United States
Environmental Protection
Agency

Environmental Monitoring Systen
Laboratory
Cincinnati OH 45268

600489001
March 1989

Research and Development

EPA

13223

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

Second Edition

XX-3

EPA/600/4-89/001
March 1989

SHORT-TERM METHODS FOR ESTIMATING THE CHRONIC TOXICITY OF EFFLUENTS AND RECEIVING WATERS TO FRESHWATER ORGANISMS

SECOND EDITION

Prepared
by

Cornelius I. Weber[1], William H. Peltier[2], Teresa J. Norberg-King[3], William B. Horning, II[1], Florence A. Kessler [4], John R. Menkedick[4], Timothy W. Neiheisel[1], Philip A. Lewis[1], Donald J. Klemm[1], Quentin H. Pickering[1], Ernest L. Robinson[1], James M. Lazorchak[1], Larry J. Wymer[4], and Ronald W. Freyberg[4]

[1]Aquatic Biology Branch, Quality Assurance Research Division
Environmental Monitoring Systems Laboratory - Cincinnati
[2]Environmental Services Division, Region 4
[3]Environmental Research Laboratory, Duluth, Minnesota
[4]Computer Sciences Corporation, Cincinnati, Ohio

ENVIRONMENTAL MONITORING SYSTEMS LABORATORY - CINCINNATI
OFFICE OF MODELING, MONITORING SYSTEMS, AND QUALITY ASSURANCE
OFFICE OF RESEARCH AND DEVELOPMENT
U. S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the chemical and biological quality of drinking water, surface waters, groundwaters, wastewaters, sediments, sludges, and solid waste. The Environmental Monitoring Systems Laboratory - Cincinnati (EMSL-Cincinnati) conducts research to:

- Develop and evaluate analytical methods to identify and measure the concentration of chemical pollutants.
- Identify and quantitate the occurrence of viruses, bacteria, and other human pathogens and indicator organisms.
- Measure the toxicity of pollutants to representative species of aquatic organisms and determine the effects of pollution on communities of indigenous freshwater, estuarine, and marine organisms, including the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.
- Develop and operate a quality assurance program to support achievement of data quality objectives for environmental measurements.

The Federal Water Pollution Control Act Amendments of 1972 (PL 92-500), the Clean Water Act (CWA) of 1977 (PL 95-217), and the Water Quality Act of 1987 (PL 100-4) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. Determination of the toxicity of effluents, therefore, plays an important role in identifying and controlling toxic discharges to surface waters. This report is a revision of EPA/600/4-85/014, and provides updated methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms for use by the U.S. Environmental Protection Agency (USEPA) regional and state programs, and National Pollutant Discharge Elimination System (NPDES) permittees.

Thomas A. Clark
Director
Environmental Monitoring Systems
Laboratory - Cincinnati

PREFACE

This manual is a revision of EPA/600/4-85/014. It was reviewed by the following members of the Bioassay Subcommittee and its parent committee, the EMSL-Cincinnati Biological Advisory Committee, representing Agency regional and headquarters programs, and research laboratories.

BIOASSAY SUBCOMMITTEE, EMSL-CINCINNATI BIOLOGICAL ADVISORY COMMITTEE

William Peltier, Chairman, Bioassay Subcommittee
Environmental Services Division, Region 4
Peter Nolan, Environmental Services Division, Region 1
Stephen Ward, Environmental Services Division, Region 2
Roland Hemmett, Environmental Services Division, Region 2
Ronald Preston, Environmental Services Division, Region 3
Robert Donaghy, Environmental Services Division, Region 3
Charles Steiner, Environmental Services Division, Region 5
Michael Bastian, Environmental Services Division, Region 6
Terry Hollister, Environmental Services Division, Region 6
Michael Tucker, Environmental Services Division, Region 7
Loys Parrish, Environmental Services Division, Region 8
Milton Tunzi, Office of Policy and Management, Region 9
Peter Husby, Office of Policy and Management, Region 9
Joseph Cummins, Environmental Services Division, Region 10
Bruce Binkley, National Enforcement Investigations Center, Denver
Wesley Kinney, Environmental Monitoring Systems Laboratory - Las Vegas
Steven Schimmel, Environmental Research Laboratory - Narragansett
Douglas Middaugh, Environmental Research Laboratory - Gulf Breeze
Teresa Norberg-King, Environmental Research Laboratory - Duluth
Larry Kapustka, Environmental Research Laboratory - Corvallis
Richard Swartz, Environmental Research Laboratory - Newport
Margarete Heber, Permits Division, Office of Water Enforcement and Permits
Edward Bender, Enforcement Division, Office of Water Enforcement and Permits
James Plafkin, Monitoring and Data Support Division, Office of Water Regulations and Standards
Chris Zarba, Criteria and Standard Division, Office of Water Regulations and Standards
Dan Rieder, Hazard Evaluation Division, Office of Pesticide Programs
Jerry Smrchek, Health and Environmental Review Division, Office of Toxic Substances
Gail Hansen, Office of Solid Waste
Royal Nadeau, Emergency Response Team, Edison, NJ

Cornelius I. Weber, Ph.D.
Chairman, Biological Advisory Committee
Acting Chief, Aquatic Biology Branch
Environmental Monitoring Systems
Laboratory - Cincinnati

ABSTRACT

This manual is a revision of EPA/600/4-85/014, and describes short-term (four- to seven-day) methods for estimating the chronic toxicity of effluents and receiving waters to the fathead minnow (*Pimephales promelas*), a cladoceran (*Ceriodaphnia dubia*), and a green alga (*Selenastrum capricornutum*). Also included are guidelines on laboratory safety, quality assurance, facilities and equipment, dilution water, effluent sampling and holding, data analysis, report preparation, and organism culturing and handling. Supplementary information on statistical techniques for test design and analysis of toxicity test data is provided in the Appendices.

CONTENTS

FIGURES
(SECTIONS 1-9)

TABLES
(SECTIONS 1-9)

ACKNOWLEDGMENTS

Materials for the first (EPA/600/4-85/014) and second editions of this manual were taken in part from the following sources: USEPA, 1975, Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians, Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA-660/3-75-009; USEPA, 1979, Handbook for Analytical Quality Control in Water and Wastewater Laboratories, Environmental Monitoring and Support Laboratory - Cincinnati, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-79/019; USEPA, 1979, Interim NPDES Compliance Biomonitoring Inspection Manual, Enforcement Division, Office of Water Enforcement, U. S. Environmental Protection Agency, Washington, D.C.; Peltier, W. H., and C. I. Weber, 1985, Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Environmental Monitoring and Support Laboratory - Cincinnati, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-85/013; Mount, D. I., and T. J. Norberg, 1984, A Seven-day Life-cycle Cladoceran Test, Environ. Toxicol. Chem. 3:425-434; Norberg, T., and D. I. Mount, 1985, A New Subchronic Fathead Minnow (Pimephales promelas) Toxicity Test, Environ. Toxicol. Chem. 4:711-718; Miller, W.E, J. C. Greene, and T. Shiroyama, 1978, The Selenastrum capricornutum Printz Algal Assay Bottle Test, Environmental Research Laboratory, U. S. Environmental Protection Agency, Corvallis, Oregon, EPA-600/9-78-018; Weber, C. I., W. B. Horning, II, D. J. Klemm, T. W. Neiheisel, P. A. Lewis, E. L. Robinson, J. Menkedick, and F. Kessler, 1988, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Environmental Monitoring and Support Laboratory - Cincinnati, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-87/028.

In addition to the contributions of the Biological Advisory Committee members, review comments received from the following persons are also gratefully acknowledged: Max Anderson, Central Regional Laboratory, USEPA, Chicago, Illinois; Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, Kentucky; Gary Collins, Environmental Monitoring Systems Laboratory, USEPA, Cincinnati, Ohio; Robert Elliott, Water Quality Monitoring Branch, USEPA, San Francisco, California; Charles Plost, Office of Modeling, Monitoring Systems and Quality Assurance, USEPA, Washington, DC; Glenn Rodrigeuz, Environmental Services Division, USEPA, Region 8, Denver, Colorado; Donald Schultz, Environmental Services Division, Region 4, Athens, Georgia; Thomas Simon, Central Regional Laboratory, USEPA, Chicago, Illinois; Albert Westerman, Natural Resources and Environmental Protection Cabinet, Frankfort, Kentucky.

The graphical displays for the statistical analyses were prepared by Minghua Grisell, Computer Sciences Corporation, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

SECTION 1

INTRODUCTION

1.1 The Federal Water Pollution Control Act (FWPCA) Amendments of 1972 (PL 92-500), 1977 (Clean Water Act, PL 95-217), and 1987 (Water Quality Act, PL 100-4), were enacted to restore and maintain the chemical, physical, and biological integrity of the Nation's waters (Section 101[a]), and contained specific or implied requirements for the collection of biomonitoring data in at least 15 sections.

1.2 The Declaration of Goals and Policy, Section 101(a)(3), in these laws, states that "it is the national goal that the discharge of toxic pollutants in toxic amounts be prohibited." To achieve the goals of this legislation, extensive effluent toxicity screening programs were conducted during the 1970s by the regions and states. Acute toxicity tests (USEPA, 1975; Peltier, 1978) were used to measure effluent toxicity and to estimate the safe concentration of toxic effluents in receiving waters. However, for those effluents that were not sufficiently toxic to cause mortality in acute (one- to four-day) tests, short-term, inexpensive methods were not available to detect the more subtle, low-level, long-term, adverse effects of effluents on aquatic organisms, such as reduction in growth and reproduction, and occurrence of terata. Fortunately, rapid developments in toxicity test methodology in this decade have resulted in the availability of several methods that permit detection of the low-level, adverse effects (chronic toxicity) of effluents in seven days or less.

1.3 As a result of the increased awareness of the value of effluent toxicity test data for toxics control in the water quality program and the National Pollutant Discharge Elimination System (NPDES) permit program, which emerged from the extensive effluent toxicity monitoring activities of the regions and states, and the availability of short-term chronic toxicity test methods, the U. S. Environmental Protection Agency (USEPA) issued a national policy statement entitled, "Policy for the Development of Water Quality-Based Permit Limitations for Toxic Pollutants," in the Federal Register, Vol. 49, No. 48, p. 9016-9019, Friday, March 9, 1984.

1.4 This policy proposed the use of toxicity data to assess and control the discharge of toxic substances to the Nation's waters through the NPDES permits program. The policy states that "biological testing of effluents is an important aspect of the water quality-based approach for controlling toxic pollutants. Effluent toxicity data, in conjunction with other data, can be used to establish control priorities, assess compliance with State water quality standards, and set permit limitations to achieve those standards." All states have water quality standards which include narrative statements prohibiting the discharge of toxic materials in toxic amounts.

1.5 A technical support document (USEPA, 1985) and a permit writer's guide (USEPA, 1987b) were prepared by the Office of Water to provide detailed guidance on the implementation of the biomonitoring policy in the discharge permit program, and the first edition of this manual (EPA/600/4-85/014) was published to provide standardized toxicity test methodology. The current (second) edition of the manual contains many improvements in culturing and test conditions, and detailed examples of the statistical analysis of test data.

1.6 The four short-term tests described in this manual are for use in the NPDES Program to estimate one or more of the following: (1) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with a standard dilution water; (2) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with dilution water consisting of non-toxic receiving water collected upstream from or outside the influence of the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same hardness as the receiving water; (3) the toxicity of receiving water downstream from or within the influence of the outfall; and (4) the effects of multiple discharges on the quality of the receiving water. The tests may also be useful in developing site-specific water quality criteria.

1.7 These methods were developed to provide the most favorable cost-benefit relationship possible, and are intended for use in effluent toxicity tests performed on-site or off-site.

The tests include:

1. A seven-day, sub-chronic, fathead minnow (Pimephales promelas), static renewal, larval survival and growth test.

2. A three-brood, seven-day, chronic, cladoceran (Ceriodaphnia dubia), static renewal, survival and reproduction test.

3. A seven-day, sub-chronic, fathead minnow (Pimephales promelas), static renewal, embryo-larval survival and teratogenicity test.

4. A four-day, chronic, algal, (Selenastrum capricornutum), static, growth test.

1.8 The first two tests were adapted from methods developed by Dr. Donald Mount and Teresa Norberg-King, Environmental Research Laboratory, USEPA, Duluth, Minnesota (Mount and Norberg, 1984; Norberg and Mount, 1985). The third test was adapted from a method developed by Drs. Wesley Birge and Jeffrey Black, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky (Birge and Black, 1981). The fourth test, a 96-h, multi-generation test utilizing the freshwater alga, Selenastrum capricornutum, was adapted from the publications of the Environmental Research Laboratory - Corvallis (USEPA, 1971; Miller et al., 1978).

1.9 The validity of the first two tests methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies on the Ottawa River, Ohio (Mount et al., 1984), Scippo Creek, Ohio (Mount and Norberg-King, 1985), Five Mile Creek, Alabama (Mount et al., 1985a), the Ohio River, West Virginia (Mount et al., 1985b), the Kanawha River, West Virginia (Mount and Norberg-King, 1986), Skeleton Creek, Oklahoma (Norberg-King and Mount, 1986), the Naugatuck River, Connecticut (Mount and Norberg-King, 1986a), and the Back River, Maryland (Mount et al., 1986b). Other field studies demonstrating the validity of the tests in this manual were carried out by Birge et al., (1989), for the Fathead Minnow Embryo-Larval Survival and Teratogenicity Test, and Eagleson, et al., (1989), for the Ceriodaphnia dubia Survival and Reproduction Test.

1.10 The tests were revised by staff from EMSL-Cincinnati, Environmental Research Laboratory-Duluth, and the regional programs to reflect the collective experience of Agency and state programs in the use of the methods during the three years since the first edition of the manual was published. The authority for promulgation of chemical, physical, and biological test procedures for the analysis of pollutants is contained in Section 304(h) of the FWPCA.

1.11 The manual was prepared in the established EMSL-Cincinnati format (Kopp, 1983) so that each method can be used independently of the other methods.

SECTION 2

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

2.1 ENDPOINTS

2.1.1 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 parameters 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 parameters, 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 primary terms in current use are as follows:

2.1.1.1 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.

2.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle 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 parameters are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

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

2.1.1.4 Maximum Acceptable Toxicant Concentration (MATC) - An undetermined concentration within the interval bounded by the NOEC and LOEC that is presumed safe by virtue of the fact that no statistically significant adverse effect was observed.

2.1.1.5. Chronic Value (ChV) - A point estimate of the presumably safe (no-effect) concentration, lying between the NOEC and LOEC, and derived by calculating the geometric mean of the NOEC and LOEC. The ChV has been referred to as the "Maximum Acceptable Toxicant Concentration."

2.1.1.6 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect (such as death, immobilization, serious incapacitation, reduced fecundity, or reduced growth) in a given percent of the test organisms, calculated by point estimation techniques. For example, the EC50 from a Probit Analysis is the estimated concentration of toxicant that would cause death, or some other observable quantal, "all or nothing," response, in 50% of the test population. If the observable effect is death (mortality), the term LC - Lethal Concentration, is used (see below). If the observable effect is a non-quantal biological measurement, the term, Inhibition Concentration (IC), may be used (see below). A certain EC, LC, or IC 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 parameters.

2.1.1.7 Lethal Concentration (LC) - Identical to EC when the observable adverse affect is death or mortality.

2.1.1.8 Inhibition Concentration (IC) - A point estimate of the toxicant concentration that would cause a given percent reduction in a non-quantal biological measurement such as fecundity or growth. For example, an IC25 would be the estimated concentration of toxicant that would cause a 25% reduction in mean young per female or some other non-quantal biological measurement.

2.1.2 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 endpoint of these tests is related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing, and LCs, ECs, and ICs are determined by point estimation techniques. There are inherent differences between the use of an NOEC, LOEC, ChV, or other estimate derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, EC, IC, or other point estimate derived from curve fitting, interpolation, etc.

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

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

2.1.4.1 In the first case, it is also assumed that adverse effects that are not "statistically observable" are also not significant from a biological

standpoint, since they are not pronounced enough to test statistically significant against some measure of the natural variability of responses.

2.1.4.2 In the second case, 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.

2.1.4.3 In either case, it is important to realize that the amount of the adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design. These aspects include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration. The sensitivity of the test, which is related to the magnitude of the adverse effect that is statistically observable, can be controlled by the experimental design and by controlling the amount of variability between responses at the given concentration.

2.1.4.4 In the first case, where the assumption of a continuous dose-response relationship is made, clearly the NOEC estimate is an estimate of some amount of adverse effect that is dependent on the experimental design. In the second case, the NOEC may be an estimate of a "safe" or "no-effect" concentration but only 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. The NOEC in that case would indeed be an estimate of a "safe" or "no-effect" concentration. If, however, the amount of adverse effect were not great enough to test as statistically different, then the NOEC might well be an estimate that again represents some amount of adverse effect which is assumed safe because it did not test as statistically significant. 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.

2.1.5 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 "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

2.2 DATA ANALYSIS

2.2.1 Role of the Statistician

2.2.1.1 The choice of a statistical method to analyze toxicity test data and 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. 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.

2.2.1.2 The recommended statistical methods presented in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. 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. Certainly there are other reasonable and defensible methods of statistical analysis of this kind of toxicity data. The methods contained in this manual have been chosen, among other reasons, because they are (1) well-tested and well-documented, (2) applicable to most different toxicity test data sets for which they are recommended, but still powerful, (3) hopefully "easily" understood by non-statisticians, and (4) amenable to use without a computer, if necessary.

2.2.2 Plotting of the Data

2.2.2.1 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 Appendix.

2.2.3 Data Transformations

2.2.3.1 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.

2.3 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

2.3.1 Statistical independence among observations is a critical assumption in the 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. A discussion of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

2.4 REPLICATION AND SENSITIVITY

2.4.1 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.

2.4.2 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. If there are only two replicates, Dunnett's Procedure may be used, but it is not possible to check the assumptions of the test.

2.5 CHOICE OF ANALYSIS AND MULTIPLE NOECs

2.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flow chart in Figure 1. An initial decision is made to use point estimation techniques and/or to use hypothesis testing. If hypothesis testing is chosen, subsequent decisions are made on the appropriate hypothesis testing procedure for a given set of data, as illustrated in the flow chart. If point estimation is chosen, the equivalent of an NOEC can be calculated. A specific flow chart is included in the analysis section for each test.

2.5.2 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 parameters 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 parameters would not be subsequently tested for an effect on some other parameter. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent statistical analysis 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.

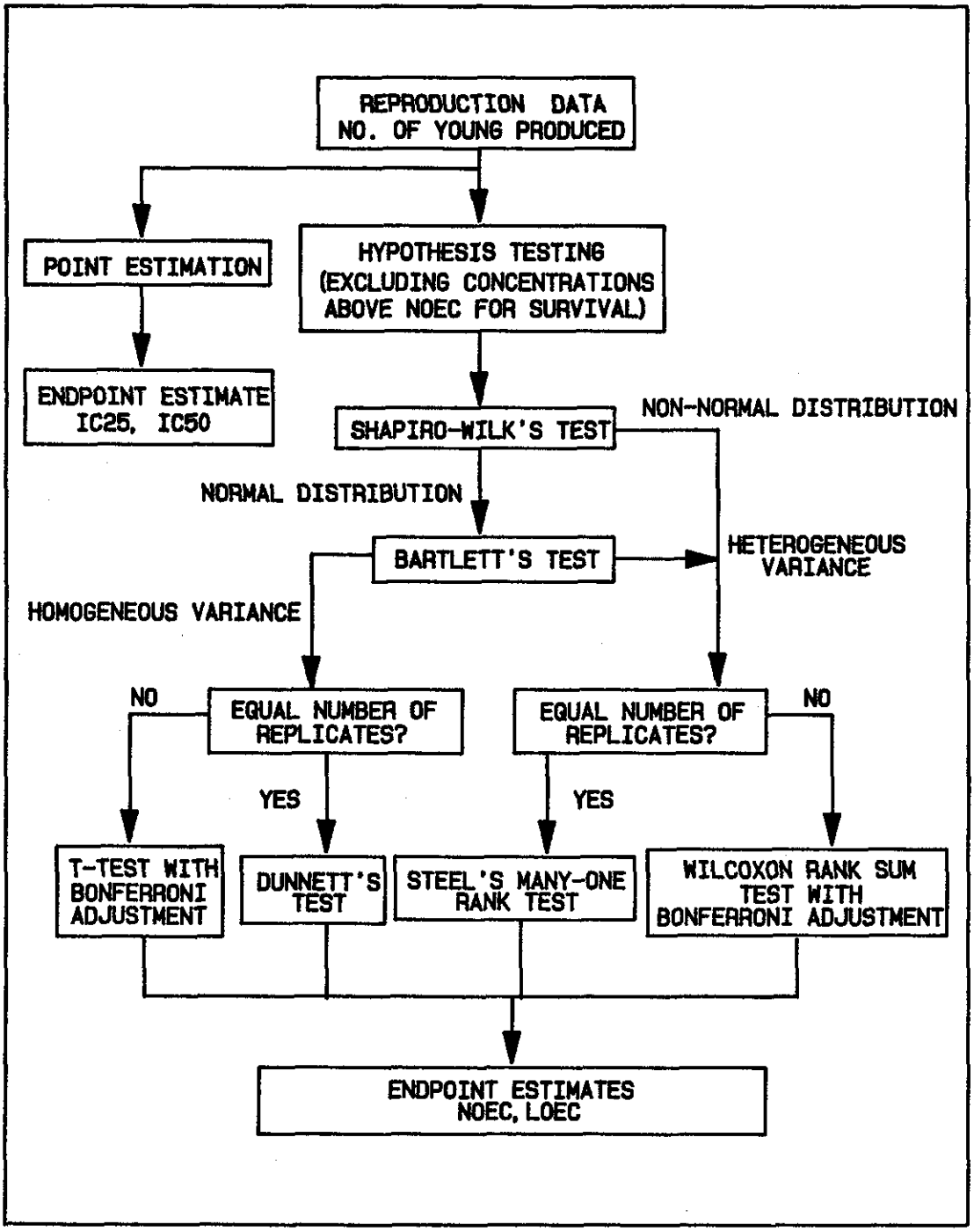

Figure 1. Flow chart for statistical analysis of test data.

2.6 ANALYSIS OF GROWTH AND REPRODUCTION DATA

2.6.1 Growth data from the fathead minnow larval survival and growth test are analyzed using hypothesis testing or point estimation techniques according to the flow chart in Figure 1. (Note that the nonparametric hypothesis tests can be used only if at least four replicates were used at each toxicant concentration).

2.6.2 Reproduction data from the Ceriodaphnia survival and reproduction test, after eliminating data from concentrations with a significant mortality effect as determined by Fisher's Exact Test, are analyzed using hypothesis testing or point estimation techniques according to the flow chart in Figure 1. (Note that the nonparametric hypothesis tests can be used only if at least four replicates were used at each toxicant concentration).

2.7 ANALYSIS OF ALGAL GROWTH RESPONSE DATA

2.7.1 The growth response data from the algal 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 flow chart in Figure 1. Point estimates, such as the EC1, EC5, EC10, or EC50, would also be appropriate in analyzing algal growth data.

2.8 ANALYSIS OF MORTALITY DATA

2.8.1 Mortality data from the fathead minnow larval survival and growth test and the fathead minnow embryo-larval survival and teratogenicity test are analyzed by Probit Analysis, if appropriate (see discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine transformation (see Appendix B), according to the flow chart in Figure 1.

2.8.2 Mortality data from the Ceriodaphnia 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 (see discussion below).

2.9 DUNNETT'S PROCEDURE

2.9.1 Dunnett's Procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison method 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 two replicates per treatment and an equal number of data points (replicates) for each concentration. However, as stated above, it is not possible to check the assumptions of the test. In cases where the number of data points 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.

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

2.9.3 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.

2.9.4 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of Dunnett's Procedure is provided in the Appendix.

2.9.5 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 per toxicant concentration. If the numbers of data points (replicates) for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

2.10 BONFERRONI'S T-TEST

2.10.1 Bonferroni's T-test (see Appendix D) 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.

2.11 STEEL'S MANY-ONE RANK TEST

2.11.1 Steel's Many-One Rank Test is a multiple comparison method 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 for 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). It is necessary to have at least four replicates per toxicant concentration to use Steel's test. The sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean.

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

2.12 WILCOXON RANK SUM TEST

2.12.1 The Wilcoxon Rank Sum Test is a nonparametric test for comparing a treatment 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.

2.12.2 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 is provided in Appendix F.

2.13 INTERPOLATION APPROACH

2.13.1 Chronic toxicity test data can be analyzed by an interpolation approach as described by DeGraeve et al. (1988, Appendix B; 1989). Precision estimates can be calculated using this approach. The round robin data (DeGraeve et al., 1988; 1989) show that the endpoints estimated by this approach are much less variable than those estimated by hypothesis testing.

2.14 PROBIT ANALYSIS

2.14.1 Probit Analysis is used to analyze percentage data from concentration-response tests. The analysis can provide an estimate of the concentration of toxicant affecting a given percent of the test organisms and provide a confidence interval for the estimate. Probit Analysis assumes a normal distribution of log tolerances and independence of the individual responses. To use Probit Analysis, at least two partial mortalities must be obtained. If a test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), a LC50 may be estimated using the graphical method, and the LC50 and confidence interval may be estimated by the moving average angle, Spearman-Karber, or other methods (see Peltier and Weber, 1985).

2.14.2 It is important to check the results of Probit Analysis to determine if the analysis is appropriate. The chi-square test for heterogeneity provides one good test of appropriateness of the analysis. In cases where there is a significant chi-square statistic, where there appears to be systematic deviation from the model, or where there are few data in the neighborhood of the point to be estimated, Probit results should be used with extreme caution.

2.14.3 The natural rate of occurrence of a measured response, such as mortality in the test organisms (referred to as the natural spontaneous response), may be used to adjust the results of the Probit Analysis if such a rate is judged to be different from zero. If a reliable, consistent estimate of the natural spontaneous response can be determined from historical data, the historical occurrence rate may be used to make the adjustment. In cases where historical data are lacking, the spontaneous occurrence rate should optimally be estimated from all the data as part of the maximum likelihood procedure. However, this can require sophisticated computer software. An acceptable alternative is to estimate the natural occurrence rate from the occurrence rate in the controls. In this instance, greater than normal replication in the controls would be beneficial.

2.14.4 A discussion of Probit Analysis and the natural occurrence rate, along with a computer program for performing the Probit Analysis, are included in Appendix I.

SECTION 3

HEALTH AND SAFETY[1]

3.1 GENERAL PRECAUTIONS

3.1.1 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.

3.1.2 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.

3.2 SAFETY EQUIPMENT

3.2.1 Personal Safety Gear

Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 Laboratory Safety Equipment

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

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, Paragraph 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 should be used whenever necessary.

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.

---

[1]Adapted from: Peltier and Weber (1985).

3.3.5. Good housekeeping contributes to safety and reliable results.

3.3.6. 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.

3.3.7. 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, and polio.

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 industrial safety manuals, including USEPA (1977) and Walters and Jameson (1984).

SECTION 4

QUALITY ASSURANCE[1]

4.1 INTRODUCTION

4.1.1 Quality Assurance (QA) practices for effluent toxicity tests consist of all aspects of the test that affect data quality, 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. For general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1979d, 1980b, and 1980c; and DeWoskin, 1984.

4.2 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.2.1 Sample holding times and temperatures must conform to conditions described in Section 8, Effluent and Receiving Water Sampling and Sample Handling.

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 cladoceran, Ceriodaphnia dubia, and the green alga, Selenastrum capricornutum. The organisms should be disease-free and should be positively identified to species. The fish and invertebrates should appear healthy, behave normally, feed well, and have low mortality in cultures and test controls.

4.4 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.4.1 Laboratory and bioassay 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 and Equipment).

4.5 ANALYTICAL METHODS

4.5.1 Routine chemical and physical analyses must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a,b).

4.6 CALIBRATION AND STANDARDIZATION

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

---

[1]Adapted from: Peltier (1978), Peltier and Weber (1985), and USEPA (1979a).

4.6.2 Wet chemical methods used to measure hardness and alkalinity must be standardized according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1, USEPA 1979b).

4.7 DILUTION WATER

4.7.1 The dilution water used in effluent toxicity tests will depend on the objectives of the study and logistical constraints, as discussed in Section 7. 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.

4.8 TEST CONDITIONS

4.8.1 Water temperature must be maintained within the limits specified for each test. Dissolved oxygen (DO) concentration and pH in fish and invertebrate test chambers should be checked daily throughout the test period, as prescribed in the methods.

4.9 ACCEPTABILITY OF SHORT-TERM CHRONIC TOXICITY TESTS

4.9.1 To be acceptable, control survival in fathead minnow and Ceriodaphnia tests must be at least 80%. At the end of the test, the average dry weight of seven-day-old fathead minnows in the controls must equal or exceed 0.250 mg. In the controls, the number of young per surviving adult Ceriodaphnia must be 15 or greater, and at least 60% must have had three broods. In algal toxicity tests, the mean cell density in the controls after 96 h must equal or exceed 2×10^5 cells/mL.

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 best professional judgment and experience of the analyst and regulatory authority. The deviation from test specifications must be noted when reporting data from the test.

4.10 TEST PRECISION

4.10.1 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 or more chronic tests with a reference toxicant. In cases where the test data are used to obtain point estimates, such as LCs, ECs, or ICs (see Section 2), 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. 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 2), precision can only be described by listing the NOEC-LOEC interval for each test. In this case, it is not possible to express precision in terms of a commonly used statistic.

For instance, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. However, the "true" no effect concentration could fall anywhere within the interval, NOEC $\pm$ (NOEC-LOEC).

4.10.2 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 $\pm$ 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of $\pm$ 100%. Other factors which can affect test precision include test organism age, condition, and sensitivity, and temperature control and feeding.

4.11 REPLICATION AND TEST SENSITIVITY

4.11.1 The sensitivity of the tests will depend in part on the number of replicates, the probability level selected, and the type of statistical analysis. The minimum recommended number of replicates varies with the test and the statistical method used, and is discussed in Section 2 and in each method. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased.

4.12 QUALITY OF TEST ORGANISMS

4.12.1 If the laboratory does not have an ongoing test organism culturing program and obtains the test organisms from an outside source, the sensitivity (quality) of test organisms will be assumed to be acceptable if a reference toxicant test is conducted side-by-side with the effluent toxicity test. If the laboratory maintains breeding cultures, the sensitivity of the offspring should be determined in a chronic toxicity test performed with a reference toxicant at least once each month. If preferred, this reference toxicant test may be performed concurrently with an effluent toxicity test.

4.13 FOOD QUALITY

4.13.1 The quality of the food for fish and invertebrates is an important factor in toxicity tests. Suitable trout chow, Artemia, and other foods must be obtained as described in the manual. Limited quantities of reference Artemia cysts, information on commerical sources of good quality Artemia cysts, and procedures for determining cyst suitability as food are available from the Quality Assurance Research Division, Environmental Monitoring Systems Laboratory - Cincinnati. The suitability of each new supply of food must be determined in a side-by-side test, using two treatments with four replicates per treatment. In this test, the response of control test organisms fed with the new food is compared with the response of organisms fed a reference food or a previously used, satisfactory food.

4.14 DOCUMENTING LABORATORY PERFORMANCE

4.14.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month for each of the toxicity test methods

commonly used in the laboratory, employing the same reference toxicant, at the same concentrations, in the same dilution water.

4.14.2 A control chart is prepared for each reference-toxicant-organism combination, and successive toxicity values are plotted and examined to determine if the results are within prescribed limits (Figure 2). In this technique, a running plot is maintained for the toxicity values (X_i) from successive tests with a given reference toxicant. The type of control chart illustrated (USEPA, 1979a) is used to evaluate the cumulative trend of the statistics from a series of tests. For point estimation techniques, the mean ($\bar{X}$) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive point, until the statistics stabilize. Outliers, which are values which fall outside the upper and lower control limits, and trends of increasing or decreasing sensitivity are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. For hypothesis testing results, it is assumed that the same concentrations of reference toxicants are used for each toxicity test. The NOEC from each successive test is entered on the control chart, and the values should fall within one concentration interval above or below the central tendency.

4.14.3 If the toxicity value from a given test with the reference toxicant does not fall in the expected range for the test organisms when using the standard dilution water, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

4.14.4 Four reference toxicants are available from EMSL-Cincinnati to establish the precision and validity of toxicity data generated by biomonitoring laboratories: sodium dodecylsulfate (SDS), copper sulfate ($CuSO_4$), sodium chloride ($NaCl$), and cadmium chloride ($CdCl_2$). The reference toxicants may be obtained by contacting the Quality Assurance Research Division, EMSL-Cincinnati, FTS 684-7325, commercial 513-569-7325. Instructions for the use and the toxicity values for the reference toxicants are provided with the samples. Note: To assure comparability of QA data on a national scale, all laboratories should use the same source of reference toxicant (EMSL-Cincinnati), and periodically (such as quarterly) use the same formulation of dilution water -- moderately hard dilution water described in Section 7, for fathead minnows and Ceriodaphnia, and algal growth medium described in Tables 1 and 2, Section 13, for Selenastrum.

4.15 RECORD KEEPING

4.15.1 Proper record keeping is required. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt, and other pertinent information relating to their history and health, and information on the calibration of equipment and instruments, test conditions employed, and test results. Annotations should be made on a real-time basis to prevent the loss of information.

