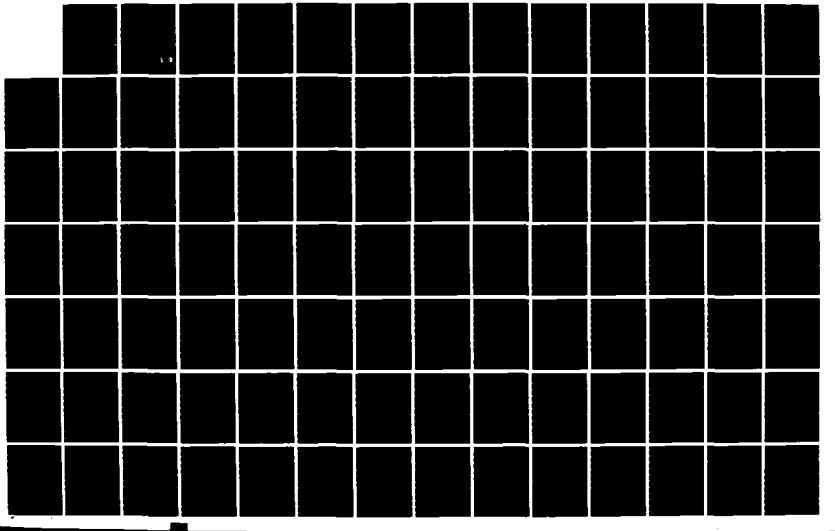
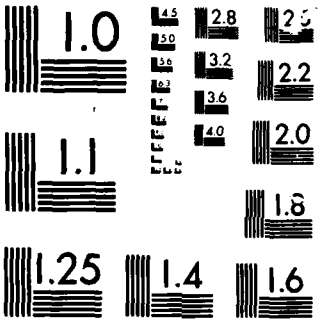
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COLLABORATIVE STUDY OF

Daphnia magna STATIC

RENEWAL ASSAYS

By

R.E. Bentley, D.C. Surprenant, and S.R. Petrocelli

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A Daphnia magna chronic test was evaluated as to its suitability as a hazardous waste testing and assessment procedure. The purpose of this project was to develop a practical and technically valid Daphnia magna chronic test protocol and then to verify the accuracy and precision of this protocol in collaborative studies conducted jointly in governmental, academic, industrial and contract testing laboratories. Based on the results of this collaborative testing, the verified test protocol would serve as a new standard test method.

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A total of 11 performing organizations including 2 governmental, 3 academic, 2 industrial and 4 contract testing laboratories were included in this collaborative study. Jointly, the laboratories attempted 45 chronic tests with 4 materials and 43 of these tests were successfully completed.

Results (both intra- and interlaboratory testing) indicated a high degree of accuracy and precision for routine tests with daphnids as the test organism. Results illustrated those effect criterion which were sensitive and reproducible measures of toxic effects and those which were not. It was also determined that environmental testing laboratories differed substantially in their individual conformance to EPA Good Laboratory Practice requirements. In summary, this study resulted in the development and validation of a technically credible protocol which when employed by competent laboratory personnel, produces reliable and useful data for evaluating the potential environmental hazard of solid waste leachates or other toxic mixtures.

EXECUTIVE SUMMARY

To effectively regulate the disposal of potentially hazardous wastes in the aquatic environment on a consistent, nation-wide basis, it is necessary to develop a waste testing and evaluation procedure which will provide accurate and precise results when performed with reasonable care by personnel in a laboratory with average facilities, capabilities and competence.

A Daphnia magna chronic test was selected for evaluation as to it's suitability as a hazardous waste testing and assessment procedure. Use of this test would result in the development of the information on the effect of the test material on the survival, growth, development and reproduction of this sensitive aquatic organism.

The purpose of this project was to develop a practical and technically valid Daphnia magna chronic test protocol and then to verify the accuracy and precision of this protocol in collaborative studies conducted jointly in governmental, academic, industrial and contract testing laboratories. Based on the results of this collaborative testing, the verified test protocol would serve as a new standard test method.

The test materials selected for this collaborative study included one organic (sodium pentachlorophenate) and one inorganic (copper chloride) chemical. These chemicals

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were tested as pure materials and also with each as a mixture with glacial acetic acid, a compound used in the extraction of potentially hazardous materials from wastes for testing purposes. Therefore, four materials were tested by each laboratory.

A total of 11 performing organizations including 2 governmental, 3 academic, 2 industrial and 4 contract testing laboratories were included in this collaborative study. Jointly, the laboratories attempted 45 chronic tests with the 4 materials and 43 of these tests were successfully completed.

Results (both intra- and inter-laboratory testing) indicated a high degree of accuracy and precision for routine tests with daphnids as the test organism. Between laboratory variability was typically a factor of 2-3 X for acute EC50 values and 2-4 X for chronic MATC values with the selected test materials. In addition, results illustrated those effect criterion which were sensitive and reproducible measures of toxic effects and those which were not. For example, survival, young per female and length were most useful, while number of reproductive days and number of molts was of lesser significance. It was also determined that environmental testing laboratories differed substantially in their individual conformance to EPA Good Laboratory Practice requirements. More emphasis on the fundamentals of laboratory quality assurance

programs for biological testing is required to ensure uniform data quality and integrity in regulatory programs.

In summary, this study resulted in the development and validation of a technically credible protocol which when employed by competent laboratory personnel produces reliable and useful data for evaluating the potential environmental hazard of solid waste leachates or other toxic mixtures.

TABLE OF CONTENTS

Executive Summary..... i

List of Tables..... vi

List of Figures.....viii

I. Introduction..... 1

II.Materials and Methods..... 3

A. Protocol Development and Preliminary Testing..... 3

B. Laboratory Solicitation and Selection..... 4

C. Test Material Selection..... 6

D. Pre-Test Meeting..... 9

E. Collaborative Testing..... 9

F. Data Compilation, Review, and Statistical Analysis.. 11

III. Results and Discussion..... 13

A. Water Quality..... 13

B. Analytical Precision and Accuracy..... 14

C. Toxicological Evaluation..... 15

D. Laboratory Quality Assurance..... 21

IV. Conclusions..... 21

V. Recommendations..... 24

VI. Literature Cited..... 26

Appendix 1 - An Assessment of the Suitability of Several Media for Culturing and Testing *Daphnia magna* 57

Appendix 2 - Protocol for the Evaluation of Waste Leachate Acute and Chronic Toxicity with Dapnnia magna..... 100

Appendix 3 - Results of precision and accuracy analyses conducted at Springborn Bionomics, Inc..... 224
Appendix 4 - Results of sample stability analyses conducted at Springborn Bionomics..... 233
Appendix 5 - Results of the analysis of concentrations measured during testing at the various collaborative laboratories..... 238
Appendix 6 - Results of fortified quality assurance blind sample analyses conducted at Springborn Bionomics, Inc..... 284

LIST OF TABLES

Table	Title	Page
1.	Panel of experts convened to review the proposed protocol entitled "Protocol for Evaluation of Waste Leachate Acute and Chronic Toxicity with *Daphnia magna*"	27
2.	Description of the major features of the protocol as accepted by the review panel	28
3.	Range of water quality parameters measured during testing with sodium pentachlorophenate	29
4.	Range of water quality parameters measured during testing with sodium pentachlorophenate/glacial acetic acid (unknown #658)	30
5.	Range of water quality parameters measured during testing with copper chloride (unknown #852)	31
6.	Range of water quality parameters measured during testing with copper chloride/glacial acetic acid (unknown #124)	32
7.	Determination of the lower and upper limits of the MATC by laboratory and compound	33
8.	Summary of the statistical determinations for the lower and upper limits for the MATC	34
9.	Summary of the statistical determinations made using the geometric mean of the MATCs	35
10.	Statistics on interlaboratory variability (Youden and Steiner, 1975)	36
11.	Comparison of control performance by laboratory for all materials tested	43
12.	Comparison of control performance by laboratory for sodium pentachlorophenate	44
13.	Comparison of control performance by laboratory for sodium pentachlorophenate/glacial acetic acid (658)	45

LIST OF TABLES (CONT.)

