.2) from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test will be used.

5. For each control and effluent concentration construct a 2x2 contingency table.

6. For the control and effluent concentration of 1% the appropriate contingency table for the test is given in Table G.3.

TABLE G.2. EXAMPLE OF FISHER'S EXACT TEST: *CERIODAPHNIA DUBIA* MORTALITY DATA

Effluent Concentration (%)	No. Dead	Total[1]
Control	1	9
1	0	10
3	0	10
6	0	10
12	0	10
25	10	10

[1] Total number of live adults at the beginning of the test.

7. Since 10/10 > 8/9, the category 'alive' is regarded as a success. For A = 10, B = 9 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 5. Since the value of b (b = 8) from the contingency table (Table G.3), is greater than the value of b (b = 5) from Table G.5, the test concludes that the proportion of survival is not significantly different for the control and 1% effluent.

8. The contingency tables for the combinations of control and effluent concentrations of 3%, 6%, 12% are identical to Table G.3. The conclusion of no significant difference in the proportion of survival for the control and the level of effluent would also remain the same.

9. For the combination of control and 25% effluent, the contingency table would be constructed as Table G.4. The category 'dead' is regarded as a success, since 10/10 > 1/9. The b value (b = 1) from the contingency table (Table G.4) is less than the b value (b = 5) from the table of significance levels of b (Table G.5). Thus, the percent mortality for 25% effluent is significantly greater than the percent mortality for the control. Thus, the NOEC and LOEC for survival are 12% and 25%, respectively.

TABLE G.3. 2×2 CONTINGENCY TABLE FOR CONTROL AND 1% EFFLUENT

	Number of		Number of Observations
	Alive	Dead	
1% Effluent	10	0	10
Control	8	1	9
Total	18	1	19

Table G.4. 2x2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		Number of Observations
	Dead	Alive	
25% Effluent	10	0	10
Control	1	8	9
Total	11	8	19

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1]

	α	0-05	0-025	0-01	0-005		α	0-05	0-025	0-01	0-005
						A=8	8	4 -038	3 -013	2 -003	2 -003
A=3 B=3	3	0 -050			——		7	2 -020	2 -020	1 -005^{+}	0 -001
							6	1 -020	1 -020	0 -003	0 -003
							5	0 -013	0 -013		
A=4 B=4	4	1 -014	1 -014	——	——		4	0 -038			
3	4	0 -029	——	——	——	7	8	3 -026	2 -007	2 -007	1 -001
							7	2 -035^{-}	1 -009	1 -009	0 -001
A=5 B=5	5	1 -024	1 -024	0 -004	0 -004		6	1 -032	0 -006	0 -006	
	4	0 -024	1 -024	——	——		5	0 -019	0 -019		
4	5	1 -048	0 -008	0 -008	——	6	8	2 -015^{-}	2 -015^{-}	1 -003	1 -003
	4	0 -040	——	——	——		7	1 -016	1 -016	0 -002	0 -002
3	5	0 -018	0 -018	——	——		6	0 -009	0 -009	0 -009	
2	5	0 -048	——	——	——		5	0 -028	——	——	
						5	8	2 -035^{-}	1 -007	1 -007	0 -001
							7	1 -032	0 -005^{-}	0 -005^{-}	0 -005^{-}
A=6 B=6	6	2 -030	1 -008	1 -008	0 -001		6	0 -016	0 -016	——	
	5	1 -040	0 -008	0 -008	——		5	0 -044	——	——	
	4	0 -030	——	——	——	4	8	1 -018	1 -018	0 -002	0 -002
5	6	1 -015^{+}	0 -015^{+}	0 -002	0 -002		7	0 -010^{+}	0 -010^{+}	——	
	5	0 -013	0 -013	——	——		6	0 -030	——	——	
	4	0 -045^{+}	——	——	——	3	8	0 -006	0 -006	0 -006	
4	6	1 -033	0 -005^{-}	0 -005^{-}	0 -005^{-}		7	0 -024	0 -024	——	
	5	0 -024	0 -024	——	——	2	8	0 -022	0 -022	——	
3	6	0 -012	0 -012	——	——	A=9	9	5 -041	4 -015^{-}	3 -005^{-}	3 -005^{-}
	5	0 -048	——	——	——		8	3 -025^{-}	3 -025^{-}	2 -008	1 -002
2	6	0 -036	——	——	——		7	2 -028	1 -008	1 -008	0 -001
							6	1 -025^{-}	1 -025^{-}	0 -005^{-}	0 -005^{-}
A=7 B=7	7	3 -035^{-}	2 -010^{+}	1 -002	1 -002		5	0 -015^{-}	0 -015^{-}	——	
	6	1 -015^{-}	1 -015^{-}	0 -002	0 -002		4	0 -041	——	——	
	5	1 -010^{+}	0 -010^{+}	——	——	8	9	4 -029	3 -009	3 -009	2 -002
	4	0 -035-	——	——	——		8	3 -043	2 -013	1 -003	1 -003
6	7	2 -021	2 -021	1 -005^{-}	1 -005^{-}		7	2 -044	1 -012	0 -002	0 -002
	6	1 -025^{+}	0 -004	0 -004	0 -004		6	1 -036	0 -007	0 -007	
	5	0 -016	0 -016	——	——		5	0 -020	0 -020	——	
	4	0 -049	——	——	——	7	9	3 -019	3 -019	2 -005	2 -005^{-}
5	7	2 -045^{+}	1 -010^{+}	0 -001	0 -001		8	2 -024	2 -024	1 -006	0 -001
	6	1 -045^{+}	0 -008	0 -008	——		7	1 -020	1 -020	0 -003	0 -003
	5	0 -027	——	——	——		6	0 -010^{+}	0 -010^{+}	——	
	7	1 -024	1 -024	——	0 -003		5	0 -029	——	——	
	6	0 -015^{+}	0 -015^{+}	0 -003	——	6	9	3 -044	2 -011	1 -002	1 -002
	5	0 -045^{+}	——	——	——		8	2 -047	1 -011	0 -001	0 -001
	7	0 -008	0 -008	0 -008	——		7	1 -035^{-}	0 -006	0 -006	
	6	0 -033	——	——	——		6	0 -017	0 -017	——	
	7	0 -028	——	——	——		5	0 -042	——	——	

[1] The table shows:(1) In bold type, for given a, A and B, the value of b ([a) which is just significant at the probability level quoted (one-tailed test); and (2) In small type, for given A, B and r = a + b, the exact probability (if there is independence) that b is equal to or less than the integer shown in bold type. From Pearson and Hartley (1962).