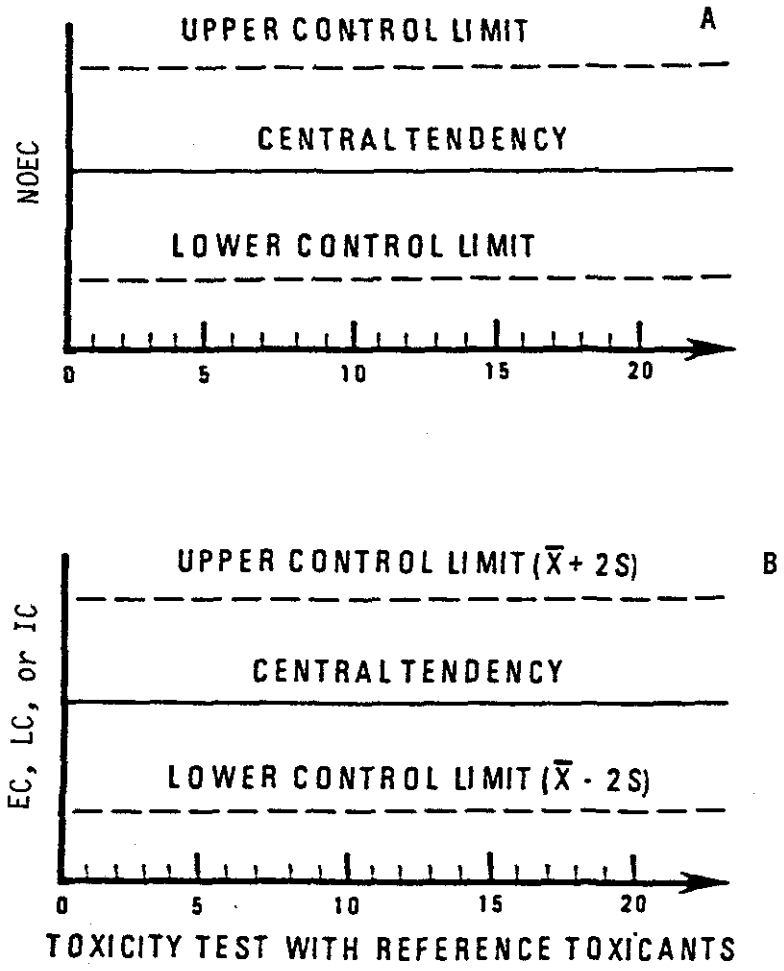

Figure 2. Control charts. (A) hypothesis testing results; (B) point estimates (EC, LC, or IC).

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

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

Where: X_i = Successive toxicity values from toxicity tests.
n = Number of tests.
$\bar{X}$ = Mean toxicity value.
S = Standard deviation.

SECTION 5

FACILITIES AND EQUIPMENT[1]

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing and holding organisms.

5.1.2 Culturing and testing areas should be separated.

5.1.3 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, surface water, dechlorinated tap water, or synthetic water. Dechlorination can be accomplished by aerating for 24 h, carbon filtration, or the use of sodium thiosulfate. Use of 1.0 mg (anhydrous) sodium thiosulfate/L will reduce 1.5 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. If air pumps are not oil-free, at a minimum the air should be filtered through cotton. Particulates can be removed from the air using BALSTON^R Grade BX or equivalent filters (Balston, Inc., Lexington, Massachusetts), and oil and other organic vapors can be removed using activated carbon filters (BALSTON^R, C-1 filter, or equivalent). The facilities must be well ventilated and free of toxic fumes. During rearing, holding, and testing, test organisms should be shielded from external disturbances.

5.1.4 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^R) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Plastics such as polyethylene, polypropylene, polyvinyl chloride, TYGON^R, etc., may be used to store and transfer effluents, but they should not be reused unless absolutely necessary, because they could carry over toxicants from one test to another if reused. The use of glass carboys is discouraged for safety reasons. Glass or disposable polystyrene containers are used for test chambers.

5.1.5 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 which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with the test media before using in the test. Copper,

[1]Adapted from: Peltier and Weber (1985).

galvanized material, rubber, brass, and lead must not come in contact with 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 test.

5.3 CLEANING

5.3.1 New plasticware used for sample collection or organism test chambers does not require cleaning. It is sufficient to rinse new sample containers once with sample before use. New, disposable, plastic test chambers normally do not have to be rinsed before use. New glassware, however, should be soaked overnight in acid (see below).

5.3.2 It is recommended that all sample containers, test vessels, tanks, and other equipment that has come in contact with effluent be washed after use in the manner described below to remove surface contaminants. Special cleaning requirements for glassware used in algal toxicity tests are described in Section 13.

1. Soak 15 min, and scrub with detergent in tap water, 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 well with deionized water.

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

SECTION 6

TEST ORGANISMS

6.1 SPECIES

6.1.1 The organisms used in the chronic tests described in this manual are the fathead minnow, Pimephales promelas, the cladoceran, Ceriodaphnia dubia (Berner, 1985), and the green alga, Selenastrum capricornutum.

6.2 SOURCE

6.2.1 The test organisms are easily cultured in the laboratory. Culturing, care, and handling procedures for Ceriodaphnia and Selenastrum are described in the respective test methods sections. A fathead minnow culturing procedure using laboratory water is described in Peltier and Weber (1985).

6.2.2 Starter cultures of Selenastrum capricornutum are available from the following sources:

1. Aquatic Biology Branch, Quality Assurance Research Division, Environmental Monitoring Systems Laboratory, USEPA, Cincinnati, Ohio 45268.

2. Environmental Research Laboratory, USEPA, 200 SW 35th Street, Corvallis, Oregon 97330.

3. American Type Culture Collection (Culture No. ATCC 22662), 12301 Parklawn Drive, Rockville, Maryland 10852.

4. Culture Collection of Algae, Botany Department, University of Texas, Austin, Texas 78712.

6.2.3 Starter cultures of fathead minnows and Ceriodaphnia can be obtained from the Aquatic Biology Branch, Quality Assurance Research Division, EMSL-Cincinnati Newtown Facility, Environmental Monitoring Systems Laboratory, USEPA, 3411 Church Street, Newtown, Ohio 45244 (Phone: FTS 684-8114; commercial 513-533-8114).

6.2.4 If there is any uncertainty concerning the identity of the test organisms, it is advisable to have them examined by a second party to confirm their identification.

6.3 SHIPMENT

6.3.1 Many states have strict regulations regarding the importation and disposal of non-native fishes. Required clearances should be obtained from state fisheries agencies before arrangements are made for the interstate shipment of fathead minnows.

6.4 DISPOSAL

6.4.1 Test organisms must be destroyed after use.

SECTION 7

DILUTION WATER

7.1 The source of dilution water used in effluent toxicity tests will depend largely on the objectives of the study:

1. If the objective of the test is to estimate the inherent chronic toxicity of the effluent, which is the primary objective of NPDES permit-related toxicity testing, a standard dilution water (moderately hard water) is used.
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 upstream and outside the influence of the the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same characteristics (pH, hardness, alkalinity, conductivity, and total suspended solids) as the receiving water. Seasonal variations in the quality of surface waters may affect effluent toxicity. Therefore, the pH, alkalinity, hardness, and conductivity of receiving water samples should be determined before each use.
3. If the objective of the test is to determine the additive effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected upstream from the outfall.

7.2 When the dilution water is to be taken from the receiving water "upstream" from the outfall, it should be collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent. 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, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3 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.

7.4 Dechlorinated water may be used as a source of dilution water if properly treated. Dechlorination can be accomplished by aerating for 24 h, carbon filtration, or the use of sodium thiosulfate. Use of 1.0 mg (anhydrous) sodium thiosulfate/L will reduce 1.5 mg chlorine/L.

7.5 Deionized water may be obtained from a MILLIPORE MILLI-Q^R System, or equivalent. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan, Continental, or equivalent system in front of the MILLI-Q^R System to extend the life of the MILLI-Q^R cartridges. The recommended order of the cartridges in a four-cartridge MILLI-Q^R System is: (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (ORGANEX-Q^R), followed by a final bacteria filter.

7.6 Synthetic, moderately hard dilution water can be prepared using reagent grade chemicals (Table 1) or mineral water (Table 2).

7.6.1 To prepare 20 L of synthetic, moderately hard water, use the reagent grade chemicals in Table 1 as follows.

1. Place 19 L of MILLI-Q^R, 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 $CaSO_4 \cdot 2H_2O$ to 1 L of MILLI-Q^R or equivalent water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved and add to the 19 L above and mix well.
5. Aerate vigorously for 24 h to dissolve the added chemicals and stabilize the medium.
6. The measured pH, hardness, etc., will be as listed in Table 1.

7.6.2 To prepare 20 L of synthetic, moderately hard water using mineral water (Table 2), follow the instructions below. Note: These instructions are specific for PERRIER^R Water. The properties of other commercially available mineral waters are not well enough known at this time to permit inclusion of recommendations for their use.

1. Place 16 L of MILLI-Q^R or equivalent water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER^R Water.
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 2.
5. The synthetic water prepared with PERRIER^R Water is referred to as 20% diluted mineral water (20% DMW) in the toxicity test methods.

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

TABLE 1. PREPARATION OF SYNTHETIC FRESH WATER USING REAGENT GRADE CHEMICALS[a]

Water Type	Reagent Added (mg/L)[b]				Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[c]	Hardness[d]	Alkalinity[d]
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	60-70
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

[a]Taken in part from Marking and Dawson (1973).
[b]Add reagent grade chemicals to deionized water.
[c]Approximate equilibrium pH after 24 h of aeration.
[d]Expressed as mg $CaCO_3$/L .

TABLE 2. PREPARATION OF SYNTHETIC FRESH WATER USING MINERAL WATER[a]

Water Type	Volume of Mineral Water Added (mL/L)[b],	Proportion of Mineral Water (%)	Final Water Quality		
			pH[c]	Hardness[d]	Alkalinity[d]
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	60-70
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[e]	---	---	---	---	---

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

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point usually should be the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge to the receiving waters, 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 prohibitive 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.

8.1.3 Sample Type

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

8.1.3.2. Grab Samples

8.1.3.2.1 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.

8.1.3.2.2 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.

8.1.3.3. Composite Samples:

8.1.3.3.1. 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 and contains all toxicity spikes.

8.1.3.3.2. 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.

8.1.4 SAMPLING RECOMMENDATIONS

8.1.4.1. When tests are conducted on-site, samples are collected daily, except for the algal tests.

8.1.4.2 When tests are conducted off-site, a minimum of three samples are collected. It is recommended that these samples not be collected on a more frequent schedule than one every other day. This collection schedule would provide fresh sample on Test Days 1, 3, and 5. The first sample would be used for test initiation, Day 1, 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.

8.1.4.3 The following effluent sampling methods are recommended:

8.1.4.3.1. Continuous Discharges

1. If the facility discharge is continuous, but the calculated retention time of a continuously discharged effluent is less than 14 days and the variability of the waste is unknown, composite samples are used.

2. If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary in chemical composition or toxicity regardless of holding time, grab samples are used.

3. The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short-circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

8.1.4.3.2. Intermittent Discharges

8.1.4.3.2.1. If the facility discharge is intermittent, composite samples are collected during the discharge period. Examples of intermittent discharges are:

1. When the effluent is continuously discharged during a single 8-h work shift or two successive 8-h work shifts.

2. When the facility retains the wastewater during an 8-h work shift, and then treats and releases the wastewater as a batch discharge.

3. When the facility discharges wastewater to an estuary only during an outgoing tide (usually during the 4 h following slack high tide).

4. At the end of the shift, clean up activities may result in the discharge of a slug of toxic waste.

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

8.2 RECEIVING WATER SAMPLING

8.2.1 It is common practice to collect grab samples for receiving water toxicity studies.

8.2.2 When non-toxic receiving water is required for a test, it may be possible to obtain it upstream from the outfall or from another surface water which is known to be uncontaminated and has properties similar to the receiving water (see Section 7). If the objective of the test is to determine the additive effects of the discharge on receiving water which may already be contaminated, the test is performed using dilution water consisting of receiving water collected daily upstream from the outfall.

8.2.3 Dilution water to be taken from the receiving water "upstream" from the outfall is collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent.

8.2.4 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.

8.3 SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.3.1 If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time from collection of a grab or composite sample and its first use for initiation of a test, or for test solution renewal, should not exceed 36 h. Composite samples should be chilled during collection, where possible, and maintained at 4°C until used.

8.3.2 Samples Used in On-Site Tests

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

8.3.3 Samples Shipped to Off-Site Facilities

8.3.3.1 Samples collected for off-site toxicity testing are to be chilled to 4°C when collected, shipped iced to the central laboratory, and there transferred to a refrigerator (4°C) until used. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory.

8.3.3.2 Samples may be shipped in 4-L (1-gal) CUBITAINERS^{R} or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use, CUBITAINERS^{R} and plastic jugs are punctured to prevent reuse.

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

8.4 SAMPLE PREPARATION

8.4.1 With the Ceriodaphnia and fathead minnow tests, effluents and surface waters must be filtered through a 60-um plankton net to remove indigenous organims that may attack or be confused with the test organisms (see Ceriodaphnia test method for details). Surface waters used in algal toxicity tests must be filtered through a 0.45-um 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 holes to remove debris and/or break up large floating or suspended solids. Caution: filtration may remove toxicity.

8.4.2 The DO concentration in the dilution water should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH.

8.4.3 If the dilution water and effluent must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved gases may become a problem. To prevent this problem, the effluent and dilution water are checked for dissolved oxygen (DO) with a probe after heating to 25°C. If the DO is greater than 100% saturation or lower than 40% saturation, the solutions are aerated moderately with a pipet tip for a few minutes until the DO is within the prescribed range.

SECTION 9

REPORT PREPARATION[1]

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

9.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contractor (if contracted)
   a. Name of firm
   b. Phone number
   c. Address

9.2 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

9.3 SOURCE OF EFFLUENT (AMBIENT) AND DILUTION WATER

1. Effluent Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data

2. Surface Water Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (at 7Q10 and at time of sampling)

[1]Adapted from: Peltier and Weber (1985).

3. Dilution Water Samples
   a. Source
   b. Collection date(s) and time(s)
   c. Pretreatment
   d. Physical and chemical characteristics

9.4 TEST METHODS

1. Toxicity test method used
2. End point(s) of test
3. Deviations 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 (mean and range)
11. Test temperature (mean and range)

9.5 TEST ORGANISMS

1. Scientific name
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)

9.6 QUALITY ASSURANCE

1. Standard toxicant used and source
2. Date and time of most recent test
3. Dilution water used in test
4. Results (LC50 or, where applicable, NOEC and/or EC1)
5. Physical and chemical methods used

9.7 RESULTS

1. Provide raw biological data in tabular form, including daily records of affected organisms in each concentration (including controls)
2. Provide table of LC50s, NOECs, etc.
3. Indicate statistical methods to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data

SECTION 10

TEST METHOD

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

1. SCOPE AND APPLICATION

1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, Pimephales promelas, 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.

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).

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

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, the concentrations of highly degradable or highly volatile toxicants, such as chlorine, present in the source may fall below detectable levels before the samples are used in a test.

1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) an abbreviated test, consisting of only one concentration such as 100% effluent or the in-stream waste concentration and a control. Abbreviated tests are used for toxicity screening or a pass/fail permit condition. Failure of the screening test usually results in a followup definitive test.

1.6 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 Larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and growth (increase in weight) of the larvae.

3. INTERFERENCES

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

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

3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

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

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.

4. SAFETY

4.1 See Section 3, Health and Safety.

5. APPARATUS AND EQUIPMENT

5.1 Fathead minnow and brine shrimp culture units -- see Peltier and Weber (1985). This test requires 180-360 larvae. It is preferable to obtain larvae from an inhouse fathead minnow culture unit. If it is not feasible to culture fish inhouse, embryos or newly hatched larvae can be shipped in well oxygenated water in insulated containers.

5.2 Samplers -- automatic sampler, preferrably with sample cooling capability, that can collect a 24-h composite sample of 2 L or more.

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

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

5.5 Water purification system -- MILLIPORE MILLI-Q^{R} or equivalent.

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

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.

5.8 Test chambers -- four (minimum of three) borosilicate glass or non-toxic disposable plastic test chambers are required for each concentration and control. Test chambers may be 1L, 500 mL, or 250 mL beakers, 220 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, 1/4 in thick).

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

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

5.12 Pipet bulbs and fillers -- Propipet^R, or equivalent.

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

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

5.15 Glass or electronic thermometers -- for measuring water temperatures.

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

5.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1, USEPA 1979b).

5.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6. REAGENTS AND CONSUMABLE MATERIALS

6.1 Reagent water -- defined as MILLIPORE MILLI-Q^R or equivalent water (see paragraph 5.5 above).

6.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling.

6.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1, USEPA 1979b).

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

6.5 Specific conductivity standards (see USEPA Method 120.1, USEPA 1979b).

6.6 Laboratory quality assurance samples and standards for the above methods.

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

6.8 Ethanol (70%) for use as a preservative for the fish larvae.

6.9 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

6.10 Brine Shrimp (Artemia) Cysts -- see Peltier and Weber (1985).

6.10.1 Although there are many commercial sources of brine shrimp eggs, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues. (A source of brine shrimp eggs that has been found to be satisfactory is Aquarium Products, 180 L Penrod Ct., Glen Burnie, MD, 21061). Each new batch of Artemia cysts should be evaluated for nutritional suitability against known suitable reference cysts by performing a larval growth test. It is recommended that a sample of newly-hatched Artemia nauplii from each new batch of cysts be chemically analyzed to determine that the concentration of total organic chlorine does not exceed 0.15 ug/g wet weight or the total concentration of organochlorine pesticides plus PCBs does not exceed 0.3 ug/g wet weight. If those values are exceeded, the Artemia should not be used.

6.10.2 Limited quantities of reference Artemia cysts, information on commerical sources of good quality Artemia cysts, and procedures for determining cyst suitability are available from the Quality Assurance Research Division, Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268.

7. TEST ORGANISMS

7.1 Fathead minnow larvae are used for the test (for fathead minnow culturing methods, see Peltier and Weber, 1985).

8. SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. TEST PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples passed through a 60 um NITEX^{R} 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, depending on the test volumes selected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (+ 300%). A dilution factor of 0.5 provides greater precision (+ 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used, beginning at 10%. If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 Based on a 0.3 dilution factor, the volume of effluent required for daily renewal of four replicates per concentration, each containing 250 mL of test solution, would be approximately 1500 mL for a screening test with 100% effluent and a control, and 2.5 L for a definitive test with five concentrations of effluent and a control. Sufficient test solution (approximately 400 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.

11.2 START OF THE TEST

11.2.1 On-site tests should be initiated within 24 h of sample collection, and off-site tests should be initiated within 36 h of sample collection. Just prior to testing, the temperature of the sample should be adjusted to (25 + 1°C) and maintained at that temperature until portions are added to the dilution water.

11.2.2 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 should not be more than 48 h old at the start of the test and should all be within 24-h of the same age.

11.2.3 Randomize the position of test chambers at the beginning of the test.

11.2.4 The larvae are pooled and placed one to four at a time into each test chamber in sequential order, until each chamber contains 15 (minimum of 10) larvae, for a total of 60 larvae (minimum of 30) for each concentration. 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 to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 uE/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 $\pm$ 1°C.

11.4 DISSOLVED OXYGEN (DO)

11.4.1 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 not fall below 40% saturation. 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, No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.5 FEEDING

11.5.1 The fish in each test chamber are fed 0.1 mL (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 mL are fed twice daily at an interval of 6 h.

11.5.2 The feeding schedule will depend on when the test solutions are renewed. If the test is initiated after 1200 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.

11.5.3 The nauplii should be rinsed with freshwater before use. The amount of food provided in each feeding should be sufficient to ensure the presence of a small amount of uneaten food at the next feeding.

11.6 DAILY CLEANING OF TEST CHAMBERS

11.6.1 At the time of the daily renewal of test solutions, uneaten and dead brine shrimp 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. A note of this should be made in the log.

11.7 TEST SOLUTION RENEWAL

11.7.1 For on-site tests, test solutions are renewed daily with freshly collected samples. For off-site tests, test solutions are also renewed daily, using the most recently collected sample. A minimum of three samples are collected, preferrably for use beginning on Days 1, 3, 5. The first sample is used for test initiation on Day 1 and test solution renewal on Day 2. The second sample is used for test solution renewal on Days 3 and 4, and the third sample is used for test solution renewals on Days 5, 6, and 7. Samples first used on Days 1, 3, and 5, are held over in the refrigerator for use on the following day(s).

11.7.2 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. For private carriers, shipping and receiving schedules on weekends vary with the carrier.

11.7.3 The test solutions are renewed immediately after cleaning the test chambers. 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 subjecting the larvae to excessive turbulence.

11.8 ROUTINE CHEMICAL AND PHYSICAL ANALYSIS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO and pH are measured at the beginning and end of each 24-h exposure period in one test chamber at the high, medium, and low test concentrations, and in the control.

11.8.1.2 Temperature should be monitored continously or observed and recorded daily for at least two locations in the environmental control system or the samples.

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

11.8.1.4 Record the data (as shown in Figure 1).

11.9 OBSERVATIONS DURING THE TEST

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

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

11.10 TERMINATION OF THE TEST

11.10.1 The test is terminated after seven days of exposure. At test termination, the surviving larvae in each test chamber (replicate) are counted and prepared as a group for dry weight determination, or are preserved in 70% ethanol for later analysis. Immediately prior to the dry weight analysis, each group of larvae is rinsed with distilled water to remove food particles, transferred to a tared weighing boat, and dried at 100°C for a minimum of 2 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 (see Figure 3). If the larvae are preserved, they must be dried and weighed within two weeks.

11.10.2 Prepare a summary table as illustrated in Figure 4.

11.11 ACCEPTABILITY OF TEST RESULTS

11.11.1 For the test results to be acceptable, survival in the controls must be at least 80%. In tests initiated with larvae less than 24-h old, the average dry weight of control larvae surviving at the end of the test should equal or exceed 0.25 mg.

11.12 SUMMARY OF TEST CONDITIONS

11.12.1 A summary of test conditions is listed in Table 1.

12. DATA ANALYSIS

12.1 GENERAL

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

12.1.2 The endpoints of toxicity tests using the fathead minnow larvae are based on the adverse effects on survival and growth. Point estimates, such as LCs and ICs, are calculated using point estimation techniques (see Section 2). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). See the Appendices for examples of the manual computations and data input and program output for the computer programs.

12.1.3 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 the Appendices. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TABLE 1. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 uE/m^2/s (50-100 ft-c)(ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	500 mL
7. Test solution volume:	250 mL/replicate
8. Renewal of test concentrations:	Daily
9. Age of test organisms:	Newly hatched larvae less than 24 h old.
10. No. larvae per test chamber:	15 (minimum of 10)
11. No. replicate chambers per concentration:	4 (minimum of 3)
12. No. larvae per concentration:	60 (minimum of 30)
13. Feeding regime:	Feed 0.1 mL newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 mL twice daily, 6 h between feedings (at the begining of the work day prior to renewal, and at the end of the work day following renewal). Sufficient larvae are added to provide an excess. Larvae are not fed during the final 12 h of the test
14. Cleaning:	Siphon daily, immediately before test solution renewal
15. Aeration:	None, unless DO concentration falls below 40% saturation. Rate should not exceed 100 bubbles/min

TABLE 1. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST (CONTINUED)

16. Dilution water:	Moderately hard synthetic water is prepared using MILLIPORE MILLI-Q^{R} or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7)
17. Effluent concentrations:	Minimum of 5 and a control
18. Dilution factor:[1]	Approximately 0.3 or 0.5
19. Test duration:	7 days
20. Endpoints:	Survival and growth (weight)
21. Test acceptability	80% or greater survival in controls; Average dry weight of surviving controls equals or exceeds 0.25 mg
22. Sampling requirement:	For on-site tests, samples are 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 are collected, and used as described in Paragraph 11.7.1
23. Sample volume required:	2.5 L/day

[1]Surface water test samples are used as collected (undiluted).

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

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

	Day							
Control:	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 larval survival and growth test. Routine chemical and physical determinations. (Continued).

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 2. Survival data for fathead minnow larval survival and growth test.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

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

Comments:

Figure 3. Weight data for fathead minnow larval survival and growth test.[1]

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

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

[1]Adapted from Hughes, et al., 1987.

Figure 4. Summary data for fathead minnow larval survival and growth test.[1]

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:

[1]Adapted from Hughes et al., 1987.

12.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW SURVIVAL DATA

12.2.1 Formal statistical analysis of the survival data is outlined in Figure 5. 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 LC1, LC5, LC10 and LC50 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 LC endpoints.

12.3.2 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 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 determine the homogeneity of variance. If either of these tests fail, 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.

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

NaPCP Conc. (ug/L)	Proportion of Survival in Replicate Chambers				Mean Prop.	Ave Dry Wgt (mg) In Replicate Chambers				Mean Dry Wgt
	A	B	C	D	Surv	A	B	C	D	(mg)
0	1.0	1.0	0.9	0.9	0.95	0.711	0.662	0.718	0.767	0.714
32	0.8	0.8	1.0	0.8	0.85	0.646	0.626	0.723	0.700	0.674
64	0.9	1.0	1.0	1.0	0.975	0.669	0.669	0.694	0.676	0.677
128	0.9	0.9	0.8	1.0	0.90	0.629	0.680	0.513	0.672	0.624
256	0.7	0.9	1.0	0.5	0.775	0.650	0.558	0.606	0.508	0.580
512	0.4	0.3	0.4	0.2	0.325	0.358	0.543	0.488	0.495	0.471

[1]Four replicates of 10 larvae each.

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

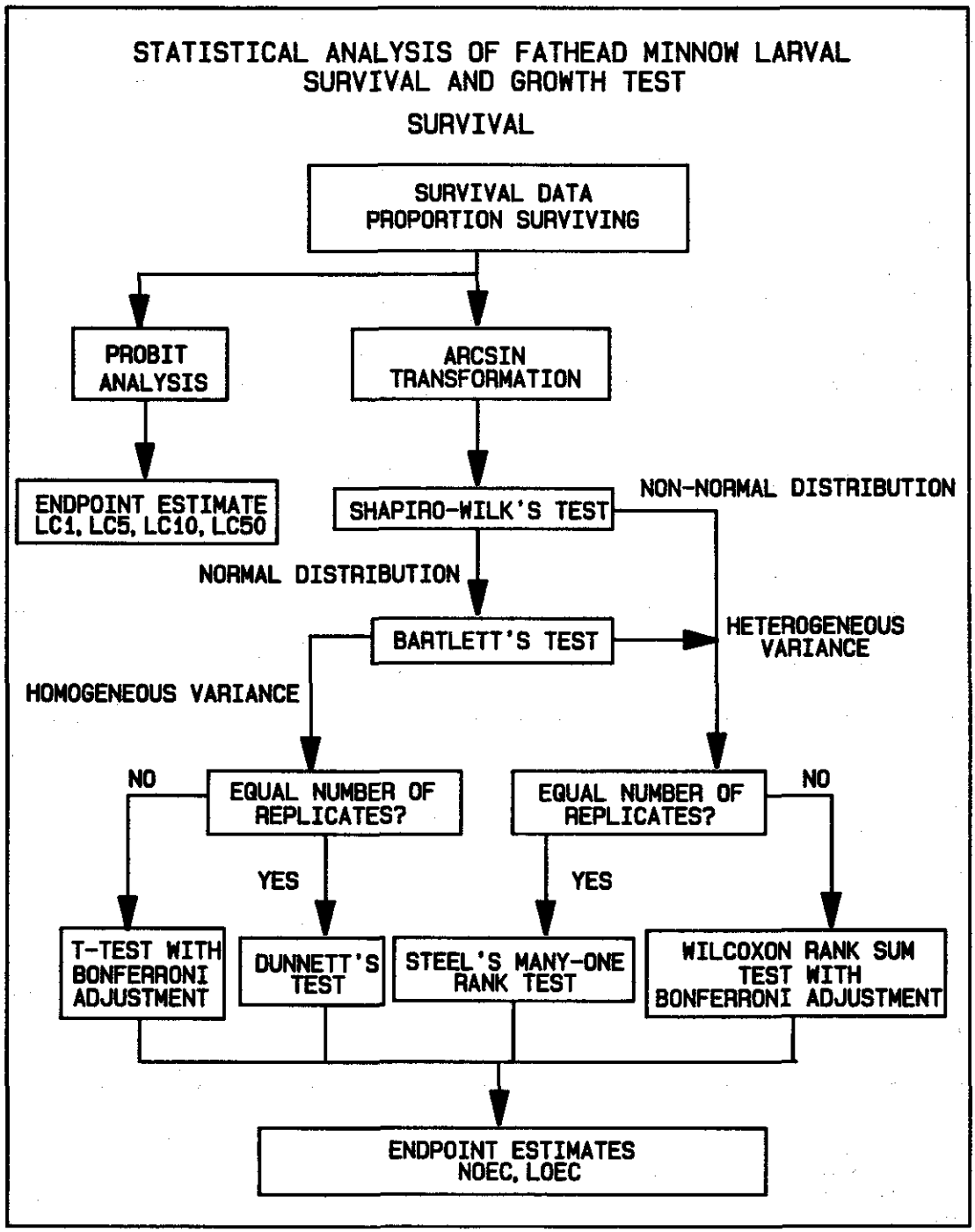

Figure 5. Flow chart for statistical analysis of fathead minnow larval survival data.

12.3.4 *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. If the data do not fit the Probit model, use the graphical or other appropriate method.

12.3.5 Example of Analysis of Survival Data

12.3.5.1 This example uses the survival data from the Fathead Minnow Larval Survival and Growth Test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and standard deviations 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 6.

TABLE 3. FATHEAD MINNOW SURVIVAL DATA

	Replicate	Control	NaPCP Concentration (ug/L) 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($\overline{Y}_i$)		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

12.2.6 Test for Normality

12.2.6.1 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.

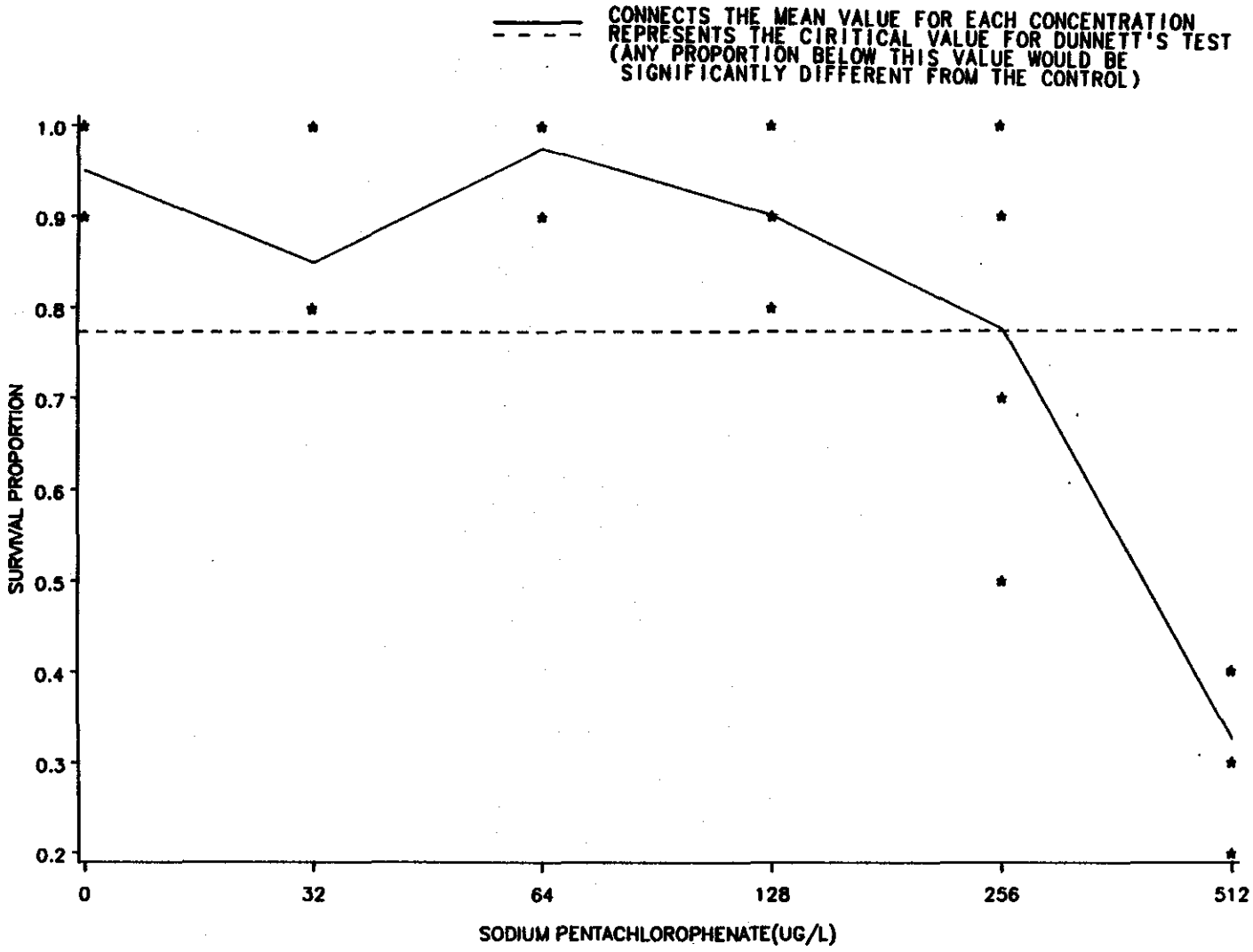

Figure 6. Plot of mean survival proportion data in Table 3.