Table	Title	Page
14.	Comparison of control performance by laboratory for copper chloride (#852.	46
15.	Comparison of control by laboratory for copper chloride/glacial acetic acid (#124).	47
16.	Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for NaPCP in testing conducted prior to and at the termination of the chronic study	48
17.	Comparison of static, acute EC50's and 95 confidence limits vs. 21-day LC50's by laboratory for compound #658 (NaPCP-GAA) in testing prior to and at the termination of the chronic study.	49
18.	Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for compound #852 ($CuCL_2$) in testing prior to and at the termination of the chronic study	50
19.	Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for compound #124 ($CuCL_2$/GAA) in testing prior to and at the termination of the chronic study	51
20.	Comparison of MATC's and effect criterion by laboratory for NaPCP	52
21.	Comparison of MATC's and effect criterion by laboratory for Compound #658 (NaPCP/GAA)	53
22.	Comparison of MATC's and effect criterion by laboratory for Compound #852 ($CuCL_2$)	54
23.	Comparison of MATC's and effect criterion by laboratory for Compound #124 ($CuCL_2$/GAA)	55
24.	Mean of geometric mean MATC's and relative importance of effect criterion per compound	56

LIST OF FIGURES

Figure	Title	Page
1.	Youden plot comparing the lower limit of the MATCs derived for NaPCP and NaPCP/GAA.	37
2.	Youden plot comparing the upper limit of the MATCs derived for NaPCP and NaPCP/GAA.	38
3.	Youden plot comparing the geometric means of the MATCs derived for NaPCP and NaPCP/GAA.	39
4.	Youden plot comparing the lower limit of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA.	40
5.	Youden plot comparing the upper limit of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA.	41
6.	Youden plot comparing the geometric means of of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA . . .	42

I. INTRODUCTION

Section 3001 of Subtitle C of RCRA requires the development and promulgation of criteria for identifying the characteristics of hazardous wastes which, due to their toxicity, pose a potential hazard to the environment. One criterion proposed for identifying wastes of a hazardous nature is the effect of the material on survival and reproduction of the freshwater invertebrate, Daphnia magna. Daphnid life-cycle toxicity tests have been used successfully to evaluate the chronic toxicity of pure compounds, commercial products and industrial wastewaters, but validated, standard procedures applicable to screening complex environmental samples are not available. To advocate the use of this toxicological assay for a broad spectrum of screening and environmental monitoring applications requires that the procedures available be standardized, verified and validated by collaborative testing.

The scope of this project encompassed three primary objectives: 1) the verification of a routine, technically practical, cost effective standard laboratory procedure for determining toxicity and evaluating the potential hazard of complex environmental samples to aquatic organisms; 2) the planning , implementation and management of an interlaboratory testing program to

determine the reliability, reproducibility and accuracy of the proposed standard procedure; and 3) the development of a descriptive standard method including information on test design considerations and constraints, scope and application of method, experimental conditions, test organism acclimation and culture procedures, quality control and quality assurance techniques, data analysis and interpretation, reporting of results and the facilities, equipment and supplies needed to perform the test.

This report presents the results of an interlaboratory validation study of a method entitled "Protocol for Evaluation of Waste Leachate Acute and Chronic Toxicity with Daphnia magna." The study was performed by Springborn Bionomics, Inc. (SBI) and included a total of eleven academic, governmental, industrial and contract testing laboratories, each of which tested one known and three unknown test materials.

II. MATERIALS AND METHODS

A. PROTOCOL DEVELOPMENT & PRELIMINARY TESTING

During 1982, a draft protocol entitled "Protocol for Evaluation of Waste Leachate Acute and Chronic Toxicity with Daphnia magna" was developed jointly by the Environmental Protection Agency (EPA), Environmental Research Laboratory - Duluth, the Environmental Monitoring Systems Laboratory - Las Vegas, and by personnel from Montana State University. The protocol was designed to estimate the acute (48-hour static exposure) and chronic (21-day static renewal exposure) toxicity of substances representing waste leachates to D. magna. This protocol was submitted to a panel composed of experts from academic, governmental, industrial and contract testing laboratories for their review and comment (Table 1). The objective of the panel review was to insure that the recommended test procedures conformed to the state of the art technical and scientific considerations. Subsequently, a meeting was convened with all parties to discuss reviewers' comments and identify any alterations to be made to the protocol. These discussions resulted in the identification of specific assignments for investigators to make the protocol more explicit. Two specific areas requiring further investigation prior to

the initiation of the interlaboratory validation study were identified. These areas encompassed both the food the daphnids were to be fed, and the medium in which the Daphnia were to be cultured and tested. This preliminary investigation was conducted at both SBI, and the U.S. Army Medical Bioengineering Research and Development Laboratory (Army), Fort Detrick, Frederick, MD, to independently determine the most appropriate food and medium for the rearing and testing of Daphnia magna (Appendix 1). The results of these studies were incorporated into the protocol, and the subcommittee chairs of the peer review panel were requested to comment. Their comments, as well as those of the principals, were compiled into a draft final protocol, which is presented in Appendix 2 and outlined in Table 2.

B. LABORATORY SOLICITATION & SELECTION

At the inception of this program, SBI was asked by the Army and the EPA to develop a collaborative study which would have broad support within the aquatic toxicology testing community. As a result, it was decided that all testing must include individuals who represented the academic, governmental, industrial, and contract testing laboratory sectors. As in any collaborative testing, it was desirable to utilize no fewer than six laboratories for statistical reliability (Williams, 1984).

Participation by other laboratories in excess of this number would provide an even better data base for statistical analysis.

Letters soliciting interest in participation were submitted to over fifty institutions. The letters requested information on the laboratories' prior experience with Daphnia magna culture and testing, the nature of the dilution water in use at each laboratory, the source and health of their organisms, and the degree of implementation of EPA Good Laboratory Practice regulations (Federal Register, 1983). Based upon the response received from this informal solicitation, a list of forty laboratories was prepared to whom a formal request for bid was sent. Of the 38 respondents, eight laboratories were selected for final qualification. These included three university, two industrial, and three contract testing laboratories. Pre-award site visits were conducted by SBI personnel at six of these labs in order to inspect test facilities, review personnel qualifications, and evaluate daphnid maintenance and quality assurance programs. Laboratories qualified in the final selection process for the collaborative program consisted of the following organizations:

Battelle Columbus Laboratories, Columbus, OH

Biospherics Inc., Rockville, MD

Environmental Research and Technology, Fort Collins, CO

Exxon Corporation, E. Millstone, NJ

Monsanto Company, St. Louis, MO

SRI International, Inc., Stanford, CA

University of South Florida, Tampa, FL

University of Wisconsin - Superior, WI

University of Wyoming - Laramie, WY

U.S. Army Medical Bioengineering Research & Development Lab, Fort Detrick, Frederick, MD

U.S. EPA - Environmental Research Laboratory - Duluth, MN

SBI personnel and facilities participated as the twelfth laboratory and referee for the collaborative study.

Since the objective of this collaborative testing was to establish the accuracy and reproducibility of the proposed method and not the laboratories performing it, laboratory names have been deleted and replaced by numeric designations in the results of this study.

C. TEST MATERIAL SELECTION

The original design for this project called for a total of six toxicants to be tested by all participating laboratories. Due to a lack of resources, testing with only four compounds was completed.

The intent of a collaborative study is greatly enhanced if the researcher is given compounds for which

no pre-judgements on appropriate ranges of toxicity can be made. However, in order to assist the laboratories in becoming familiar with the proposed test methods, one material was identified to all collaborative laboratories. This material, sodium pentachlorophenate (NaPCP), served as the reference toxicant, and enabled the participating laboratory to perform a test using the proposed method with a compound of known toxicity. This approach also allowed the referee (SBI) to evaluate the interlaboratory variability for this material and corroborate these results with values found in the open literature. The other three compounds were tested as unknowns, and were coded as compounds #658, #852, and #124. Since the extraction procedure used to prepare leachates for testing incorporates the use of glacial acetic acid (GAA), two of these materials had an amount of GAA roughly equivalent to the concentration expected in extracted samples (500 uL/L). While the effect of this small quantity of glacial acetic acid was not initially known, it was assumed that it would have little or no effect on the toxicity of any pure material to be tested. It was therefore decided that compound #658 would be NaPCP with glacial acetic acid (NaPCP/GAA), compound #852 would be copper (as copper chloride, $CuCl_2$), and compound #124 would be copper with glacial acetic acid ($CuCl_2$). The desired result was to have two tests with NaPCP and two with copper to improve

the statistical analysis of the data derived from the testing. The materials tested during this study were selected based on solubility and stability in water, ability to be analytically quantified in water, and to be representative of both organic and inorganic materials. Due to the apparent stability of the compounds selected, and in order to facilitate chemical analysis, a non-toxic tracer was added to all samples of the test materials. This material, fluorescein (LC50 >1000 mg/L), was selected in order to streamline the analysis of the compounds by permitting simple, colorimetric analysis rather than high pressure liquid chromatographic (for NaPCP) or atomic absorption spectrophotometric (for copper) analyses, and was the analyte which was used to quantitate the concentrations of the compounds tested. All test materials were supplied as stock solutions to the participating laboratories to ensure uniformity of mixtures. By protocol, all laboratories were required to sample all test exposure solutions on days 0, 7, 14, and 21, package these water samples, and ship them to SBI for analysis. Fortified quality assurance blind samples (QA) were prepared by SBI and analyzed on each day test sample analyses were performed.

Prior to testing with specific materials by the collaborating laboratories, precision and accuracy analyses were conducted at SBI. Results of these analyses

helped to determine the precision which the analyst might be expected to achieve, and the overall accuracy (or recovery) of the methodology employed. Additionally, storage stability of the samples was determined over a minimum of a 120-day period to account for the possible need to store samples from the collaborating laboratories prior to analysis.