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=9 B=5	9	2 -027	1 -005⁻	1 -005⁻	1 -005⁻	A=10 B=4	10	1 -011	1 -011	0 -001	0 -001
	8	1 -023	1 -023	0 -003	0 -003		9	1 -041	0 -005⁻	0 -005⁻	0 -005⁻
	7	0 -	0 -010⁺	——	——		8	0 -	0 -015⁻	——	——
	6	0 -028	——	——	——		7	0 -	——	——	——
4	9	1 -014	1 -014	0 -001	0 -001	3	10	1 -038	0 -003	0 -003	0 -003
	8	0 -007	0 -007	0 -007	——		9	0 -014	0 -014	——	——
	7	0 -021	0 -021	——	——		8	0 -	——	——	——
	6	0 -049	——	0 -005	——	2	10	0 -	0 -015⁺	——	——
3	9	1 -	0 -005⁻	0 -005⁻	0 -005⁻		9	0 -	——	——	——
	8	0 -018	0 -018	——	——						
	7	0 -	——	——	——						
2	9	0 -018	0 -018	——	——	A=11 B=11	11	7 -	6 -018	5 -006	4 -002
							10	5 -032	4 -012	3 -004	3 -004
							9	4 -040	3 -015⁻	2 -004	2 -004
A=10 B=10	10	6 -043	5 -016	4 -005⁺	3 -002		8	3 -043	2 -015⁻	1 -004	1 -004
	9	4 -029	3 -010⁻	3 -010	2 -003		7	2 -040	1 -012	0 -002	0 -002
	8	3 -	2 -012	1 -003	1 -003		6	1 -032	0 -006	0 -006	——
	7	2 -	1 -010⁻	1 -010⁻	0 -002		5	0 -018	0 -018	——	——
	6	1 -029	0 -005⁺	0 -005⁺	——		4	0 -	——	——	——
	5	0 -016	0 -016	——	——	10	11	6 -	5 -012	4 -004	4 -004
	4	0 -043	——	——	——		10	4 -021	4 -021	3 -007	2 -002
9	10	5 -033	4 -011	3 -003	3 -003		9	3 -024	3 -024	2 -007	1 -002
	9	4 -	3 -017	2 -005⁻	2 -005⁻		8	2 -023	2 -023	1 -006	0 -001
	8	2 -019	2 -019	1 -004	1 -004		7	1 -017	1 -017	0 -003	0 -003
	7	1 -	1 -015⁻	0 -002	0 -002		6	1 -043	0 -009	0 -009	——
	6	1 -040	0 -008	0 -008	——		5	0 -023	0 -023	——	——
	5	0 -022	0 -022	——	——	9	11	5 -026	4 -008	4 -008	3 -002
8	10	4 -023	4 -023	3 -007	2 -002		10	4 -038	3 -012	2 -003	2 -003
	9	3 -032	2 -009	2 -009	1 -002		9	3 -040	2 -012	1 -003	1 -003
	8	2 -031	1 -008	1 -008	0 -001		8	2 -	1 -009	1 -009	0 -001
	7	1 -023	1 -023	0 -004	0 -004		7	1 -	1 -025⁻	0 -004	0 -004
	6	0 -011	0 -011	——	——		6	0 -012	0 -012	——	——
	5	0 -029	——	——	——		5	0 -030	——	——	——
7	10	3 -	3 -015⁻	2 -003	2 -003	8	11	4 -018	4 -018	3 -005⁻	3 -005⁻
	9	2 -018	2 -018	1 -004	1 -004		10	3 -024	3 -024	2 -006	1 -001
	8	1 -013	1 -013	0 -002	0 -002		9	2 -022	2 -022	1 -005⁻	1 -005⁻
	7	1 -036	0 -006	0 -006	——		8	1 -	1 -015⁻	0 -002	0 -002
	6	0 -017	0 -017	——	——		7	1 -037	0 -007	0 -007	——
	5	0 -041	——	——	——		6	0 -017	0 -017	——	——
6	10	3 -036	2 -008	2 -008	1 -001		5	0 -040	——	——	——
	9	2 -036	1 -008	1 -008	0 -001	7	11	4 -043	3 -011	2 -002	2 -002
	8	1 -024	1 -024	0 -003	0 -003		10	3 -047	2 -013	1 002	1 -002
	7	0 -	0 -010⁺	——	——		9	2 -039	1 -009	1 -009	0 -001
	6	0 -026	——	——	——		8	1 -	1 -025⁻	0 -004	0 -004
5	10	2 -022	2 -022	1 -004	1 -004		7	0 -	0 -010⁺	——	——
	9	1 -017	1 -017	0 -002	0 -002		6	0 -	0 -025⁻	——	——
	8	1 -	0 -007	0 -007	——	6	11	3 -	2 006	2 -006	1 -001
	7	0 -	0 -019	——	——		10	2 -	1 -005⁺	1 -005⁺	0 -001
	6	0 -	——	——	——		9	1 -	1 -018	0 -002	0 -002