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

Replicate	Control	NaPCP Concentration (ug/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

12.2.6.2 Calculate the denominator, D, of the statistic:

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

Where X_i = the ith centered observation
$\overline{X}$ = the overall mean of the centered observations
n = the total number of centered observations

12.2.6.3 For this set of data: $n = 24$

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

$$D = 0.4265$$

12.2.6.4 Order the centered observations from smallest to largest

$$X(1) - X(2) - \ldots - X(n)$$

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

12.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$ where k is approximately n/2. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 6.

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

12.2.6.6 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,

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

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	0.0	$x_{(13)} - x_{(12)}$

12.2.6.7 The decision rule for this test is to compare W as calculated in 12.2.6.6 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.

12.2.7 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i.

ln = $\log_e$

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

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

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

12.2.7.2 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.

12.2.7.3 Bartlett's statistic is therefore:

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

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

$$= 6.8178/1.1296$$

$$= 6.036$$

12.2.7.4 B is approximately distributed as chi square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 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.

12.2.8 Dunnett's Procedure

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

TABLE 7. ANOVA TABLE

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

Where: p = number toxicant concentrations including the control
N = total number of observations $n_1 + n_2 \ldots + n_p$
n_i = number of observations in concentration i

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

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

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

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

T_i = the total of the replicate measurements for concentration "i"

Y_{ij} = the jth observation for concentration "i" (represents the proportion surviving for toxicant concentration i in test chamber j)

12.2.8.2 For the data in this example:

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

$N = 24$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$

$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$

$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$

$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$

$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

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

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

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

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

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

$SSW = SST - SSB = 2.000 - 1.574 = 0.426$

$S_B^2 = SSB/p\text{-}1 = 1.574/6\text{-}1 = 0.315$

$S_W^2 = SSW/N\text{-}p = 0.426/24\text{-}6 = 0.024$

12.2.8.3 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	

12.2.8.4 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 within mean sqaure
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

12.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 ug/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(ug/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

12.2.8.6 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 ug/L concentration has significantly lower survival than the control. Hence the NOEC and the LOEC for survival are 256 ug/L and 512 ug/L, respectively.

12.2.8.7 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 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_i = the number of replicates in the control.

12.2.8.8 In this example:

$$MSD = 2.41\ (0.155) \sqrt{(1/4) + (1/4)}$$
$$= 2.41\ (0.155)(0.707)$$
$$= 0.264$$

12.2.8.9 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.

$$MSD_u = 0.943 - 0.766 = 0.177$$

12.2.8.10 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.

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

12.2.9 Probit Analysis

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

12.2.9.2 For this example, since there is 100% survival in the controls, there is no need to adjust for control mortality. The test for heterogeneity was not significant, thus Probit Analysis appears appropriate for this data.

TABLE 10. DATA FOR PROBIT ANALYSIS

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

TABLE 11. OUTPUT FROM EPA PROBIT ANALYSIS PROGRAM, VERSION 1.4, USED FOR CALCULATING EC VALUES

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING EC VALUES
Version 1.4

Probit Analysis of Fathead Minnow Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Adjusted Proportion Responding	Predicted Proportion Responding
Control	40	2	0.0500	0.0000	0.0782
32.0000	40	6	0.1500	0.0779	0.0000
64.0000	40	1	0.0250	-.0577	0.0001
128.0000	40	4	0.1000	0.0237	0.0101
256.0000	40	9	0.2250	0.1593	0.1650
512.0000	40	27	0.6750	0.6474	0.6452

Chi - Square Heterogeneity = 4.522

Mu = 2.626029
Sigma = 0.223555

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-6.746692	3.112017	(-12.846246, -0.647139)
Slope	4.473178	1.196026	(2.128967, 6.817389)
Spontaneous Response Rate	0.078182	0.022541	(0.034002, 0.122363)

Estimated EC Values and Confidence Limits

Point	Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
EC 1.00	127.6359	34.5885	195.4335
EC 5.00	181.2605	71.4914	248.7074
EC10.00	218.5347	104.8182	284.0806
EC15.00	247.9341	135.2143	311.8864
EC50.00	422.6964	345.7290	531.0254
EC85.00	720.6437	562.5553	1420.7512
EC90.00	817.5905	616.4054	1836.3506
EC95.00	985.7196	702.9269	2696.8005
EC99.00	1399.8575	893.3054	5581.7588

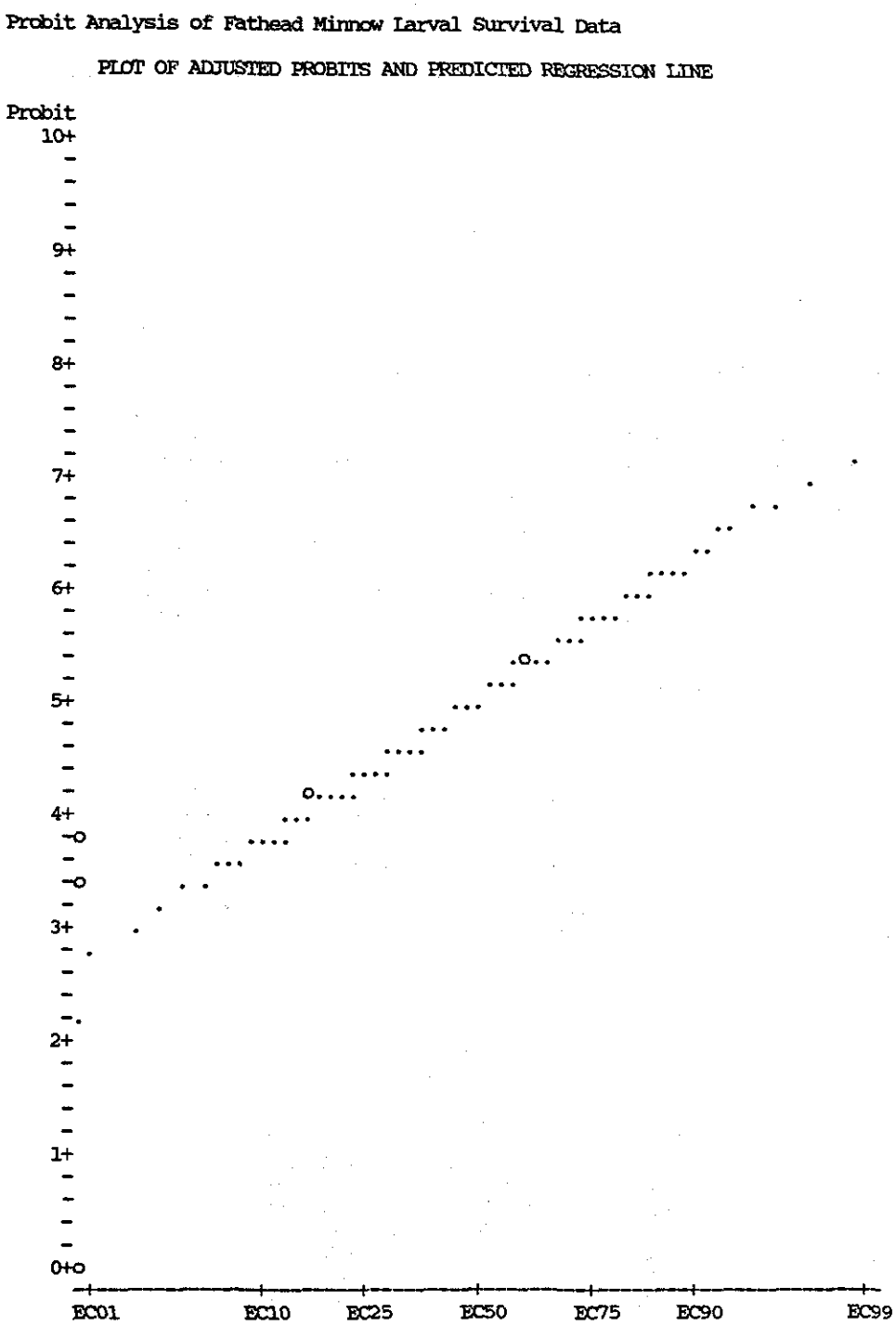

Figure 7. Plot of adjusted probits and predicted regression line from EPA Probit Program.

12.3 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW GROWTH DATA

12.3.1 Formal statistical analysis of the growth data is outlined in Figure 8. The response used in the statistical analysis is mean weight per replicate. An IC estimate can be calculated for the growth data via a point estimation technique (see Section 2). Hypothesis testing can be used to obtain a NOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

12.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a non-parametric 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 fail, the non-parametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

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

12.3.5 The data, mean and standard deviation of the observations at each concentration including the control are listed in Table 12. A plot of the mean weights for each treatment is provided in Figure 9. Since there is significant mortality in the 512 ug/L concentration, its effect on growth is not considered.

TABLE 12. FATHEAD MINNOW GROWTH DATA

		NaPCP Concentration (ug/L)				
Replicate	Control	32	64	128	256	512
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($\overline{Y}_i$)	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	6

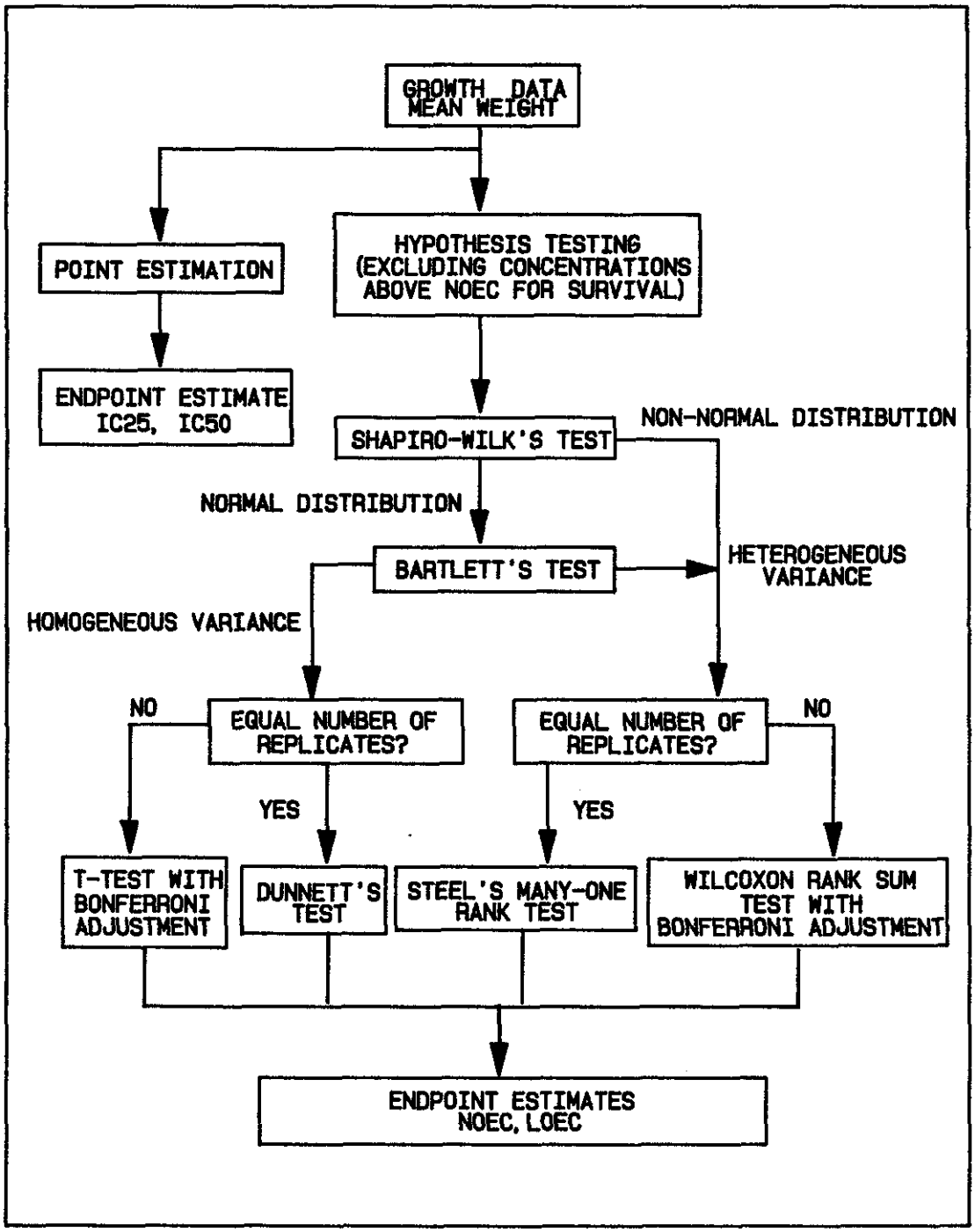

Figure 8. Flow chart for statistical analysis of fathead minnow larval growth data.

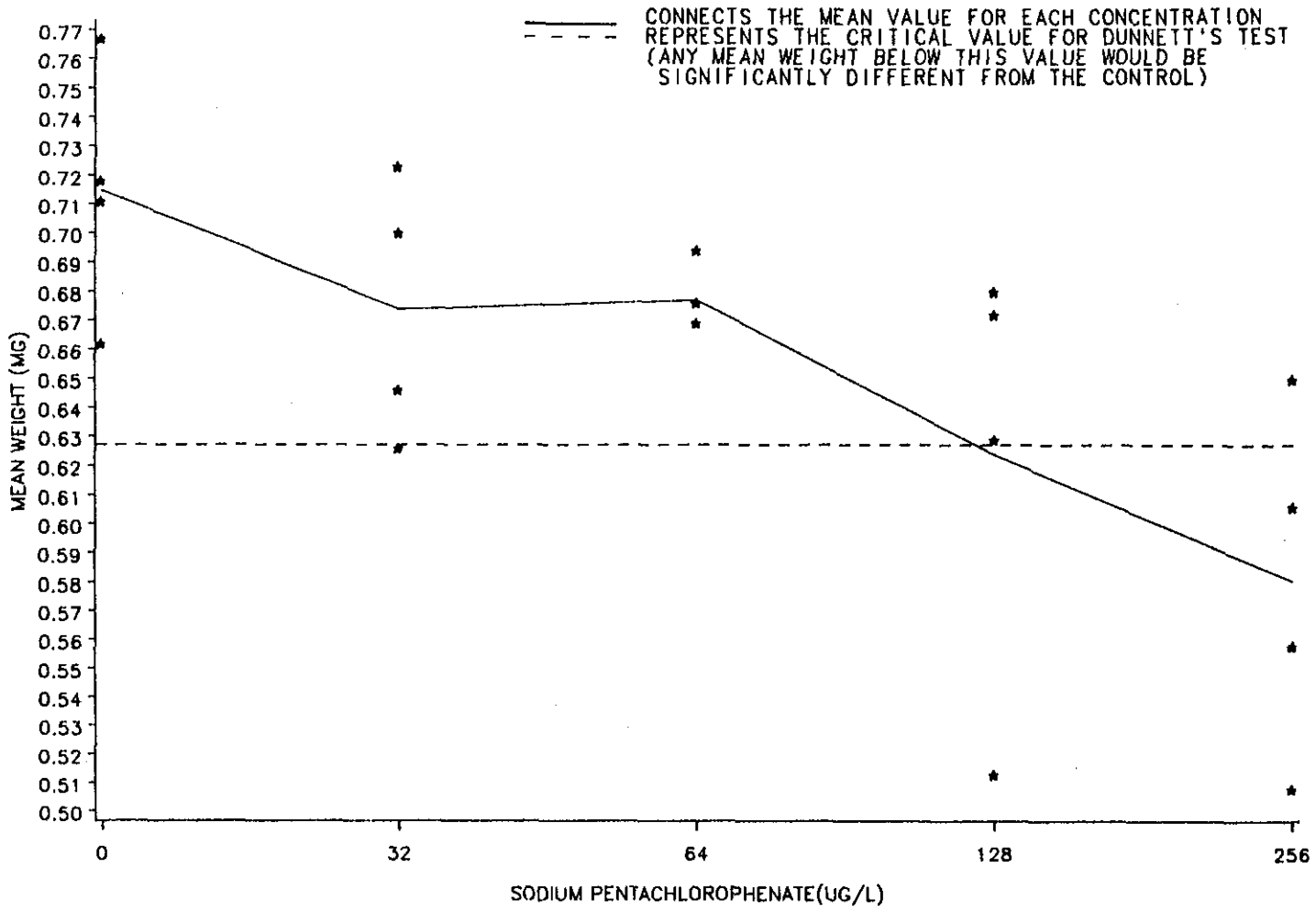

Figure 9. Plot of mean weight data from fathead minnow larval survival and growth test.

12.3.6 Test for Normality

12.3.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 13.

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

		NaPCP Concentration (ug/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

12.3.6.2 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, $n = 20$

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

$$D = 0.0412$$

12.3.6.3 Order the centered observations from smallest to largest

$$X(1) - X(2) - \ldots - X(n)$$

Where $X(i)$ is the ith ordered observation. These ordered observations are listed in Table 14.

12.3.6.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is approximately n/2. For the data in this example, $n = 20$, $k = 10$. The a_i values are listed in Table 15.

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

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

12.3.6.5 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 15.

For this set of data:

$$W = \frac{1}{0.0412} (0.1988)^2 = 0.959$$

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

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	$x(11) - x(10)$

12.3.6.6 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 = 0.959 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

12.3.7 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i.

ln = $\log_e$

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

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

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

12.3.7.2 For the data in this example, (See Table 12) 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.

12.3.7.3 Bartlett's statistic is therefore:

$$B = [(15)\ln(0.0027) - 3\sum_{i=1}^{p} \ln(S_i^2)]/1.133$$

$$= [15(-5.9145) - 3(-32.4771]/1.133$$

$$= 8.7138/1.133$$

$$= 7.691$$

12.3.7.4 B is approximately distributed as chi square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.277. Since B = 7.691 is less than the critical value of 13.277, conclude that the variances are not different.

12.3.8 Dunnett's Procedure

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

TABLE 16. ANOVA TABLE

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

Where: p = number toxicant concentrations including the control
N = total number of observations $n_1 + n_2 \ldots + n_p$
n_i = number of observations in concentration i

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

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

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

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

T_i = the total of the replicate measurements for concentration "i"

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

12.3.8.2 For the data in this example:

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

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.858$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.695$

$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.708$

$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.494$

$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 2.322$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 13.077$

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

$$= \frac{1}{4}(34.376) - \frac{(13.077)2}{20} = 0.044$$

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

$$= 8.635 - \frac{(13.077)2}{20} = 0.085$$

$SSW = SST - SSB = 0.085 - 0.044 = 0.041$

$S_B^2 = SSB/p\text{-}1 = 0.044/5\text{-}1 = 0.011$

$S_w^2 = SSW/N\text{-}p = 0.041/20\text{-}5 = 0.0027$

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

TABLE 17. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	0.044	0.011
Within	15	0.041	0.0027
Total	19	0.085	

12.3.8.4 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 dry weight for toxicant concentration i
$\bar{Y}_1$ = mean dry weight for the control
S_W = square root of within mean sqaure
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

12.3.8.5 Table 18 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 ug/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.714 - 0.674)}{[0.052 \sqrt{(1/4) + (1/4)}]} = 1.081$$

TABLE 18. CALCULATED T VALUES

NaPCP Concentration (ug/L)	i	t_i
32	2	1.081
64	3	1.000
128	4	2.432
256	5	3.622

12.3.8.6 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_4 and t_5 are greater than 2.36, the 128 ug/L and 256 ug/L concentrations have significantly lower growth than the control. Hence the NOEC and the LOEC for growth are 64 ug/L and 128 ug/L, respectively.

12.3.8.7 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.

12.3.8.8 In this example:

$$MSD = 2.36\ (0.052)\ \sqrt{(1/4) + (1/4)}$$
$$= 2.36\ (0.052)(0.707)$$
$$= 0.087$$

12.3.8.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.087 mg.

12.3.8.10 This represents a 12% reduction in mean weight from the control.

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 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.

13.1.2 An interlaboratory study of Method 1000.0 described in the first edition of this manual (Horning and Weber, 1985), 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 now included in this revised edition of the method, had been applied to the results of the interlaboratory study, 40 of the 135 completed tests would have been considered unacceptable (Norberg-King, 1988).

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests can not be determined.

TABLE 19. PRECISION OF THE FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[a,b]

Test	NOEC (ug/L)	LOEC (ug/L)	Chronic Value (ug/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181

[a]From Pickering, 1988.

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

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

Sample	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
	Median	± 1[b]	>2[c]	Median	± 1[b]	>2[c]
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

[a]From DeGraeve et. al., 1988.
[b]Percent of values within one concentration intervals of the median.
[c]Percent of values within two or more concentrations intervals of the median.

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

Sample	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
	Median	± 1[b]	>2[c]	Median	± 1[b]	>2[c]
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

[a]From DeGraeve et. al., 1988.
[b]Percent of values within one concentration intervals of the median.
[c]Percent of values within two or more concentrations intervals of the median.

SECTION 11

TEST METHOD

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

1. SCOPE AND APPLICATION

1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, Pimephales promelas, using embryos and larvae in an 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.

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

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 degradeable and highly volatile toxicants, such as chlorine, in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) an abbreviated test, consisting of only one test concentration, such as 100% effluent or the instream waste concentration, and a control. Abbreviated tests are used for toxicity screening or a pass/fail permit condition. Failure of the screening test usually results in a followup definitive test.

2. SUMMARY OF METHOD

2.1 Fathead minnow embryos and larvae are exposed to different concentrations of effluent or to receiving water in a static renewal system 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).

3. INTERFERENCES

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

7. Test Organisms

7.1 Fathead minnow embryos, less than 36-h old, are used for the test (for fathead minnow culturing methods (see Peltier and Weber, 1985).

7.2 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 um NITEX^{R} 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. It is recommended that when possible, the embryos be obtained from local sources. Receipt of embryos via Express Mail, air express, or other carrier, from a reliable outside source, is an acceptable alternative.

7.3 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^{o}C. Sudden changes in pH, dissolved ions, osmotic strength, and DO should be avoided.

7.4 The test is conducted with four (minimum of three) test chambers at each toxicant concentration and control. Fifteen (minimum of 10) embryos are placed in each replicate test chamber. Thus, 60 (minumum of 30) embryos are exposed per test concentration.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. TEST PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected. Four (minimum of three) replicate test chambers are used for each surface water sample.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (+ 300%). A dilution factor of 0.5 provides greater precision (+ 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5.

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%).

11.1.2.3 Based on a 0.3 dilution factor, the volume of effluent required for daily renewal of four replicates per concentration, each containing 200 mL of test solution, is 1200 mL for a screening test with 100% effluent and a control, and 1800 mL for a definitive test with five effluent concentrations and a control. Sufficient test solution (approximately 1200 mL) at each effluent concentration is prepared 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.

11.1.2.4 The hardness of the test solutions must exceed 25 mg/L ($CaCO_3$) to insure hatching success. If the hardness of the effluent is less than 25 mg $CaCO_3$/L, adjust the hardness by adding reagents for synthetic softwater listed in Table 1, Section 7.

11.2 START OF THE TEST

11.2.1 On-site tests should be initiated within 24 h of sample collection, and off-site tests should be initiated within 36 h of sample collection. Just prior to testing, the temperature of the sample should be adjusted to (25 + 1°C) and maintained at that temperature until portions are added to the dilution water.

11.2.2 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.

11.2.3 Add 10-15 embryos to each test chamber using a small bore (2mm) glass tube calibrated to contain approximately the desired number of embryos. Repeat the process until the required number of embryos have been added to each chamber.

11.2.4 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.

11.2.5 Randomize the position of the test chambers at the beginning of the test.

11.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 uE/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 test solution temperature should be maintained at 25 $\pm$ 1°C.

11.4 DISSOLVED OXYGEN (DO)

11.4.1 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 not fall below 40% saturation. If it is necessary to aerate, all concentrations and the control should be aerated. The rate should not exceed 100 bubbles/min, using a pipet with a 1-2 mm orifice, such as a 1-mL Kimax Serological Pipet No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the fish.

11.5 FEEDING

11.5.1 Feeding is not required.

11.6 DAILY CLEANING OF TEST CHAMBERS

11.6.1 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.

11.7 TEST SOLUTION RENEWAL

11.7.1 For on-site tests, test solutions are renewed daily with freshly collected samples. For off-site tests, test solutions are also renewed daily, using the most recently collected sample. A minimum of three samples are collected, preferrably for use beginning on Days 1, 3, 5. The first sample is used for test initiation on Day 1 and test solution renewal on Day 2. The second sample is used for test solution renewal on Days 3 and 4, and the third sample is used for test solution renewals on Days 5, 6, and 7. Samples first used on Days 1, 3, and 5, are held over in the refrigerator for use on the following day(s).

11.7.2 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. For private carriers, shipping and receiving schedules on weekends vary with the carrier.

11.7.3 The test solutions are renewed immediately after removing dead embryos and/or larvae. During the daily renewal process, a small amount of water is left in the chamber to ensure that the embryos and larvae remain submerged during the renewal process. New test solution should be added slowly to avoid subjecting the embryos and larvae to excessive turbulence.

11.8 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO and pH are measured at the beginning and end of each 24-h exposure period in one test chamber at the high, medium, and low test concentrations, and in the control.

11.8.1.2 Temperature should be monitored continously or observed and recorded daily for at least two locations in the environmental control system or the samples.

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

11.8.1.4 Record the data (as shown in Figure 1).

11.9 OBSERVATIONS DURING THE TEST

11.9.1 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.

11.9.2 At 25°C, hatching begins on about 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.

11.9.3 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 the test organisms remain immersed during the performance of the above operations.

11.10 TERMINATION OF THE TEST

11.10.1 The test is terminated after seven days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each. 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.

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

11.11 ACCEPTABILITY OF TEST RESULTS

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

11.12 SUMMARY OF TEST CONDITIONS

11.12.1 A summary of test conditions is listed in Table 1.

12. DATA ANALYSIS

12.1 GENERAL

12.1.1 Tabulate and summarize the data.

12.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, and dead and deformed larvae. Point estimates, such as LC1, LC5, LC10 and LC50, are calculated using Probit Analysis (Finney, 1971). LOEC and NOEC values, for total mortality, are obtained using a hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). See the Appendices for examples of the manual computations and examples of data input and output for the computer programs.

12.1.3 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.

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

12.2.1 Formal statistical analysis of the total mortality data is outlined 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 LC1, LC5, LC10 and LC50 endpoints. 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 LC endpoints.

TABLE 1. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE FATHEAD MINNOW (PIMEPHALES PROMELAS) EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST

1. Test type:	Static renewal
2. Temperature:	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 uE/m^2/s or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test chamber size:	150-500 mL
7. Test solution volume:	70-200 mL
8. Renewal of test concentration:	Daily
9. Age of test organisms:	Less than 36-h old embryos
10. No. embryos per test chamber:	15 (minimum of 10)
11. No. Replicate test chambers per concentration:	4 (minimum of 3)
12. No. Embryos per concentration:	60 (minimum of 30)
13. Feeding regime:	Feeding not required
14. Aeration:	None unless DO falls below 40% saturation
15. Dilution water:	Moderately hard synthetic water is prepared using MILLIPORE MILLI-Q^R or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7). The hardness of the test solutions must equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching.
16. Effluent test concentrations:	5 and a control
17. Dilution factor:[1]	Approximately 0.3 or 0.5

[1]Surface water test samples are used as collected (undiluted).

TABLE 1. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR FATHEAD MINNOW (PIMEPHALES PROMELAS) EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST (CONTINUED)

18. Test duration:	7 days
19. Endpoint:	Combined mortality (dead and deformed organisms)
20. Test acceptability	80% or greater survival in controls
21. Sampling requirement:	For on-site tests, samples are 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 are collected and used as described in Paragraph 11.7.1.
22. Sample volume required:	2.5 L/day

Figure 1. Data form for the fathead minnow embryo/larval survival and teratogenicity test. Routine chemical and physical determinations.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

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

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

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

Figure 1. Data form for the fathead minnow embryo/larval survival and teratogenicity test. Routine chemical and physical determinations. (Continued)

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

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

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

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

Figure 2. Data form for the fathead minnow embryo/larval survival and teratogenicity test. Survival and terata data.

Discharger: ____________________ Test Dates: ____________________

Location: ____________________ Analyst: ____________________

	Rep	Condition of Embryos/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							
Treat:	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treat:	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treat:	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Figure 2. Data form for the fathead minnow embryo/larval survival and teratogenicity test. Survival and terata data. (Continued)

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

	Rep	Embryos-Larvae	Day 1	2	3	4	5	6	7
Treat:	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treat:	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Figure 3. Data form for the fathead minnow embryo/larval survival and teratogencity test. Summary data.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

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

Comments:

12.2.2 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 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 determine the homogeneity of variance. If either of these tests fail, 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.

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

12.2.4 Probit Analysis (Finney, 1971) is used to estimate the concentration that causes a specified percent increase in total mortality from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined.

12.2.5 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 transformation procedure described in Appendix B. The raw and transformed data, means and standard deviations 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 both replicates for the 16.0% concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

12.2.6 Test for Normality

12.2.6.1 Since only two replicates were run at each concentration level, the test for normality is invalid. Additionally, a non-parametric alternative to Dunnett's Procedure is not available with only duplicates. Thus, the only information that can be derived from the data is from Dunnett's Procedure. However, the results from this test should be interpreted with caution since the assumptions of the test are in question.

12.2.7 Dunnett's Procedure

12.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 4.

TABLE 2. DATA FROM FATHEAD MINNOW EMBRYO-LARVAL TOXICITY TEST WITH TRICKLING FILTER WASTE

A. REPLICATES A AND B (USED IN DUNNETT'S PROCEDURE)

Repl.	Effl. Conc. (%)	No. Eggs at Start	Dead at Hatching No.	(%)	Dead + Deform. at hatching No.	(%)	Dead at Test Termination No.	(%)	Dead + Deform. at termination No.	(%)
A	Cont.	51	5	10	6	12	6	12	7	14
	3	50	5	10	5	10	5	10	5	10
	5	52	5	10	6	12	5	10	6	12
	7	50	2	4	8	16	9	18	15	30
	11	50	10	20	25	50	17	34	32	64
	16	49	39	80	39	80	49	100	49	100
B	Cont.	49	9	18	9	18	10	20	10	20
	3	50	6	12	6	12	9	18	9	18
	5	50	10	20	10	20	10	20	10	20
	7	50	6	12	10	20	16	32	20	40
	11	49	30	61	37	76	33	66	40	82
	16	50	29	58	34	68	45	90	50	100

B. COMBINED DATA FROM REPLICATES A AND B (USED IN PROBIT ANALYSIS)

Repl.	Effl. Conc. (%)	No. Eggs at Start	Dead at Hatching No.	(%)	Dead + Deform. at hatching No.	(%)	Dead at Test Termination No.	(%)	Dead + Deform. at termination No.	(%)
A&B	Cont.	100	14	14	15	15	16	16	17	17
	3	100	11	11	11	11	14	14	14	14
	5	102	15	15	16	16	15	15	16	16
	7	100	8	8	18	18	25	25	35	35
	11	99	40	40	62	62	50	50	72	73
	16	99	68	69	73	74	94	95	99	100

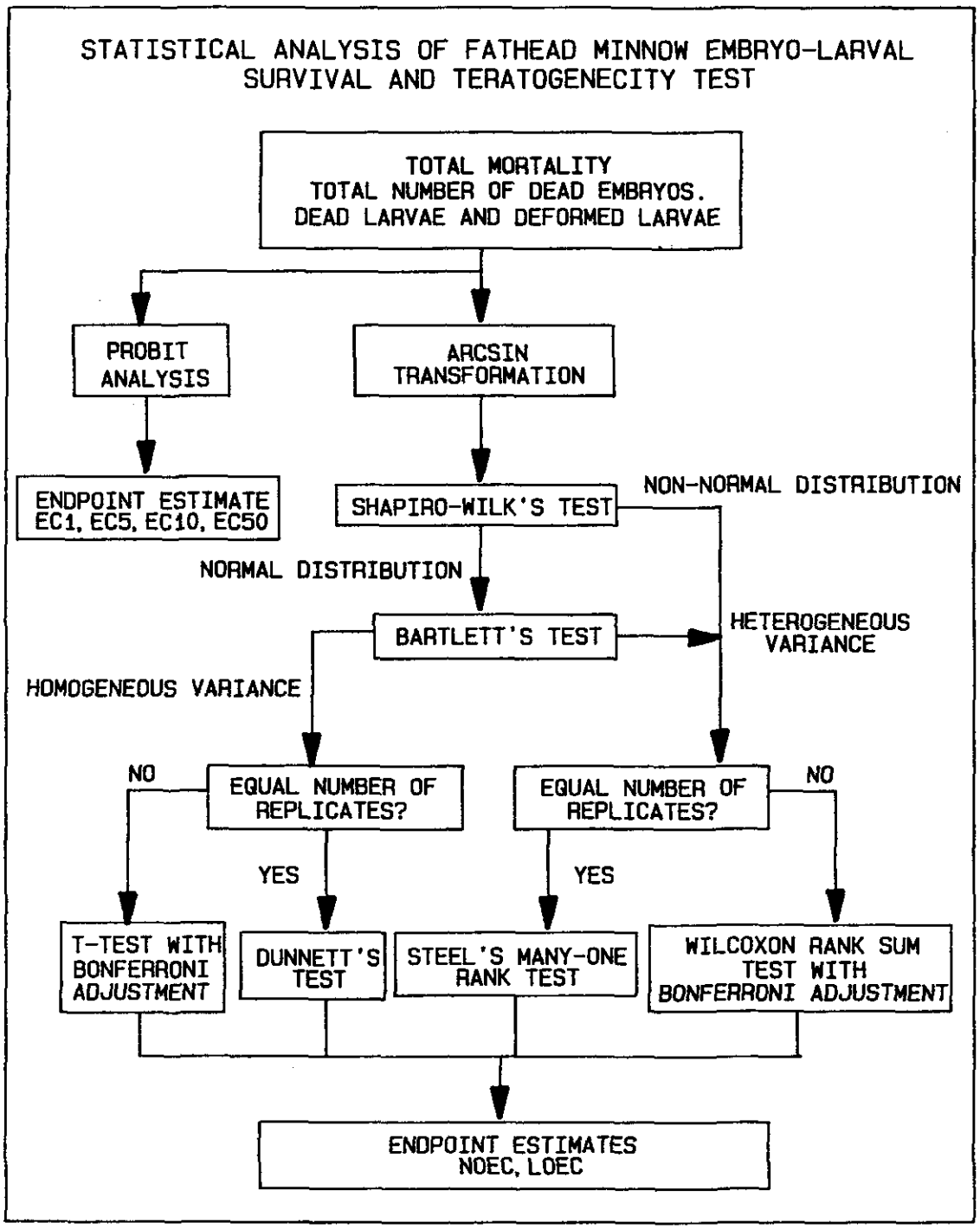

Figure 4. Flow chart for statistical analysis of fathead minnow embryo-larval data.