D. PRE-TEST MEETING

In order to ensure that all collaborating laboratories had a complete and thorough understanding of the protocol, the Scope of Work required that a representative of each performing organization be present at a pre-test meeting held at SBI's laboratory. This meeting covered in depth the requirements of the protocol, the standardized data forms to be used in recording data, the data submission requirements, and the quality assurance requirements. A significant amount of time was allowed for questions from the participants to clarify the requirements of the protocol. All collaborators were instructed to contact SBI personnel if they encountered any problem areas or had any questions.

E. COLLABORATIVE TESTING

The preliminary food and culture/testing medium study (Appendix 1), conducted as a prerequisite to this

collaborative study, established that for acceptable results and practicality, the standardized medium would be a modification of Marking's and Dawson's formulation for hard reconstituted water, while the food would be a combination of trout chow and the alga, Selenastrum capricornutum. Each laboratory received fresh stocks of trout chow, algae, and Daphnia prior to the initiation of testing. Each labotatory demonstrated that daphnids could be cultured successfully under the prescribed conditions.

Prior to initiating the collaborative tests, a new population of Daphnia magna as well as a quantity of fish food (standard trout chow) large enough for the entire duration of these studies was submitted to each participating laboratory from the EPA, Environmental Research Laboratory, Duluth, MN. In addition, all laboratories were supplied agar slants of the alga, Selenastrum capricornutum, to be used as a food supplement from Springborn Bionomics, Inc. The original culture was from the Army Bioengineering Research & Development Laboratory, who obtained their culture from the American Type Culture Collection. Prior to initiating the testing phases of the program, all participating laboratories were required to acclimate the daphnids to the conditions described in the protocol.

The testing was designed to occur in phases. The first phase was a familiarization period during which

daphnids were acclimated to the requirements of the protocol, and the "known" test compound, sodium pentachlorophenate, was tested. The testing with the three unknowns was not initiated until the results of this test were received and reviewed by SBI personnel. If the preliminary results were consistent with the results obtained from the testing which had been previously conducted at SBI, the laboratories were authorized to proceed with tests of the unknowns. If there were any problems with the familiarization phase testing or the data were contradictory, efforts were immediately made to locate the source of the problem and to implement the appropriate corrections. All participants during the testing were required to submit monthly progress reports to SBI and as stated previously, were encouraged to call and discuss any problem areas, or areas of confusion.

F. DATA COMPILATION, REVIEW AND STATISTICAL ANALYSIS

During testing, all data were to be recorded on forms provided by SBI with the protocol to promote uniformity of data submission, and to facilitate the final tabulation and analysis of the results of each laboratory's tests. Upon completion of each set of tests, the data were submitted to SBI for analysis and archiving.

Data submitted by all collaborating laboratories and analyzed by SBI personnel included the results of physical

measurements (pH, dissolved oxygen, temperature, lighting regime, hardness and alkalinity) and the biological measurements (survival, number of reproductive days, time to first brood, cumulative young produced per female, cumulative young produced per female per reproductive day and length). The length measurement was stated as an optional measurement in the protocol; however, for the purposes of this study, it was a required measurement.

EC50 values were calculated by moving average angle analysis, probit analysis or binomial probability with non-linear interpolation according to a program developed by Stephan (1982, personal communication).

Survival data from each concentration were compared to the survival in the controls using the Fisher exact test with a one-sided 5% significance level. In order to control for multiple comparisons, no concentration was considered significantly different from the control if all higher concentrations were not significantly different from the control (Marcus et al., 1976). All other biological measurements were subjected to a one-way analysis of variance (Steel and Torrie, 1960), and where treatment results differed from the control, results were analyzed by Dunnett's procedure. These results were used to estimate the maximum acceptable toxicant concentration (MATC). The MATC is defined as the maximum concentration of test material which would not elicit an adverse

response from the exposed organisms which was significantly different from that of the control organisms.

In order to determine whether systematic or interlaboratory variabilities existed, the lower and upper levels of the MATC's as well as the geometric means of the MATC's were analyzed by the methods of Youden and Steiner (1975) utilizing NaPCP and #658 (NaPCP/GAA), and #852 ($CuCl_2$) and #124 ($CuCl_2$/GAA) as pairs.

III. RESULTS AND DISCUSSION

A. WATER QUALITY

Water quality measurements made during this testing were pH, dissolved oxygen (D.O.), temperature, hardness and alkalinity (Tables 3-6). For all four tests at each laboratory and among all laboratories, pH's ranged from 7.3-8.9, D.O's from 4.6-9.7 mg/L, temperature from 18-23 C, hardness from 110-216 mg/L, and alkalinity from 100-136 mg/L. These data illustrate that the recommended dilution water, while in some cases being more variable than allowed by the protocol, provided a test medium which could be prepared with a minimal variability in water quality. There was virtually no difference observed between the measured water quality parameters measured on

newly formulated solutions or the solutions which had daphnids (2-3 day old samples).

B. ANALYTICAL PRECISION AND ACCURACY

The results of the analytical precision and accuracy studies conducted at SBI are presented in Appendix 3. Mean recoveries (or accuracy) of all test materials from water ranged from 91-104%, indicating satisfactory recovery of these compounds. The results of the sample stability analyses yielded satisfactory recoveries throughout the desirable holding time, and in some instances, for substantially longer periods than required (Appendix 4). The tracer, fluorescein, which was used in conjunction with all test materials proved a much more cost effective means of measuring the concentration of these test materials in water than conventional instrumental methods. All analyses were conducted by colorimetric technique on a Technicon AutoAnalyzer. The results of each compound's analysis by SBI of each of the collaborative laboratories' are presented in Appendix 5. Results of fortified quality assurance blind samples generally yielded results within the range considered to be acceptable ($\pm$ 2 standard deviations) (Appendix 6). In certain of the analyses, it was determined that the quality assurance samples had been fortified incorrectly. In these cases, all data were further analyzed to assure

that the reported results were accurate. It is standard operating procedure at SBI to run a full set of standards before and after the analysis of each group of samples. In many cases, standards are also inserted among the samples during the analysis. Results of the analysis of these standards are plotted by a 1st order linear regression equation to ascertain overall linearity (as indicated by the correlation index). If the correlation index does not fall within the range established at SBI (>0.985), the analysis is further examined, and the entire set of analyses may be eventually discarded. In the cases where the QA blind samples were not correctly fortified, it has been determined through this procedure that the analyses are correct as reported.

C. TOXICOLOGICAL EVALUATION

Eleven of the twelve laboratories selected to initiate the collaborative study completed the required testing. One laboratory elected not to complete the testing program as required in the contractual agreement.

Forty-five chronic tests were attempted during the collaborative study - 43 were successfully completed. A test was considered successful based upon the following criteria: control organism survival of $\geq$ 80% at test termination, control reproduction $\geq$ 40 young per female surviving at test termination, and a meaningful

concentration-effect relationship. One laboratory (by virtue of unacceptably low young production among control organisms) was unable to successfully complete 2 of the 4 required tests. After reviewing the data and information provided by this laboratory, it is believed that the poor young production was a result of the use of Selenastrum solutions which were contaminated (bacteria), and therefore provided less than the required nutritional level to the test organisms.

The statistical analysis of the data derived from these studies is presented in Tables 7-10 and Figures 1-6. Table 7 presents the lower and upper limits of the MATC by laboratory. Tables 8 and 9 present the mean, standard deviation, minimum, maximum, range (in orders of magnitude) and standard deviation (in orders of magnitude) for the lower and upper limits of the MATC and the geometric mean, respectively. Table 10 presents summary statistics on interlaboratory variability. According to the protocol, each test is supposed to bracket the MATC. In a limited number of the tests conducted during this testing, the MATC was not bracketed. These data were used in the interlaboratory comparisons since this use provided a more conservative approach to the analysis of interlaboratory variation. With the exception of NaPCP, the range of values for all compounds was less than one order of magnitude. The range of the lower limit of the

MATC for NaPCP was large due to an outlying value of 56 reported by laboratory 11. The standard deviation varied from 0.13 to 0.39 orders of magnitude for all determinations.

If interlaboratory variability was due to systematic differences, a laboratory will have both values (NaPCP and NaPCP/GAA or $CuCl_2$ and $CuCl_2$/GAA) higher than the average or lower than the average, and points will cluster along the 45° line in the Youden plots. If the variability is due to random variation the two values will not be correlated and the points will lie in an eliptical region. The Youden plots do not show points which cluster along the 45° line. For NaPCP, systematic error was estimated to be 27%, 32% and 19% of the variance of the lower and upper limits of the MATC and the geometric mean, respectively. These values were not significantly different than zero. The low values found by laboratory 8 appears to account for most of this variation. For $CuCl_2$, there was no appreciable systematic error. The ranking test did not indicate that there were any laboratories that had consistently high, low or variable values.

A comparison of all the data submitted by the collaborative laboratories demonstrated significant variability in the number and frequency of molts generated by the test organisms. This variability suggested that a consistent understanding and method of measuring this

parameter did not exist among the laboratories participating in this study. Based upon the lack of reproducible measurements of this parameter, the number of molts produced by the test organisms was not evaluated when determining the MATC for each of the test materials.