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=11 B=6	8	1 -043	0 -007	0 -007	——	A=12 B=9	7	1 -037	0 -007	0 -007	——
	7	0 -017	0 -017	——	——		6	0 -017	0 -017	——	——
	6	0 -037	——	——	——		5	0 -039	——	——	——
5	11	2 -018	2 -018	1 -003	1 -003	8	12	5 -049	4 -014	3 -004	3 -004
	10	1 -013	1 -013	0 -001	0 -001		11	3 -018	3 -018	2 -004	2 -004
	9	1 -036	0 -005⁻	0 -005⁻	0 -005⁻		10	2 -015⁺	2 -015⁺	1 -003	1 -003
	8	0 -013	0 -013	——	——		9	2 -040	1 -010⁻	1 -010⁻	0 -001
	7	0 -029	——	——	——		8	1 -025⁻	1 -025⁻	0 -004	0 -004
4	11	1 -009	1 -009	1 -009	0 -001		7	0 -010⁺	0 -010⁺	——	——
	10	1 -033	0 -004	0 -004	0 -004		6	0 -024	0 -024	——	——
	9	0 -011	0 -011	——	——	7	12	4 -036	3 -009	3 -009	2 -002
	8	0 -026	——	——	——		11	3 -038	2 -010⁻	2 -010⁻	1 -002
3	11	1 -033	0 -003	0 -003	0 -003		10	2 -029	1 -006	1 -006	0 -001
	10	0 -011	0 -011	——	——		9	1 -017	1 -017	0 -002	0 -002
	9	0 -027	——	——	——		8	1 -040	0 -007	0 -007	——
2	11	0 -013	0 -013	——	——		7	0 -016	0 -016	——	——
	10	0 -038	——	——	——		6	0 -034	——	——	——
						6	12	3 -025⁻	3 -025⁻	2 -005⁻	2 -005⁻
							11	2 -022	2 -022	1 -004	1 -004
A=12 B=12	12	8 -047	7 -019	6 -007	5 -002		10	1 -013	1 -013	0 -002	0 -002
	11	6 -034	5 -014	4 -005⁻	4 -005⁻		9	1 -032	0 -005⁻	0 -005⁻	0 -005⁻
	10	5 -045⁻	4 -018	3 -006	2 -002		8	0 -011	0 -011	——	——
	9	4 -050⁻	3 -020	2 -006	1 -001		7	0 -025⁻	0 -025⁻	——	——
	8	3 -050⁻	2 -018	1 -005⁻	1 -005⁻		6	0 -050⁻	——	——	——
	7	2 -045⁻	1 -014	0 -002	0 -002	5	12	2 -015⁻	2 -015	1 -002	1 -002
	6	1 -034	0 -007	0 -007	——		11	1 -010⁻	1 -010⁻	1 -010⁻	0 -001
	5	0 -019	0 -019	——	——		10	1 -028	0 -003	0 -003	0 -003
	4	0 -047	——	——	——		9	0 -009	0 -009	0 -009	——
11	12	7 -037	6 -014	5 -005⁻	5 -005⁻		8	0 -020	0 -020	——	——
	11	5 -024	5 -024	4 -008	3 -002		7	0 -041	——	——	——
	10	4 -029	3 -010⁺	2 -003	2 -003	4	12	2 -050	1 -007	1 -007	0 -001
	9	3 -030	2 -009	2 -009	1 -002		11	1 -027	0 -003	0 -003	0 -003
	8	2 -026	1 -007	1 -007	0 -001		10	0 -008	0 -008	0 -008	——
	7	1 -019	1 -019	0 -003	0 -003		9	0 -019	0 -019	——	——
	6	1 -045⁻	0 -009	0 -009	——		8	0 -038	——	——	——
	5	0 -024	0 -024	——	——	3	12	1 -029	0 -002	0 -002	0 -002
10	12	6 -029	5 -010	5 -010⁻	4 -003		11	0 -009	0 -009	0 -009	——
	11	5 -043	4 -015⁺	3 -005⁻	3 -005⁻		10	0 -022	0 -022	——	——
	10	4 -048	3 -017	2 -005⁻	2 -005⁻		9	0 -044	——	——	——
	9	3 -046	2 -015⁻	1 -004	1 -004	2	12	0 -011	0 -011	——	——
	8	2 -038	1 -010⁺	0 -002	0 -002		11	0 -033	——	——	——
	7	1 -026	0 -005⁻	0 -005⁻	0 -005-						
	6	0 -012	0 -012	——	——						
	5	0 -030	——	——	——	A=13 B=13	13	9 -048	8 -020	7 -007	6 -003
9	12	5 -021	5 -021	4 -006	3 -002		12	7 -037	6 -015⁺	5 -006	4 -002
	11	4 -029	3 -009	3 -009	2 -002		11	6 -048	5 -021	4 -008	3 -002
	10	3 -029	2 -008	2 -008	1 -002		10	4 -024	4 -024	3 -008	2 -002
	9	2 -024	2 -024	1 -006	0 -001		9	3 -024	3 -024	2 -008	1 -002
	8	1 -016	1 -016	0 -002	0 -002		8	2 -021	2 -021	1 -006	0 -001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=13 B=13	7	2 -048	1 -015+	0 -003	0 -003	A=13 B=7	11	2 -022	2 -022	1 -004	1 -004
	6	1 -037	0 -007	0 -007	——		10	1 -012	1 -012	0 -002	0 -002
	5	0 -020	0 -020	——	——		9	1 -029	0 -004	0 -004	0 -004
	4	0 -048		——	——		8	0 -010+	0 -010+	——	——
12	1	8 -039	7 -015-	6 -005+	5 -002		7	0 -022	0 -022	——	——
	1	6 -027	5 -010-	5 -010-	4 -003		6	0 -044	——	——	——
	1	5 -033	4 -013	3 -004	3 -004	6	13	3 -021	3 -021	2 -004	2 -004
	1	4 -036	3 -013	2 -004	2 -004		12	2 -017	2 -017	1 -003	1 -003
	9	3 -034	2 -011	1 -003	1 -003		11	2 -046	1 -010-	1 -010-	0 -001
	8	2 -029	1 -008	1 -008	0 -001		10	1 -024	1 -024	0 -003	0 -003
	7	1 -020	1 -020	0 -004	0 -004		9	1 -050-	0 -008	0 -008	——
	6	1 -046	0 -010-	0 -010-	——		8	0 -017	0 -017	——	——
	5	0 -024	0 -024	——	——		7	0 -034	——	——	——
11	1	7 -031	6 -011	5 -003	5 -003	5	13	2 -012	2 -012	1 -002	1 -002
	1	6 -048	5 -018	4 -006	3 -002		12	2 -044	1 -008	1 -008	0 -001
	1	4 -021	4 -021	3 -007	2 -002		11	1 -022	1 -022	0 -002	0 -002
	1	3 -021	3 -021	2 -006	1 -001		10	1 -047	0 -007	0 -007	——
	9	3 -050-	2 -017	1 -004	1 -004		9	0 -015-	0 -015-	——	——
	8	2 -040	1 -011	0 -002	0 -002		8	0 -029	——	——	——
	7	1 -027	0 -005-	0 -005-	0 -005-	4	13	2 -044	1 -006	1 -006	0 -000
	6	0 -013	0 -013	——	——		12	1 -022	1 -022	0 -002	0 -002
		0 -030		——	——		11	0 -006	0 -006	0 -006	——
10	1	6 -024	6 -024	5 -007	4 -002		10	0 -015-	0 -015-	——	——
	1	5 -035-	4 -012	3 -003	3 -003		9	0 -029	——	——	——
	1	4 -037	3 -012	2 -003	2 -003	3	13	1 -025	1 -025	0 -002	0 -002