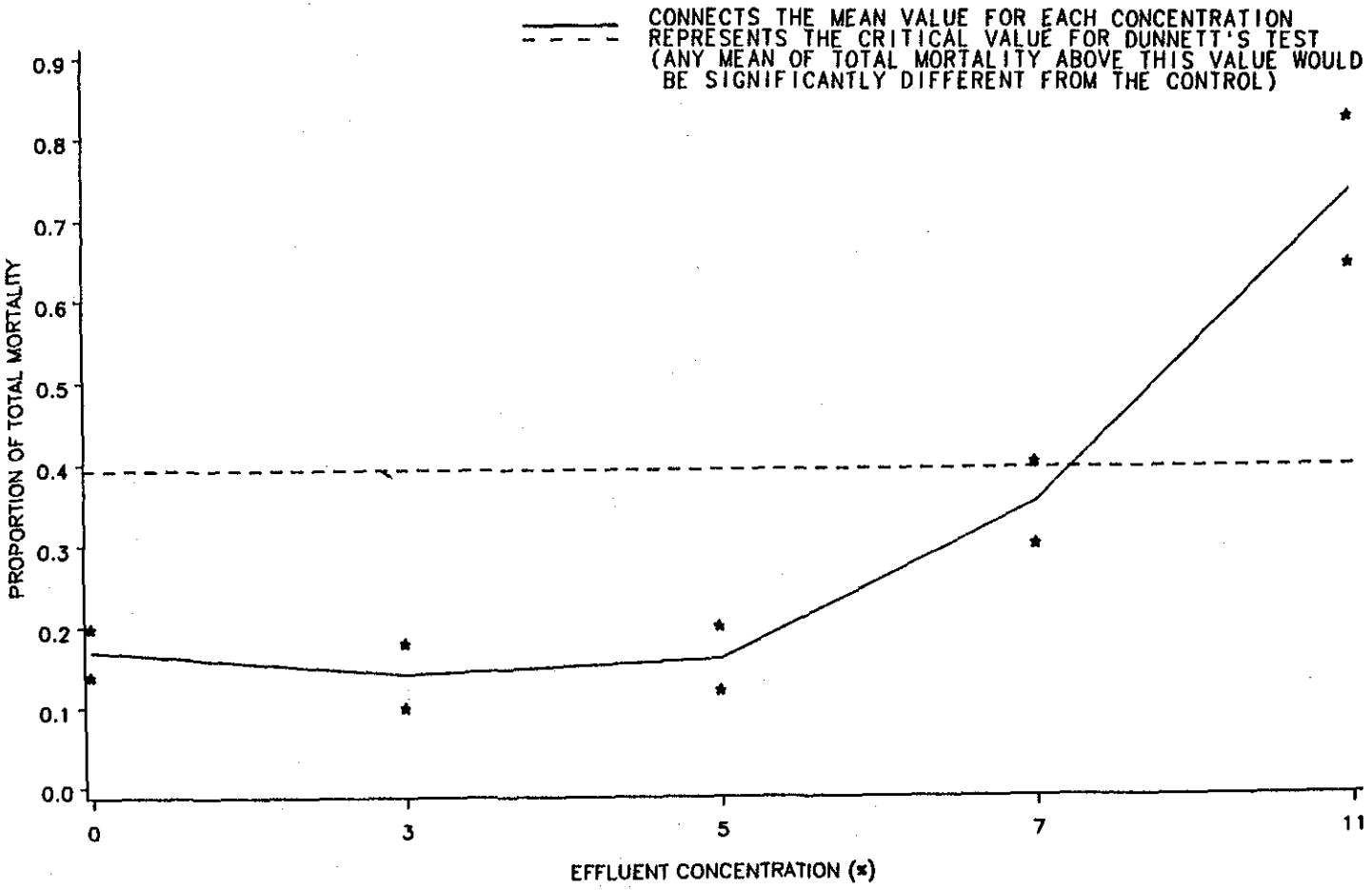

Figure 5. Plot of fathead minnow total mortality data from the embryo-larval test.

TABLE 3. FATHEAD MINNOW EMBRYO-LARVAL TOTAL MORTALITY DATA

	Replicate	Control	Effluent Concentration (%) 3.0	5.0	7.0	11.0	16.0
RAW	A	0.14	0.10	0.12	0.30	0.64	1.0
	B	0.20	0.18	0.20	0.40	0.82	1.0
ARC SINE TRANS-FORMED	A	0.384	0.322	0.354	0.580	0.927	-
	B	0.464	0.438	0.464	0.685	1.133	-
MEAN($\bar{Y}_i$)		0.424	0.380	0.409	0.632	1.030	
S_i^2		0.003	0.007	0.006	0.006	0.021	
i		1	2	3	4	5	

TABLE 4. 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 effluent concentration levels including the control

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

n_i = number of observations in concentration i

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

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

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

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

T_i = the total of the replicate measurements for concentration "i"

Y_{ij} = the jth observation for concentration "i" (represents the proportion of total mortality for effluent concentration i in test chamber j)

12.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 2$

$N = 10$

$T_1 = Y_{11} + Y_{12} = 0.848$

$T_2 = Y_{21} + Y_{22} = 0.760$

$T_3 = Y_{31} + Y_{32} = 0.818$

$T_4 = Y_{41} + Y_{42} = 1.265$

$T_5 = Y_{51} + Y_{52} = 2.060$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 5.751$

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

$$= \frac{1}{2}(7.810) - \frac{(5.751)^2}{10} = 0.598$$

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

$$= 3.948 - \frac{(5.751)^2}{10} = 0.640$$

$SSW = SST - SSB = 0.640 - 0.598 = 0.042$

$S_B^2 = SSB/p-1 = 0.598/5-1 = 0.1495$

$S_W^2 = SSW/N-p = 0.042/10-5 = 0.008$

12.2.7.3 Summarize these calculations in an ANOVA table (Table 5).

TABLE 5. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	0.598	0.1495
Within	5	0.042	0.008
Total	9	0.640	

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

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

Where $\overline{Y}i$ = mean proportion of total mortality for concentration i
$\overline{Y}_1$ = mean proportion of total mortality for the control
S_W = square root of within mean sqaure
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

Since we are looking for an increased response in percent of total mortality over control, the control mean is subtracted from the mean at a concentration.

12.2.7.5 Table 6 includes the calculated t values for each concentration and control combination. In this example, comparing the 3.0% concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.380 - 0.424)}{[0.089 \sqrt{(1/2) + (1/2)}]} = - 0.494$$

TABLE 6. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
3.0	2	-0.494
5.0	3	-0.168
7.0	4	2.337
11.0	5	6.809

12.2.7.6 Since the purpose of this test is to detect a significant increase in total mortality, 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, five degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.85. The mean proportion of total mortality for concentration "i" is considered significantly less than the mean proportion of total mortality for the control if t_i is greater than the critical value. Therefore, only the 11.0% concentration has a significantly higher mean proportion of total mortality than the control. Hence the NOEC is 7.0% and the LOEC is 11.0%.

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

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

Where: d = the critical value for 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.

12.2.7.8 In this example:

$$MSD = 2.85\ (0.089) \sqrt{(1/2) + (1/2)}$$
$$= 2.85\ (0.089)(1.0)$$
$$= 0.254$$

12.2.7.9 The MSD (0.254) is in transformed units. To determine the MSD in terms of percent total mortality, carry out the following conversion.

1. Add the MSD to the transformed control mean.

$$0.424 + 0.254 = 0.678$$

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

$$[\text{Sine}\ (\ 0.424)\ ]^2 = 0.169$$
$$[\text{Sine}\ (\ 0.678)\ ]^2 = 0.393$$

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

$$MSD_u = 0.393 - 0.169 = 0.224$$

12.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of total mortality between the control and any effluent concentration that can be detected as statistically significant is 0.224.

12.2.8 Probit Analysis

12.2.8.1 The data used for the Probit Analysis is summarized in Table 7. For the Probit Analysis, the effluent concentration with 100% mortality in both replicates is considered. To perform the Probit Analysis, run the EPA Probit Analysis Program provided in Appendix I. Examples of the program output are illustrated in Table 8 and Figure 6.

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

TABLE 7. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	3.0	5.0	7.0	11.0	16.0
Number Dead	17	14	16	35	72	99
Number Exposed	100	100	102	100	99	99

TABLE 8. OUTPUT FROM EPA PROBIT ANALYSIS PROGRAM, VERSION 1.4, USED FOR CALCULATING EC VALUES.

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Adjusted Proportion Responding	Predicted Proportion Responding
Control	100	17	0.1700	0.0000	0.1560
3.0000	100	14	0.1400	-.0190	0.0000
5.0000	102	16	0.1569	0.0010	0.0174
7.0000	100	35	0.3500	0.2298	0.1765
11.0000	99	72	0.7273	0.6769	0.7449
16.0000	99	99	1.0000	1.0000	0.9759

Chi - Square Heterogeneity = 5.286

Mu = 0.959956
Sigma = 0.123640

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-2.764127	1.002530	(-4.729086, -0.799168)
Slope	8.088003	0.990954	(6.145732, 10.030273)
Spontaneous Response Rate	0.156014	0.022593	(0.111732, 0.200296)

Estimated EC Values and Confidence Limits

Point	Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
EC 1.00	4.7025	3.6073	5.5567
EC 5.00	5.7093	4.6408	6.5196
EC10.00	6.3314	5.3031	7.1058
EC15.00	6.7892	5.7994	7.5354
EC50.00	9.1192	8.3614	9.7763
EC85.00	12.2489	11.4157	13.3942
EC90.00	13.1345	12.1697	14.5708
EC95.00	14.5657	13.3302	16.5676
EC99.00	17.6840	15.7134	21.2145

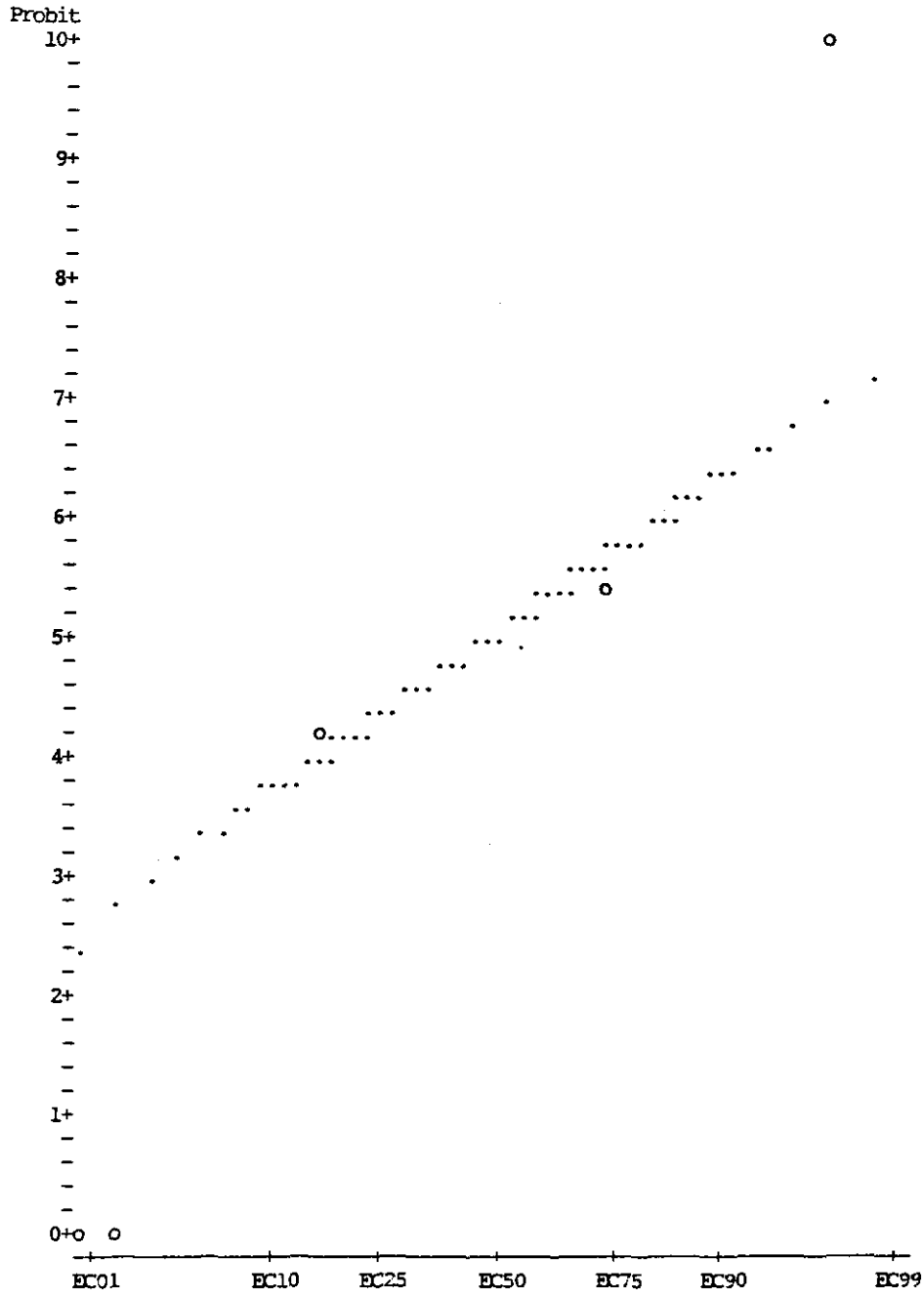

Figure 6. Plot of adjusted probits and predicted regression line.

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Data shown in Tables 9 and 10 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 9), and 41% for Diquat (Table 10).

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

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 9. PRECISION OF THE FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[a,b]

Test	LC1[c] (mg/L)	95% Confidence Limits	NOEC[d] (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		
Mean	0.0068		
SD	0.0042		
CV(%)	62		

[a]Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Aquatic Biology Branch, Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio (Cornelius I. Weber, Project Officer).

[b]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.

[c]Determined by Probit Analysis.

[d]Highest no-observed-effect concentration determined by independent statistical analysis (2x2 Chi-square Fisher's Exact Test).

TABLE 10. PRECISION OF THE FATHEAD MINNOW, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[a,b]

Test	LC1[c] (mg/L)	95% Confidence Limits
1	0.58	0.32 - 0.86
2	2.31	d
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	

[a]Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Aquatic Biology Branch, Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio (Cornelius I. Weber, Project Officer).

[b]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.

[c]Determined by Probit analysis.

[d]Not calculatable.

TABLE 11. PRECISION OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[a,b,c]

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

[a]Data provided by Timothy Neiheisel, Aquatic Biology Branch, Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio.

[b]Effluent concentrations used: 3, 5, 7, 11 and 16%.

[c]Maximum precision achieved in terms of NOEC-LOEC interval. For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

SECTION 12

TEST METHOD

CLADOCERAN, CERIODAPHNIA DUBIA, SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

1. SCOPE AND APPLICATION

1.1 This method measures the chronic toxicity of whole effluents and receiving water to the cladoceran, Ceriodaphnia dubia, during a three-brood (seven-day), static renewal exposure. 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.

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).

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

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, such as chlorine, in the source may not be detected in the test.

1.5 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

1.6 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) an abbreviated test, consisting of only one concentration such as 100% effluent or the instream waste concentration and a control. Abbreviated tests are used for toxicity screening or a pass/fail permit condition. Failure of the screening test is usually followed by a definitive test.

2. SUMMARY OF METHOD

2.1 Ceriodaphnia are exposed in a static renewal system to different concentrations of effluent, or to receiving water until 60% of surviving control organisms have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the control organisms should produce three broods of young during a seven-day period.

3. INTERFERENCES

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

3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

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

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

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.

4. SAFETY

4.1 See Section 3, Health and Safety.

5. APPARATUS AND EQUIPMENT

5.1 Ceriodaphnia and algal culture units -- See culturing methods below.

5.2 Samplers -- automatic sampler, preferrably with sample cooling capability, capable of collecting a 24-h composite sample of 2 L.

5.3 Sample containers -- for sample shipment and storage (See Section 8, Effluent and Receiving Water Sampling and Sample Handling).

5.4 Environmental chambers, incubators, or equivalent facilities with temperature control (25$\pm$ 1°C; Fisher # 11-679-66 or equivalent).

5.5 Water purification system -- MILLIPORE MILLI-Q^R system or equivalent.

5.6 Balance -- Analytical, capable of accurately weighing 0.0001 g.

5.7 Reference weights, Class S -- for checking performance of balance.

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 (salad dressing) cups are recommended because they will fit in the viewing field of most stereoscopic microscopes. Glass beakers are rinsed thoroughly with dilution water before use. Plastic cups do not require rinsing.

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

5.10 Light meter -- with a range of 0-200 uE/m^2/s (0-1000 ft-c).

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

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

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

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

5.15 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope).

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

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

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

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

5.20 Racks (boards) for test chambers -- Racks 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 in this Section).

5.21 Dissecting microscope with substage lighting -- for examining Ceriodaphnia in the test chambers.

5.22 Light box -- for illuminating organisms during examination.

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.

5.24 Pipettors, adjustable volume repeating dispensers -- Pipettors such as the Gilson REPETMAN^R, 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.

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

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

5.27 Pipet bulbs and fillers -- Propipet^R, or equivalent.

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

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

5.30 Glass or electronic thermometers -- for measuring water temperatures.

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

5.32 National Bureau of Standards Certified thermometer -- see EPA Method 170.1, USEPA 1979b.

5.33 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6. REAGENTS AND CONSUMABLE MATERIALS

6.1 Reagent water -- defined as MILLIPORE MILLI-Q^R water, or equivalent; carbon-filtered, deionized water which does not contain substances which are toxic to the test organisms (see paragraph 5.5 above).

6.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling. Dilution water that contains undesirable organisms, that may attack the test organisms should be filtered through a fine mesh net (30-um or smaller openings).

6.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

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

6.5 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

6.6 Laboratory quality assurance samples and standards for the above methods.

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

6.8 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7. TEST ORGANISMS

7.1 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.

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

7.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 40 animals and 3 mL of food (see below) are placed in a 1-L bottle filled full with culture water. 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.

7.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope 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 (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-9/9AF Medium[1], prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.
5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a 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 CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1978, and Berner, 1985).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

[1]CMCP-9 and 9AF are available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, Pennsylvania, 18976 (215-343-6484).

7.5 MASS CULTURE

7.5.1 Mass cultures are used only as a "backup" reservoir of organisms. Neonates from mass cultures are not to be used directly in toxicity tests (see Paragraph 12.2.3 below).

7.5.2 One-liter or 2L 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.

7.5.4 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal of the medium and brood organisms. Each culture is started by adding 40-50 neonates per liter of medium. The stocked organisms should be transferred to new culture medium at least twice a week for two weeks. At each nenewal, 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 each week.

7.5.3 Reserve cultures also may be maintained in large (80-L) aquaria or other large tanks.

7.6 INDIVIDUAL CULTURE

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

7.6.2 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 in this Section).

7.6.3 Organisms are fed daily 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.

7.6.4 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.

7.6.5 Cultures which are properly maintained should produce at least 15 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.

7.6.6 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 15 young per adult on a board during a one-week period would indicate problems, such as poor quality of culture media or food. Organisms on that board should not be used as a source of test organisms.

7.7 CULTURE MEDIUM

7.7.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q^{R} or equivalent deionized water and reagent grade chemicals or 20% DWM is recommended as a standard culture medium (see Section 7, Dilution Water).

7.8 CULTURE CONDITIONS

7.8.1 Ceriodaphnia should be cultured at a temperature of 25 ± 1°C.

7.8.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

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

7.8.4 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.

8. FOOD PREPARATION AND FEEDING

8.1 Feeding the proper amount of the right food is extremely important in Ceriodaphnia 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^{R}, and Trout chow (YCT), along with the unicellular green alga, Selenastrum capricornutum, will provide suitable nutrition if fed daily.

8.2 The YCT and algae are prepared as follows:

8.2.1 Digested trout chow:

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications. Suppliers of trout chow include Zeigler Bros., Inc., P. O. Box 95, Gardners, Pennsylvania, 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, Minnesota, 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, Utah 84107 (800-521-9092).

2. Add 5.0 g of trout chow pellets to 1 L of MILLI-Q^R 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^R 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL^R and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

8.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S^R to 1 L of MILLI-Q^R 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^R preparations (below). Discard excess material.

8.2.3 CEROPHYLL^R (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. (Available as "CEREAL LEAVES," from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, (800-325-3010); or as CEROPHYLL^R, from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, New York, 14692-9012, (716-359-2502). Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q^R 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.

8.2.4 Combined YCT Food:

1. 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.

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.

8.2.5 Algal (Selenastrum) Food

8.2.5.1 Algal Culture Medium

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^{R} water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the 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.
3. Immediately filter the pH-adjusted medium through a 0.45um 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.

8.2.5.2 Algal Cultures

8.2.5.2.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of Selenastrum capricornutum.

8.2.5.2.2 Two types of algal cultures are maintained: (1) stock cultures, and (2) "food" cultures.

8.2.5.2.2.1 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 toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the Ceriodaphnia 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 uE/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 X 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) every four to six months.

8.2.5.2.2.2 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for Ceriodaphnia cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 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 the excess 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 uE/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.

8.2.5.2.3 Preparing Algal Concentrate for Use as Ceriodaphnia 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 approximately two-to-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 13), and used to determine the

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES.

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL MILLI-QR Water
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
2	$NaNO_3$	12.75 g
3	$MgSO_4 \cdot 7H_2O$	7.35 g
4	K_2HPO_4	0.522 g
5	$NaHCO_3$	7.50 g

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

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

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

[d] $CuCl_2 \cdot 2H_2O$ - 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 #1.

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 (ug/L)	Element	Concentration (ug/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	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	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----

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 tests.
4. Algal concentrate may be stored in the refrigerator for one month.

8.3 FEEDING

8.3.1 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 number of males, and ephippial females. Also, their offspring may produce few young when used in toxicity tests.

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

8.4 FOOD QUALITY

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

9. SAMPLE COLLECTION, PRESERVATION AND HANDLING

9.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

10. CALIBRATION AND STANDARDIZATION

10.1 See Section 4, Quality Assurance.

11. QUALITY CONTROL

11.1 See Section 4, Quality Assurance.

12. TEST PROCEDURES

12.1 PREPARATION OF TEST SOLUTIONS

Figure 1. Examples of a board with pos chambers, (2) die for each p test board pl the position the board.

12.1.1 Surface Waters

12.1.1.1 Surface water t
collected. Approximately
assuming 10 replicates of
analysis.

12.1.2 Effluents

12.1.2.1 The selection o
the objectives of the stu
or 0.5, is commonly used.
testing between 100% and
(100%, 30%, 10%, 3%, and
effort, but because of th
poor test precision (+ 30
precision (+ 100%), but r
range of effluent concent
as the dilution factor is

12.1.2.2 If the effluent
range of effluent concent
and 0.1%). If a high rat
of the test, additional d
concentrations can be add

12.1.2.3 A volume of 15 r
and will provide a depth
stereomicroscope with a m
for each effluent dilutio
required for daily renewa
15 mL of test solution, w
Prepare enough test solut
concentration to provide

12.2 START OF THE TEST

12.2.1 On-site tests sho
and off-site tests should
prior to testing, the tem
1°C and maintained at tha
dilution water.

12.2.2 The test solution
treatments and a control,
(Figure 1 of this Section
randomized block design,
beginning of the test. A
that the same template is

12.2.3 Neonates less tha
required to begin the tes

cultures using brood boards, as described above. Neonates are taken only from adults 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).

12.2.4 Ten brood cups, each with 8 or more young, are selected from a brood board for use in setting up a test. To start the test, one neonate from the first brood cup is transferred to each of the six test chambers in the first row on the test board (Figure 1, 3). A second brood cup is selected, and one neonate from this cup is 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.

12.2.5 This blocking procedure allows the performance of each female to be tracked. If a female produces one weak offspring or male, the likelihood of producing all weak offspring or all males is greater. By using this known parentage technique, poor performance of young from a given female can be omitted from all concentrations.

12.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

12.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 uE/m^2/s, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. It is critical that the test water temperature be maintained at 25 $\pm$ 1°C to obtain three broods in seven days.

12.4 DISSOLVED OXYGEN (DO)

12.4.1 Low DO concentrations may be important when running effluent toxicity tests. However, aeration is not practical for the Ceriodaphnia test. If the DO in the effluent and/or dilution water is low, aerate before preparing the test solutions.

12.5 FEEDING

11.5.1 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/15 mL test solution and 0.1 mL Selenastrum 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 X10^5 cells/mL in the test chamber).

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

12.6 TEST SOLUTION RENEWAL

12.6.1 For on-site tests, test solutions are renewed daily with freshly collected samples. For off-site tests, test solutions are also renewed daily,

using the most recently collected sample. A minimum of three samples are collected, preferrably for use beginning on Days 1, 3, 5. The first sample is used for test initiation on Day 1 and test solution renewal on Day 2. The second sample is used for test solution renewal on Days 3 and 4, and the third sample is used for test solution renewals on Days 5, 6, and 7. Samples first used on Days 1, 3, and 5, are held over in the refrigerator at 4°C for use on the following day(s).

12.6.2 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. For private carriers, shipping and receiving schedules on weekends vary with the carrier.

12.6.3 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.

12.7 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

12.7.1 At a minimum, the following measurements are made:

12.7.1.1 DO and pH are measured at the beginning and end of each 24-h exposure period in the high, medium, and low test concentrations, and in the control.

12.7.1.2 Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples.

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

12.7.1.4 Record the data (as shown in Figure 1).

12.8 OBSERVATIONS DURING THE TEST

12.8.1 Three broods are usually obtained in the controls in a seven-day test conducted at 25 $\pm$ 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, soon after the adults are transferred to fresh test solutions. Successive broods are released every 36 to 48 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.

12.8.2 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 4, just prior to test solution renewal, and the fifth released just after renewal, and counted on Day 5. Partial broods, released over a two-day period, should be counted as one brood.

12.8.3 Each day, the live adults are transferred to fresh test solutions, and the numbers of live young are recorded (see data form, Figure 2). If difficultly is encountered in counting the live young because of their erratic motion, two drops of 1N HCl can be added to the chamber (except the chambers used for DO and pH measurements) after the adult has been transferred. Upon addition of acid, the young die quickly and settle to the bottom of the test chamber where they may be counted with a minimum of effort and error. The young are discarded after counting.

12.8.4 The young are best counted with the aid of a stereomicroscope with substage lighting. If counts are made without the aid of a stereomicroscope, it is helpful to place the test chambers on a black strip of tape on a light box.

12.8.5 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.

12.9 TERMINATION OF THE TEST

12.9.1 Tests should be terminated when 60% or more of the surviving females in the controls have produced their third brood. Because of the rapid rate of development of Ceriodaphnia, 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.

12.9.2 The data recorded in Figure 2 is summarized as illustrated in Figure 3.

12.10. ACCEPTABILITY OF TEST RESULTS

12.10.1 For the test results to be acceptable, survival in the controls must be at least 80%, and reproduction in the controls must average 15 or more young per surviving female.

12.11 SUMMARY OF TEST CONDITONS

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

13. DATA ANALYSIS

13.1 GENERAL

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

TABLE 3. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 uE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test chamber size:	30 mL
7. Test solution volume:	15 mL
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Less than 24 h; and all released within a 8-h period
10. No. neonates per test chamber:	1
11. No. replicate test chambers per Concentration:	10
12. No. neonates per test concentration:	10
13. Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily.
14. Aeration:	None
15. Dilution water:	Moderately hard synthetic water is prepared using MILLIPORE MILLI-Q^R or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7).
16. Effluent concentrations:	Minimum of 5 effluent concentrations and a control.

TABLE 3. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST (CONTINUED)

17. Dilution factor:[1]	Approximately 0.3 or 0.5
18. Test duration:	Until 60% of control females have three broods (may require more or less than 7 days).
19. Endpoints:	Survival and reproduction
20. Test acceptability:	80% or greater survival and an average of 15 or more young/surviving female in the control solutions. At least 60% of surviving females in controls should have produced their third brood.
21. Sampling requirements:	For on-site tests, samples are 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 are collected, and used as described in Paragraph 12.6.1.
22. Sample volume required:	1 L

[1]Surface water test samples are used undiluted.

Figure 2. Data form for the Ceriodaphnia survival and reproduction test. Daily record. See Figure 1 for key to positions on randomized test board. (The chart on the right was reduced to save space).

Discharger: _____

Location: _____

Date Sample Collected: _____

Analyst: _____

Test Dates: _____

Template No.: _____

Dilution Water: _____

Test Chambers (glass/plastic): _____

Food: _____

Test Temp: _____

Test Organisms (age): _____

Comments: _____

_____

_____

_____

_____

_____

_____

_____

√ = Test organism alive
x = Test organism dead
0 = Number of live young
(-0) = Number of dead young
M = Lost or missing
y = Male

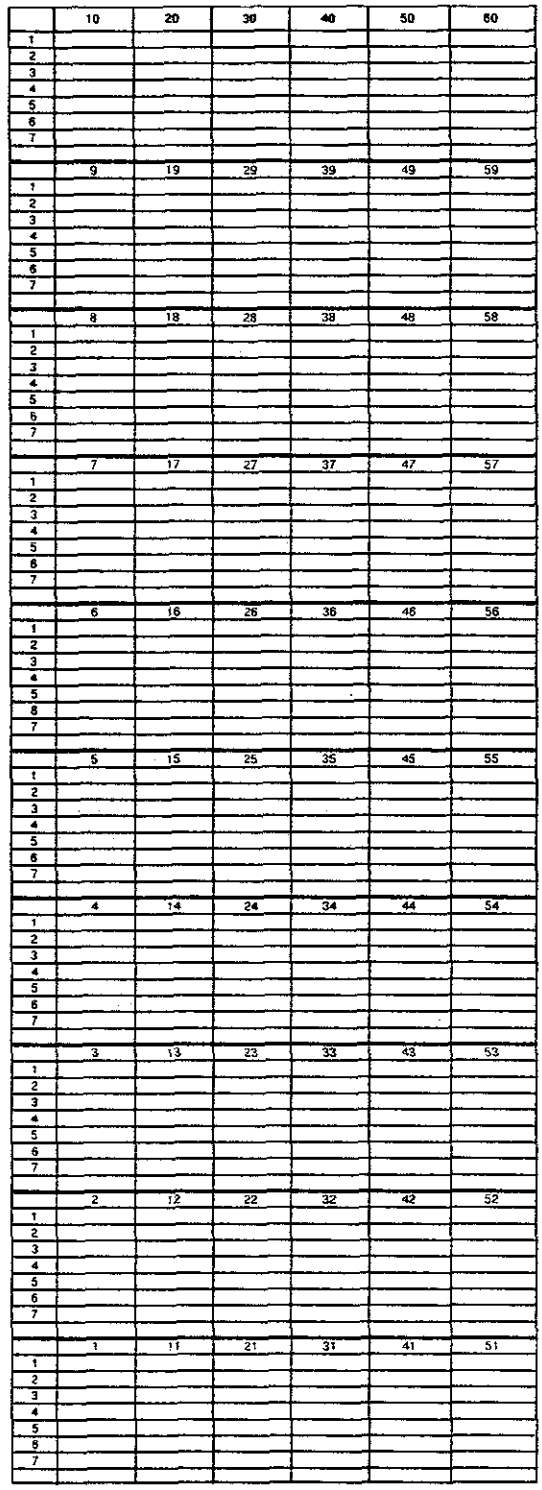

Figure 3. Data form for the <u>Ceriodaphnia</u> survival and reproduction test. Summary of data from form in Figure 2.

Discharger: ____________________ Analyst: ____________________
Location: ____________________ Test Start-Date/Time: ____________________
Date Sample Collected: ____________________ Test Stop -Date/Time: ____________________

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

Figure 3. Data form for the *Ceriodaphnia* survival and reproduction test. Summary of data from form in Figure 2. (Continued)

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

Conc.	Day	Replicate 1	2	3	4	5	6	7	8	9	10	No. of Young	No. of Adults	Young per Adult
	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Total													

13.1.2 The endpoints of toxicity tests using Ceriodaphnia are based on the adverse effects on survival and reproduction. Point estimates, such as LCs and ICs, are calculated using point estimation techniques (see Section 2). LOEC and NOEC values, for survival and growth, 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 the Appendices for examples of the manual computations and data input and output for the computer programs.