A comparison of the performance by laboratory for controls throughout the testing is presented in Tables 11-15. For all four tests and across all laboratories, the control survival ranged from 80-100%. Number of reproductive days were 11-14; cumulative number of offspring, 60-101; cumulative number of offspring per reproductive day, 4.8-7.6; and length ranged from 3 6-4.2 mm. While some variability is evident, these data suggest that the requirements in the protocol for these measurements should be retained.

All chronic testing was preceded by a 48-hour static acute toxicity test, which served as a range-finding test for the selection of chronic test concentrations. For all laboratories with NaPCP and #658 (NaPCP/GAA), the mean and standard deviation EC50's were 987 $\pm$ 309 and 1088 $\pm$ 361 ug/L. The ranges of EC50 values for these compounds were from 484-1612 ug/L, and from 500-1595 ug/L. The mean and standard deviation EC50 values for compounds #852 (copper) and #124 (copper/GAA) were 271 $\pm$ 173 and 205 $\pm$ 42 ug/L. The ranges of EC50 values for these compounds were from 48-656 ug/L and 140-287 ug/L (Tables 16-19). The 21-day

LC50 values which were calculated based upon the number of animals surviving at test termination were 657 ± 154 ug/L and 585 ± 163 ug/L for NaPCP and #658, and 93 ± 20 ug/L and 102 ±38 ug/L for #852 and #124 (Tables 16-19). While evincing some difference, these values tend to corroborate the similarity in toxicity between the test material alone, and the test material mixed with glacial acetic acid, and lend further credence to the direct comparison of the resulting data.

Another comparison of reproducibility between laboratories is found in the geometric mean MATC's shown in Tables 20-23. As before, while the ranges suggest some "normal" biological variability, the geometric mean MATC of all values for each compound are virtually the same.

A comparison of all MATC values derived during this testing indicates that the most important biological measurements are survival, young per female, and length, in that order. Assuming that the range-finding test is accurate, it is normally expected that there will be an effect on the survival of the organisms. As can be seen in Table 24, survival, in the case of NaPCP and NaPCP/GAA, was clearly the most important effect criterion and yielded good reproducibility between these compounds. The other criteria measured were of much less importance for these two compounds.

Cumulative number of offspring per female appears to be another parameter of significance. Besides yielding important information on the overall health of the daphnids, in certain cases, it proved to be the only effect criterion. Length, while more difficult to measure, was a similarly important effect criterion. Care must be taken to assure that the investigator is measuring correctly (and an explicit procedure must be written to assure this), but the data clearly suggest that the growth measurements have the potential to provide more information about the long term effects of a material on this organism than certain other measurements. Correspondingly, it is obvious that exposure to copper provided a significant number of "sub-lethal" effects, with young per female, young per female per day, and length being very important. Since young/female/day is a function of young/female, it is probably not necessary to make both measurements.

The other effect criterion, number of reproductive days, and time to first brood appear to be of lesser importance in evaluating exposure effects as these criterion were never the sole indicator of the MATC. In fact, in many cases these two criterion did not yield an accurate estimation of the MATC.

D. LABORATORY QUALITY ASSURANCE

The protocol specifically required adherence to the most recent Good Laboratory Practice guidelines (as published by the EPA in November 1983). Based upon SBI's pre-award audit of the facilities and statements made during the pre-test meeting stressing the necessity for strict adherence to GLP's, it was expected that the laboratories would be substantially in compliance with the intent of the guidelines. While certain of the laboratories were meticulous in their record-keeping, it was found that some labs were not in compliance with the regulations. Among the deviations observed were the use of pencil to record data, the use of correcting fluid to revise data, total obliteration of entries, lack of initialed and dated data entries, and others. While these problems do not appear to have altered the final outcome of the testing, they suggest that the laboratories varied widely with respect to the importance or attention given to even the most fundamental quality assurance requirements.

IV. CONCLUSIONS

In conclusion, this collaborative study entailed the testing of four different materials in pairs of two which were similar enough to each other to enable direct comparison of the data derived from the testing. Of 45 tests attempted, 43 were completed successfully.

The data derived from these tests yielded a high degree of precision both within and between laboratories, and particularly when compared to previous daphnid interlaboratory studies. Static, acute toxicity tests, chronic control performance, MATC's, and even effect criterion provided reproducible data. Between laboratory variability was typically a factor of 2-3 X for acute EC50 values and 2-4 X for chronic MATC values with the selected test materials. In addition, results illustrated those effect criteria which were sensitive and reproducible measures of toxic effects and those which were not. For example, survival, young per female and length were most useful, while number of reproductive days and number of molts was of lesser significance.

Based upon the ultimate significance of the biological effect measurements, it appears that survival, young per female, and length measurements should be required criteria for assessing the toxic effects of the test material to D. magna. The other measurements yielded data which were not required for an estimate of the MATC and in some cases, actually confounded the estimate.

A review of the data packages submitted clearly suggests the need for greater adherence to the Good Laboratory Practice regulations.

Finally, it is our belief that the protocol as written presents a workable, explicit methodology for

performing a static, renewal chronic toxicity test with Daphnia magna to provide an estimate of the MATC.

V. RECOMMENDATIONS

In general, "Interim Procedures for Conducting the Daphnia magna Toxicity Assay", as followed in the preceding studies was considered acceptable as a routine, standard test methodology.

Following are comments - both positive and negative - pertaining to the protocol, and suggestions for change or improvement.

1. There was general agreement between all laboratories that the acclimation of the test organisms was very important. The quality of the daphnids derived from the acclimation cultures was consistently high. One laboratory commented that the criterion, added during the pre-test meeting, that cultures must produce $\geq$ 3 young per female per reproductive day prior to use, should be increased to 5 young per female per reproductive day. It was their opinion that cultures producing only 3 young per female per reproductive day could be unhealthy.

2. Many laboratories felt that the glassware cleaning procedures were unnecessarily cumbersome, and needed, at least, to be consistent throughout the protocol. It was suggested that once the test vessels are initially cleaned with soap, acid and acetone, that beakers should then be

able to be scrubbed with a brush and rinsed with deionized water.

3. Many of the laboratories remarked that the two-stage randomization procedure for both the acute and chronic tests exposed the daphnids to excessive handling. All of these laboratories felt that a one-stage randomization procedure would be adequate.

4. Some laboratories indicated that the protocol requirements for dilution water hardness and alkalinity were too restrictive. Several found it difficult to consistently meet the ranges as provided, although most were able to maintain their waters within the appropriate range. Based upon the new/old solution water quality measurements, it appears that the inclusion of a requirement to measure old solutions should be deleted.

5. One laboratory commented that further thought should be given to the algae culturing requirements. Vitamins and selenium were suggested as additions while sodium silicate was suggested as being unnecessary.

In view of the above suggestions, it appears that certain changes to the test protocol as presently constituted are warranted. The practical considerations described above will enhance the ultimate outcome of the test by eliminating some of the more restrictive areas.

VI. Literature Cited

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Williams, L.R., 1984. Guidelines for conducting collaborative testing of biological test methods. U.S. EPA Environmental Monitoring Systems Laboratory, Las Vegas, NV.

Youden, W.J. and E.H. Steiner, 1975. Statistical Manual of the Association of Official Analytical Chemists. AOAC, Arlington, VA.

Table 1. Panel of experts convened to review the proposed protocol entitled "Protocol for Evaluation of Waste Leachate Acute and Chronic Toxicity with Daphnia magna".

Government

Dr. Cornelius Weber - EPA

Dr. Lewellyn Williams - EPA

Mr. J. Gareth Pearson - EPA

Mr. Stephen Ells - EPA

Mr. Todd Kimmell - EPA

Dr. Kenneth Biesinger - EPA

Dr. William van der Schalie - U.S.A.M.B.R.D.L.

Industrial Laboratories

Dr. Michael Lewis - Procter & Gamble Company

Dr. Alan Maki - Exxon Company

Dr. Carl Muska - Haskell Laboratory for Toxicology & Industrial Medicine

Mr. Mark Palmieri - Allied Chemical Corporation

Dr. William Adams - Monsanto Company

Commercial Laboratories

Mr. William McAllister - Analytical Bio-Chemistry Laboratories, Inc.

Mr. Benjamin Parkhurst - Western Aquatics, Inc.

Mr. Gerald LeBlanc - Springborn Bionomics, Inc.

Mr. Robert Bentley - Springborn Bionomics, Inc.

Dr. Kenneth Duke - Battelle Columbus Laboratories

Academic

Dr. Karen Porter - University of Georgia

Dr. Arthur Buikema - Virginia Polytechnical Institute and State University

Dr. Clyde Goulden - The Academy of National Sciences

Dr. Kathleen Keating - Rutgers University

Table 2. Description of the major features of the protocol as accepted by the review panel.

·DESIGN

- 5 toxicant concentrations
- 50% dilutions
- 10 replicates per concentration
- test vessel - 100mL beaker with 80 mL solution
- one organism per replicate
- test duration - 21 days

·CONDITIONS

- Static renewal (M, W, F)
- dilution water - Marking's & Dawson's hard reconstituted water
- food - 5mg/L SD-9 fish food/1.8 mg/L ($1X10^5$ cells/mℓ) green alga per feeding
- lighting 50-100 footcandles, 16 hr. L
- organism age - ≤24 hours @ test initiation

·END POINTS

- survival
- reproduction
- growth

Table 3. Range of water quality parameters measured during testing with sodium pentachlorophenate.