	1	3 -033	2 -010+	1 -002	1 -002		12	0 -007	0 -007	0 -007	——
	9	2 -026	1 -006	1 -006	0 -001		11	0 -018	0 -018	——	——
	8	1 -017	1 -017	0 -003	0 -003		10	0 -036	——	——	——
	7	1 -038	0 -007	0 -007	——	2	13	0 -010-	0 -010-	0 -010-	——
	6	0 -017	0 -017	——	——		12	0 -029	——	——	——
	5	0 -038		——	——						
9	1	5 -017	5 -017	4 -005-	4 -005-						
	1	4 -023	4 -023	3 -007	2 -001	A=14	14	10 -049	9 -020	8 -008	7 -003
	1	3 -022	3 -022	2 -006	1 -001		13	8 -038	7 -016	6 -006	5 -002
	1	2 -017	2 -017	1 -004	1 -004		12	6 -023	6 -023	5 -009	4 -003
	9	2 -040	1 -010+	0 -001	0 -001		11	5 -027	4 -011	3 -004	3 -004
	8	1 -025-	1 -025-	0 -004	0 -004		10	4 -028	3 -011	2 -003	2 -003
	7	0'-010+	0 -010+	——	——		9	3 -027	2 -009	2 -009	1 -002
	6	0 -023	0 -023	——	——		8	2 -023	2 -023	1 -006	0 -001
	5	0 -049		——	——		7	1 -016	1 -016	0 -003	0 -003
8	1	5 -042	4 -012	3 -003	3 -003		6	1 -038	0 -008	0 -008	——
	1	4 -047	3 -014	2 -003	2 -003		5	0 -020	0 -020	——	——
	1	3 -041	2 -011	1 -002	1 -002		4	0 -049	——	——	——
	1	2 -029	1 -007	1 -007	0 -001	13	14	9 -041	8 -016	7 -006	6 -002
	9	1 -017	1 -017	0 -002	0 -002		13	7 -029	6 -011	5 -004	5 -004
	8	1 -037	0 -006	0 -006	——		12	6 -037	5 -015+	4 -005+	3 -002
	7	0 -015-	0 -015-	——	——		11	5 -041	4 -017	3 -006	2 -001
	6	0 -032		——	——		10	4 -041	3 -016	2 -005-	2 -005-
7	1	4 -031	3 -007	3 -007	2 -001		9	3 -038	2 -013	1 -003	1 -003
	1	3 -031	2 -007	2 -007	1 -001		8	2 -031	1 -009	1 -009	0 -001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=14	7	1 -021	1 -021	0 -004	0 -004	A=14 B=7	14	4 -026	3 -006	3 -006	2 -001
	6	1 -048	0 -010+	——	——		13	3 -025	2 -006	2 -006	1 -001
	5	0 -025-	0 -025-	——	——		12	2 -017	2 -017	1 -003	1 -003
12	1	8 -033	7 -012	6 -004	6 -004		11	2 -041	1 -009	1 -009	0 -001
	1	6 -021	6 -021	5 -007	4 -002		10	1 -021	1 -021	0 -003	0 -003
	1	5 -025+	4 -009	4 -009	3 -003		9	1 -043	0 -007	0 -007	——
	1	4 -026	3 -009	3 -009	2 -002		8	0 -015-	0 -015-	——	——
	1	3 -024	3 -024	2 -007	1 -002		7	0 -030	——	——	——
	9	2 -019	2 -019	1 -005-	1 -005-	6	14	3 -018	3 -018	2 -003	2 -003
	8	2 -042	1 -012	0 -002	0 -002		13	2 -014	2 -014	1 -002	1 -002
	7	1 -028	0 -005+	0 -005+	——		12	2 -037	1 -007	1 -007	0 -001
	6	0 -013	0 -013	——	——		11	1 -018	1 -018	0 -002	0 -002
	5	0 -030	——	——	——		10	1 -038	0 -005+	0 -005+	——
11	1	7 -026	6 -009	6 -009	5 -003		9	0 -012	0 -012	——	——
	1	6 -039	5 -014	4 -004	4 -004		8	0 -024	0 -024	——	——
	1	5 -043	4 -016	3 -005-	3 -005-		7	0 -044	——	——	——
	1	4 -042	3 -015-	2 -004	2 -004	5	14	2 -010+	2 -010+	1 -001	1 -001
	1	3 -036	2 -011	1 -003	1 -003		13	2 -037	1 -006	1 -006	0 -001
	9	2 -027	1 -007	1 -007	0 -001		12	1 -017	1 -017	0 -002	0 -002
	8	1 -017	1 -017	0 -003	0 -003		11	1 -038	0 -005-	0 -005-	0 -005-
	7	1 -038	0 -007	0 -007	——		10	0 -011	0 -011	——	——
	6	0 -017	0 -017	——	——		9	0 -022	0 -022	——	——
	5	0 -038	——	——	——		8	0 -040	——	——	——
10	1	6 -020	6 -020	5 -006	4 -002	4	14	2 -039	1 -005-	1 -005-	1 -005-
	1	5 -028	4 -009	4 -009	3 -002		13	1 -019	1 -019	0 -002	0 -002
	1	4 -028	3 -009	3 -009	2 -002		12	1 -044	0 -005-	0 -005-	0 -005-
	1	3 -024	3 -024	2 -007	1 -001		11	0 -011	0 -011	——	——
	1	2 -018	2 -018	1 -004	1 -004		10	0 -023	0 -023	——	——
	9	2 -040	1 -011	0 -002	0 -002		9	0 -041	——	——	——
	8	1 -024	1 -024	0 -004	0 -004	3	14	1 -022	1 -022	0 -001	0 -001
	7	0 -010-	0 -010-	0 -010-	——		13	0 -006	0 -006	0 -006	——
	6	0 -022	0 -022	——	——		12	0 -015-	0 -015-	——	——
	5	0 -047	——	——	——		11	0 -029	——	——	——
9	1	6 -047	5 -014	4 -004	4 -004	2	14	0 -008	0 -008	0 -008	——
	1	4 -018	4 -018	3 -005-	3 -005-		13	0 -025	0 -025	——	——
	1	3 -017	3 -017	2 -004	2 -004		12	0 -050	——	——	——
	1	3 -042	2 -012	1 -002	1 -002						
	1	2 -029	1 -007	1 -007	0 -001						
	9	1 -017	1 -017	0 -002	0 -002						
	8	1 -036	0 -006	0 -006	——	A=15 B=15	15	11 -050-	10 -021	9 -008	8 -003
	7	0 -014	0 -014	——	——		14	9 -040	8 -018	7 -007	6 -003
	6	0 -030	——	——	——		13	7 -025+	6 -010+	5 -004	5 -004
8	1	5 -036	4 -010-	4 -010-	3 -002		12	6 -030	5 -013	4 -005-	4 -005-
	1	4 -039	3 -011	2 -002	2 -002		11	5 -033	4 -013	3 -005-	3 -005-
	1	3 -032	2 -008	2 -008	1 -001		10	4 -033	3 -013	2 -004	2 -004
	1	2 -022	2 -022	1 -005-	1 -005-		9	3 -030	2 -010+	1 -003	1 -003
	1	2 -048	1 -012	0 -002	0 -002		8	2 -025+	1 -007	1 -007	0 -001
	9	1 -026	0 -004	0 -004	0 -004		7	1 -018	1 -018	0 -003	0 -003
	8	0 -009	0 -009	0 -009	——		6	1 -040	0 -008	0 -008	——
	7	0 -020	0 -020	——	——		5	0 -021	0 -012	——	——
	6	0 -040	——	——	——		4	0 -050-	——	——	——