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.

13.2 EXAMPLE OF ANALYSIS OF CERIODAPHNIA SURVIVAL DATA

13.2.1 Formal statistical analysis of the survival data is outlined 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 LC1, LC5, LC10 and LC50 endpoints. Concentrations at which there is no survival are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the LC endpoints.

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 population. Additional information on Fisher's Exact Test is provided in Appendix G.

13.2.3 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 number dead at a given concentration is the response.

13.2.4 Example of Analysis of Survival Data

13.2.4.1 The data in Table 4 will be used to illustrate the analysis of survival data from the Ceriodaphnia 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.

13.2.5 Fisher's Exact Test

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

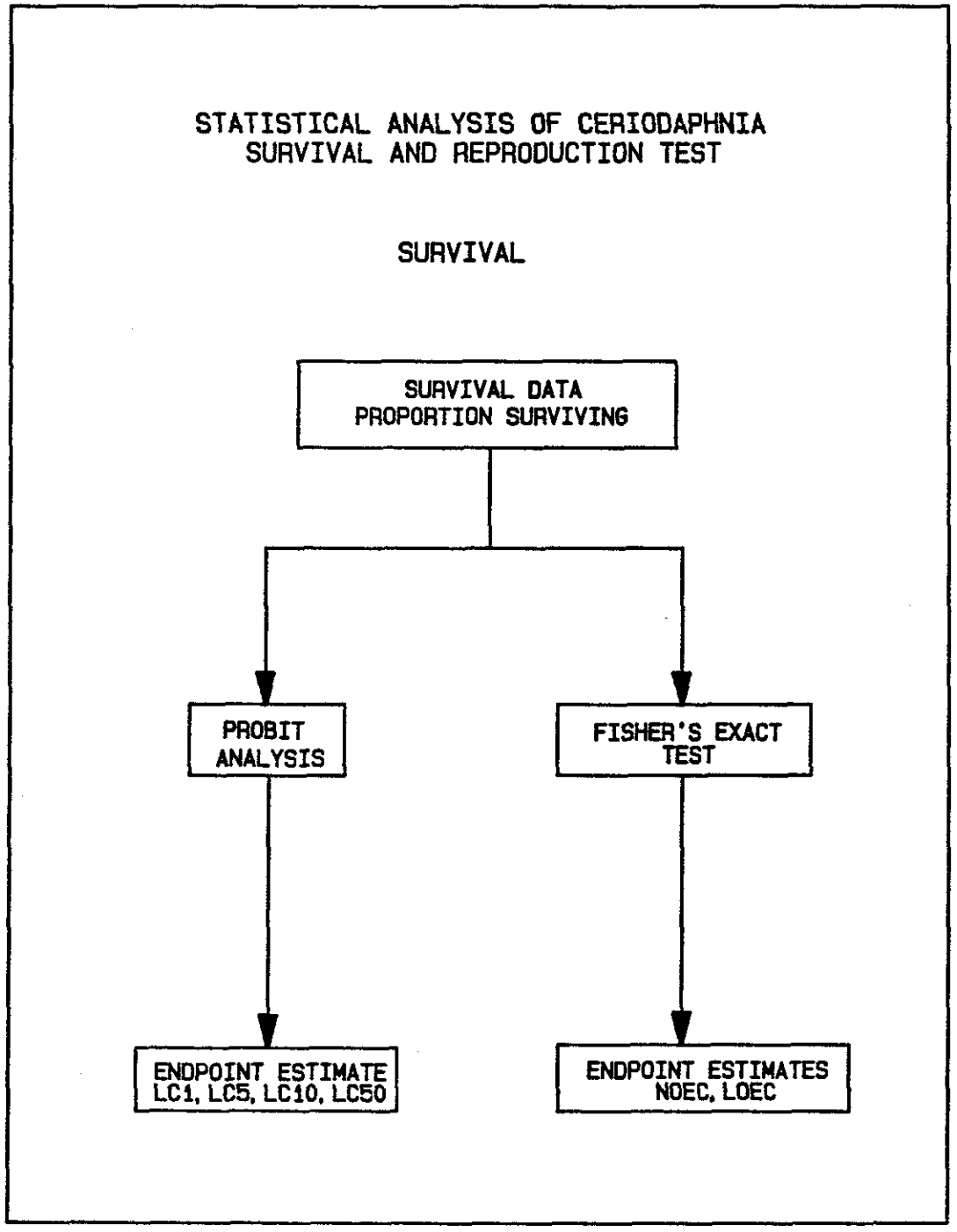

Figure 4. Flow chart for statistical analysis of Ceriodaphnia survival data.

TABLE 4. SUMMARY OF SURVIVAL AND REPRODUCTION DATA FOR CERIODAPHNIA 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	3

TABLE 5. FORMAT OF THE 2X2 CONTINGENCY TABLE

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

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 this 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.

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

TABLE 6. 2X2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		Number of Observations
	Alive	Dead	
Control	10	0	10
25% Effluent	3	7	10
Total	13	7	20

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.4 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%.

13.2.6 Probit Analysis

13.2.6.1 The data used for the probit analysis are summarized in Table 7. For the probit analysis, the data from all concentrations are considered. To perform the probit analysis, run the EPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

13.2.6.2 For this example there is only one partial mortality, and Probit analysis is not appropriate.

TABLE 7. DATA FOR PROBIT ANALYSIS

	Effluent Concentration (%)					
	Control	1.56	3.12	6.25	12.5	25.0
Number Dead	0	0	0	0	0	7
Number Exposed	10	10	10	10	10	10

13.3 EXAMPLE OF ANALYSIS OF CERIODAPHNIA REPRODUCTION DATA

13.3.1 Formal statistical analysis of the reproduction data is outlined in Figure 5. 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. 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 2). Hypothesis testing can be used to obtain a NOEC for reproduction. Concentrations above the NOEC for survival are excluded from the hypothesis test for reproduction effects.

13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a non-parametric 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 fail, a non-parametric 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.

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

13.3.5 The data, mean and standard deviation 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 6. Since there is significant mortality in the 25% effluent concentration, its effect on reproduction is not considered.

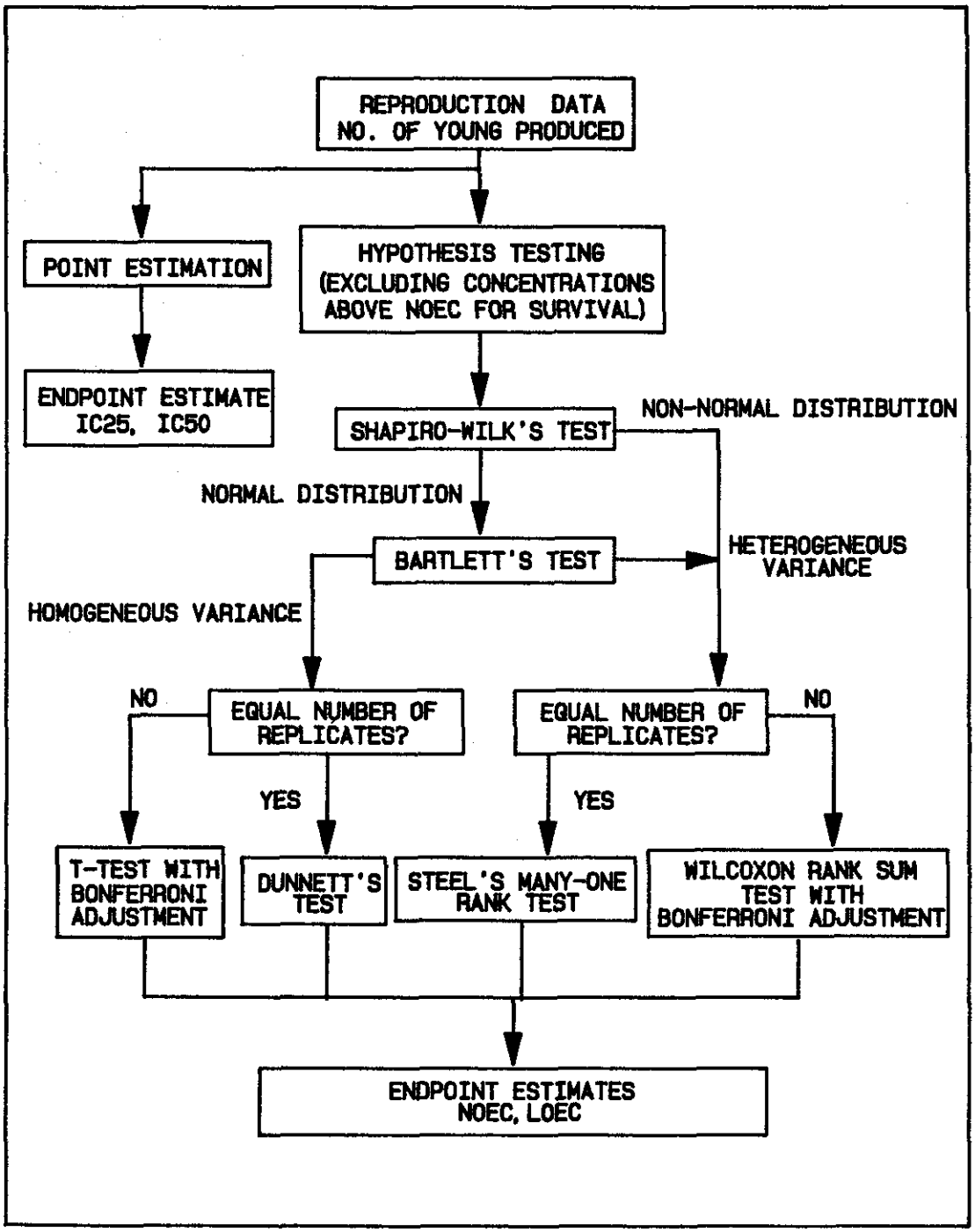

Figure 5. Flow chart for statistical analysis of Ceriodaphnia reproduction data.

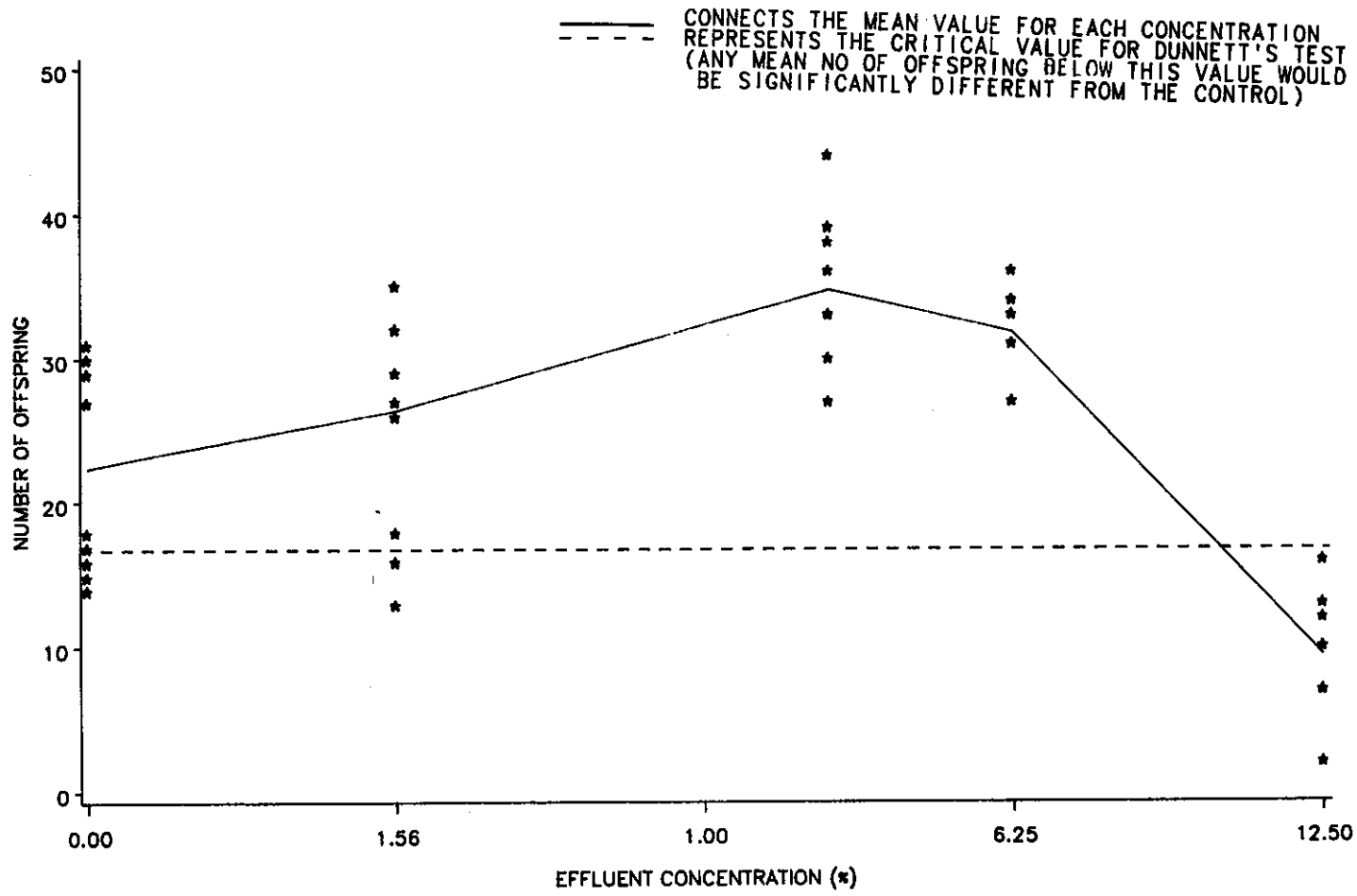

Figure 6. Plot of number of young per adult female from a Ceriodaphnia survival and reproduction test.

TABLE 8. CERIODAPHNIA REPRODUCTION DATA

Replicate	Control	Effluent Concentration (%) 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($\bar{Y}_i$)	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

13.3.6 Test for Normality

13.3.6.1 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.

13.3.6.2 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

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

13.3.6.3 Order the centered observations from smallest to largest

$$X(1) - X(2) - \ldots - X(n)$$

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

13.3.6.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is approximately n/2. For the data in this example, n = 50, k = 25. The a_i values are listed in Table 11.

13.3.6.5 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:

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

13.3.6.6 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.

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)

13.3.7 Test for Homogeneity of Variance

13.3.7.1 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, including control
n_i = the number of replicates for concentration i
$\ln = \log_e$
$i = 1, 2, \ldots, p$ where p is the number of concentrations including the control

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

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

13.3.7.2 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.

13.3.7.3 Bartlett's statistic is therefore:

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

$$= [45(3.5) - 9(16.1)]/1.04$$

$$= 12.6/1.04$$

$$= 12.1$$

13.3.7.4 B is approximately distributed as chi square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = 12.1 is less than the critical value of 13.3, conclude that the variances are not different.

13.3.8 Dunnett's Procedure

13.3.8.1 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	$S_B^2 = SSB/(p-1)$
Within	N - p	SSW	$S_W^2 = SSW/(N-p)$
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

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

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

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

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

T_i = the total of the replicate measurements for concentration "i"

Y_{ij} = the jth observation for concentration "i" (represents the number of young produced by female j in effluent concentration i)

13.3.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 10$
$N = 50$
$T_1 = Y_{11} + Y_{12} + \ldots + Y_{110} = 224$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{210} = 263$
$T_3 = Y_{31} + Y_{32} + \ldots + Y_{310} = 346$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{410} = 317$
$T_5 = Y_{51} + Y_{52} + \ldots + Y_{510} = 94$

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

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

$$= \frac{1}{10}(348,386) - \frac{(1244)2}{50} = 3887.88$$

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

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

$SSW = SST - SSB = 5321.28 - 3887.88 = 1433.40$
$S_B^2 = SSB/p-1 = 3887.88/5-1 = 971.97$
$S_w^2 = SSW/N-p = 1433.40/50-5 = 31.85$

13.3.8.3 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	

13.3.8.4 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 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 sqaure
n_1 = number of replicates for 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.

13.3.8.5 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:

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

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

13.3.8.6 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.

13.3.8.7 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_i = the number of replicates in the control.

13.3.8.8 In this example:

$$MSD = 2.23\ (5.64)\ \sqrt{(1/10) + (1/10)}$$
$$= 2.23\ (5.64)(0.45)$$
$$= 5.66$$

13.3.8.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 5.66.

13.3.8.10 This represents a 25% decrease in mean reproduction from the control.

14. PRECISION AND ACCURACY

14.1 PRECISION

14.1.1 Single Laboratory Precision

14.1.1.1 Information on the single laboratory precision of the Ceriodaphnia reproduction test based on the NOEC and LOEC values from nine tests with the reference toxicant NaPCP is provided in Table 15. The NOECs and LOECs of all tests fell in the same concentration range, indicating maximum possible precision.

14.1.2 Multilaboratory Precision

14.1.2.1 An interlaboratory study was performed by the Aquatic Biology Branch, EMSL-Cincinnati in 1985, involving a total of 11 analysts in 10 different laboratories (Neiheisel et. al., 1988a). 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. Some deviations from the standard protocol were reported by the participating laboratories.

14.1.2.2 Ten sets of data from six laboratories met the acceptability criteria, and were statistically analyzed using non-parametric procedures to determine NOECs and LOECs. 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 16).

14.1.2.3 An second interlaboratory study of Method 1002.0 (using the first edition of this manual; Horning and Weber, 1985), was coordinated by Battelle, Columbus Division, and involved 11 participating laboratories (DeGraeve et al., 1989). All participants used 10% DMW (10% PERRIER^{R} Water) as the culture and dilution water, and used their own formulation of food for culturing and testing the Ceriodaphnia. 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). The overall precision (CV) of the test was 27% for the survival data (7-day LC50s) and 40% for the reproduction data (IC50s).

14.2 ACCURACY

14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 15. SINGLE LABORATORY PRECISION OF THE CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[a,b]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[c]	0.25	0.50	0.35
2[d]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[e]	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

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

[b]Data from tests performed by Philip Lewis, Aquatic Biology Branch, EMSL-Cincinnati. Tests were conducted in reconstituted hard water (hardness = 180 mg $CaCO_3$/L; pH = 8.1).

[c]Concentrations used in Test 1 were: 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

[d]Concentrations used in Tests 2 and 3 were: 0.007, 0.022, 0.067, 0.20, 0.60 mg NaPCP/L.

[e]Concentrations used in Tests 4 through 9 were: 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

TABLE 16. INTERLABORATORY PRECISION OF *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST[1]

Analyst	Test	Endpoints (% Effluent)			
		Reproduction		Survival	
		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

[1]From Neiheisel et al., 1988a.

SECTION 13

TEST METHOD

ALGAL, SELENASTRUM CAPRICORNUTUM, GROWTH TEST
METHOD 1003.0

1. SCOPE AND APPLICATION

1.1 This method measures the chronic toxicity of whole effluents and receiving water to the fresh water alga, Selenastrum capricornutum, during a four-day, static exposure. 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.

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

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 degradeable and volatile toxicants, such as chlorine, in the source may not be detected in the test.

1.4 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.

1.5 This method is restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 A Selenastrum 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.

3. INTERFERENCES

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

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.

3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

3.4 Pathogenic organisms and/or planktivores in the dilution water and effluent may affect test organism survival and growth, and confound test results.

3.5 Nutrients in the effluent or dilution water may confound test results.

4. SAFETY

4.1 See Section 3, Safety and Health.

5. APPARATUS AND EQUIPMENT

5.1 Laboratory Selenastrum culture unit -- See culturing methods below. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

5.2 Samplers -- Automatic sampler capable of collecting a 24-h composite sample of 1 L.

5.3 Sample containers -- for sample shipment and storage see Section 8, Effluent and Receiving Water Sampling and Sample Handling.

5.4 Environmental chamber, incubator, or equivalent facility -- with "cool-white" fluorescent illumination (86 $\pm$ 8.6 uE/m^2/s, or 400 $\pm$ 40 ft-c) and temperature control (25 $\pm$ 1°C, for compatibility with other tests).

5.5 Mechanical shaker -- Capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

5.6 Light meter -- with a range of 0-200 uE/m^2/s (0-1000 ft-c).

5.7 Water purification system -- MILLIPORE MILLI-Q^R or equivalent.

5.8 Balance -- Analytical, capable of accurately weighing 0.0001 g.

5.9 Reference weights, Class S -- for checking performance of balance.

5.10 Glass or electronic thermometers -- for measuring water temperatures.

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

5.12 National Bureau of Standards Certified thermometer (see EPA Method 170.1, USEPA 1979b).

5.13 Meters: pH and specific conductivity -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

5.14 Tissue grinder -- for chlorophyll extraction.

5.15 Fluorometer (Optional) -- Equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

5.16 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

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

5.18 Electronic particle counter (Optional) -- Coulter Counter, Model ZBI, or equivalent, with mean cell (particle) volume determination.

5.19 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope).

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

5.21 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

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

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

5.24 Volumetric flasks and graduated cylinders -- Class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

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

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

5.27 Pipet bulbs and fillers -- Propipet^{R}, or equivalent.

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

5.29 Culture chambers -- 1-4 L borosilicate, Erlenmeyer flasks.

5.30 Test chambers -- 125 or 250 mL borosilicate, Erlenmeyer flasks, with stainless steel closures.

5.31 Preparation of glassware -- prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal bioassays as follows:

5.31.1 Wash with non-phosphate detergent solution, preferably heated to 50°C or hotter. 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.

5.31.2 Rinse with tap water.

5.31.3 Test flasks should be thoroughly rinsed with 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.

5.31.4 Rinse twice with MILLI-Q^R water.

5.31.5 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 5.31.7. If acetone is used, go to 5.31.6.

5.31.6 Rinse thoroughly with MILLI-Q^R water, and dry in an 105°C oven.

5.31.7 Cover the mouth of each chamber with aluminum foil or other closure, as appropriate, before storing.

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

6. REAGENTS AND CONSUMABLE MATERIALS

6.1 Reagent water -- defined as MILLIPORE MILLI-Q^R or equivalent water (see paragraph 5.7 above).

6.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling.

6.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

6.4 Standard particles -- polymer microspheres, 5.0 $\pm$ 0.03 um diameter, 65.4 um^3 volume, for calibration of electronic particle counters (available from Duke Scientific Co., 1135D, San Antonio Road, Palo Alto, California, 94303).

6.5 Standard pH buffers 4, 7, 8 and 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1, USEPA 1979b).

6.6 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

6.7 Laboratory quality assurance samples and standards for the above methods.

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

6.9 Acetone -- pesticide-grade or equivalent.

6.10 Dilute (10%) hydrochloric acid -- carefully add 10 mL of concentrated HCl to 90 mL of MILLI-Q^R water.

7. TEST ORGANISMS

7.1 Log-phase-growth Selenastrum capricornutum are used for the test.

7.2 CULTURE MEDIUM

7.2.1 The culture medium is used to maintain stock cultures of the test organisms.

7.2.2 Prepare five stock nutrient solutions using reagent grade chemicals as described in Table 1.

7.2.3 Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q^R 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 sodium hydroxide or hydrochloric acid, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

7.2.4 Immediately filter the pH-adjusted medium through a 0.45um 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 prior to use by passing 500 mL of distilled water through it.

7.2.5 If the filtration is carried out with sterile apparatus, filtered medium can be placed immediately into sterile culture flasks, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving before placing in the culture flasks. However, the pH should be checked after autoclaving to determine if it was changed.

7.2.6 Unused sterile medium should not be stored in the (250 mL) test culture flasks more than one week prior to use, because there may be substantial loss of water by evaporation.

7.3 ALGAL CULTURES

7.3.1 Test organisms -- Selenastrum capricornutum, a unicellular coccoid green alga. See Section 6, Test Organisms, for information on sources of "starter" cultures.

7.3.2 Stock algal cultures

7.3.2.1 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

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES.

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL Distilled Water
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
2	$NaNO_3$	12.750 g
3	$MgSO_4 \cdot 7H_2O$	7.350 g
4	K_2HPO_4	0.522 g
5	$NaHCO_3$	7.50 g

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

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

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

[d] $CuCl_2 \cdot 2H_2O$ - 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 #1.

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 (ug/L)	Element	Concentration (ug/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	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	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----

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.

7.3.2.2 Maintain the stock cultures at 25 ± 1°C, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 uE/m^2/s (400 ± 40 ft-c). Shake continuously at 100 cpm or twice daily by hand.

7.3.2.3 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.

7.3.2.4 Viable unialgal culture material may be maintained long periods of time if placed in a refrigerator at 4°C.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. TEST PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5.

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%).

11.1.2.3 The volume of effluent required for the test is 600 - 1000 mL. Prepare enough test solution at each effluent concentration (approximately 700 mL) to provide 50 - 100 mL of test solution for each of three replicate test flasks and 400 mL for chemical analyses.

11.1.3 Dilution water may consist of stock culture medium without EDTA, or other water such as surface water, depending on the objectives of the test. However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution (except for EDTA) should be added per liter of dilution water. Surface 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 um particle size retention.

11.1.4 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 (except EDTA) is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain as a minimum the concentration of nutrients in stock culture medium.

11.1.5 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 um 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.

11.1.6 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.

11.2 PREPARATION OF INOCULUM

11.2.1 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. Estimate the volume of stock culture required to prepare the inoculum as described in the following example:

> If the seven-to 10-day stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 18 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 18,000,000 cells or 12.5 mL of stock solution

(18,000,000/2,000,000) 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.

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 distilled or deionized water.
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:

$$\text{Volume (mL) of Stock Culture Required} = \frac{\text{Number of flasks to be used} \times \text{Volume of Test Solution/Flask} \times 10{,}000 \text{ cells/mL}}{\text{Cell density (cells/mL) in the stock culture}}$$

$$= \frac{18 \text{ flasks} \times 100 \text{ mL/flask} \times 10{,}000 \text{ cells/mL}}{2{,}000{,}000 \text{ cells/mL}}$$

$$= 9.0 \text{ mL Stock Culture}$$

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.

11.3 START OF THE TEST

11.3.1 On-site tests should be initiated within 24 h of sample collection, and off-site tests should be initiated within 36 h of sample collection. Just prior to testing, the temperature of the sample should be adjusted to (25 ± 1°C) and maintained at that temperature until portions are added to the dilution water.

11.3.2 The test begins when the algae are added to the test flasks.

1. Mix the inoculum well, and add 1 mL to the test solution in each flask.
2. Make a final check of the cell density in three of the test solutions at time "zero " (within 2 h of the inoculation).

11.4 LIGHT, PHOTOPERIOD, AND TEMPERATURE

11.4.1 Test flasks are incubated under continuous illumination at 86 ± 8.6 uE/m^2/s (400 ± 40 ft-c), at 25 ± 1°C, and should be shaken continously 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.

11.5 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.5.1 At a minimum, the following measurements are made:

11.5.1.1 Temperature should be monitored continously or observed and recorded daily for at least two locations in the environmental control system or the samples.

11.5.1.2 pH, 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 (see Figure 1).

11.6 OBSERVATIONS DURING THE TEST

11.6.1 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-to-two days in the test. It may be desirable, therefore, to determine the algal growth response daily.

11.7 TERMINATION OF THE TEST

11.7.1 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).

11.7.2 Cell counts

11.7.2.1 Automatic Particle Counters

11.7.2.1.1 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 um^3/cell. The Coulter Counter is widely used and is discussed in detail by Miller et al., 1978.

11.7.2.1.2 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.

11.7.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a 1% sodium chloride electrolyte solution, such as Isoton^R, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-um diameter aperature. 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^{R}).
4. Determine the cell density (and MCV, if desired).

11.7.2.2 Manual microscope counting methods

11.7.2.2.1 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, 1985, and Weber, 1973. Whenever feasible, 400 cells per replicate are counted to obtain $\pm$ 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

11.7.3 Chlorophyll Content

11.7.3.1 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).

11.7.3.2 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 um 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.

11.7.3.3 For chlorophyll measurement methods, see APHA, 1985.

11.7.4 Turbidity (Absorbance)

11.7.4.1 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.

11.7.4.2 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.

11.7.5. Record the data as indicated in Figure 2.

11.8 SUMMARY OF TEST CONDITIONS

11.8.1 A summary of test conditions is listed in Table 3.

11.9 ACCEPTABILITY OF TEST RESULTS

11.9.1 The test results are acceptable if the algal cell density in the control flasks (without EDTA) exceeds 2 X 10^5 cells/mL at the end of the test, and does not vary more than 20% among replicates.

12. DATA ANALYSIS

12.1 GENERAL

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

12.1.2 The endpoints of toxicity tests using Selenastrum capricornutum are based on the adverse effect on cell growth (see Section 2). LOEC and NOEC values, for growth, are obtained using a hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). Point estimates, such as EC1, EC5, EC10 and EC50, would also be appropriate in analyzing algal growth response data. See the Appendices for examples of the manual computations and examples of computer program data input and output.

12.1.3 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.

12.2 EXAMPLE OF ANALYSIS OF ALGAL GROWTH DATA

12.2.1 Formal statistical analysis of the growth data is outlined in Figure 3. The response used in the statistical analysis is the number of cells per milliliter per replicate.

12.2.2 The statistical analysis consists of a parametric test, Dunnett's Procedure, and a non-parametric 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 fail, the non-parametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

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

12.2.4 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.

TABLE 3. SUMMARY OF RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE ALGAL (SELENASTRUM CAPRICORNUTUM) GROWTH TEST

1. Test type:	Static
2. Temperature:	25 ± 1°C
3. Light quality:	"Cool white" fluorescent lighting
4. Light intensity:	86 ± 8.6 uE/m^2/s (400 ± 40 ft-c)
5. Photoperiod:	Continuous illumination
6. Test chamber size:	125 mL or 250 mL
7. Test solution volume:	50 mL or 100 mL
8. Renewal of test solutions:	None
9. Age of test organisms:	4 to 7 days
9. Initial cell density in test chambers:	10,000 cells/mL
10. No. replicate chambers/concentration:	3
11. Shaking rate:	100 cpm continuous, or twice daily by hand
12. Dilution water:	Algal stock culture medium without EDTA or enriched surface water
13. Effluent concentrations:	Minimum of 5 and a control
14. Dilution factor[1]:	Approximately 0.3 or 0.5
15. Test duration:	96 h
16. Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, biomass)
17. Test acceptability:	2 X 10^5 cells/mL in the controls; Variability of controls should not exceed 20%
18. Sample volume required:	1 L (one sample for test initation)

[1]Surface water samples for toxicity tests are used undiluted.

Figure 1. Data form for algal growth test. Routine chemical and physical determinations.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

Treatment	Contr	Effluent Concentration					Remarks
Temp.							
pH							
Alkalinity							
Hardness							
Salinity							
Conductivity							
Chlorine							

Figure 2. Data form for algal growth test. Cell density determinations.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

Conc:	Cell Density Measurement Replicate			Treatment Mean	Comments
	1	2	3		
Control					
Conc:					
Conc:					
Conc:					
Conc:					
Conc:					

Comments:

TABLE 4. ALGAL GROWTH RESPONSE DATA

	Replicate	Control	Toxicant Concentration (ug Cd/L) 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} Trans-	A	3.082	3.084	2.917	2.693	2.104	1.693
formed	B	3.072	3.074	2.798	2.619	2.167	1.602
	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
S_i^2		0.0009	0.00003	0.0045	0.0019	0.0013	0.0021
i		1	2	3	4	5	6

12.2.5 Test for Normality

12.2.5.1 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

Replicate	Control	Toxicant Concentration (ug Cd/L) 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

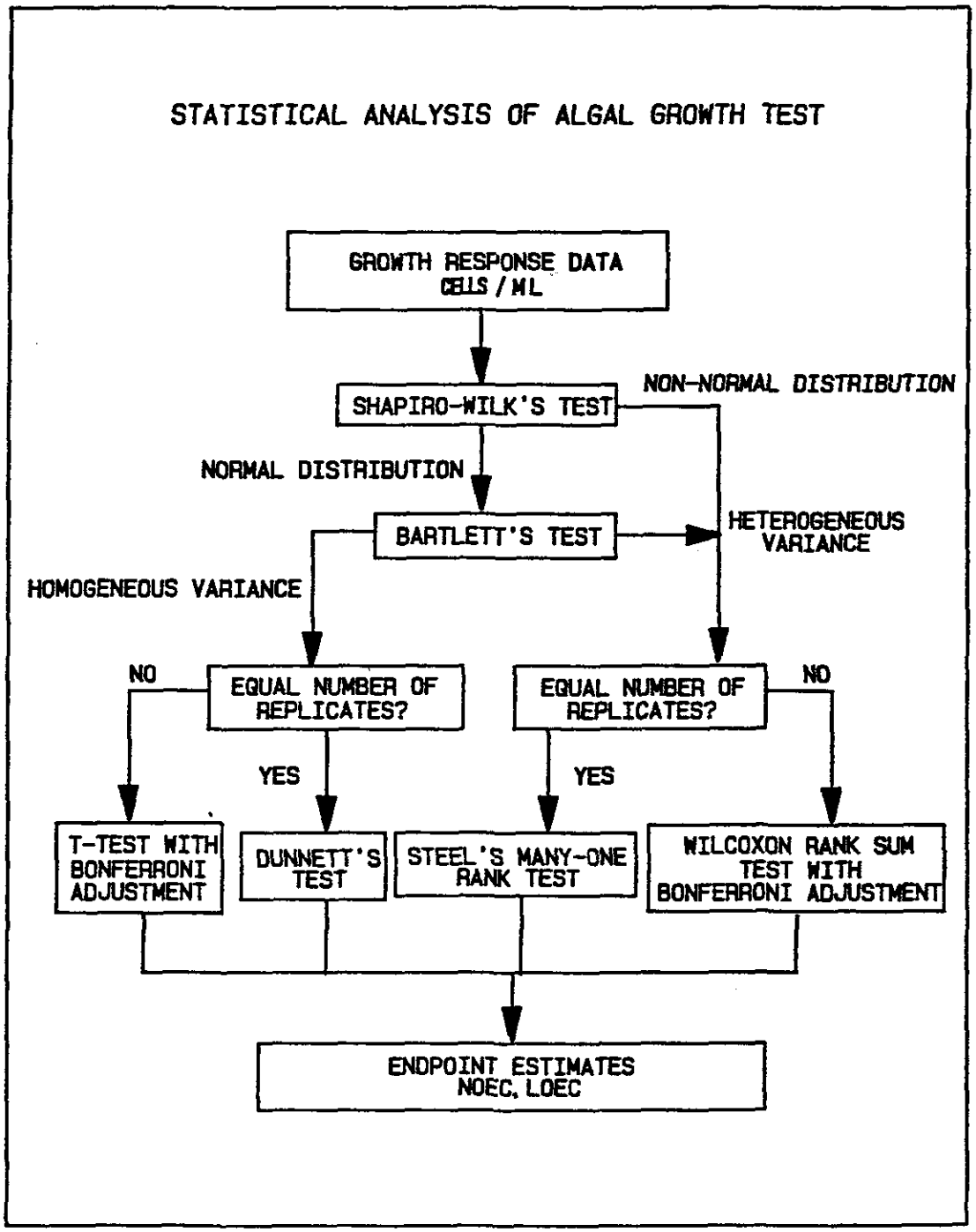

Figure 3. Flow chart for statistical analysis of algal growth response data.