Laboratory	pH	D.O.(mg/L)	Temp.(°C)[a]	Hardness(mg/L $CaCO_3$)[b]	Alkalinity(mg/L $CaCO_3$)[b]
1	8.2 - 8.5	7.5 - 8.7[c]	19.8 - 20.1	165 - 177	111 - 119
2	7.7 - 8.3	7.0 - 8.7	18.7 - 20.9	164 - 204	112 - 144
3	8.2 - 8.4	8.0 - 9.0	19.8 - 23.2	155 - 171	110 - 122
4	8.1 - 8.3	6.5 - 7.3	20.2 - 20.4	155 - 172	110 - 120
5	8.4 - 8.6	8.2 - 9.0	19.1 - 20.1	164 - 178	100 - 128
6	7.6 - 8.1	8.6 - 9.5	18.8 - 22.0	178 - 192	116 - 134
7[d]	8.0 - 8.4	8.0 - 9.2	20.0	160 - 190	110 - 130
7[d]	8.0 - 8.4	8.1 - 9.2	20.0 - 21.0	160 - 190	110 - 130
8	8.0 - 8.2	8.3 - 8.8	20.0 - 22.0	180	140
9	8.0 - 8.4	7.3 - 8.2	18.0 - 20.0	160 - 180	110 - 120
10	8.0 - 8.5	8.1 - 9.0	19.0 - 20.6	170 - 212	108 - 136
11	7.8 - 8.3	6.2 - 7.2	19.5 - 20.5	169 - 189	110 - 119

[a]Range of daily temperature measurements for both new and old solutions.

[b]Range of weekly measurements for new solutions.

[c]4.8 (1 value).

[d]Test conducted twice by this laboratory.

Table 4. Range of water quality parameters measured during testing with sodium pentachlorophenate/ glacial acetic acid (unknown #658).

Laboratory	pH	D.O.(mg/L)	Temp.(°C) [a]	Hardness(mg/L $CaCO_3$) [b]	Alkalinity(mg/L $CaCO_3$) [b]
1	8.1 - 8.4	7.7 - 8.5	20.0	169 - 177	111 - 115
2	7.5 - 8.3	7.8 - 8.6	19.1 - 21.8	172 - 176	116 - 124
3	8.2 - 8.4	8.2 - 8.9	19.5 - 20.9	159 - 171	107 - 127
4	8.0 - 8.4	4.6 - 6.8	20.6	155 - 172	114 - 128
5	8.2 - 8.4	7.4 - 8.3	19.5 - 20.6	156 - 182	110 - 135
6	7.8 - 8.1	8.2 - 9.4	19.6 - 22.4	170 - 184	114 - 134
7	8.0 - 8.4	7.8 - 9.2	20.0 - 21.0	160 - 180	110 - 130
8	-	-	-	-	-
9	8.1 - 8.3	7.6 - 8.2	19.0 - 20.0	160 - 170	110
10	7.9 - 8.3	7.8 - 9.7	19.0 - 21.0	170 - 197	110 - 123
11	7.7 - 8.5	6.0 - 8.1	19.5 - 20.5	164 - 184	111 - 121

[a] Range of daily temperature measurements for both new and old solutions.

[b] Range of weekly measurements for new solutions.

Table 5. Range of water quality parameters measured during testing with copper chloride (unknown #852).

Laboratory	pH	D.O.(mg/L)	Temp(°C) [a]	Hardness(mg/L $CaCO_3$) [b]	Alkalinity(mg/L $CaCO_3$) [b]
1	8.0 - 8.4	7.8 - 8.5	20.0	173 - 177	111 - 115
2	7.3 - 8.2	7.3 - 8.8	19.5 - 22.0	168 - 176	112 - 120
3	8.2 - 8.4	8.6 - 9.1	19.8 - 20.9	163 - 171	109 - 119
4	8.0 - 8.4	6.3 - 7.0	20.3 - 20.5	158 - 168	113 - 118
5	8.2 - 8.5	7.8 - 8.4	19.5 - 20.3	158 - 188	100 - 132
6	7.9 - 8.9	8.0 - 9.3	18.4 - 22.8	172 - 202	112 - 130
7	7.9 - 8.2	8.2 - 8.6	20.0 - 20.5	160 - 170	108 - 116
8	-	-	-	-	-
9	7.9 - 8.2	7.6 - 8.0	19.0 - 20.0	160 - 170	110 - 120
10	7.9 - 8.5	7.9 - 9.0	19.4 - 21.5	170 - 203	111 - 122
11	7.4 - 8.6	5.8 - 7.6	19.5 - 20.5	166 - 182	110 - 118

[a] Range of daily temperature measurements for both new and old solutions.

[b] Range of weekly measurements for new solutions.

Table 6. Range of water quality parameters measured during testing with copper chloride/glacial acetic acid (unknown #124).

Laboratory	pH	D.O.(mg/L)	Temp.(°C)[a]	Hardness(mg/L $CaCO_3$)[b]	Alkalinity(mg/L $CaCO_3$)[b]
1	8.1 - 8.4	8.1 - 8.5	20.0 - 20.5	163 - 169	107 - 115
2	7.4 - 8.3	7.8 - 8.5	18.6 - 21.3	168 - 176	116 - 144
3	8.1 - 8.4	8.6 - 8.9	19.8 - 21.5	160 - 188	115 - 132
4	8.0 - 8.4	6.5 - 7.4	20.0 - 20.1	160 - 174	112 - 125
5	8.2 - 8.6	8.0 - 9.1	19.0 - 20.6	144 - 192	108 - 124
6	8.1 - 8.4	8.2 - 9.6	18.3 - 21.6	168 - 192	110 - 122
7	8.0 - 8.2	8.2 - 8.6	20.0	160 - 180	110 - 120
8	8.0 - 8.3	8.2 - 8.8	20.0 - 22.0	180	140
9	7.9 - 8.2	7.9 - 8.1	19.0 - 20.0	110 - 180	110 - 120
10	8.0 - 8.5	8.0 - 9.1	19.0 - 21.0	177 - 216	110 - 136
11	8.1 - 8.4	5.6 - 7.2	19.5 - 20.0	166 - 180	110 - 113

[a]Range of daily temperature measurements for both new and old solutions.

[b]Range of weekly measurements for new solutions.

Table 7. Determination of the lower and upper limits of the MATC by laboratory and compound. All values are presented in µg/L.

	NaPCP		NaPCP/GAA		$CuCl_2$		$CuCL_2$/GAA	
Laboratory	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
1	469	983	464	917	10	26	68	130
2	471	898	386	737	--	28	--	17
3	425	875	397	793	25	61	29	53
4	317	580	535	1049	44	84	12	25
5	390	808	427	835	69	129	16	30
6	506	1050	425	797	59	107	34	71
7	576	1450	406	743	18	33	31	77
7A	243	467	---	---	--	--	--	--
8	83	170	---	---	--	27	9.8	21
9	220	458	221	447	11	80	43	87
10	194	444	---	119	38	93	19	41
11	---	56	234	483	48	56	56	107

Table 8. Summary of the statistical determinations for the lower and upper limits for the MATC. All values are presented in ug/L.

Compound	Mean	S.D.	Minimum	Maximum	Range in Orders of Magnitude	S.D. in Orders of Magnitude
Lower Limit						
NaPCP	354.00	153.73	83	576	0.841	0.25
NaPCP/GAA	388.33	101.42	221	535	0.384	0.13
$CuCL_2$	35.78	21.14	10	69	0.839	0.31
$CuCL_2$/GAA	31.78	19.22	9.8	68	0.841	0.28
Upper Limit						
NaPCP	686.58	396.90	56	1450	1.413	0.39
NaPCP/GAA	692.00	270.06	119	1049	0.945	0.28
$CuCL_2$	66.80	37.37	26	129	0.696	0.27
$CuCL_2$/GAA	59.91	37.62	17	130	0.883	0.30

Table 9. Summary of the statistical determinations made using the geometric mean of the MATCs. All values are presented in µg/L.

Compound	Mean	S.D.	Minimum	Maximum	Range in Orders of Magnitude	S.D. in Orders of Magnitude
NaPCP	512.61	233.54	118.79	913.89	0.886	0.25
NaPCP/GAA	541.64	138.93	314.30	749.14	0.377	0.13
$CuCL_2$	50.37	27.97	16.13	94.35	0.767	0.29
$CuCL_2$/GAA	45.13	26.48	14.35	94.02	0.816	0.28

Table 10. Statistics on interlaboratory variability (Youden and Stiener, 1975).

Compound	S_R	S_D	F ratio	S_{bias}	D.F.
NaPCP/NaPCP:GAA					
Lower limit	80.5	118.5	2.17	61.5	8
Upper limit	243.0	106.7	2.80	230.6	10
Geometric mean	136.2	173.5	1.62	75.9	8
$CuCl_2/CuCl_2$:GAA					
Lower limit	24.3	14.3	<1	0	——
Upper limit	42.6	31.1	<1	0	——
Geometric mean	33.1	18.6	<1	0	——

Figure 1. Youden plot comparing the lower limit of the MATCs derived for NaPCP and NaPCP/GAA.