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=15 B=14	15	10 -042	9 -017	8 -006	7 -002	A=15 B=9	13	4 -042	3 -013	2 -003	2 -003
	14	8 -031	7 -013	6 -005-	6 -005-		12	3 -032	2 -009	2 -009	1 -002
	13	7 -041	6 -017	5 -007	4 -002		11	2 -021	2 -021	1 -005-	1 -005-
	12	6 -046	5 -020	4 -007	3 -002		10	2 -045-	1 -011	0 -002	0 -002
	11	5 -048	4 -020	3 -007	2 -002		9	1 -024	1 -024	0 -004	0 -004
	10	4 -046	3 -018	2 -006	1 -001		8	1 -048	0 -009	0 -009	——
	9	3 -041	2 -014	1 -004	1 -004		7	0 -019	0 -019	——	——
	8	2 -033	1 -009	1 -009	0 -001		6	0 -037	——	——	——
	7	1 -022	1 -022	0 -004	0 -004	8	15	5 -032	4 -008	4 -008	3 -002
	6	1 -049	0 -011	——	——		14	4 -033	3 -009	3 -009	2 -002
	5	0 -025+	——	——	——		13	3 -026	2 -006	2 -006	1 -001
13	15	9 -035-	8 -013	7 -005-	7 -005-		12	2 -017	2 -017	1 -003	1 -003
	14	7 -023	7 -023	6 -009	5 -003		11	2 -037	1 -008	1 -008	0 -001
	13	6 -029	5 -011	4 -004	4 -004		10	1 -019	1 -019	0 -003	0 -003
	12	5 -031	4 -012	3 -004	3 -004		9	1 -038	0 -006	0 -006	——
	11	4 -030	3 -011	2 -003	2 -003		8	0 -013	0 -013	——	——
	10	3 -026	2 -008	2 -008	1 -002		7	0 -026	——	——	——
	9	2 -020	2 -020	1 -005+	0 -001		6	0 -050-	——	——	——
	8	2 -043	1 -013	0 -002	0 -002	7	15	4 -023	4 -023	3 -005-	3 -005-
	7	1 -029	0 -005+	0 -005+	——		14	3 -021	3 -021	2 -004	2 -004
	6	0 -013	0 -013	——	——		13	2 -014	2 -014	1 -002	1 -002
	5	0 -031	——	——	——		12	2 -032	1 -007	1 -007	0 -001
12	15	8 -028	7 -010-	7 -010-	6 -003		11	1 -015+	1 -015+	0 -002	0 -002
	14	7 -043	6 -016	5 -006	4 -002		10	1 -032	0 -005-	0 -005-	0 -005-
	13	6 -049	5 -019	4 -007	3 -002		9	0 -010+	0 -010+	——	——
	12	5 -049	4 -019	3 -006	2 -002		8	0 -020	0 -020	——	——
	11	4 -045+	3 -017	20-005-	2 -005-		7	-038	——	——	——
	10	3 -038	2 -012	1 -003	1 -003	6	15	3 -015+	3 -015+	2 -003	2 -003
	9	2 -028	1 -007	1 -007	0 -001		14	2 -011	2 -011	1 -002	1 -002
	8	1 -018	1 -018	0 -003	0 -003		13	2 -031	1 -006	1 -006	0 -001
	7	1 -038	0 -007	0 -007	——		12	1 -014	1 -014	0 -002	0 -002
	6	0 -017	0 -017	——	——		11	1 -029	0 -004	0 -004	0 -004
	5	0 -037	——	——	——		10	0 -009	0 -009	0 -009	——
11	15	7 -022	7 -022	6 -007	5 -002		9	0 -017	0 -017	——	——
	14	6 -032	5 -011	4 -003	4 -003		8	0 -032	——	——	——
	13	5 -034	4 -012	3 -003	3 -003	5	15	2 -009	2 -009	2 -009	1 -001
	12	4 -032	3 -010+	2 -003	2 -003		14	2 -032	1 -005-	1 -005-	1 -005-
	11	3 -026	2 -008	2 -008	1 002		13	1 -014	1 -014	0 -001	0 -001
	10	2 -019	2 -019	1 -004	1 -004		12	1 -031	0 -004	0 -004	0 -004
	9	2 -040	1 -011	0 -002	0 -002		11	0 -008	0 -008	0 -008	——
	8	1 -024	1 -024	0 -004	0 -004		10	0 -016	0 -016	——	——
	7	1 -049	0 -010-	0 -010-	——		9	0 -030	——	——	——
	6	0 -022	0 -022	——	——	4	15	2 -035+	1 -004	1 -004	1 -004
	5	0 -046	——	——	——		14	1 -016	1 -016	0 -001	0 -001
10	15	6 -017	6 -017	5 -005-	5 -005-		13	1 -037	0 -004	0 -004	0 -004
	14	5 -023	5 -023	4 -007	3 -002		12	0 -009	0 -009	0 -009	——
	13	4 -022	4 -022	3 -007	2 -001		11	0 -018	0 -018	——	——
	12	3 -018	3 -018	2 -005-	2 -005-		10	0 -033	——	——	——
	11	3 -042	2 -013	1 -003	1 -003	3	15	1 -020	1 -020	0 -001	0 -001
	10	2 -029	1 -007	1 -007	0 -001		14	0 -005-	0 -005-	0 -005-	0 -005-
	9	1 -016	0 -016	0 -002	0 -002		13	0 -012	0 -012	——	——
	8	1 -034	0 -006	0 -006	——		12	0 -025-	0 -025-	——	——
	7	0 -013	1 -013	——	——		11	0 -043	——	——	——
	6	0 -028	——	——	——	2	15	0 -007	0 -007	0 -007	——
9	15	6 -042	5 -012	4 -003	4 -003		14	0 -022	0 -022	——	——
	14	5 -047	4 -015-	3 -004	3 -004		13	0 -044	——	——	——