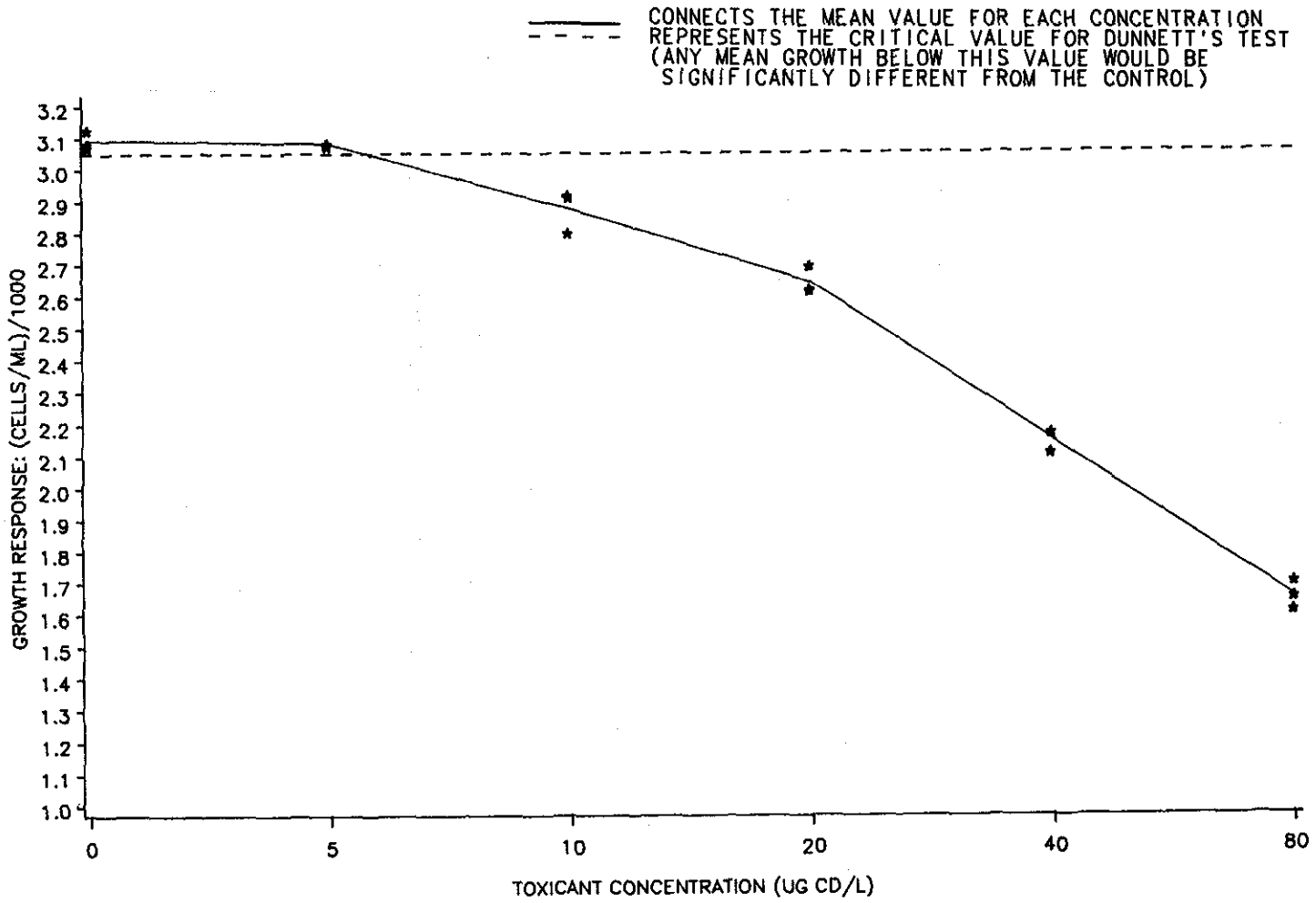

Figure 4. Plot of $\log_{10}$ transformed cell count data from algal growth response test (Table 4).

12.2.5.2 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, $n = 18$

$$\overline{X} = \frac{1}{18}(0.000) = 0.000$$

$$D = 0.0214$$

12.2.5.3 Order the centered observations from smallest to largest:

$$X(1) - X(2) - \ldots - X(n)$$

Where $X(i)$ is the ith ordered observation. These ordered observations are listed in Table 6.

12.2.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is approximately n/2. For the data in this example, n = 18, k = 9. The a_i values are listed in Table 7.

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

12.2.5.5 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.

For this set of data:

$$W = \frac{1}{0.0214}(0.1436)^2 = 0.964$$

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	$X_{(10)} - X_{(9)}$

12.2.5.6 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.

12.2.6 Test for Homogeneity of Variance

12.2.6.1 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:

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

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

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

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

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

12.2.6.2 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.

12.2.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(0.0018) - 2 \sum_{i=1}^{p} \ln(S_i^2)]/1.194$$

$$= [12(-6.3200) - 2(-41.9082)]/1.194$$

$$= 7.9764/1.194$$

$$= 6.6804$$

12.2.6.4 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.

12.2.7 Dunnett's Procedure

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

TABLE 8. ANOVA TABLE

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

Where:
p = number of toxicant concentrations including the control
N = total number of observations $n_1 + n_2 \ldots + n_p$
n_i = number of observations in concentration i

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

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

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

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$
T_i = the total of the replicate measurements for concentration "i"
Y_{ij} = the jth observation for concentration "i" (represents the cell count for toxicant concentration i in test chamber j)

12.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$
$N = 18$
$T_1 = Y_{11} + Y_{12} + Y_{13} = 9.281$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 9.239$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 8.627$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 7.928$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 6.438$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 4.938$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 46.451$

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

$$= \frac{1}{3}(374.606) - \frac{(46.451)^2}{18} = 4.997$$

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

$$= 124.890 - \frac{(46.451)^2}{18} = 5.018$$

$SSW = SST - SSB = 5.018 - 4.997 = 0.021$
$S_B^2 = SSB/p\text{-}1 = 4.996/6\text{-}1 = 0.999$
$S_W^2 = SSW/N\text{-}p = 0.021/18\text{-}6 = 0.0018$

12.2.7.3 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	

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

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

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 within mean sqaure
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

12.2.7.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 5 ug/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 (ug 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

12.2.7.6 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 ug/L concentrations have significantly lower mean cell counts than the control. Hence the NOEC and the LOEC for the test are 5 ug/L and 10 ug/L, respectively.

12.2.7.7 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 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_i = the number of replicates in the control.

12.2.7.8 In this example:

$$MSD = 2.50\ (0.0424)\ \sqrt{(1/3) + (1/3)}$$
$$= 2.50\ (0.0424)(0.8165)$$
$$= 0.086$$

12.2.7.9 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.

   $MSU_u = 1241.6 - 1018.6 = 223$

12.2.7.10 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.

12.2.7.11 This represents a decrease in growth of 18% from the control.

12.3 BIOSTIMULATION

12.3.1 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., 1980b):

$$S(\%) = \frac{T - C}{C} \times 100$$

13. TEST PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Data from repetitive 96-h toxicity tests conducted with three reference toxicants, using medium containing EDTA, are shown in Table 11. The relative standard deviation (coefficient of variation) of the LC1s ranged from 47% to 83%.

13.2 ACCURACY

3.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 11. PRECISION OF THE SELENASTRUM CAPRICORNUTUM, 96-H TOXICITY TEST, USING REFERENCE TOXICANTS

Test No.	Toxicant: Cadmium Chloride EC1[a] (ug Cd/L)	UL	LL	NOEC[b] (ug Cd/L)	Sodium Pentachlorophenate EC1 (ug/L)	UL	LL	NOEC (ug/L)	Sodium Dodecyl Sulfate EC1 (mg/L)	UL	LL	NOEC (mg/L)
1	0.201	0.272	0.181	LT 0.49[c]	20.7	27.6	13.9	62.5	2.57	3.13	1.97	5.0
2	0.647	1.33	0.198	LT 10.0	NC	---	---	80.0	1.32	1.77	0.890	2.5
3	.372	5.45	0.220	1.0	16.7	21.6	11.9	40.0	5.57	6.60	4.30	10.0
4	.242	0.446	0.0981	LT 2.0	41.3	46.8	34.8	66.0	6.41	7.52	4.98	7.5
5	.638	0.972	0.352	LT 2.0	40.2	45.5	33.9	LT 66.0	1.26	1.81	0.766	LT 5.0
6	2.37	3.27	1.54	LT 8.0	47.0	53.1	39.8	82.0	2.85	2.98	2.72	5.0
7	2.27	2.98	1.59	5.0	43.4	48.7	37.1	LT 66.0				
8	1.23	1.78	0.748	5.0	84.3	90.0	76.3	102				
9	0.347	0.652	0.137	LT 5.0	40.5	48.4	30.8	LT 66.0				
10	.608	1.01	0.296	LT 5.0	33.4	40.6	25.5	82.0				
11	1.72	2.38	1.11	5.0								
N	11				9				6			
Mean	0.968				40.8				3.33			
SD	0.806				19.3				1.98			
CV	83%				47%				60%			

[a] EC1 (threshold concentration) and upper (UL) and lower (LL) confidence limits determined by Probit Analysis.

[b] NOEC determined with Dunnett's Test.

[c] LT = NOEC less than the lowest concentration tested.

Reference toxicant concentrations used in the toxicity tests are listed below:

Cadmium Chloride (ug Cd/L):

1: 0.49, 0.95, 1.88, 3.77, 7.27
2: 10.0, 20.0, 40.0, 80.0
3: 1.0, 2.0, 4.0, 8.0, 16.0
4: 2.0, 4.0, 8.0, 16.0, 32.0
5: 2.0, 4.0, 8.0, 16.0, 32.0
6: 8.0, 16.0, 32.0, 64.0, 128
7: 5.0, 10.0, 20.0, 40.0, 80.0
8: 5.0, 10.0, 20.0, 40.0, 80.0
9: 5.0, 10.0, 20.0, 40.0, 80.0
10: 5.0, 10.0, 20.0, 40.0, 80.0
11: 5.0, 10.0, 20.0, 40.0, 80.0

Sodium Pentachlorophenate (ug/L):

1: 62.5, 125, 250, 500, 1000
2: 40.0, 80.0, 160, 320, 640
3: 40.0, 80.0, 160, 320, 640
4: 66.0, 82.0, 102, 128, 160
5: 66.0, 82.0, 102, 128, 160
6: 66.0, 82.0, 102, 128, 160
7: 66.0, 82.0, 102, 128, 160
8: 66.0, 82.0, 102, 128, 160
9: 66.0, 82.0, 102, 128, 160
10: 66.0, 82.0, 102, 128, 320

Sodium Dodecyl Sulfate (mg/L):

1: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0
2: 2.5, 5.0, 10.0, 12.5, 15.0, 20
3: 2.5, 10.0, 12.5, 15.0, 20.0
4: 5.0, 7.5, 12.5, 15.0, 20.0
5: 5.0, 12.5, 20.0, 40.0, 80.0
6: 2.5, 5.0, 12.5, 15.0, 20.0, 40.0

SELECTED REFERENCES

Adams, N., et al. 1985. Toxicity of eight water-soluble organic chemicals to Selenastrum capricornutum: A study of methods for calculating toxic values using different growth parameters. Arch. Environ. Contam. Toxicol. 14:333.

Adams, N., et al. 1986. Effect of acetone on the toxicity of four chemicals to Selenastrum capricornutum. Bull. Environ. Contam. Toxicol. 36:254-259.

Akasenova, Y. I., G. I. Bogucharskova, and M. I. Zozulina. 1969. The role of phytoplankton and bacterioplankton in the food of the dominant Cladocera of the Lower Don. Hydrobiol. 5(5):19-26.

APHA. 1985. Standard Methods for the Examination of Water and Wastewater. 16th Ed. American Public Health Association, Washington, D.C.

ASTM. 1988. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. ASTM E-729-88, American Society for Testing and Materials, Philadelphia, Pennsylvania.

ASTM. 1988. Standard guide for conducting acute toxicity tests on aqueous effluents with fishes, macroinvertebrates, and amphibians. ASTM E-1192-88, American Society for Testing and Materials, Philadelphia, Pennsylvania.

ASTM. 1988. Standard guide for conducting early life-stage toxicity tests with fishes. ASTM E-1241-88, American Society for Testing and Materials, Philadelphia, Pennsylvania.

Atkins, W. R. G. 1923. The phosphate content of fresh and salt waters in its relationship to the growth of algal plankton. J. Mar. Biol. Assoc. 13:119-150.

Barron, M. G., and I. R. Adelman. 1985. Temporal characterization of growth of fathead minnow (Pimephales promelas) larvae during sublethal hydrogen cyanide exposure. Comp. Biochem. Physiol. C. 81:341.

Bartlett, M.S. 1937. Some examples of statistical methods of research in agriculture and applied biology. J. Royal Statist. Soc. Suppl. 4:137-183.

Beckett, D. C., and P. A. Lewis. 1982. An efficient procedure for slide mounting of larval chironomids. Trans. Amer. Fish. Soc. 101(1):96-99.

Benoit, D. A. 1982. User's guide for conducting life-cycle chronic toxicity tests with fathead minnows (Pimephales promelas). Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA-600/8-81-011.

Benoit, D. A., F. A. Puglisi, and D. L. Olson. 1982. A fathead minnow, Pimephales promelas, early life stage toxicity test method evaluation and exposure to four organic chemicals. Environ. Pollut. (Series A) 28:189-197.

Berner, D. B. 1985. The taxonomy of Ceriodaphnia (Crustacea:Cladocera) in U. S. Environmental Agency Cultures. Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268, EPA/600/4-86/032.

Birge, W. J., and J. A. Black. 1981. In situ acute/chronic toxicological monitoring of industrial effluents for the NPDES biomonitoring program using fish and amphibian embryo/larval stages as test organisms. Office of Water Enforcement and Permits, U. S. Environmental Protection Agency, Washington, DC, OWEP-82-001.

Birge, W. J., J. A. Black, and B. A. Ramey. 1981. The reproductive toxicology of aquatic contaminants. Hazard assessments of chemicals, current developments, Vol. 1, Academic Press, Inc., p. 59-114.

Birge, W. J., J. A. Black, T. M. Short, and A. G. Westerman. 1989. A comparative ecological and toxicological investigation of an STP effluent and its receiving stream. Environ. Toxicol. Chem. 8(5): In Press.

Birge, W. J., J. A. Black, and A. G. Westerman. 1979. Evaluation of aquatic pollutants using fish and amphibian eggs as bioassay organisms. National Academy of Sciences, Washington, D.C., p. 108-118.

Birge, W. J., J. A. Black, and A. G. Westerman. 1985. Short-term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and complex effluents. Environ. Tox. Chem. 4:807-821.

Birge, W. J., J.A., Black, A. G., Westerman, and B. A. Ramey. 1983. Fish and amphibian embryos - A model system for evaluating teratogenicity. Fundam. Appl. Toxicol. 3: 237-242.

Birge, W. J., and R. A. Cassidy. 1983. Importance of structure-activity relationships in aquatic toxicology. Fundam. Appl. Toxicol. 3:359-368.

Black, J. A., W. J. Birge, A. G. Westerman, and P. C. Francis. 1983. Comparative aquatic toxicology of aromatic hydrocarbons. Fundam. Appl. Toxicol. 3: 353-358.

Blaise, C. R. 1986. Micromethod for acute aquatic toxicity assessment using the green alga Selenatrum capricornutum. Toxic. Assess. 1:377-385.

Burton, G. A., Jr., D. Nimmo, D. Murphey, and F. Payne. 1987. Stream profile determinations using microbial activity assays and Ceriodaphnia. Environ. Toxicol. Chem. 6:505-513.

Burton, G. A., Jr., A. Drotar, J. Lazorchak, and L. Bahls. 1987. Relationship of microbial activity and Ceriodphnia responses to mining

impacts on the Clark Fork River, Montana. Arch. Environ. Contam. Toxicol. 16:523-530.

Chengalath, R. 1982. A faunistic and ecological survey of the littoral Cladocera of Canada. Can. J. Zool. 60:2668-2682.

Chiaudani, G., and M. Vighi. 1974. The N:P ratio and tests with Selenastrum to predict eutrophication in lakes. Water Res. 8:1063-1069.

Conit, R. J. 1972. Phosphorus and algal growth in the Spokane River. Northwest Sci. 46(3):177-189.

Conover, W. J. 1980. Practical nonparametric statistics. Second edition. John Wiley and Sons, New York. p. 466-467.

Cooney, J. D., G. M. DeGraeve, E. L. Moore, T. L. Pollock, and W. D. Palmer. 1986. Effects of food and water quality on the culturing and toxicity testing of Ceriodaphnia. Draft Report, No. 526-P-5372, Battelle Laboratories, Columbus, Ohio.

Cowgill, U. M. 1987. Critical analysis of factors affecting the sensitivity of zooplankton and the reproducibility of toxicity test results. Wat. Res. 21(12):1453-1462.

Cowgill, U. M., K. I. Keating, and I. T. Tokahashi. 1985. Fecundity and longevity of Ceriodaphnia dubia/affinis in relation to diet at two different temperatures. J. Crust. Biol. 5(3):420-429.

Cowgill, U. M., D. P. Milazzo, and C. K. Meagher. 1988. New diet for Ceriodaphnia dubia. Bull. Environ. Contam. Toxicol. 41:304-309.

Cowgill, U. M., I. T. Takahashi, and S. L. Applegath. 1985. A comparison of the effect of four benchmark chemicals on Daphnia magna and Ceriodaphnia dubia-affinis tested at two different temperatures. Environ. Toxicol. Chem. 4:415-422.

Czeczuga, B., and E. Bobiatynskaksok. 1972. The extent of consumption of the energy contained in the food suspension by Ceriodaphnia reticulata (Jurine). In: Z. Kajak, and A. Hillbricht-Ilkowska, eds., Productivity Problems of Freshwaters, Proc. IBP-UNESCO Symposium, Kazimierz Dolny, Poland, May 6-12, 1970. Krakow, Poland.

DeGraeve, G. M. and J. D. Cooney. 1987. Ceriodaphnia: an update on effluent toxicity testing and research needs. Environ. Toxicol. Chem. 6:331-333.

DeGraeve, G. M., J. D. Cooney, T. L. Pollock, N. G. Reichenbach, J. H. Dean, M. D. Marcus, and D. O. McIntyre. 1988. Fathead minnow 7-day test: round robin study. Intra- and interlaboratory study to determine the reproducibility of the seven-day fathead minnow larval survival and growth test. Columbus, Battelle, Columbus, Ohio. Available from: American Petroleum Institute, 1220 L Street, NW, Washington, DC 20005, Rept. No. 4486.

DeGraeve, G. M., J. D. Cooney, B. H. Marsh, T. L. Pollock, and N. G. Reichenbach. 1989. Intra- and Interlaboratory study to determine the reproducibility of the seven-day Ceriodaphnia dubia survival and reproduction tests. Battelle, Columbus Division, Columbus, Ohio (In preparation).

DeWoskin, R. S. 1984. Good laboratory practice regulations: a comparison. Research Triangle Institute, Research Triangle Park, North Carolina. 63 pp.

Dixon, W. J., and F. J. Massey, Jr. 1983. Introduction to statistical analysis. Fourth edition. McGraw Hill, New York.

Draper, N. R., and J. A. John. 1981. Influential observations and outliers in regression. Technometrics 23:21-26.

Dunnett, C. W. 1955. Multiple comparison procedure for comparing several treatments with a control. J. Amer. Statist. Assoc. 50:1096-1121.

Dunnett, C. W. 1964. New table for multiple comparisons with a control. Biometrics 20:482.

Eagleson, K. W., D. L. Lenat, L. Ausley, and F. Winborne. 1988. Comparison of measured in-stream biological responses with responses predicted by Ceriodaphnia chronic toxicity test. North Carolina Division of Environmental Management, Raleigh, North Carolina (In Preparation).

Elnabarawy, M. T., A. N. Welter, and R. R. Tobideau. 1986. Relative sensitivity of three daphnid species to selected organic and inorganic chemicals. Environ. Toxicol. Chem. 5:393-398.

Eloranta, V., and O. Laitinen. 1982. The usefulness of the Selenastrum capricornutum algal assay to evaluate the toxic effects of pulp and paper mill effluents in lake water. Vatten 38:317-331.

FDA. 1978. Good laboratory practices for nonclinical laboratory studies. Part 58, Fed. Reg. 43(247):60013-60020, December 22, 1978.

Ferris, J. J., S. Kobyashi, and N. L. Clesceri. 1974. Growth of Selenastrum capricornutum in natural waters augmented with detergent products in wastewaters. Water Res. 8(12):1013-1020.

Filip, D. S., and E. J. Middelbrooks. 1975. Evaluation of sample preparation techniques for algal bioassays. Water Res. 9:581-585.

Finney, D. J. 1948. The Fisher-Yates test of significance in 2X2 contingency tables. Biometrika 35:145-156.

Finney, D. J. 1971. Probit analysis. Third Edition. Cambridge Press, New York. 668 pp.

Fitzgerald, G. P. 1972. Bioassay analysis of nutrient availability. In: Allen, H.E., and J.R. Kramer, eds. Nutrients in natural waters. John Wiley & Sons, Inc., New York. pp. 147-165.

Forsberg, C. G. 1972. Algal assay procedure. JWPCF 44(8):1623-1628.

Forsberg, C. G., and A. Forsberg. 1972. Algal growth potential test improves sewage settlement control. Ambio 1(1):26-29.

Gast, M. H., and W. A. Brungs. 1973. A procedure for separating eggs of the fathead minnow. Prog. Fish. Cult. 35:54.

Goldman, C. R., M. G. Tunzi, and R. Armstrong. 1969. Carbon-14 uptake as a sensitive measure of the growth of algal cultures. In: Middlebrooks, E. J., T. E. Maloney, C. F. Powers, and L. M. Kaack, eds., Proc. of the Eutroph. Bioassess. Workshop, 19-21 June, 1969. U.S. Pacific Northwest Water Laboratory, Corvallis, Oregon. p. 158-170.

Gophen, M. 1976. Temperature dependence on food intake, ammonia excretion and respiration in Ceriodaphnia reticulata (Jurine) (Lake Kinneret, Israel). Freshw. Biol. 6(5):451-455.

Gophen, M. 1977. Food and feeding habits of Mesocylops leuckarti (Claus) in Lake Kinneret (Israel). Freshwat. Biol. 7:513-518.

Gophen, M. 1979. Bathymetrical distribution and diurnal migrations of zooplankton in Lake Kinneret (Israel) with particular emphasis on Mesocyclop leuckarti (Claus). Hydrobiologia 64(3):199-208.

Gophen, M., B. Z. Cavari, and T. Berman. 1974. Zooplankton feeding on differentially labelled algae and bacteria. Nature 247:393-394.

Gotham, I. J., and G. Rhee. 1982. Effects of a hexachlorobiphenyl and pentachlorophenol on growth and photosynthesis of phytoplankton. J. Great Lakes Res. 8(2):328-335.

Gray, R. H., R. W. Hanf, D. D. Dauble, and J. R. Skalaski. 1982. Chronic effects of a coal liquid on a freshwater alga, Selenastrum capricornutum. Environ. Sci. Techn. 16(4):225-229.

Green, J. 1976. Changes in the zooplankton of Lakes Mutanda, Bunyonyi and Mulehe (Uganda). Freshw. Biol. 6:433-436.

Greene, J. C., C. L. Bartels, W. J. Warren-Hicks, B. R. Parkhurst, G. L. Linder, S. A. Peterson, and W. E. Miller. 1988. Protocols for short-term toxicity screening of hazardous waste sites. Environmental Research Laboratory, U. S. Environmental Protection Agency, Corvallis, Oregon, EPA/600/3-88/029.

Greene, J. C., W. E. Miller, T. Shiroyama, and E. Merwin. 1975. Toxicity of zinc to the green alga Selenastrum capricornutum as a function of phosphorus or ionic strength. In: Middlebrooks, E. J., T. E. Maloney,

C. F. Powers, and L. M. Kaack, eds., Proc. of the Eutroph. Bioassessment Workshop, 19-21 June, 1969. U. S. Pacific Northwest Water Laboratory, Corvallis, Oregon. EPA-660/3-75-034, p. 28-43.

Greene, J. C., R. A. Soltero, W. E. Miller, A. F. Gasperino, and T. Shiroyama. 1976. The relationship of laboratory algal assays to measurements of indigenous phytoplankton in Long Lake, Washington. In: Middlebrooks, E. J., E. H. Falkenborg, and T. E. Maloney, eds., Biostimulation and nutrient assessment. Ann Arbor Science Publ., Ann Arbor, Mich. p. 93-126.

Hall, W. S., R. L. Paulson, L W. Hall, Jr., and D. T. Burton. 1986. Acute toxicity of cadmium and sodium pentachlorophenate to daphnids and fish. Bull. Environ. Contam. Toxicol. 37:308-316.

Hamilton, M. A. 1986. Statistical analysis of cladoceran reproductivity test. Environ. Toxicol. Chem. 5:205-212.

Hart, W. B., Doudordoff, P. and Greenbank, J. 1945. The Evaluation of the toxicity of industrial wastes, chemicals and other substances to fresh-water fishes. The Atlantic Refining Company, Philadelphia, Pennsylvania.

Hedtke, S. F., C. W. West, K. N. Allen, T. J. Norberg-King, and D. I. Mount. 1986. Toxicity of pentachlorophenol to aquatic organisms under naturally varying and controlled environmental conditions. Environ. Toxicol. Chem. 5:531-542.

Hodges, J.L., Jr., and E.L. Lehmann. 1956. The efficiency of some nonparametric competitors of the t-test. Ann. Math. Statist. 31:625-642.

Hodgkiss, I. J., and L. T. H. Chan. 1976. Studies on Plover Cove Reservoir, Hong Kong. Part 4. The composition and spatial distribution of the crustacean zooplankton. Freshw. Biol. 6(4):301-315.

Horning, W. B., II, and C. I. Weber. 1985 Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Environmental Monitoring and Support Laboratory - Cincinnati, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-85/014.

Hughes, M. M., M. A. Heber, S. C. Schimmel, and W. J. Berry. 1987. Guidance manual for conducting complex effluent and receiving water larval fish growth-survival studies with the sheepshead minnow (Cyprinodon variegatus). Contribution No. X104. In: Schimmel, S. C., ed., Users guide to the conduct and interpretation of complex effluent toxicity tests at estuarine/marine sites. Environmental Research Laboratory, U. S. Environmental Protection Agency, Narragansett, Rhode Island, Contribution 796, 265 pp.

Keller, A. E., R. J. Dutton, G. Bitton, and T. L. Crisman. 1988. Chronic toxicity of hydrothol-191 to Ceriodaphnia dubia at 25 and 15°C. Bull. Environ. Contam. Toxicol. 41:233-240.

Kititsyna, L. A., and O. A. Sergeeva. 1976. Effect of temperature increases on the size and weight of some invertebrate populations in the cooling reservoir of the Kurakhovsk State Regional Electric Power Plant. Ekologiya 5:99-102.

Knight, J. T., and W. T. Waller. 1987. Incorporating Daphnia magna into the seven-day Ceriodaphnia effluent toxicity test method. Environ. Toxicol. Chem. 6:635-645.

Kopp, J. F. 1983. Guidelines and format for EMSL-Cincinnati methods. Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/8-83-020.

Lewis, M. A. 1986 Comparison of the effects of surfactants on freshwater phytoplankton communities in experimental enclosures and on algal population growth in the laboratory. Environ. Toxicol. Chem. 5:319-332.

Lynch, M. 1979. Predation, competition, and zooplankton community structure: An experimental study. Limnol. Oceanogr. 24(2):253-272.

Macek, K. J., and B. H. Sleight. 1977. Utility of toxicity tests with embryos and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes. In: F. L. Mayer and J. L. Hamelink, eds., Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, American Society for Testing and Materials, Philadelphia, Pennsylvania. p. 137-146.

Marking, L. L., and V. K. Dawson. 1973. Toxicity of quinaldine sulfate to fish. Invest. Fish Contr. No. 48, U. S. Fish and Wildlife Service, Washington, D.C. 8 pp.

Mayer, F. L., Jr., K. S. Mayer, and M. R. Ellersieck. 1986. Relation of survival to other endpoints in chronic toxicity tests with fish. Environ. Toxicol. Chem. 5:737-748.

Mayes, M. A., H. C. Alexander, D. C. Hopkins, and P. B. Latavitis. 1986. Acute and chronic toxicity of ammonia to freshwater fish: A site-specific study. Environ. Toxicol. Chem. 5:437-442.

McKim, J. M. 1977. Evaluation of tests with the early life stages of fish for predicting long-term toxicity. J. Fish. Res. Board Can. 34:1148-1154.

McNaught, D. C., and D. I. Mount. 1985. Appropriate durations and measures for Ceriodaphnia toxicity tests. In: R. C. Bahner and D. J. Hansen, eds. Aquatic Toxicology and Hazard Assessment: Eighth Symposium. ASTM STP 891. American Society for Testing and Materials, Philadelphia, Pennsylvania, p. 375-381.

McPhee, C. 1961. Bioassay of algal production in chemically altered waters. Limnol. Oceanogr. 6:416-422.

Michaels, R. A., R. G. Rowland, and C. F. Wurster. 1982. Polychlorinated biphenyls (PCB) inhibit photosynthesis per cell in the marine diatom Thalassiosira pseudonana. Environ. Pollut. (Ser. A.) 27:9-14.

Miller, R. G. 1981. Simultaneous statistical inference. Springer-Verlag, New York. 299 pp.

Miller, W. E., J. C. Greene, and T. Shiroyama. 1978. The Selenastrum capricornutum Printz algal assay bottle test. Environmental Research Laboratory, U. S. Environmental Protection Agency, Corvallis, Oregon, EPA-600/9-78-018.

Miller, W. E., and T. E. Maloney. 1971. Effects of secondary and tertiary wastewater effluents on algal growth in a lake-river system. JWPCF 43(12):2361-2365.

Mount, D. I., and T. J. Norberg. 1984. A seven-day life-cycle cladoceran test. Environ. Toxicol. Chem. 3:425-434.

Mount, D. I., and T. J. Norberg-King, eds. 1985. Validity of effluent and ambient toxicity tests for predicting biological impact, Scippo Creek, Circleville, Ohio. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/3-85/044.

Mount, D. I., and T. J. Norberg-King. 1986. Validity of effluent and ambient toxicity tests for predicting biological impact, Kanawha River, Charleston, West Virginia. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/3-86/006.

Mount, D. I., T. J. Norberg-King, R. Keen, and J. T. Taraldsen. 1987. A reference tests water for cladocerans. Abstract, 11th Annual Symposium, Aquatic Toxicology and Hazard Assessment, American Society for Testing and Materials, May 10-12, 1987, Cincinnati, Ohio.

Mount, D. I., T. J. Norberg-King, and A. E. Steen. 1986a. Validity of effluent and ambient toxicity tests for predicting biological impact, Naugatuck River, Connecticut. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/8-86/005.

Mount, D. I., A. E. Steen, and T. J. Norberg-King, eds. 1985b. Validity of effluent and ambient toxicity testing for predicting biological impact on Five Mile Creek, Birmingham, Alabama. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/8-85/015.

Mount, D. I., A. E. Steen, and T. J. Norberg-King, eds. 1985b. Validity of effluent and ambient toxicity tests for predicting biological impact, Ohio River, near Wheeling, West Virginia. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/3-85/071.

Mount, D. I., A. E. Steen, and T. J. Norberg-King. 1986b. Validity of effluent and ambient toxicity tests for predicting biological impact, Back River, Baltimore Harbor, Maryland. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/8-86/001.

Mount, D. I., and C. E. Stephan. 1967. A method for establishing acceptable toxicant limits for fish - Malathion and 2,4-D. Trans. Am. Fish. Soc. 96:185-193.