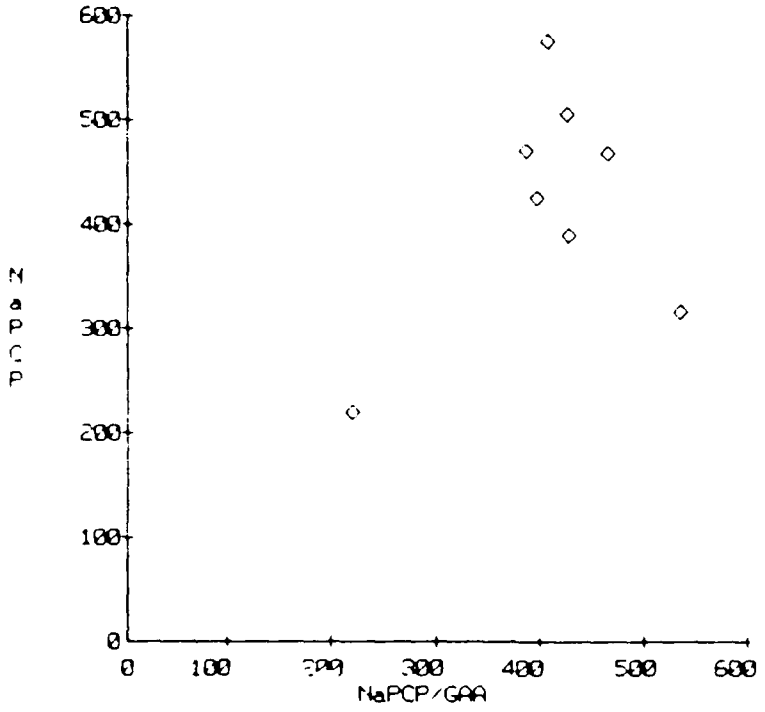


Figure 2. Youden plot comparing the upper limit of the MATCs derived for NaPCP and NaPCP/GAA.

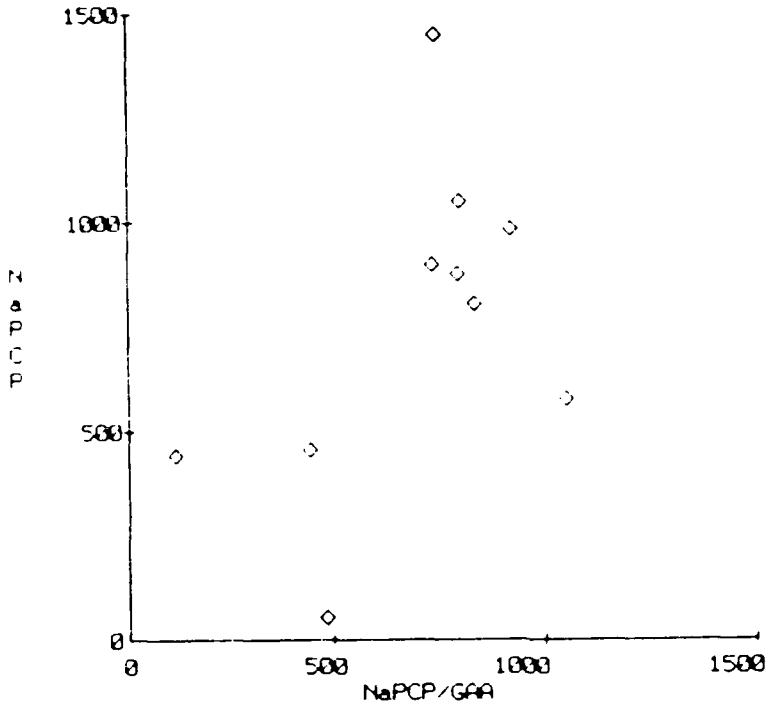


Figure 3. Youden plot comparing the geometric means of the MATCs derived for NaPCP and NaPCP/GAA.

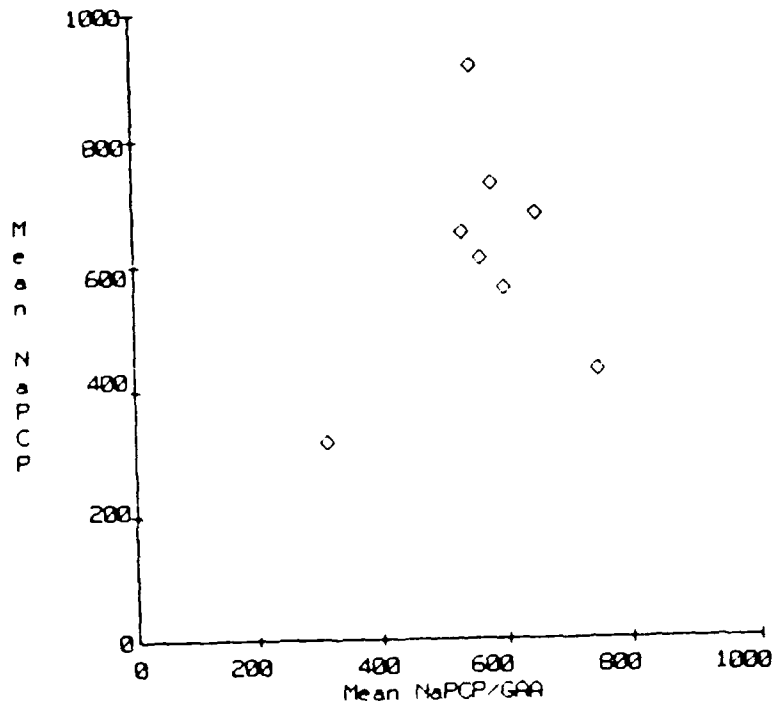


Figure 4. Youden plot comparing the lower limit of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA.

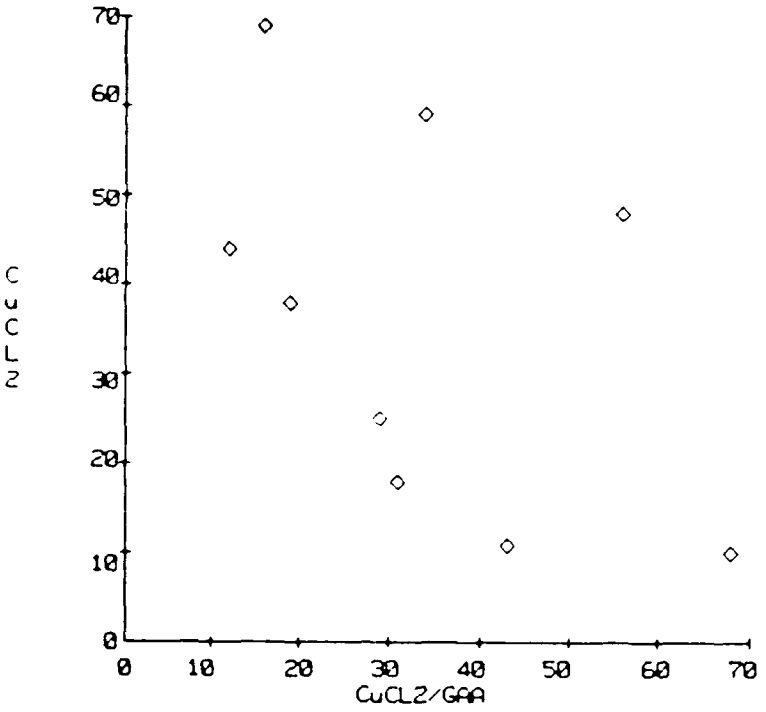


Figure 5. Youden plot comparing the upper limit of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA.

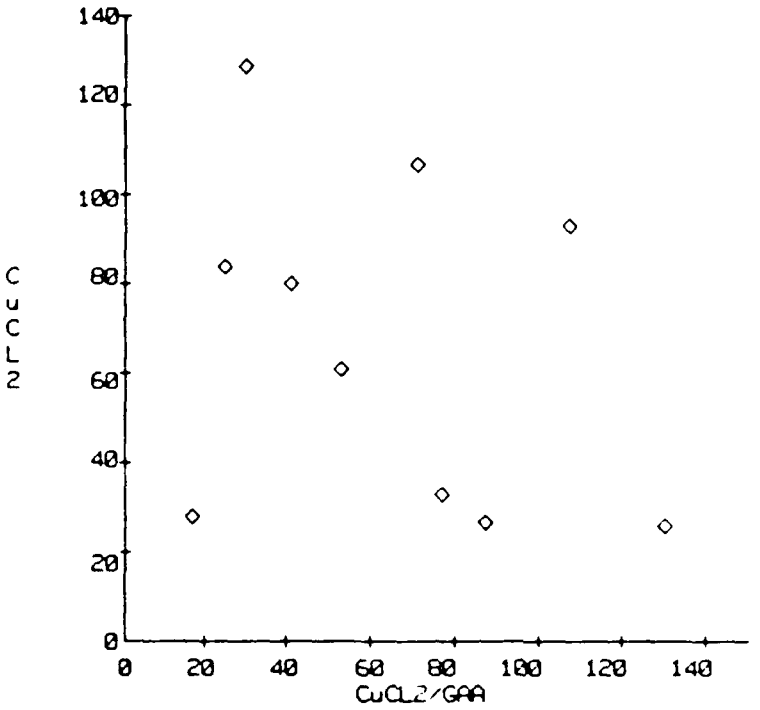


Figure 6. Youden plot comparing the geometric means of the MATCs derived for $CuCL_2$ and $CuCL_2$/GAA.