APPENDIX H

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a t-test is the recommended analysis. The t-test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro Wilk's test may be used to test the normality assumption (see Appendix B for details). If the data do not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values.

3. The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed F test at the 0.01 level of significance, put the larger of the two variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \text{ where } S_1^2 > S_2^2$$

5. Compare F with the 0.005 level of a tabled F value with $n_1 - 1$ and $n_2 - 1$ degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of *Ceriodaphnia dubia* reproduction data from an effluent screening test will be used to illustrate the F test. The raw data, mean and variance for the control and 100% effluent are given in Table H.1.

TABLE H.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FROM AN EFFLUENT SCREENING TEST

	Replicate											
	1	2	3	4	5	6	7	8	9	10	$\overline{X}$	S^2
Control	36	38	35	35	28	41	37	33	.	.	35.4	14.5
100% Effluent	23	14	21	7	12	17	23	8	18	.	15.9	36.6

7. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{36.61}{14.55}$$

8. There are 9 replicates for the effluent concentration and 8 replicates for the control. Thus, the numerator degrees of freedom is 8 and the denominator degrees of freedom is 7. For a two-tailed test at the 0.01 level of

significance, the critical F value is obtained from a table of the F distribution (Snedecor and Cochran, 1980). The critical F value for this test is 8.68. Since 2.52 is not greater than 8.68, the conclusion is that the variances of the control and 100% effluent are homogeneous.

9. **EQUAL VARIANCE T-TEST**

9.1 To perform the t-test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

9.2 Since we are usually concerned with a decreased response from the control, such as a decrease in survival or a decrease in reproduction, a one-tailed test is appropriate. Thus, compare the calculated t with a critical t, where the critical t is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

9.3 Using the data from Table H.1 to illustrate the t-test, the calculation of t is as follows:

$$t = \frac{35.4 - 15.9}{5.13\sqrt{\frac{1}{8} + \frac{1}{9}}} = 7.82$$

Where:

$$S_p = \sqrt{\frac{(8 - 1)14.5 + (9 - 1)36.6}{(8 + 9 - 2)}} = 5.13$$

9.4 For an 0.05 level of significance test with 15 degrees of freedom the critical t is 1.754 (Note: Table D.5 for K = 1 includes the critical t values for comparing two groups). Since 7.82 is greater than 1.754, the conclusion is that the reproduction in the 100% effluent concentration is significantly lower than the control reproduction.

10. **UNEQUAL VARIANCE T-TEST**

10.1 If the F test for equality of variance fails, the t-test is still a valid test. However, the denominator of the t statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t-test is then conducted as the equal variance t-test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX I

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 Data input is illustrated by a set of total mortality data (Figure I.1) from a fathead minnow embryo-larval survival and teratogenicity test. The program requests the following input:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

2.2 The program output for the abbreviated output includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls (see Figure I.2).
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. Estimated LC1 and LC50 values and associated 95% confidence intervals (see Figure I.2).