Mount, D. I., N. A. Thomas, T. J. Norberg, M. T. Barbour, T. H. Roush, and W. F. Brandes. 1984. Effluent and ambient toxicity testing and instream community response on the Ottawa River, Lima, Ohio. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/3-84/080.

Neiheisel, T. W., J. R. Menkendick, F. A. Kessler, W. B. Horning, and C. I. Weber. 1988a. Multi-laboratory study of Ceriodaphnia chronic toxicity test. In Press.

Neiheisel, T. M., W. B. Horning, II, B. M. Austern, D. F. Bishop, T. L. Reed, and J. F. Estenik. 1988b. Toxicity reduction at municipal wastewater treatment plants. JWPCF 60(1):57-67.

Norberg, T. J., and D. I. Mount. 1983. A seven-day larval growth test. Presented at the Annual Meeting, Society of Environmental Toxicology and Chemistry, November 6-9, 1983, Arlington, Virginia.

Norberg, T. J., and D. I. Mount. 1985. A new fathead minnow (Pimephales promelas) subchronic toxicity test. Environ. Toxicol. Chem. 4(5):711-718.

Norberg, T. J., and D. I. Mount. 1985. Diets for Ceriodaphnia reticulata life-cycle tests. In: R. D. Cardwell, R. Purdy, and R. C. Bahner, eds., Aquatic Toxicology and Hazard Assessment, Seventh Symposium, ASTM STP 854, American Society for Testing and Materials, Philadelphia, p. 42-52.

Norberg-King, T. 1987. Relative sensitivity of the fathead minnow (Pimephales promelas) seven day subchronic test to the early life stage and life cycle tests. M. S. Thesis, Montana State Univ, Bozeman, Montana.

Norberg-King, I. 1989. An evaluation of the fathead minnow seven-day subchronic test for estimating chronic toxicity. Environ. Toxicol. Chem. In Press.

Norberg-King, T. J., and D. I. Mount, eds. 1986 Validity of effluent and ambient toxicity tests for predicting biological impact, Skeleton Creek, Enid, Oklahoma. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA/600/8-86/002.

O'Brien, W. J. 1972. Limiting factors in phytoplankton algae: their meaning and measurement. Science 178:616.

O'Brien, W. J. 1974. Filtering rate of Ceriodaphnia reticulata in pond waters of varying phytoplankton concentration. Amer. Midl. Nat. 91(2):509-512.

Owsley, J. A., and D. E. McCauley. 1986. Effect of extended sublethal exposure to sodium selenite on Ceriodaphnia affinis. Bull. Environ. Contam. Toxicol. 36:876-880.

Parker, M. 1977. The use of algal bioassays to predict the short and long term changes in algal standing crop which result from altered phosphorus and nitrogen loadings. Water Res. 11:719-725.

Pearson, E.S., and T.O. Hartley. 1962. Biometrika tables for statisticians. Vol. 1. Cambridge Univ. Press, England. p. 65-70.

Peltier, W. 1978. Methods for measuring the acute toxicity of effluents to aquatic organisms. Second edition. Environmental Monitoring and Support Laboratory - Cincinnati, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-78-012.

Peltier, W., and C. I. Weber. eds. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. Third edition. Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA 600/4-85-013, 230 pp.

Pennak, R. W. 1978. Freshwater invertebrates of the United States. Second edition. Ronald Press, New York, 769 pp.

Pickering, Q. H. 1988. Evaluation and comparison of two short-term fathead minnow tests for estimating chronic toxicity. Wat. Res. 22(7):883-893.

Rehnberg, B. G., D. A. Schultz, and R. L. Raschke. 1982. Limitations of electronic counting in reference to algal assays. JWPCF 54(2):181-186.

Sachdev, D. R., and N. L. Clesceri. 1978. Effects of organic fractions from secondary effluent on Selenastrum capricornutum (Kutz.). JWPCF 52:1810-1820.

Scheffe, H. 1959. The analysis of variance. John Wiley and Sons, New York, 477 pp.

Schoenberg, S. A., and A. E. Maccubbin. 1985. Relative feeding rates of free and particule-bound bacteria by freshwater macrozooplankton. Limnol. Oceanogr. 30(5):1084-1090.

Shapiro, S. S., and M. B. Wilk. 1965. An analysis of variance test for normality (complete samples). Biometrika 52:591-611.

Shuba, T., and R. R. Costa. 1982. Development and growth of Ceriodaphnia reticulata embryos. Trans. Amer. Microsc. Soc. 9(3):429-435.

Skulberg, O. 1964. Algal problems related to the eutrophication of European water supplies, and a bioassay method to assess fertilizing influences of pollution on inland waters. In: D. F. Jackson (ed.), Algae and man. Plenum Press, New York, p. 262-299.

Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. Seventh edition. Iowa State University Press, Ames, 593 pp.

Spencer, D. F., and L. H. Nichols. 1983. Free nickel ion inhibits growth of two species of green algae. Environ. Pollut. (Ser. A.) 31:97-104.

Steel, R. G. D. 1959. A multiple comparison rank sum test: treatments versus control. Biometrics 15:560-572.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics with special reference to biological sciences. McGraw-Hill Publ., New York.

Takahashi, I. T., U. M. Cowgill, and P. G. Murphy. 1987. Comparison of ethanol toxicity to Daphnia magna and Ceriodaphnia dubia tested at two different temperatures: static acute toxicity test results. Bull. Environ. Contam. Toxicol. 39:229-236.

Tallqvist, T. 1973. Algal assay procedure (Bottle Test) at the Norwegian Institute for Water Research. In: Algal assays in water pollution research: Proceedings from a Nordic Symposium, Oslow, 25-29 October, 1972. Nordforsk. Secretariat of Environm. Sci., Helsinki. Publ. 1973-2, p. 5-17.

Tarzwell, C. M. 1971. Bioassays to determine allowable waste concentrations in the aquatic environment. In: H. A. Cole (Organizer), A discussion on biological effects of pollution in the sea. Proc. Roy. Soc. Lond. B 177(1048):279-285.

Thomas, R. E., and R. L. Smith. 1975. Assessing treatment process efficiency with the algal assay test. In: E. J. Middlebrooks, T. E. Maloney, C. F. Powers, and L. M. Kaack, eds., Proc. of the Eutroph. Bioassessment Workshop, 19-21 June, 1969. U. S. Pacific Northwest Water Laboratory, Corvallis, Oregon, EPA-660/3-75-034, p. 244-248.

Thurston, R. V., R. C. Russo, E. L. Meyn, and R. K. Zaidel. 1986. Chronic toxicity of ammonia to fathead minnows. Trans. Amer. Fish. Soc. 115:196-207.

USEPA. 1971. Algal assay procedures: Bottle test. National Eutrophication Program. U. S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, Oregon, 82 pp.

USEPA. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota, EPA-660/3-75-009.

USEPA. 1977. Occupational health and safety manual. Office of Planning and Management, U. S. Environmental Protection Agency, Washington, DC.

USEPA. 1979a. Handbook for analytical quality control in water and wastewater laboratories. U. S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, EPA-600/4-79-019.

USEPA. 1979b. Methods for chemical analysis of water and wastes. Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-79-020.

USEPA. 1979c. Interim NPDES compliance biomonitoring inspection manual. Office of Water Enforcement, U. S. Environmental Protection Agency, Washington, DC, MCD-62.

USEPA. 1979d. Good laboratory practice standards for health effects. Paragraph 772.110-1, Part 772 - Standards for development of test data. Fed. Reg. 44:27362-27375, May 9, 1979.

USEPA. 1980a. Appendix B - Guidelines for Deriving Water Quality Criteria for the Protection of Aquatic Life and Its Uses. Federal Register, Vol. 45, No. 231, Friday, November 28, 1980.

USEPA. 1980b. Proposed good laboratory practice guidelines for toxicity testing. Paragraph 163.60-6. Fed. Reg. 45:26377-26382, April 18, 1980.

USEPA. 1980c. Physical, chemical, persistence, and ecological effects testing; good laboratory practice standards (proposed rule). 40 CFR 772, Fed. Reg. 45:77353-77365, November 21, 1980.

USEPA. 1985. Technical Support Document for Water Quality-based Control. Office of Water, U. S. Environmental Protection Agency, Washington, DC.

USEPA. 1986. Ambient water quality criteria for pentachlorophenol. Criteria and Standards Division, Office of Water Regulations and Standards, U. S. Environmental Protection Agency, Washington, DC, EPA/440/5-86/009.

USEPA. 1987a. Ambient water quality criteria for zinc. Criteria and Standards Division, Office of Water Regulations and Standards, U. S. Environmental Protection Agency, Washington, DC, EPA/440/5-87/003.

USEPA. 1987b. Permit writer's guide to water quality-based permitting for toxic pollutants. Office of Water, U. S. Environmental Protection Agency, Washington, DC, EPA/440/4-87/005.

Vigerstad, T. J., and L. J. Tilly. 1977. Hyperthermal effluent effects on heleoplanktonic Cladocera and the influence of submerged macrophytes. Hydrobiol. 55(1):81-86.

Walsh, G. E., and S. V. Alexander. 1980. A marine algal bioassay method: Results with pesticides and industrial wastes. Water, Air, and Soil Pollut. 13:45-55.

Walsh, G. E., L. H. Bahner, and W. B. Horning. 1980. Toxicity of textile mill effluents to freshwater and estuarine algae, crustaceans, and fishes. Environ. Pollut. (Ser. A.) 21:169-179.

Walters, D. B., and C. W. Jameson. 1984. Health and safety for toxicity testing. Butterworth Publ., Woburn, Massachusetts.

Ward, G. S., and P. R. Parrish. 1980. Evaluation of early life stage toxicity tests with embryos and juveniles of sheepshead minnows. In: J. G. Eaton, P. R. Parrish and A. C. Hendricks, Aquatic Toxicology, ASTM STP 707, American Society for Testing and Materials, Philadelphia, Pennsylvania, p. 243-247.

Weber, C. I., ed. 1973. Biological field and laboratory methods for measuring the quality of surface waters and wastes. Office of Research and Development, U. S. Environmental Protection Agency, Cincinnati, Ohio, EPA 670/4-73-001, 200 pp.

Weiss, C. M. 1975. Field investigation of the algal assay procedure on surface waters of North Carolina. In: Middlebrooks, E. J., E. H. Falkenborg, and T. E. Maloney, eds., Biostimulation and nutrient assessment. Ann Arbor Science Publ., Ann Arbor, Michigan, p. 93-126.

Weiss, C. M. 1976. Evaluation of the algal assay procedure. U. S. Environmental Protection Agency, Corvallis, Oregon, EPA-660/3-76-064.

Weiss, C. M., and R. W. Helms. 1971. Interlaboratory precision test: An eight-laboratory evaluation of the Provisional Algal Assay Procedure Bottle Test. National Eutrophication Research Program. U. S. Environmental Protection Agency, Corvallis, Oregon.

Winner, R. W. 1988. Evaluation of the relative sensitivities of 7-D Daphnia magna and Ceriodaphnia dubia toxicity tests for cadmium and sodium pentachlorophenate. Environ. Toxicol. Chem. 7:153-159.

Woltering, D. M. 1984. The growth response in fish chronic and early life stage toxicity tests: A critical review. Aquat. Toxicol. 5:1-21.

Wong, P. T. S., Y. K. Chau, and P. L. Luxon. 1978. Toxicity of a mixture of metals on freshwater algae. J. Fish. Res. Board. Can. 35:479-481.

Wong, C. K. 1981. Predatory feeding behavior of Epischura lacustris (Copepoda:Calanoida) and prey defense. Can. J. Fish Aquat. Sci. 38(3):275-279.

Wright, J. Jr., F. A. Camp, and J. Cairns, Jr. 1974. Preliminary algal bioassays to determine nutrients limiting algal productivity in Mountain Lake, Virginia. Assoc. SE Biol. Bull. 21(2):92.

Yoshioka, Y., et al. 1986. Correlation of the five test methods to assess chemical toxicity and relation to physical properties. Ecotoxicol. Environ. Saf. 12:15.

Young, T. C., J. V. DePinto, S. E. Flint, M. S. Switzenbaum, and J. K. Edzwald. 1982. Algal availability of phosphorus in municipal wastewater. JWPCF 54(11):1505-1516.

APPENDICES

APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS[1]

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and Bonferroni's T-test 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 deal with (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 insure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test vessels, and the arrangement of treatments and replicate vessels is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially by level of concentration 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 is described using the Fathead Minnow Larval Survival and Growth test. For a test design with five treatments, a control, and four replicates at each treatment, there would be 24 experimental units, i.e., 24 positions to be randomized. There are several ways to randomly assign the positions. Random numbers may be selected from a random numbers table or may be generated by computer software.

2.3 In this example, the first four random numbers selected would be used for the four control replicates. The selection of random numbers would continue, four at a time, each group being assigned to the four replicates of a given test concentration, progressing from the lowest concentration to the highest. The rank ordering of these random numbers would determine the relative positioning for the controls and concentration levels.

2.4 The result of this randomization procedure is presented in Table A.1, using an effluent concentration series of 1.0%, 3.2%, 10.0%, 32.0%, and 100%.

TABLE A.1. RANDOMIZATION OF THE POSITIONS OF EXPERIMENTAL UNITS USING A DESIGN OF FOUR ROWS AND SIX COLUMNS

100%	10.0%	1.0%	100%	3.2%	1.0%
3.2%	3.2%	100%	32.0%	Control	Control
10.0%	Control	1.0%	32.0%	10.0%	32.0%
3.2%	Control	10.0%	32.0%	100%	1.0%

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).

TABLE A.2. TABLE OF RANDOM NUMBERS[1]

10	09	73	25	33	76	52	01	35	86	34	67	35	48	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	17	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	54	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	58	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	89	39

[1]From Dixon and Massey, 1983.

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS[1]

1. INTRODUCTION

1.1 Dunnett's Procedure and Bonferroni's T-test 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 non-parametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or non-parametric 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 A formal test for normality is the Shapiro-Wilk's Test. 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 Kolomogorov "D" statistic 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 and the mean and standard deviation of the observations at each concentration, including the control, are listed in Table B.1.

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.

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

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

Where: X_i = the centered observations and $\bar{X}$ is the overall mean of the centered observations. For this set of data, $\bar{X} = 0$, and D = 0.0412.

2.5 Order the centered observations from smallest to largest.

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

Where $X^{(i)}$ denotes the ith ordered observation. The ordered observations are listed in Table B.3.

2.6 From Table B.4, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$, where k is approximately n/2. For the data in this example, n = 20, k = 10. The a_i values are listed in Table B.5.

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. Thus, the conclusion of the test is that the data are normally distributed.

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.

TABLE B.1. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) FOR THE SHAPIRO-WILK'S TEST

		NaPCP Concentration (ug/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

TABLE B.2. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

		NaPCP Concentration (ug/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

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

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST[1]

i \ n	2	3	4	5	6	7	8	9	10
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
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	0.3290	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
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	0.0823	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

[1]Taken from: Conover, 1980.

TABLE B.4 COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Continued)

i \ n	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	0.1683	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	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	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	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

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	$X(11) - X(10)$

TABLE B.6 QUANTILES OF THE SHAPIRO-WILK'S TEST STATISTIC[1]

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

[1]Taken from Conover, 1980.

3. TEST FOR HOMOGENEITY OF VARIANCE

3.1 For Dunnett's Procedure and Bonferroni's T-test, 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.7, together with the calculated variance for the control and each toxicant concentration.

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

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

Where: V_i = Degrees of freedom for each toxicant concentration and control

p = Number of levels of toxicant concentration including the control

$\overline{S}^2$ = The average of the individual variances.

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

ln = Log_e

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, $v_i = 3$, $p = 5$, $\overline{S}^2 = 0.0027$, and $C = 1.133$. The calculated B value is:

$$B = \frac{(15)[\ln(0.0027)] - 3 \sum_{i=1} \ln(S_i^2)}{1.133}$$

$$= \frac{15(-5.9145) - 3(-32.4771)]}{1.133}$$

$$= 7.691$$

3.5 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.

TABLE B.7. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		NaPCP Concentration (ug/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

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 a non-parametric 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[1]

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the i-th 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

[1]From: Peltier and Weber (1985).

or Bonferroni's, 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 dead or "affected" organisms)/(number exposed).

Example: If 8 of 20 animals in a given treatment die:

$$RP = 8/20$$
$$= 0.40$$

4.2.4 Transform each RP to arc sine, as follows.

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = arc sine $(RP)^{0.5}$.

Example: If RP = 0.40:

$$\text{Angle} = \text{arc sine } (0.40)^{0.5}$$
$$= \text{arc sine } 0.6325$$
$$= 0.6847 \text{ radians}$$

4.2.4.2 Modification of the arc sine when RP = 0.

Angle (in radians) = arc sine $(1/4N)^{0.5}$

Where: N = Number of animals/treatment

Example: If 20 animals are used:

Angle = arc sine $(1/80)^{0.5}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine 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]

1.1 Dunnett's Procedure 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, Bonferroni's T-test 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. 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:

TABLE C.1. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR DUNNETT'S PROCEDURE

		NaPCP Concentration (ug/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
Total(T_i)	2.858	2.695	2.708	2.494	2.322

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:

Total Sum of Squares: $SST = \sum_{ij} Y_{ij}^2 - G^2/N$

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

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

Where: G = The grand total of all sample observations; $G = \sum_{i} T_i$

N = The total sample size; $N = \sum_{i} n_i$

n_i = The number of replicates for concentration "i".

T_i = The total of the replicate measurements for concentration "i".

Y_{ij} = The jth observation for concentration "i".

1.4 Calculations:

Total Sum of Squares: $SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 8.635 - (13.077)^2/20$

$= 0.085$

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

$= 8.594 - (13.077)^2/20$

$= 0.044$

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

$= 0.085 - 0.044$

$= 0.041$

1.5 Prepare the ANOVA table as follows:

TABLE C.2 GENERALIZED 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	

*p = Number of different concentrations, including the control.

1.6 The completed ANOVA table for this data is provided below:

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	DF	SS	Mean Square
Between	5 - 1 = 4	0.044	0.011
Within	20 - 5 = 15	0.041	0.0027
Total	19	0.085	

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

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

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".

1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES.

NaPCP Concentration (ug/L)	i	t_i
32	2	1.081
64	3	1.000
128	4	2.432
256	5	3.622

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_4 and t_5 are greater than 2.36, the 128 ug/L and 256 ug/L concentrations have significantly lower growth than the control. Hence the NOEC and LOEC for growth are 64 ug/L and 128 ug/L, respectively.

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:

$$MSD = 2.36\ (0.052)[\sqrt{(1/4) + (1/4)}] = 2.36\ (0.052)(\sqrt{2/4})$$
$$= 2.36\ (0.052)(0.707)$$
$$= 0.087$$

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 12% 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:

$$0.714 - 0.087 = 0.627$$

Step 2. Obtain untransformed values for the control mean (0.714) and the difference (0.627) obtained in Step 1, above.

$$[Sine(0.714)]^2 = 0.429$$
$$[Sine(0.627)]^2 = 0.344$$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$$MSD_u = 0.429 - 0.344 = 0.085$$

In this case, the MSD would represent a 19.8% decrease in survival from the control [(0.085/0.429)(100)].

1.12 Table of Dunnett's "t" values.

TABLE C.5. DUNNETT'S "T" VALUES[1]

(One-tailed) $d^{\alpha}_{k,\nu}$

	$\alpha = .05$									$\alpha = .01$								
ν \ k	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.68	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.60	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	3.88	4.07	4.21	4.33	4.43	4.51	4.59
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.66	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92	2.90	3.29	3.51	3.67	3.79	3.88	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	3.66	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	3.48	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	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	2.66	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.51	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	2.88	3.05	3.17	3.26	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	3.42
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	2.39	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57	2.49	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	2.82	2.92	2.99	3.05	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	3.00	3.04	3.08	3.12
120	1.66	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.89	2.94	2.99	3.03	3.06
∞	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.38	2.42	2.33	2.56	2.68	2.77	2.84	2.89	2.93	2.97	3.00

[1]From: Miller, 1981.

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 based on Dunnett, C. W., 1955, "Multiple Comparison Procedure for Comparing Several Treatments with a Control," J. Amer. Statist. Assoc. 50:1096-1121.

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, Bonferroni's T-test is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN (XT and AT) by D. L. Weiner, Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, Ohio 45268. A complete listing of the program is contained in EPA/600/4-87/028. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.6 Data Input and Output

2.6.1 Reproduction data from a Ceriodaphnia survival and reproduction test (Table C.6) are used to illustrate the data input and output for this program.

2.6.2 Data Input

2.6.2.1 When the program is entered, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform ANOVA (analysis) on existing data set
4. Exit the program

TABLE C.6. SAMPLE DATA FOR DUNNETT'S PROGRAM.
CERIODAPHNIA 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

2.6.2.2 When Option 1 (Create a data file) is selected, the program prompts the user for the following information:

1. Number of groups, including control
2. For each group:
   - Number of observations
   - Data for each observation

2.6.2.3 After the data have been entered, the user may save the file on a disk, and the program returns to the main menu (see below).

2.6.2.4 Sample data input is shown below.

```
 1) Create a data file
 2) Edit a data file
 3) Perform ANOVA on existing data set
 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
```

2.6.3 Program Output

2.6.3.1 When Option 3 (Perform ANOVA on existing data set) is selected from the main 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 below).

```
 1) Create a data file
 2) Edit a data file
 3) Perform ANOVA on existing data set
 4) Stop

Your choice ? 3


File name ? cerio


 Available Transformations
     1)  no transform
     2)  square root
     3)  log10
     4)  arcsine square root

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
```

2.6.3.2 Summary statistics 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.

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

Minumum detectable difference for Dunnett's test = -5.628560
This difference corresponds to -25.13 percent of control

Between groups 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

APPENDIX D

BONFERRONI'S T-TEST

1. Bonferroni's T-test 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. Bonferroni's T-test 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 Bonferroni's T-test is provided below. The data used in the example are the same as in Appendix C, except that the third replicate from the 256 ug/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 LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BONFERRONI'S T-TEST

		NaPCP Concentration (ug/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	(LOST)
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.572
Total(T_i)	2.858	2.695	2.708	2.494	1.716

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:

Total Sum of Squares: $SST = \sum_{ij} Y_{ij}^2 - G^2/N$

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

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

Where: G = The grand total of all sample observations; $G = \sum_{i} T_i$

N = The total sample size; $N = \sum_{i} n_i$

n_i = The number of replicates for concentration "i".

T_i = The total of the replicate measurements for concentration "i".

Y_{ij} = The jth observation for concentration "i".

3.2 Calculations:

Total Sum of Squares: $SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 8.268 - (12.471)^2/19$

$= 0.082$

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

$= 8.228 - (12.471)^2/19$

$= 0.042$

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

$= 0.082 - 0.042$

$= 0.040$

3.3 Prepare the ANOVA table as follows:

TABLE D.2. GENERALIZED 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	

*p = Number of different concentrations, including the control.

3.4 *The completed ANOVA table for this data is provided below:*

TABLE D.3. COMPLETED ANOVA TABLE FOR BONFERRONI'S T-TEST

Source	DF	SS	Mean Square
Between	5 - 1 = 4	0.042	0.0105
Within	19 - 5 = 14	0.040	0.0028
Total	18	0.082	

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 (ug/L)	i	t_i
32	2	1.067
64	3	0.987
128	4	2.402
256	5	3.507

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 ug/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 ug/L and 256 ug/L respectively.

TABLE D.5. CRITICAL VALUES FOR BONFERRONI'S "T"
P = 0.05 CRITICAL LEVEL, ONE TAILED

D.F.	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	3.107	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	2.466	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.201	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	2.780	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	2.958	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	2.286	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
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	2.524	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
INF.	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]

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to the Dunnett's Procedure, and may be applied to the 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 critical value in Table E.5. 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 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.

4. For each control and concentration combination, combine the data and arrange the observations 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 the 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.

6. For this set of data, we wish to 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, we are only concerned with comparing 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 2.3 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.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: DATA FOR CERIODAPHNIA 7-DAY CHRONIC TEST

Effluent Concentration	Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
Cont	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

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[a]	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)

[a]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	63.5
12	76
25	55

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES

	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	271	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

From Steel, 1959.

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a non-parametric 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 vs. 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 below. 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 the observation.

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 vs. 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, we wish to 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, we are only concerned with comparing 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.1. EXAMPLE OF WILCOXON'S RANK SUM TEST: DATA FOR CERIODAPHNIA 7-DAY CHRONIC TEST

Effluent Concentration	Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
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

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[a]	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)

[a]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 VS. 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 VS. 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 VS. 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
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]

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 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 pairwise comparison error rate of 0.05 is suggested rather that an experimentwise error rate.

2. The basis for Fisher's Exact Test is a 2x2 contingency table. From the 2x2 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 (Table G.5). However, in order to use this table the contingency table must be arranged in the following format:

TABLE G.1. FORMAT FOR CONTINGENCY TABLE

	Number of		Number of Observations
	Successes	Failures	
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 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 Ceriodaphnia 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 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.

TABLE G.3. 2X2 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

7. Since $10/10 \geq 8/9$, 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 $10/10 \geq 1/9$. 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.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]

	a	Probability			
		0·05	0·025	0·01	0·005
A=3 B=3	3	**0** ·050	—	—	—
A=4 B=4	4	**0** ·014	**0** ·014	—	—
3	4	**0** ·029	—	—	—
A=5 B=5	5	**1** ·024	**1** ·024	**0** ·004	**0** ·004
	4	**0** ·024	**0** ·024	—	—
4	5	**1** ·048	**0** ·008	**0** ·008	—
	4	**0** ·040	—	—	—
3	5	**0** ·018	**0** ·018	—	—
2	5	**0** ·048	—	—	—
A=6 B=6	6	**2** ·030	**1** ·008	**1** ·008	**0** ·001
	5	**1** ·040	**0** ·008	**0** ·008	—
	4	**0** ·030	—	—	—
5	6	**1** ·015⁺	**0** ·015⁺	**0** ·002	**0** ·002
	5	**0** ·013	**0** ·013	—	—
	4	**0** ·045⁺	—	—	—
4	6	**1** ·033	**0** ·005⁻	**0** ·005⁻	**0** ·005⁻
	5	**0** ·024	**0** ·024	—	—
3	6	**0** ·012	**0** ·012	—	—
	5	**0** ·048	—	—	—
2	6	**0** ·036	—	—	—
A=7 B=7	7	**3** ·035⁻	**2** ·010⁺	**1** ·002	**1** ·002
	6	**1** ·015⁻	**1** ·015⁻	**0** ·002	**0** ·002
	5	**0** ·010⁺	**0** ·010⁺	—	—
	4	**0** ·035⁻	—	—	—
6	7	**2** ·021	**2** ·021	**1** ·005⁻	**1** ·005⁻
	6	**1** ·025⁺	**0** ·004	**0** ·004	**0** ·004
	5	**0** ·016	**0** ·016	—	—
	4	**0** ·049	—	—	—
5	7	**2** ·045⁺	**1** ·010⁺	**0** ·001	**0** ·001
	6	**1** ·045⁺	**0** ·008	**0** ·008	—
	5	**0** ·027	—	—	—
4	7	**1** ·024	**1** ·024	**0** ·003	**0** ·003
	6	**0** ·015⁺	**0** ·015⁺	—	—
	5	**0** ·045⁺	—	—	—
3	7	**0** ·008	**0** ·008	**0** ·008	—
	6	**0** ·033	—	—	—
2	7	**0** ·028	—	—	—
A=8 B=8	8	**4** ·038	**3** ·013	**2** ·003	**2** ·003
	7	**2** ·020	**2** ·020	**1** ·005⁺	**0** ·001
	6	**1** ·020	**1** ·020	**0** ·003	**0** ·003
	5	**0** ·013	**0** ·013	—	—
	4	**0** ·038	—	—	—
7	8	**3** ·026	**2** ·007	**2** ·007	**1** ·001
	7	**2** ·035⁻	**1** ·009	**1** ·009	**0** ·001
	6	**1** ·032	**0** ·006	**0** ·006	—
	5	**0** ·019	**0** ·019	—	—
6	8	**2** ·015⁻	**2** ·015⁻	**1** ·003	**1** ·003
	7	**1** ·016	**1** ·016	**0** ·002	**0** ·002
	6	**0** ·009	**0** ·009	**0** ·009	—
	5	**0** ·028	—	—	—
5	8	**2** ·035⁻	**1** ·007	**1** ·007	**0** ·001
	7	**1** ·032	**0** ·005⁻	**0** ·005⁻	**0** ·005⁻
	6	**0** ·016	**0** ·016	—	—
	5	**0** ·044	—	—	—
4	8	**1** ·018	**1** ·018	**0** ·002	**0** ·002
	7	**0** ·010⁺	**0** ·010⁺	—	—
	6	**0** ·030	—	—	—
3	8	**0** ·006	**0** ·006	**0** ·006	—
	7	**0** ·024	**0** ·024	—	—
2	8	**0** ·022	**0** ·022	—	—
A=9 B=9	9	**5** ·041	**4** ·015⁻	**3** ·005⁻	**3** ·005⁻
	8	**3** ·025⁻	**3** ·025⁻	**2** ·008	**1** ·002
	7	**2** ·028	**1** ·008	**1** ·008	**0** ·001
	6	**1** ·025⁻	**1** ·025⁻	**0** ·005⁻	**0** ·005⁻
	5	**0** ·015⁻	**0** ·015⁻	—	—
	4	**0** ·041	—	—	—
8	9	**4** ·029	**3** ·009	**3** ·009	**2** ·002
	8	**3** ·043	**2** ·013	**1** ·003	**1** ·003
	7	**2** ·044	**1** ·012	**0** ·002	**0** ·002
	6	**1** ·036	**0** ·007	**0** ·007	—
	5	**0** ·020	**0** ·020	—	—
7	9	**3** ·019	**3** ·019	**2** ·005⁻	**2** ·005⁻
	8	**2** ·024	**2** ·024	**1** ·006	**0** ·001
	7	**1** ·020	**1** ·020	**0** ·003	**0** ·003
	6	**0** ·010⁺	**0** ·010⁺	—	—
	5	**0** ·029	—	—	—
6	9	**3** ·044	**2** ·011	**1** ·002	**1** ·002
	8	**2** ·047	**1** ·011	**0** ·001	**0** ·001
	7	**1** ·035⁻	**0** ·006	**0** ·006	—
	6	**0** ·017	**0** ·017	—	—
	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) (CONTINUED)

	a	Probability			
		0·05	0·025	0·01	0·005
A=9 B=5	9	2 ·027	1 ·005⁻	1 ·005⁻	1 ·005⁻
	8	1 ·023	1 ·023	0 ·003	0 ·003
	7	0 ·010⁺	0 ·010⁺	—	—
	6	0 ·028	—	—	—
4	9	1 ·014	1 ·014	0 ·001	0 ·001
	8	0 ·007	0 ·007	0 ·007	—
	7	0 ·021	0 ·021	—	—
	6	0 ·049	—	—	—
3	9	1 ·045⁺	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·018	0 ·018	—	—
	7	0 ·045⁺	—	—	—
2	9	0 ·018	0 ·018	—	—
A=10 B=10	10	6 ·043	5 ·016	4 ·005⁺	3 ·002
	9	4 ·029	3 ·010⁻	3 ·010⁻	2 ·003
	8	3 ·035⁻	2 ·012	1 ·003	1 ·003
	7	2 ·035⁻	1 ·010⁻	1 ·010⁻	0 ·002
	6	1 ·029	0 ·005⁺	0 ·005⁺	—
	5	0 ·016	0 ·016	—	—
	4	0 ·043	—	—	—
9	10	5 ·033	4 ·011	3 ·003	3 ·003
	9	4 ·050⁻	3 ·017	2 ·005⁻	2 ·005⁻
	8	2 ·019	2 ·019	1 ·004	1 ·004
	7	1 ·015⁻	1 ·015⁻	0 ·002	0 ·002
	6	1 ·040	0 ·008	0 ·008	—
	5	0 ·022	0 ·022	—	—
8	10	4 ·023	4 ·023	3 ·007	2 ·002
	9	3 ·032	2 ·009	2 ·009	1 ·002
	8	2 ·031	1 ·008	1 ·008	0 ·001
	7	1 ·023	1 ·023	0 ·004	0 ·004
	6	0 ·011	0 ·011	—	—
	5	0 ·029	—	—	—
7	10	3 ·015⁻	3 ·015⁻	2 ·003	2 ·003
	9	2 ·018	2 ·018	1 ·004	1 ·004
	8	1 ·013	1 ·013	0 ·002	0 ·002
	7	1 ·036	0 ·006	0 ·006	—
	6	0 ·017	0 ·017	—	—
	5	0 ·041	—	—	—
6	10	3 ·036	2 ·008	2 ·008	1 ·001
	9	2 ·036	1 ·008	1 ·008	0 ·001
	8	1 ·024	1 ·024	0 ·003	0 ·003
	7	0 ·010⁺	0 ·010⁺	—	—
	6	0 ·026	—	—	—
5	10	2 ·022	2 ·022	1 ·004	1 ·004
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·047	0 ·007	0 ·007	—
	7	0 ·019	0 ·019	—	—
	6	0 ·042	—	—	—

	a	Probability			
		0·05	0·025	0·01	0·005
A=10 B=4	10	1 ·011	1 ·011	0 ·001	0 ·001
	9	1 ·041	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·015⁻	0 ·015⁻	—	—
	7	0 ·035⁻	—	—	—
3	10	1 ·038	0 ·003	0 ·003	0 ·003
	9	0 ·014	0 ·014	—	—
	8	0 ·035⁻	—	—	—
2	10	0 ·015⁺	0 ·015⁺	—	—
	9	0 ·045⁺	—	—	—
A=11 B=11	11	7 ·045⁺	6 ·018	5 ·006	4 ·002
	10	5 ·032	4 ·012	3 ·004	3 ·004
	9	4 ·040	3 ·015⁻	2 ·004	2 ·004
	8	3 ·043	2 ·015⁻	1 ·004	1 ·004
	7	2 ·040	1 ·012	0 ·002	0 ·002
	6	1 ·032	0 ·006	0 ·006	—
	5	0 ·018	0 ·018	—	—
	4	0 ·045⁺	—	—	—
10	11	6 ·035⁻	5 ·012	4 ·004	4 ·004
	10	4 ·021	4 ·021	3 ·007	2 ·002
	9	3 ·024	3 ·024	2 ·007	1 ·002
	8	2 ·023	2 ·023	1 ·006	0 ·001
	7	1 ·017	1 ·017	0 ·003	0 ·003
	6	1 ·043	0 ·009	0 ·009	—
	5	0 ·023	0 ·023	—	—
9	11	5 ·026	4 ·008	4 ·008	3 ·002
	10	4 ·038	3 ·012	2 ·003	2 ·003
	9	3 ·040	2 ·012	1 ·003	1 ·003
	8	2 ·035⁻	1 ·009	1 ·009	0 ·001
	7	1 ·025⁻	1 ·025⁻	0 ·004	0 ·004
	6	0 ·012	0 ·012	—	—
	5	0 ·030	—	—	—
8	11	4 ·018	4 ·018	3 ·005⁻	3 ·005⁻
	10	3 ·024	3 ·024	2 ·006	1 ·001
	9	2 ·022	2 ·022	1 ·005⁻	1 ·005⁻
	8	1 ·015⁻	1 ·015⁻	0 ·002	0 ·002
	7	1 ·037	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·040	—	—	—
7	11	4 ·043	3 ·011	2 ·002	2 ·002
	10	3 ·047	2 ·013	1 ·002	1 ·002
	9	2 ·039	1 ·009	1 ·009	0 ·001
	8	1 ·025⁻	1 ·025⁻	0 ·004	0 ·004
	7	0 ·010⁺	0 ·010⁺	—	—
	6	0 ·025⁻	0 ·025⁻	—	—
6	11	3 ·029	2 ·006	2 ·006	1 ·001
	10	2 ·028	1 ·005⁺	1 ·005⁺	0 ·001
	9	1 ·018	1 ·018	0 ·002	0 ·002