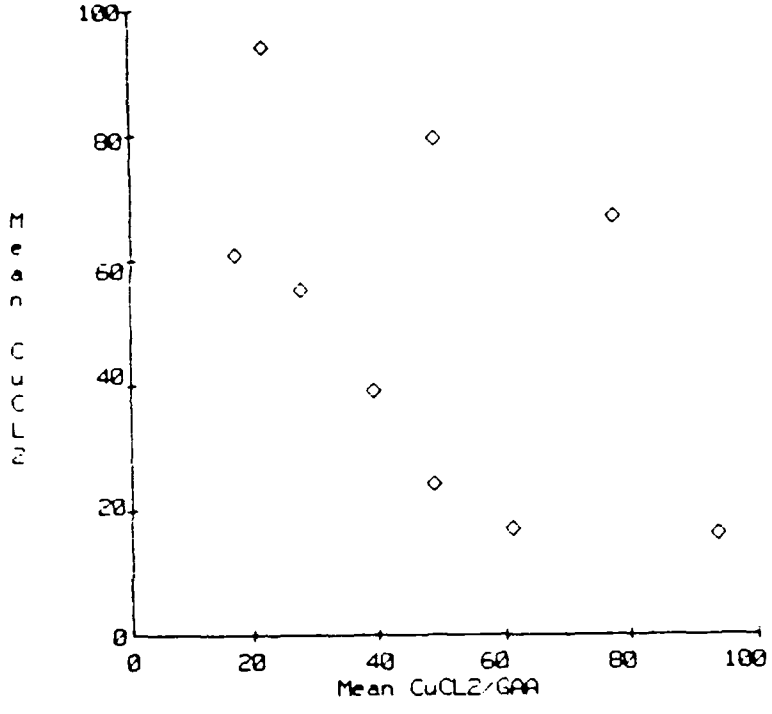


Table 11. Comparison of control performance by laboratory for all materials tested.

Laboratory	Survival	No. Reproduct. Days	Cum. No. Offspring	No. Offspring/ Reproduct. Day	Length
1	95(6) [a]	13(1)	82(10)	6.2(0.7)	4.2(0.1)
2	100(0)	12(1)	70(10)	5.8(0.5)	3.6(0.1)
3	100(0)	12(1)	61(6)	5.2(0.3)	3.9(0.5)
4	100(0)	12(1)	60(6)	4.8(0.2)	4.0(0.5)
5	95(6)	12(1)	94(29)	7.5(2.0)	4.2(0.2)
6	98(5)	13(1)	66(3)	5.2(0.2)	3.8(0.1)
7	98(5)	13(2)	84(23)	6.3(1.0)	4.2(0.1)
8 [b]	100(0)	12(0)	89(10)	7.6(0.6)	4.2(0.1)
9	90(0)	11(2)	72(14)	6.4(0.8)	3.9(0.1)
10	95(10)	12(2)	74(13)	6.2(0.7)	4.1(0.4)
11	100(0)	14(0.6)	101(16)	7.5(1.0)	- [c]

[a] Standard deviation.

[b] Represents values from two tests only.

[c] There was a discrepancy in the measurement technique at this laboratory. Numbers are therefore not presented. (See text)

Table 12. Comparison of control performance by laboratory for sodium pentachlorophenate.

Laboratory	Survival (%)	No. Reproduct. Days	Cum. No. Offspring per ♀	No. Offspring/ Reproduct. Day	Length (mm)
1	90(32)	14(0)	76(10)	5.4(0.7)	4.1(0.1)
2	100(0)	13(1)	83(10)	6.4(0.8)	3.7(0.1)
3	100(0)	12(1)	61(8)	4.9(0.8)	4.5(0.3)
4	100(0)	13(1)	61(12)	4.8(0.8)	5.7(0.1)
5	100(0)	12(1)	77(8)	6.3(1.0)	4.1(0.1)
6	100(0)	13(2)	64(15)	5.0(1.0)	3.7(0.4)
7	100(0)	14(1)	72(9)	5.3(0.6)	4.2(0.1)
7	100(0)	15(0)	108(14)	7.0(0)	4.4(0.1)
8	100(0)	12(2)	82(13)	7.1(0.4)	4.1(0.1)
9	90(32)	10(0)	62(7)	6.2(0.7)	3.9(0.1)
10	100(0)	13(0)	77(7)	5.9(0.6)	4.3(0.1)
11	100(0)	14(1)	107(11)	8.0(0.6)	-[a]

[a]There was a discrepancy in the measurement technique at this laboratory. Numbers are therefore not presented.

Table 13. Comparison of control performance by laboratory for sodium pentachlorophenate/glacial acetic acid (#658).

Laboratory	Survival	No. Reproduct. Days	Cum. No. Offspring	No. Offspring/ Reproduct. Day	Length
1	100(0)	12(1)	71(6)	6.0(0.4)	4.2(0.1)
2	100(0)	11(2)	59(10)	5.3(0.8)	3.6(0.1)
3	100(0)	11(1)	63(5)	5.6(0.7)	3.6(0.2)
4	100(0)	11(1)	52(12)	4.7(0.9)	4.2(0.3)
5	100(0)	13(1)	133(24)	10(1)	4.4(0.1)
6	100(0)	13(1)	66(6)	5.2(0.4)	3.9(0.1)
7	90(32)	15(1)	113(17)	7.6(1.0)	4.3(0.1)
8[a]	-	-	-	-	-
9	90(32)	10(0)	74(11)	7.4(1.1)	3.9(0.1)
10	100(0)	9.6(1.5)	66(17)	7.1(2.5)	3.9(0.1)
11	100(0)	13(1)	82(22)	6.1(1.5)	-[b]

[a] Invalid test.

[b] There was a discrepancy in the measurement technique at this laboratory. Numbers are therefore not presented.

Table 14. Comparison of control performance by laboratory for copper chloride (#852).

Laboratory	Survival	No. Reproduct. Days	Cum. No. Offspring	No. Offspring/ Reproduct. Day	Length
1	100(0)	14(1)	90(12)	6.5(0.8)	4.1(0.1)
2	100(0)	12(0)	67(6)	5.6(0.5)	3.5(0.1)
3	100(0)	10(1)	52(8)	5.1(0.7)	3.5(0.2)
4	100(0)	14(1)	65(8)	4.7(0.6)	4.6(0.3)
5	90(32)	12(1)	99(14)	8.1(1.2)	4.3(0.1)
6	100(0)	12(0)	64(9)	5.2(0.6)	4.0(0.1)
7	100(0)	12(1)	61(12)	5.0(1.1)	4.2(0.1)
8[a]	-	-	-	-	-
9	90(32)	11(2)	61(5)	5.4(0.6)	3.9(0.1)
10	80(42)	12(1)	61(8)	5.4(1.1)	3.7(0.1)
11	100(0)	14(0)	119(13)	8.5(0.9)	-[b]

[a] Invalid test.

[b] There was a discrepancy in the measurement technique at this laboratory. Numbers are therefore not presented.

Table 15. Comparison of control performance by laboratory for copper chloride/glacial acetic acid (#124).

Laboratory	Survival	No. Reproduct. Days	Cum. No. Offspring	No. Offspring/ Reproduct. Day	Length
1	90(32)	13(1)	90(10)	7.0(1.2)	4.2(0.1)
2	100(0)	12(1)	72(10)	5.8(1.0)	3.4(0.1)
3	100(0)	13(1)	67(6)	5.3(0.6)	4.1(0.1)
4	100(0)	12(0)	63(5)	5.2(0.4)	3.6(0.1)
5	90(32)	12(1)	66(11)	5.5(1.3)	3.9(0.1)
6	90(32)	13(1)	71(6)	5.5(0.4)	3.8(0.2)
7	100(0)	12(0)	90(13)	7.4(1.2)	4.1(0.1)
8	100(0)	12(0)	96(8)	8.0(0.6)	4.2(0.1)
9	90(32)	14(1)	92(12)	6.7(0.8)	4.0(0.1)
10	100(0)	14(1)	90(7)	6.5(0.5)	4.6(0.1)
11	100(0)	13(1)	97(22)	7.3(1.3)	-[a]

[a] There was a discrepancy in the measurement technique at this laboratory. Numbers are therefore not presented.

Table 16. Comparison of static, acute EC50'S and 95% confidence limits vs. 21-day LC50's by laboratory for NaPCP in testing conducted prior to and at the termination of the chronic study.