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

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis

Number responding in the control group = ? 2
Number of animals exposed in the concurrent control group = ? 20
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 0.5
Number responding = ? 2
Number exposed = ? 20

Concentration = ? 1.0
Number responding = ? 1
Number exposed = ? 20

Concentration = ? 2.0
Number responding = ? 4
Number exposed = ? 20

Concentration = ? 4.0
Number responding = ? 16
Number exposed = ? 20

Concentration = ? 8.0
Number responding = ? 20
Number exposed = ? 20

Number	Number Conc.	Number Resp.	Exposed
1	0.5000	2	20
2	1.0000	1	20
3	2.0000	4	20
4	4.0000	16	20
5	8.0000	20	20

Do you wish to modify your data ? N

The number of control animals which responded = 2
The number of control animals exposed = 20
Do you wish to modify these values ? N

Figure I.1. Sample Data Input for USEPA Probit Analysis program, Version 1.5.

Example of Probit Analysis

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC 1.00	1.346	0.453	1.922
LC/EC 50.00	3.018	2.268	3.672

Figure I.2. USEPA Probit Analysis Program Used for Calculating LC/EC Values, Version 1.5.

APPENDIX J

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Fathead Minnow Larval Survival and Growth test. These data are listed in Table J.1.

TABLE J.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25%	2	0.05
12.5%	0	0.00
25.0%	0	0.00
50.0%	26	0.65
100.0%	40	1.00

7. Let $p_0, p_1, ..., p_k$ denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^{s} = p_i^{s} = \frac{(p_i + p_{i-1})}{2}$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% and 25.0% effluent concentrations, the responses for these four groups must be averaged:

$$p_0^{s} = p_1^{s} = p_2^{s} = p_3^{s} = \frac{0.05+0.05+0.00+0.00}{4} = \frac{0.10}{4} = 0.025$$

7.2 Since $p_4 = 0.65$ is larger than p_3^s, set $p_4^s = 0.65$. Similarly, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table J.2.

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$

p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

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

$$p_0^{a} = p_1^{a} = p_2^{a} = p_3^{a} = \frac{p_1^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{0.025 - 0.025}{1 - 0.025} = \frac{0.0}{0.975} = 0.0$$

$$p_4^{a} = \frac{p_4^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{0.650 - 0.025}{1 - 0.025} = \frac{0.0625}{0.975} = 0.641$$

$$p_5^{a} = \frac{p_5^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{1.000 - 0.025}{1 - 0.025} = \frac{0.975}{0.975} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table J.2. A plot of the smoothed, adjusted data is shown in Figure J.1.

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

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^{a})(x_i + x_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

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

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

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

m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
[(0.000 - 0.000) (1.0969 + 1.3979)]/2 +
[(0.641 - 0.000) (1.3979 + 1.6990)]/2 +
[(1.000 - 0.641) (1.6990 + 2.0000)]/2

= 1.656527

TABLE J.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.025	0.000
6.25%	0.05	0.025	0.000
12.5%	0.00	0.025	0.000
25.0%	0.00	0.025	0.000
50.0%	0.65	0.650	0.641
100.0%	1.00	1.000	1.000

10. Calculate the estimated variance of m as follows:

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

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

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

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

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

V(m) = $(0.000)(1.000)(1.3979 - 0.7959)^2/4(39)$ +
$(0.000)(1.000)(1.6990 - 1.0969)^2/4(39)$ +
$(0.641)(0.359)(2.0000 - 1.3979)^2/4(39)$

= 0.00053477

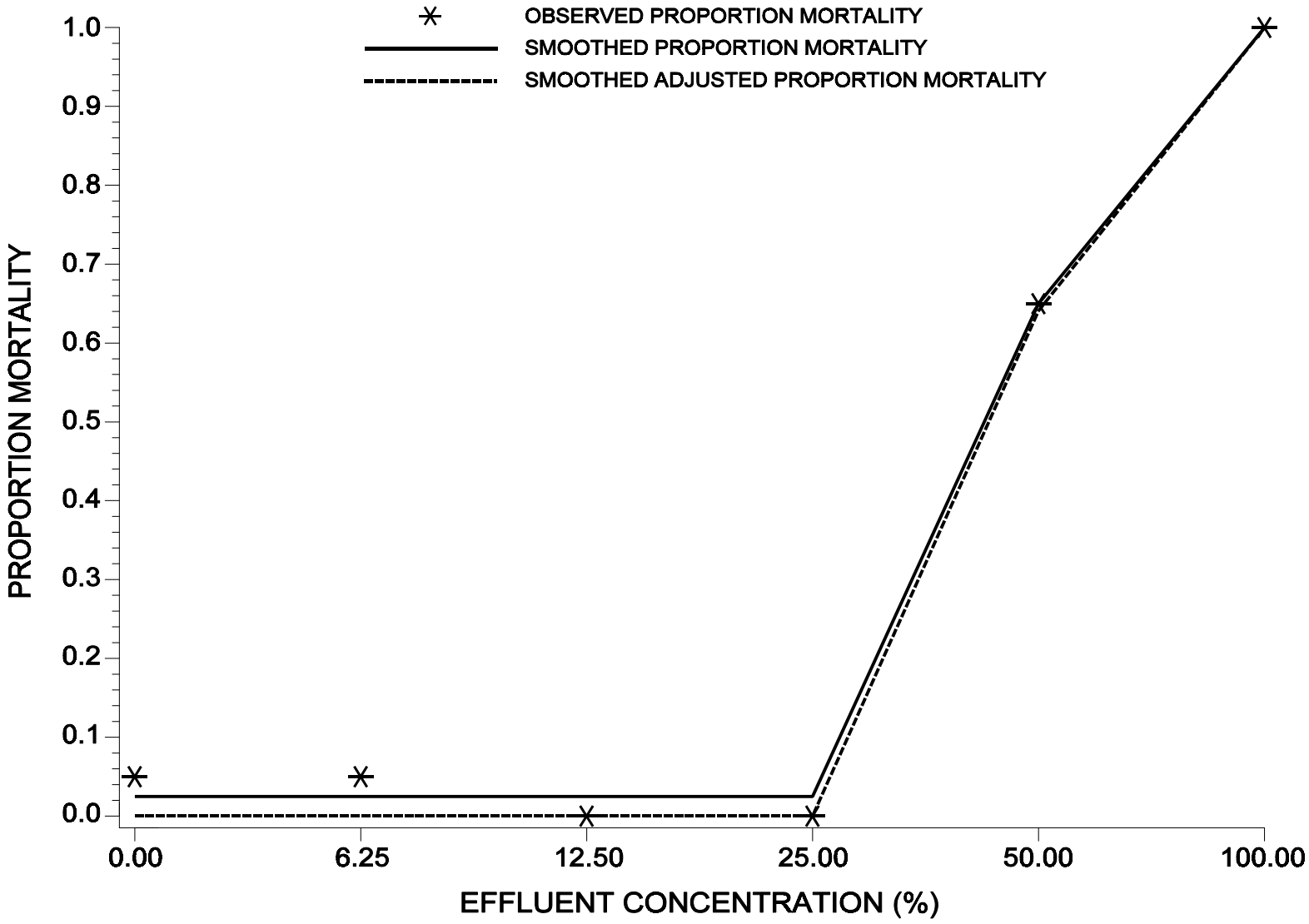

Figure J.1. Plot of the smoothed, adjusted data for the fathead minnow larval survival and growth test.

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

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

$$1.656527 \pm 2\sqrt{0.00053477} = (1.610277,\ 1.702777)$$

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

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

LC50 = antilog(m) = antilog(1.656527) = 45.3%.