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED)

	a	Probability			
		0·05	0·025	0·01	0·005
A=11 B=6	8	1 ·043	0 ·007	0 ·007	—
	7	0 ·017	0 ·017	—	—
	6	0 ·037	—	—	—
5	11	2 ·018	2 ·018	1 ·003	1 ·003
	10	1 ·013	1 ·013	0 ·001	0 ·001
	9	1 ·036	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·013	0 ·013	—	—
	7	0 ·029	—	—	—
4	11	1 ·009	1 ·009	1 ·009	0 ·001
	10	1 ·033	0 ·004	0 ·004	0 ·004
	9	0 ·011	0 ·011	—	—
	8	0 ·026	—	—	—
3	11	1 ·033	0 ·003	0 ·003	0 ·003
	10	0 ·011	0 ·011	—	—
	9	0 ·027	—	—	—
2	11	0 ·013	0 ·013	—	—
	10	0 ·038	—	—	—
A=12 B=12	12	8 ·047	7 ·019	6 ·007	5 ·002
	11	6 ·034	5 ·014	4 ·005⁻	4 ·005⁻
	10	5 ·045⁻	4 ·018	3 ·006	2 ·002
	9	4 ·050⁻	3 ·020	2 ·006	1 ·001
	8	3 ·050⁻	2 ·018	1 ·005⁻	1 ·005⁻
	7	2 ·045⁻	1 ·014	0 ·002	0 ·002
	6	1 ·034	0 ·007	0 ·007	—
	5	0 ·019	0 ·019	—	—
	4	0 ·047	—	—	—
11	12	7 ·037	6 ·014	5 ·005⁻	5 ·005⁻
	11	5 ·024	5 ·024	4 ·008	3 ·002
	10	4 ·029	3 ·010⁺	2 ·003	2 ·003
	9	3 ·030	2 ·009	2 ·009	1 ·002
	8	2 ·026	1 ·007	1 ·007	0 ·001
	7	1 ·019	1 ·019	0 ·003	0 ·003
	6	1 ·045⁻	0 ·009	0 ·009	—
	5	0 ·024	0 ·024	—	—
10	12	6 ·029	5 ·010⁻	5 ·010⁻	4 ·003
	11	5 ·043	4 ·015⁺	3 ·005⁻	3 ·005⁻
	10	4 ·048	3 ·017	2 ·005⁻	2 ·005⁻
	9	3 ·046	2 ·015⁻	1 ·004	1 ·004
	8	2 ·038	1 ·010⁺	0 ·002	0 ·002
	7	1 ·026	0 ·005⁻	0 ·005⁻	0 ·005⁻
	6	0 ·012	0 ·012	—	—
	5	0 ·030	—	—	—
9	12	5 ·021	5 ·021	4 ·006	3 ·002
	11	4 ·029	3 ·009	3 ·009	2 ·002
	10	3 ·029	2 ·008	2 ·008	1 ·002
	9	2 ·024	2 ·024	1 ·006	0 ·001
	8	1 ·016	1 ·016	0 ·002	0 ·002

	a	Probability			
		0·05	0·025	0·01	0·005
A=12 B=9	7	1 ·037	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·039	—	—	—
8	12	5 ·049	4 ·014	3 ·004	3 ·004
	11	3 ·018	3 ·018	2 ·003	2 ·003
	10	2 ·015⁺	2 ·015⁺	1 ·003	1 ·003
	9	2 ·040	1 ·010⁻	1 ·010⁻	0 ·001
	8	1 ·025⁻	1 ·025⁻	0 ·004	0 ·004
	7	0 ·010⁺	0 ·010⁺	—	—
	6	0 ·024	0 ·024	—	—
7	12	4 ·036	3 ·009	3 ·009	2 ·002
	11	3 ·038	2 ·010⁻	2 ·010⁻	1 ·002
	10	2 ·029	1 ·006	1 ·006	0 ·001
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·040	0 ·007	0 ·007	—
	7	0 ·016	0 ·016	—	—
	6	0 ·034	—	—	—
6	12	3 ·025⁻	3 ·025⁻	2 ·005⁻	2 ·005⁻
	11	2 ·022	2 ·022	1 ·004	1 ·004
	10	1 ·013	1 ·013	0 ·002	0 ·002
	9	1 ·032	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·011	0 ·011	—	—
	7	0 ·025⁻	0 ·025⁻	—	—
	6	0 ·050⁻	—	—	—
5	12	2 ·015⁻	2 ·015⁻	1 ·002	1 ·002
	11	1 ·010⁻	1 ·010⁻	1 ·010⁻	0 ·001
	10	1 ·028	0 ·003	0 ·003	0 ·003
	9	0 ·009	0 ·009	0 ·009	—
	8	0 ·020	0 ·020	—	—
	7	0 ·041	—	—	—
4	12	2 ·050	1 ·007	1 ·007	0 ·001
	11	1 ·027	0 ·003	0 ·003	0 ·003
	10	0 ·008	0 ·008	0 ·008	—
	9	0 ·019	0 ·019	—	—
	8	0 ·038	—	—	—
3	12	1 ·029	0 ·002	0 ·002	0 ·002
	11	0 ·009	0 ·009	0 ·009	—
	10	0 ·022	0 ·022	—	—
	9	0 ·044	—	—	—
2	12	0 ·011	0 ·011	—	—
	11	0 ·033	—	—	—
A=13 B=13	13	9 ·048	8 ·020	7 ·007	6 ·003
	12	7 ·037	6 ·015⁺	5 ·006	4 ·002
	11	6 ·048	5 ·021	4 ·008	3 ·002
	10	4 ·024	4 ·024	3 ·008	2 ·002
	9	3 ·024	3 ·024	2 ·008	1 ·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) (CONTINUED)

	a	Probability					a	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	13	8 ·039	7 ·015⁻	6 ·005⁺	5 ·002		7	0 ·022	0 ·022	—	—
	12	6 ·027	5 ·010⁻	5 ·010⁻	4 ·003		6	0 ·044	—	—	—
	11	5 ·033	4 ·013	3 ·004	3 ·004	6	13	3 ·021	3 ·021	2 ·004	2 ·004
	10	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	13	7 ·031	6 ·011	5 ·003	5 ·003	5	13	2 ·012	2 ·012	1 ·002	1 ·002
	12	6 ·048	5 ·018	4 ·006	3 ·002		12	2 ·044	1 ·008	1 ·008	0 ·001
	11	4 ·021	4 ·021	3 ·007	2 ·002		11	1 ·022	1 ·022	0 ·002	0 ·002
	10	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
	5	0 ·030	—	—	—		11	0 ·006	0 ·006	0 ·006	—
10	13	6 ·024	6 ·024	5 ·007	4 ·002		10	0 ·015⁻	0 ·015⁻	—	—
	12	5 ·035⁻	4 ·012	3 ·003	3 ·003		9	0 ·029	—	—	—
	11	4 ·037	3 ·012	2 ·003	2 ·003	3	13	1 ·025	1 ·025	0 ·002	0 ·002
	10	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	13	5 ·017	5 ·017	4 ·005⁻	4 ·005⁻						
	12	4 ·023	4 ·023	3 ·007	2 ·001	A=14 B=14	14	10 ·049	9 ·020	8 ·008	7 ·003
	11	3 ·022	3 ·022	2 ·006	1 ·001		13	8 ·038	7 ·016	6 ·006	5 ·002
	10	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	13	5 ·042	4 ·012	3 ·003	3 ·003		6	1 ·038	0 ·008	0 ·008	—
	12	4 ·047	3 ·014	2 ·003	2 ·003		5	0 ·020	0 ·020	—	—
	11	3 ·041	2 ·011	1 ·002	1 ·002		4	0 ·049	—	—	—
	10	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	13	4 ·031	3 ·007	3 ·007	2 ·001		9	3 ·038	2 ·013	1 ·003	1 ·003
	12	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) (CONTINUED)

	a	Probability			
		0·05	0·025	0·01	0·005
A=14 B=13	7	1 ·021	1 ·021	0 ·004	0 ·004
	6	1 ·048	0 ·010⁺	—	—
	5	0 ·025⁻	0 ·025⁻	—	—
12	14	8 ·033	7 ·012	6 ·004	6 ·004
	13	6 ·021	6 ·021	5 ·007	4 ·002
	12	5 ·025⁺	4 ·009	4 ·009	3 ·003
	11	4 ·026	3 ·009	3 ·009	2 ·002
	10	3 ·024	3 ·024	2 ·007	1 ·002
	9	2 ·019	2 ·019	1 ·005⁻	1 ·005⁻
	8	2 ·042	1 ·012	0 ·002	0 ·002
	7	1 ·028	0 ·005⁺	0 ·005⁺	—
	6	0 ·013	0 ·013	—	—
	5	0 ·030	—	—	—
11	14	7 ·026	6 ·009	6 ·009	5 ·003
	13	6 ·039	5 ·014	4 ·004	4 ·004
	12	5 ·043	4 ·016	3 ·005⁻	3 ·005⁻
	11	4 ·042	3 ·015⁻	2 ·004	2 ·004
	10	3 ·036	2 ·011	1 ·003	1 ·003
	9	2 ·027	1 ·007	1 ·007	0 ·001
	8	1 ·017	1 ·017	0 ·003	0 ·003
	7	1 ·038	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·038	—	—	—
10	14	6 ·020	6 ·020	5 ·006	4 ·002
	13	5 ·028	4 ·009	4 ·009	3 ·002
	12	4 ·028	3 ·009	3 ·009	2 ·002
	11	3 ·024	3 ·024	2 ·007	1 ·001
	10	2 ·018	2 ·018	1 ·004	1 ·004
	9	2 ·040	1 ·011	0 ·002	0 ·002
	8	1 ·024	1 ·024	0 ·004	0 ·004
	7	0 ·010⁻	0 ·010⁻	0 ·010⁻	—
	6	0 ·022	0 ·022	—	—
	5	0 ·047	—	—	—
9	14	6 ·047	5 ·014	4 ·004	4 ·004
	13	4 ·018	4 ·018	3 ·005⁻	3 ·005⁻
	12	3 ·017	3 ·017	2 ·004	2 ·004
	11	3 ·042	2 ·012	1 ·002	1 ·002
	10	2 ·029	1 ·007	1 ·007	0 ·001
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·036	0 ·006	0 ·006	—
	7	0 ·014	0 ·014	—	—
	6	0 ·030	—	—	—
8	14	5 ·036	4 ·010⁻	4 ·010⁻	3 ·002
	13	4 ·039	3 ·011	2 ·002	2 ·002
	12	3 ·032	2 ·008	2 ·008	1 ·001
	11	2 ·022	2 ·022	1 ·005⁻	1 ·005⁻
	10	2 ·048	1 ·012	0 ·002	0 ·002
	9	1 ·026	0 ·004	0 ·004	0 ·004
	8	0 ·009	0 ·009	0 ·009	—
	7	0 ·020	0 ·020	—	—
	6	0 ·040	—	—	—

	a	Probability			
		0·05	0·025	0·01	0·005
A=14 B=7	14	4 ·026	3 ·006	3 ·006	2 ·001
	13	3 ·025	2 ·006	2 ·006	1 ·001
	12	2 ·017	2 ·017	1 ·003	1 ·003
	11	2 ·041	1 ·009	1 ·009	0 ·001
	10	1 ·021	1 ·021	0 ·003	0 ·003
	9	1 ·043	0 ·007	0 ·007	—
	8	0 ·015⁻	0 ·015⁻	—	—
	7	0 ·030	—	—	—
6	14	3 ·018	3 ·018	2 ·003	2 ·003
	13	2 ·014	2 ·014	1 ·002	1 ·002
	12	2 ·037	1 ·007	1 ·007	0 ·001
	11	1 ·018	1 ·018	0 ·002	0 ·002
	10	1 ·038	0 ·005⁺	0 ·005⁺	—
	9	0 ·012	0 ·012	—	—
	8	0 ·024	0 ·024	—	—
	7	0 ·044	—	—	—
5	14	2 ·010⁺	2 ·010⁻	1 ·001	1 ·001
	13	2 ·037	1 ·006	1 ·006	0 ·001
	12	1 ·017	1 ·017	0 ·002	0 ·002
	11	1 ·038	0 ·005⁻	0 ·005⁻	0 ·005⁻
	10	0 ·011	0 ·011	—	—
	9	0 ·022	0 ·022	—	—
	8	0 ·040	—	—	—
4	14	2 ·039	1 ·005⁻	1 ·005⁻	1 ·005⁻
	13	1 ·019	1 ·019	0 ·002	0 ·002
	12	1 ·044	0 ·005⁻	0 ·005⁻	0 ·005⁻
	11	0 ·011	0 ·011	—	—
	10	0 ·023	0 ·023	—	—
	9	0 ·041	—	—	—
3	14	1 ·022	1 ·022	0 ·001	0 ·001
	13	0 ·006	0 ·006	0 ·006	—
	12	0 ·015⁻	0 ·015⁻	—	—
	11	0 ·029	—	—	—
2	14	0 ·008	0 ·008	0 ·008	—
	13	0 ·025	0 ·025	—	—
	12	0 ·050	—	—	—
A=15 B=15	15	11 ·050⁻	10 ·021	9 ·008	8 ·003
	14	9 ·040	8 ·018	7 ·007	6 ·003
	13	7 ·025⁺	6 ·010⁺	5 ·004	5 ·004
	12	6 ·030	5 ·013	4 ·005⁻	4 ·005⁻
	11	5 ·033	4 ·013	3 ·005⁻	3 ·005⁻
	10	4 ·033	3 ·013	2 ·004	2 ·004
	9	3 ·030	2 ·010⁺	1 ·003	1 ·003
	8	2 ·025⁺	1 ·007	1 ·007	0 ·001
	7	1 ·018	1 ·018	0 ·003	0 ·003
	6	1 ·040	0 ·008	0 ·008	—
	5	0 ·021	0 ·021	—	—
	4	0 ·050⁻	—	—	—

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED)

	a	Probability			
		0·05	0·025	0·01	0·005
A=15 B=14	15	10 ·042	9 ·017	8 ·006	7 ·002
	14	8 ·031	7 ·013	6 ·005⁻	6 ·005⁻
	13	7 ·041	6 ·017	5 ·007	4 ·002
	12	6 ·046	5 ·020	4 ·007	3 ·002
	11	5 ·048	4 ·020	3 ·007	2 ·002
	10	4 ·046	3 ·018	2 ·006	1 ·001
	9	3 ·041	2 ·014	1 ·004	1 ·004
	8	2 ·033	1 ·009	1 ·009	0 ·001
	7	1 ·022	1 ·022	0 ·004	0 ·004
	6	1 ·049	0 ·011	—	—
	5	0 ·025⁺	—	—	—
13	15	9 ·035⁻	8 ·013	7 ·005⁻	7 ·005⁻
	14	7 ·023	7 ·023	6 ·009	5 ·003
	13	6 ·029	5 ·011	4 ·004	4 ·004
	12	5 ·031	4 ·012	3 ·004	3 ·004
	11	4 ·030	3 ·011	2 ·003	2 ·003
	10	3 ·026	2 ·008	2 ·008	1 ·002
	9	2 ·020	2 ·020	1 ·003⁺	0 ·001
	8	2 ·043	1 ·013	0 ·002	0 ·002
	7	1 ·029	0 ·005⁺	0 ·005⁺	—
	6	0 ·013	0 ·013	—	—
	5	0 ·031	—	—	—
12	15	8 ·028	7 ·010⁻	7 ·010⁻	6 ·003
	14	7 ·043	6 ·016	5 ·006	4 ·002
	13	6 ·049	5 ·019	4 ·007	3 ·002
	12	5 ·049	4 ·019	3 ·006	2 ·002
	11	4 ·045⁺	3 ·017	2 ·005⁻	2 ·005⁻
	10	3 ·038	2 ·012	1 ·003	1 ·003
	9	2 ·028	1 ·007	1 ·007	0 ·001
	8	1 ·018	1 ·018	0 ·003	0 ·003
	7	1 ·038	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·037	—	—	—
11	15	7 ·022	7 ·022	6 ·007	5 ·002
	14	6 ·032	5 ·011	4 ·003	4 ·003
	13	5 ·034	4 ·012	3 ·003	3 ·003
	12	4 ·032	3 ·010⁺	2 ·003	2 ·003
	11	3 ·026	2 ·008	2 ·008	1 ·002
	10	2 ·019	2 ·019	1 ·004	1 ·004
	9	2 ·040	1 ·011	0 ·002	0 ·002
	8	1 ·024	1 ·024	0 ·004	0 ·004
	7	1 ·049	0 ·010⁻	0 ·010⁻	—
	6	0 ·022	0 ·022	—	—
	5	0 ·046	—	—	—
10	15	6 ·017	6 ·017	5 ·005⁻	5 ·005⁻
	14	5 ·023	5 ·023	4 ·007	3 ·002
	13	4 ·022	4 ·022	3 ·007	2 ·001
	12	3 ·018	3 ·018	2 ·005⁻	2 ·005⁻
	11	3 ·042	2 ·013	1 ·003	1 ·003
	10	2 ·029	1 ·007	1 ·007	0 ·001
	9	1 ·016	1 ·016	0 ·002	0 ·002
	8	1 ·034	0 ·006	0 ·006	—
	7	0 ·013	0 ·013	—	—
	6	0 ·028	—	—	—
9	15	6 ·042	5 ·012	4 ·003	4 ·003
	14	5 ·047	4 ·015⁻	3 ·004	3 ·004

	a	Probability			
		0·05	0·025	0·01	0·005
A=15 B=9	13	4 ·042	3 ·013	2 ·003	2 ·003
	12	3 ·032	2 ·009	2 ·009	1 ·002
	11	2 ·021	2 ·021	1 ·005⁻	1 ·005⁻
	10	2 ·045⁻	1 ·011	0 ·002	0 ·002
	9	1 ·024	1 ·024	0 ·004	0 ·004
	8	1 ·048	0 ·009	0 ·009	—
	7	0 ·019	0 ·019	—	—
	6	0 ·037	—	—	—
8	15	5 ·032	4 ·008	4 ·008	3 ·002
	14	4 ·033	3 ·009	3 ·009	2 ·002
	13	3 ·026	2 ·006	2 ·006	1 ·001
	12	2 ·017	2 ·017	1 ·003	1 ·003
	11	2 ·037	1 ·008	1 ·008	0 ·001
	10	1 ·019	1 ·019	0 ·003	0 ·003
	9	1 ·038	0 ·006	0 ·006	—
	8	0 ·013	0 ·013	—	—
	7	0 ·026	—	—	—
	6	0 ·050⁻	—	—	—
7	15	4 ·023	4 ·023	3 ·005⁻	3 ·005⁻
	14	3 ·021	3 ·021	2 ·004	2 ·004
	13	2 ·014	2 ·014	1 ·002	1 ·002
	12	2 ·032	1 ·007	1 ·007	0 ·001
	11	1 ·015⁺	1 ·015⁺	0 ·002	0 ·002
	10	1 ·032	0 ·005⁻	0 ·005⁻	0 ·005⁻
	9	0 ·010⁺	0 ·010⁺	—	—
	8	0 ·020	0 ·020	—	—
	7	0 ·038	—	—	—
6	15	3 ·015⁺	3 ·015⁺	2 ·003	2 ·003
	14	2 ·011	2 ·011	1 ·002	1 ·002
	13	2 ·031	1 ·006	1 ·006	0 ·001
	12	1 ·014	1 ·014	0 ·002	0 ·002
	11	1 ·029	0 ·004	0 ·004	0 ·004
	10	0 ·009	0 ·009	0 ·009	—
	9	0 ·017	0 ·017	—	—
	8	0 ·032	—	—	—
5	15	2 ·009	2 ·009	2 ·009	1 ·001
	14	2 ·032	1 ·005⁻	1 ·005⁻	1 ·005⁻
	13	1 ·014	1 ·014	0 ·001	0 ·001
	12	1 ·031	0 ·004	0 ·004	0 ·004
	11	0 ·008	0 ·008	0 ·008	—
	10	0 ·016	0 ·016	—	—
	9	0 ·030	—	—	—
4	15	2 ·035⁺	1 ·004	1 ·004	1 ·004
	14	1 ·016	1 ·016	0 ·001	0 ·001
	13	1 ·037	0 ·004	0 ·004	0 ·004
	12	0 ·009	0 ·009	0 ·009	—
	11	0 ·018	0 ·018	—	—
	10	0 ·033	—	—	—
3	15	1 ·020	1 ·020	0 ·001	0 ·001
	14	0 ·005⁻	0 ·005⁻	0 ·005⁻	0 ·005⁻
	13	0 ·012	0 ·012	—	—
	12	0 ·025⁻	0 ·025⁻	—	—
	11	0 ·043	—	—	—
2	15	0 ·007	0 ·007	0 ·007	—
	14	0 ·022	0 ·022	—	—
	13	0 ·044	—	—	—

APPENDIX H

TOXICITY SCREENING TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR INSTREAM WASTE CONCENTRATION

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 non-parametric 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.

$$F = \frac{S_1^2}{S_2^2} \quad \text{where } S_1^2 > S_2^2$$

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 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 REPRODUCTION DATA FROM AN EFFLUENT SCREENING TEST

	Replicate											
	1	2	3	4	5	6	7	8	9	10	$\bar{X}$	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.

$$F = \frac{36.61}{14.55}$$

$$= 2.52$$

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:

$$t = \frac{\overline{Y}_1 - \overline{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where:

$\overline{Y}_1$ = Mean for the control

$\overline{Y}_2$ = 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, you would 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:

$$S_p = \sqrt{\frac{(8 - 1)\ 14.5 + (9 - 1)\ 36.6}{8 + 9 - 2}} = 5.13$$

9.3 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:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_1^2}{n_2}}}$$

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

1.1 This program calculates the EC50, EC15, EC10, EC5, and EC1 values, and associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by D. L. Weiner, Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, Ohio 45268. A full listing of the program is contained in EPA/600/4-87/028. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.1 Data input is illustrated by a set of total mortality data from a fathead minnow embryo-larval survival and teratogenicity test. The program begins with a request for the following information:

1. Output designation (P = printer, D = disk file).
2. Title for the output.
3. A selection of model fitting options (see sample output for a detailed description of options). If Option 2 is selected, the theoretical lower threshold needs to be entered. If option 3 is selected, the program requests the number of animals responding in the control group and the total number of original animals in the control group be entered.
4. The number of test concentrations.

2.2. The program then requests information on the results at each concentration, beginning with the lowest concentration.

1. Concentration.
2. Number of organisms responding.
3. Total number of exposed organisms.

2.2.1. See sample data input on the next page.

2.2.1 Sample Data Input.

```
ÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜ
Ü                                                            Ü
Ü                EPA PROBIT ANALYSIS PROGRAM                 Ü
Ü              USED FOR CALCULATING EC VALUES                Ü
Ü                       Version 1.4                          Ü
ÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜÜ


Output to printer or disk file (P / D)? p
Title ? Example for Probit Analysis


        Model Fitting Options Which Are Available

1) Fit a model which includes two parameters: an intercept and a
   slope.  This model assumes that the spontaneous response
   (in controls) is zero.  No control data are entered if this
   option is specified.

2) Fit a model which includes three parameters: an intercept, a
   slope and a theoretical lower threshold which represents the
   level of spontaneous response (in controls).  This option
   requires the user to input the theoretical lower threshold
   (the value must be between 0.0 and 0.99).  No control data is
   entered if this option is specified.

3) Fit a model which includes three parameters, an intercept, a
   slope and a lower threshold.  The lower threshold is estimated
   based on control data which are input by the user. If the number
   responding in the control group is zero, then this option is
   indentical to option two (above).

   Your choice (1, 2, or 3)? 3


Number of responders in the control group = ? 17
Number of animals exposed in the concurrent control group = ? 100
Number of administered concentrations ? 5
```

2.2.1 Sample Data Input (Continued).

```
Input data starting with the lowest concentration

Concentration = ? 3.0
Number responding = ? 14
Number exposed = ? 100

Concentration = ? 5.0
Number responding = ? 16
Number exposed = ? 102

Concentration = ? 7.0
Number responding = ? 35
Number exposed = ? 100

Concentration = ? 11.0
Number responding = ? 72
Number exposed = ? 99

Concentration = ? 16.0
Number responding = ? 99
Number exposed = ? 99
```

Number	Conc.	Number Resp.	Number Exposed
1	3.0000	14	100
2	5.0000	16	102
3	7.0000	35	100
4	11.0000	72	99
5	16.0000	99	99

```
Do you wish to modify your data ? n


The number of control animals which responded =  17
The number of control animals exposed  =  100
Do you wish to modify these values ? n
```

2.3 Sample Data Output

2.3.1 The program output includes the following:

2.3.1.1 Statistical table (Table I.1.)

1. The observed, adjusted (using Abbott's formula) and predicted proportions responding at each concentration.
2. Chi-square statistic for heterogeneity. This test is one indicator of how well the data fit the model.
3. Estimates of the mean (mu) and standard deviation (sigma) of the underlying tolerance distribution.
4. Estimates and standard errors of the intercept and slope of the fitted probit regression line.
5. Estimate and standard error of the lower threshold (if requested - requires control data on input).
6. A list of estimated EC values and 95% confidence limits. Please note that EC, effective concentration, is a broad term and applies to any response, such as fertilization, death or immobilization. If mortality data is entered in the program as the response, the EC estimates are equivalent to LC (lethal concentration) estimates.

2.3.1.2 Plot (Figure I.1.)

1. A plot of the fitted probit regression line with observed data overlaid on the plot.

TABLE I.1. OUTPUT FROM PROBIT PROGRAM

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Adjusted Proportion Responding	Predicted Proportion Responding
Control	100	17	0.1700	0.0000	0.1560
3.0000	100	14	0.1400	-.0190	0.0000
5.0000	102	16	0.1569	0.0010	0.0174
7.0000	100	35	0.3500	0.2298	0.1765
11.0000	99	72	0.7273	0.6769	0.7449
16.0000	99	99	1.0000	1.0000	0.9759

Chi - Square Heterogeneity = 5.286

Mu = 0.959956
Sigma = 0.123640

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-2.764127	1.002530	(-4.729086, -0.799168)
Slope	8.088003	0.990954	(6.145732, 10.030273)
Spontaneous Response Rate	0.156014	0.022593	(0.111732, 0.200296)

Estimated EC Values and Confidence Limits

Point	Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
EC 1.00	4.7025	3.6073	5.5567
EC 5.00	5.7093	4.6408	6.5196
EC10.00	6.3314	5.3031	7.1058
EC15.00	6.7892	5.7994	7.5354
EC50.00	9.1192	8.3614	9.7763
EC85.00	12.2489	11.4157	13.3942
EC90.00	13.1345	12.1697	14.5708
EC95.00	14.5657	13.3302	16.5676
EC99.00	17.6840	15.7134	21.2145

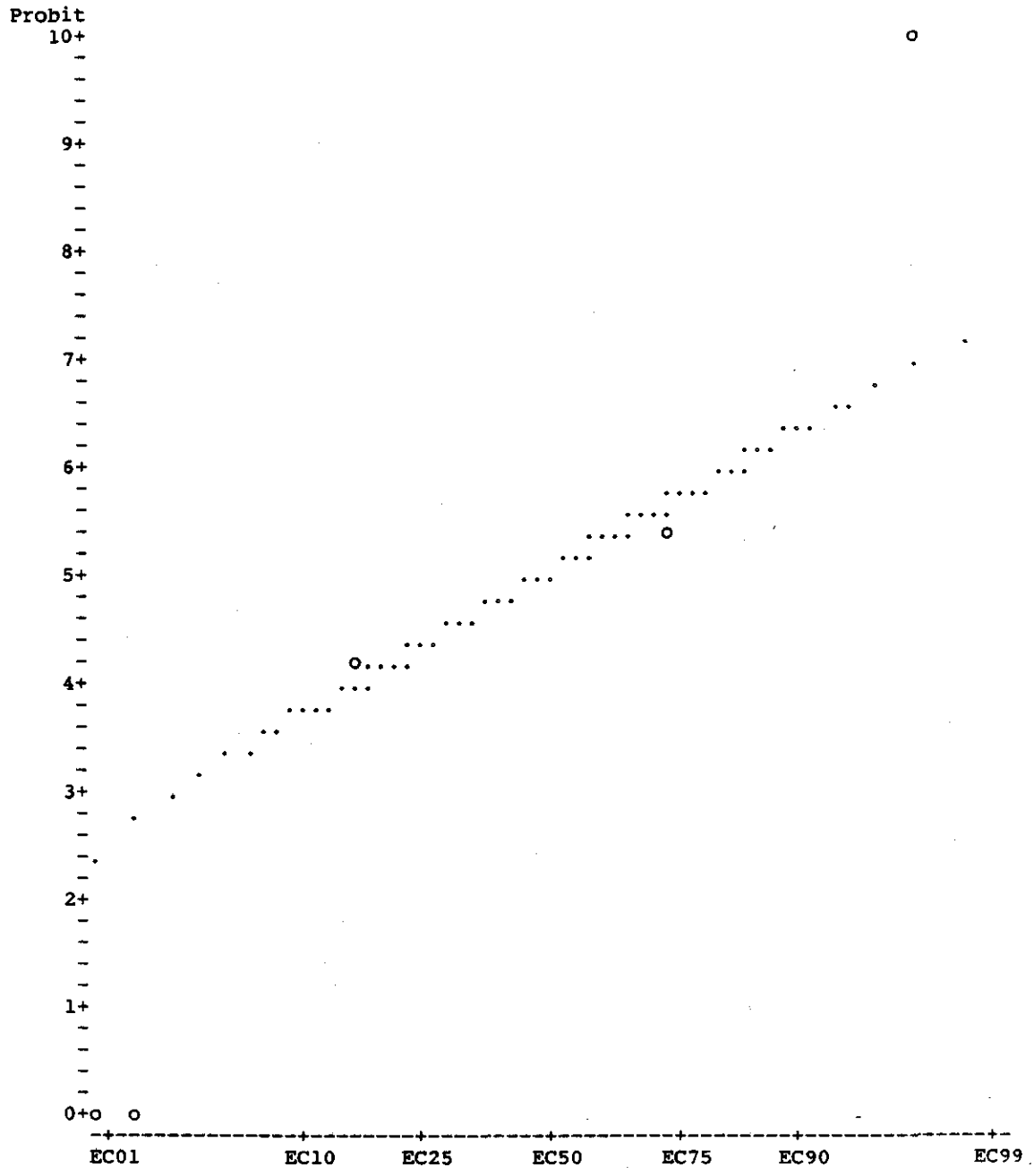

Figure I.1. Plot of Adjusted Probits and Predicted Regression Line.

☆ U.S. GOVERNMENT PRINTING OFFICE: 1989— 6 4 8 - 1 6 3 / 8 7 1 0 0

Environmental Protection Agency
Information
Cincinnati OH 45268

Official Business
Penalty for Private Use, $300

Please make all necessary changes on the above label, detach or copy, and return to the address in the upper left-hand corner.

If you do not wish to receive these reports CHECK HERE □; detach, or copy this cover, and return to the address in the upper left-hand corner.

EPA/600/4-89/001