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

lower limit: antilog(1.610277) = 40.8%

upper limit: antilog(1.702777) = 50.4%

APPENDIX K

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton et al, 1977). Appendix The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Analysis is recommended only when the requirements for the Probit Method and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$, with their average. For example, if p_i is less than p_{i-1} then:

Where: $p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$

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

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

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

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

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

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

Where: $Trim = \max(p^a_1, 1-p^a_k)$

p^a_1 = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p^a_k = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained from EMSL-Cincinnati by sending a written request to EMSL, 3411 Church Street, Cincinnati, OH 45244.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Fathead Minnow Larval Survival and Growth test will be used. The data are listed in Table K.1.

TABLE K.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	2	0.05
25.0	0	0.00
50.0	0	0.00
100.0	32	0.80

12.1 The program requests the following input (Figure K.1):

a. Output destination (D = disk file, P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure K.2):

a. A table of the concentrations tested, number of organisms exposed, and mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>spearman

 TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

ENTER DATE OF TEST:
1
ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
 L
ENTER TEST SPECIES NAME:
Fathead minnow
ENTER TOXICANT  NAME:
Effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
 40
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
2
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:        40

ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:        0 2 0 0 32

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
 y
```

Figure K.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: fathead minnow

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	40	2
6.25	40	0
12.50	40	2
25.00	40	0
50.00	40	0
100.00	40	32

SPEARMAN-KARBER TRIM: 20.41%

SPEARMAN-KARBER ESTIMATES: LC50: 77.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure K.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX L

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from an Fathead Minnow Larval Survival and Growth test. These data are listed in Table L.1.

TABLE L.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	40	1.00
100.0	40	1.00

4. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^s = p_1^s = p_2^s = p_3^s = \frac{0.05+0.00+0.00+0.00}{4} = \frac{0.05}{4} = 0.0125$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.0125, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table L.2.

TABLE L.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.0125	0.00
6.25	0.00	0.0125	0.00
12.5	0.00	0.0125	0.00
25.0	0.00	0.0125	0.00
50.0	1.00	1.0000	1.00
100.0	1.00	1.0000	1.00

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0125 - 0.0125}{1 - 0.0125} = \frac{0.0}{0.9875} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.0125}{1 - 0.0125} = \frac{0.9875}{0.9875} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations are shown in Table L.2.

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure L.1.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

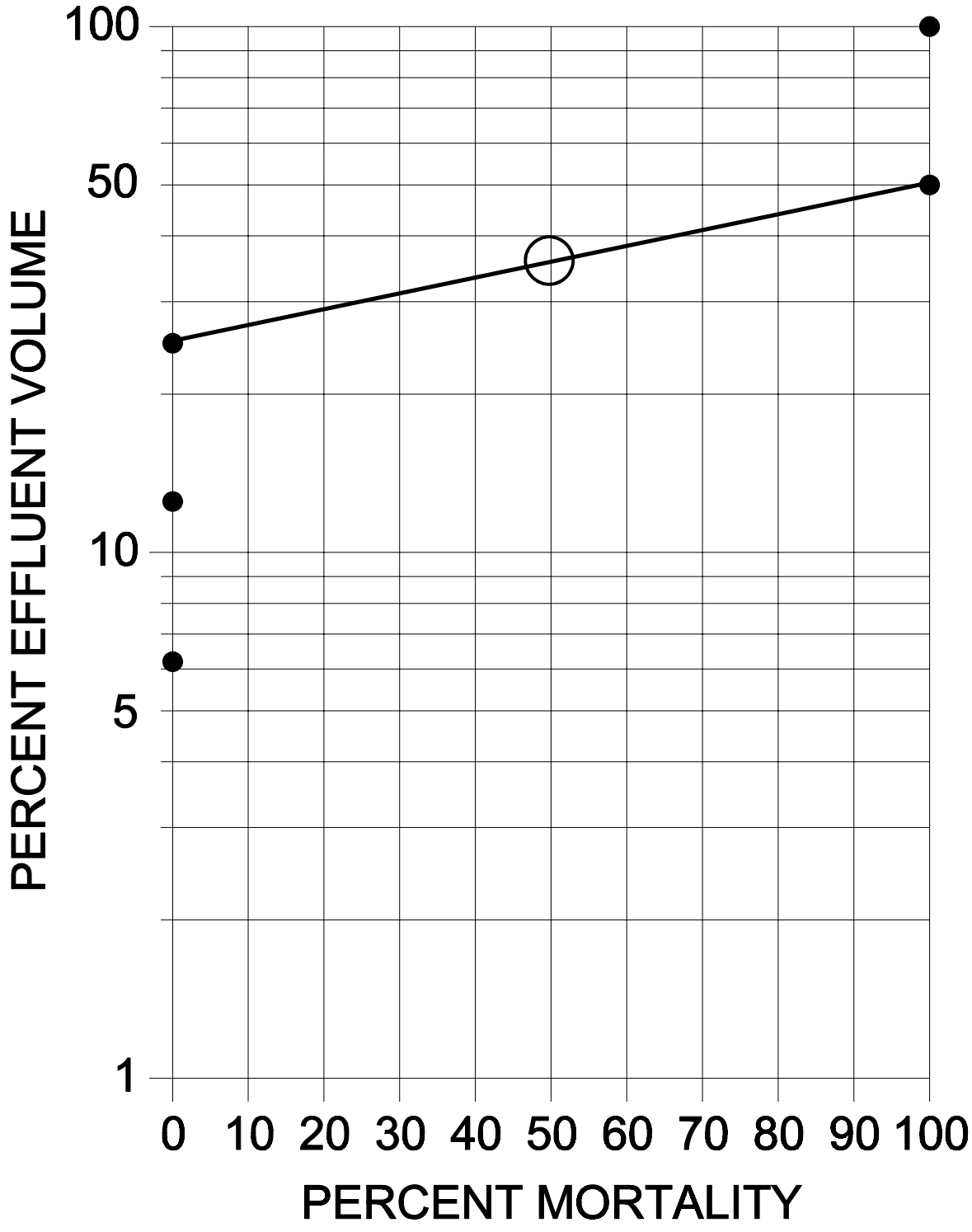

Figure L.1 Plot of the smoothed adjusted response proportions for fathead minnow, *Pimephales promelas*, survival data.

APPENDIX M

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically nonincreasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

4. LINEAR INTERPOLATION METHOD

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control

response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [\ M_1\ (1 - p/100) - M_J\ ]\ \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response. The ICp is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the ICp would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. **CONFIDENCE INTERVALS**

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the ICp (ICp*) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 time, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the

BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" which is described below in subsection 7.

6. **MANUAL CALCULATIONS**

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the *Ceriodaphnia dubia* reproduction data used in the example in Section 13. Table M.1 includes the raw data and the mean reproduction for each concentration. Data are included for all animals tested regardless of death of the organism. If an animal died during the test without producing young, a zero is entered. If death occurred after producing young, the number of young produced prior to death is entered. A plot of the data is provided in Figure M.1.

TABLE M.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)				
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	10	0
2	30	35	30	34	13	0
3	29	32	33	36	7	0
4	31	26	33	34	7	0
5	16	18	36	31	7	0
6	15	29	33	27	10	0
7	18	27	33	33	10	0
8	17	16	27	31	16	0
9	14	35	38	33	12	0
10	27	13	44	31	2	0
Mean ($\overline{Y}_i$)	22.4	26.3	34.6	31.7	9.4	0
i	1	2	3	4	5	6

6.2 MONOTONICITY

6.2.1 As can be seen from the plot in Figure M.1, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

6.2.2 Starting with the control mean $\overline{Y}_1 = 22.4$ and $\overline{Y}_2 = 26.3$, we see that $\overline{Y}_1 < \overline{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 24.35$$

6.2.3 Since $\overline{Y}_3 = 34.6$ is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

6.2.4 Additionally, $\overline{Y}_4 = 31.7$ is larger than M_3, and is pooled with the first three means. Thus,

$$M_1 = M_2 = M_3 = M_4 = (M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7$$

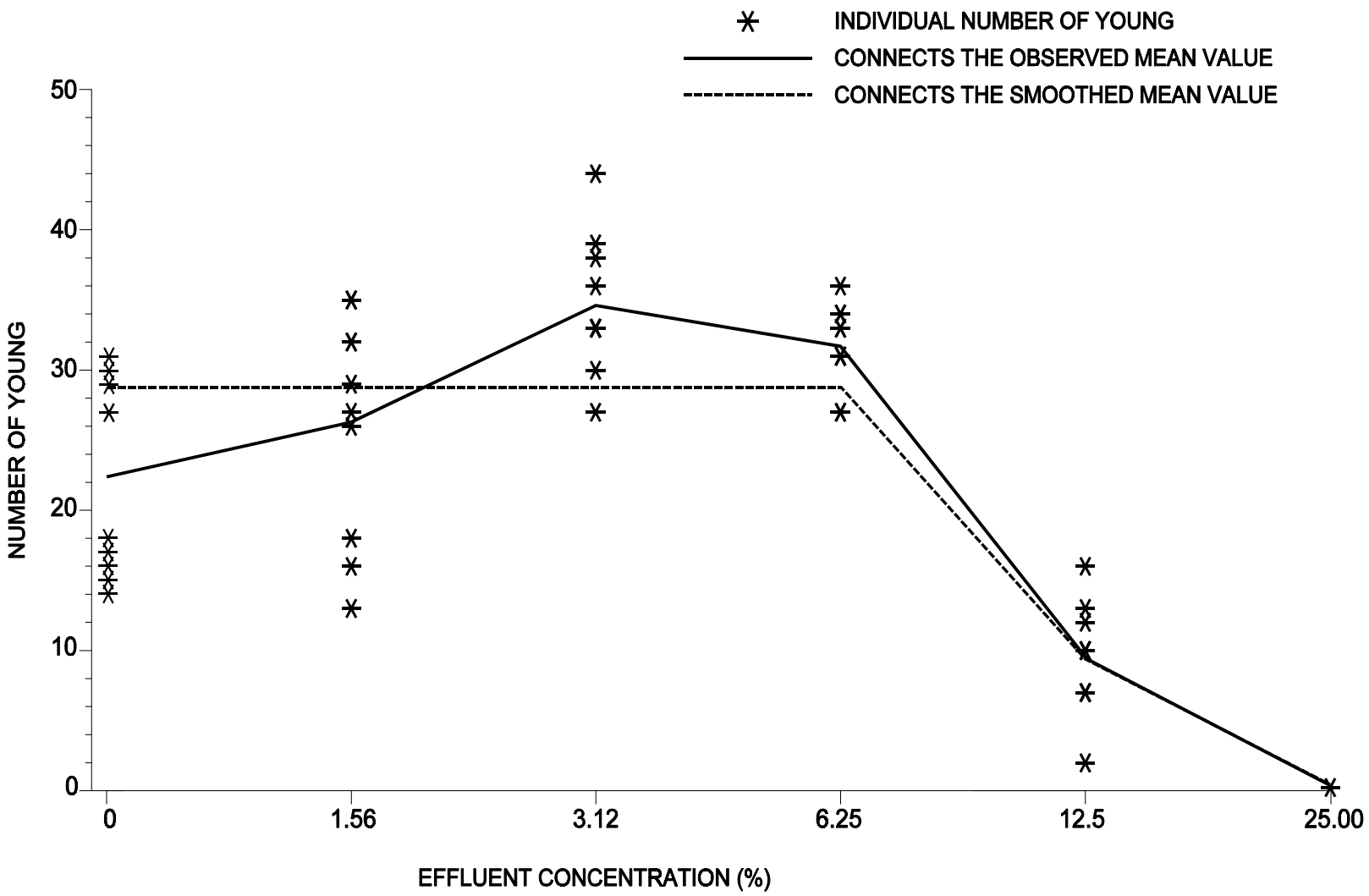

Figure M.1. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

TABLE M.2. *CERIODAPHNIA DUBIA* REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Effluent Concentration %	i	Response Mean (Y_i) (Young/female)	Smoothed Mean (M_i) (Young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

6.2.5 Since $M_4 > \overline{Y}_5 = 9.4$, set $M_5 = 9.4$. Likewise, $M_5 > \overline{Y}_6 = 0$ and M_6 becomes 0. Table M.2 contains the smoothed means and Figure M.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 Estimates of the IC25 and IC50 are calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1-p/100) = 28.75(1-25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1-p/100) = 28.75(1-50/100)$. Examining the smoothed means and their associated concentrations (Table M.2), the two effluent concentrations bracketing the reproduction of 21.56 young per adult are $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent.

6.3.2 Using Equation 1 from 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J + [\, M_1\,(1 - p/100) - M_J\,] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 6.25 + [28.75\,(1 - 25/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 8.57% effluent

6.3.3 Using the equation from section 4.2, the estimate of the IC50 is calculated as follows:

$$ICp = C_J + [\, M_1\,(1 - p/100) - M_J\,] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC50 = 6.25 + [28.75\,(1 - 50/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 10.89% effluent

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Method was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). To obtain the program and supporting documentation, send a written request to EMSL-Cincinnati at 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means (Y_i) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be used (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure M.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method (Norberg-King, 1993).

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT.

7.5.1 The program output includes the following (Figures M.3 and M.4):

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means ($\bar{Y}_i$), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is

over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the *Ceriodaphnia dubia* reproduction data in Table M.1 is provided in Figures M.3 and M.4.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.57% effluent. The empirical 95% confidence intervals for the true mean were 8.30% to 8.85% effluent.

7.6.2 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 10.89% effluent. The empirical 95% confidence intervals for the true mean were 10.36% to 11.62% effluent.

ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use arrow Keys to Switch Fields

Figure M.2. ICp data entry/edit screen. Twelve concentrating identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 8.5715 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.6014 Standard Deviation: 0.1467
Original Confidence Limits: Lower: 8.3040 Upper: 8.8496
Resampling time in Seconds: 2.53 Random Seed: -1652543090

Figure M.3. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 10.8931 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.9108 Standard Deviation: 0.3267
Original Confidence Limits: Lower: 10.3618 Upper: 11.6201
Resampling time in Seconds: 2.58 Random Seed: 340510286

Figure M.4. Example of ICPIN program output for the IC50.

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