Laboratory	EC50 95% confidence limit (ug/L)	LC50 95% confidence limit (ug/L)
1	926 (869-1005)	757 (714-808)
2	1512 (1379-1648)	650 (471-898)
3	1612 (1000-2000)	875 (---[a])
4	1017 (922-1119)	580 (317-1200)
5	898 (824-985)	490 (390-808)
6	870 (790-978)	675 (506-1050)
7	850 (630-1300)	914 (576-1450)
7	1100 (960-1200)	672 (467-1175)
8	740 (622-892)	710 (500-1000)
9	484 (418-576)	404 (309-562)
10	904 (750-1500)	566 (354-674)
11	931 (563-1900)	687 (484-980)

[a]Confidence limits unable to be calculated.

Table 17. Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for compound 658 (MaPCP/GAA) in testing prior to and at the termination of the chronic study.

Laboratory	EC50 95% confidence limit (ug/L)	LC50 95% confidence limit (ug/L)
1	[illegible] (1000-2000)	652 (464-917)
2	1367 (1269-1470)	679 (386-1580)
3	1500 (1250-2500)	933 (750-1500)
4	925 (625-1240)	624 (267-1049)
5	1150 (1030-1310)	527 (208-835)
6	730 (572-899)	582 (425-797)
7	910 (820-1000)	438 (107-743)
8	---[a]	---[a]
9	500 (426-598)	404 (286-648)
10	1595 (1250-2500)	378 (206-611)
11	848 (781-928)	637 (483-973)

[illegible] test

Table 18. Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for compound 852 ($CuCL_2$) in testing prior to and at the termination of the chronic study.

Laboratory	EC50 95% confidence limit (ug/L)	LC50 95 confidence limit (ug/L)
1	207 (166-252)	69 (26-110)
2	656 (500-1000)	86 (65-110)
3	459 (250-1000)	66 (46-91)
4	48 (36-61)	130 (96-224)
5	222 (173-282)	100 (69-129)
6	200 (151-296)	82 (61-110)
7	202 (177-231)	90 (76-117)
8	---[a]	---[a]
9	181 (155-211)	90 (68-122)
10	192 (158-238)	96 (80-142)
11	344 (307-392)	119 (84-168)

[a] Invalid test

Table 19. Comparison of static, acute EC50's and 95% confidence limits vs. 21-day LC50's by laboratory for compound 124 ($CuCL_2$/GAA) in testing prior to and at the termination of the chronic study.

Laboratory	EC50 95% confidence limit (ug/L)	LC50 95% confidence limit (ug/L)
1	252 (206-308)	92 (67-136)
2	185 (168-200)	100 (66-131)
3	140 (113-185)	80 (53-121)
4	158 (129-200)	72 (52-101)
5	287 (252-336)	84 (55-110)
6	174 (130-237)	95 (71-126)
7	212 (180-247)	88 (77-101)
8	224 (185-268)	199 (160-320)
9	213 (183-253)	120 (87-166)
10	212 (150-300)	64 (51-106)
11	202 (163-273)	133 (56-215)

Comparison of MATC's and effect criterion by laboratory for NaPCP.

	Geometric Mean MATC	Effect Criterion
	679	Survival
	650	Survival
	610	Survival
	429	Survival
	561	Survival
	729	Survival
	914	Survival
	336	Length
	119	# of reprod. days and time to 1st brood
	317	y/♀ and y/♀/d
	293	Survival
	< 56	y/♀ and y/♀/d

Mean and standard deviation geometric mean MATC - 512 ± 234 ug/L.

Table 21. Comparison of MATC's and effect criterion by laboratory for Compound 658 (NaPCP/GAA)

Laboratory	Geometric Mean MATC	Effect Criterion
1	652	Survival
2	533	Survival
3	561	Survival
4	749	Survival
5	597	Survival
6	582	Survival
7	549	Survival
8	---	---
9	314	Survival, y/♀, y/♀/d
10	< 119	y/♀
11	336	y/♀, y/♀/d

Mean and standard deviation geometric mean MATC - 541 ± 139 ug/L.

Table 22. Comparison of MATC's and effect criterion by laboratory for Compound 852 ($CuCl_2$)

Laboratory	Geometric Mean MATC	Effect Criterion
1	16	y/♀, time to 1st brood
2	<28	Y/♀ and y/♀/d
3	39	y/♀, y/♀/d, length
4	61	Survival
5	94	a
6	79	Survival, y/♀ and length
7	24	Length
8	--	----
9	17	Length
10	55	y/♀, y/♀/d, length
11	67	y/♀, y/♀/d

Mean and standard deviation geometric mean MATC - 50 ± 28 ug/L

[a]Effect criterion - statistical significance was demonstrated in all parameters of interest.

Table 23. Comparison of MATC's and effect criterion by laboratory for Compound 124 ($CuCL_2$/GAA).

Laboratory	Geometric mean MATC	Effect Criterion
1	94	a
2	<17	Length
3	39	y/♀/d and # of reprod. days
4	17	Length
5	22	time to 1st brood
6	49	b
7	49	Length
8	14	Length
9	61	Length
10	28	y/♀, y/♀/d, Length
11	77	# of reprod. days, y/♀ and y/♀/d
Mean and Standard deviation geometric mean MATC - 45 ± 26 ug/L		

[a] Effect criterion - statistical signif.cance was demonstrated in all parameters of interest.

[b] Effect criterion - # of reproductive days, y/♀, y/♀/d, time to 1st brood and length.

Table 24. Mean of geometric mean MATC's and relative importance of effect criterion per compound.

	NaPCP	NaPCP/GAA(658)	$CuCl_2$(852)	$CuCl_2$/GAA(124)	Total [a]
Geometric Mean MATC (ug/L)	512	541	50	45	
Standard deviation	234	139	28	26	
(Range)	(119 - 914)	(314 - 749)	(16 - 94)	(14 - 94)	
Effect Criterion					
Survival	8	9	3	1	21
♂/♀	2	2	7	4	15
♂/♀/d	2	2	5	5	14
Length	0	0	6	8	14
Reprod. days	1	0	1	4	6
Time to 1st brood	1	0	2	3	6

[a] Values in this column may exceed the number of studies conducted due to the overlap of effect criteria at the MATC.

APPENDIX 1

An Assessment of the Suitability of Several Media for Culturing and Testing *Daphnia magna*

AN ASSESSMENT OF THE SUITABILITY OF SEVERAL COMBINATIONS OF MEDIA AND DIETS FOR CULTURING AND TESTING *Daphnia magna*

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INTRODUCTION

The U.S. Army and U.S. EPA are currently developing a standard test procedure to assess the toxicity of solid waste leachates. The test is of static, renewal design and employs Daphnia magna as the test organism. A major consideration in the development of this test was the culture and test medium and the diet to be recommended. The purpose of this study was to assess the suitability of three combinations of test media and diets and recommend one combination for use in the test procedure.

The three media-diet combinations were selected at a meeting in Cleveland, Ohio, in February 1983, attended by recognized experts in aquatic toxicology and cladoceran biology. The suitability of the media was assessed by culturing successive generations of D. magna under acclimation and test specifications outlined in the solid waste leachate toxicity test protocol. This test was performed concurrently at the Aquatic Toxicology Laboratory of Springborn Bionomics, Wareham, Massachusetts, and at the U.S. Army Medical Bioengineering Research and Development Laboratory, Ft. Detrick, Maryland.

MATERIALS AND METHODS

This study was conducted according to the protocol entitled "Protocol for assessing the suitability of various Daphnia magna culture and test media (EG&G, 1983)." Daphnia used in this study were obtained from the USEPA, Duluth, Minnesota.

Culture Media

Three medium-diet combinations were assessed by Bionomics and Ft. Detrick. Marking's and Dawson's (MD) medium-diet combination of distilled, deionized water reconstituted to a total hardness of 160-180 mg/L as $CaCO_3$ according to Marking and Dawson (1973)[1] (Appendix I). Added to the reconstituted water was 5.0 mg/L of SD-9 fish food suspension (Appendix II) and 1.8 mg/L of the alga, Selenastrum capricornutum (1×10^5 cells/mL) cultured in micronutrient supplemented MBL medium (Appendix III). Modified MS medium-diet combination consisted of an inorganic medium modified from the MS medium developed by Dr. K.I. Keating, Rutgers University (Appendix IV) and 6.8 mg/L of the alga, Chlamydomonas reinhardtii (1.3×10^5 cells/L L). The Chlamydomonas reinhardtii was cultured in a medium similar to the MS medium (Appendix V). The supplemental MD medium-diet pair consisted of the same reconstituted water and food as the MD medium-diet combination supplemented with the micronutrients used in the preparation of the modified MS medium. In addition to these medium-diet pairs, Bionomics tested its standard daphnid medium and diet (BM) which consisted of well water fortified to a total hardness of 160-180 mg/L as $CaCO_3$ accord-

[1] Marking, L.L. and V.K. Dawson. 1973. Toxicity of quinaldine sulfate to fish. Invest. Fish Control No. 10. U.S. Fish. Wildl. Serv., Washington, DC. 10 p.

ing to Marking and Dawson (1973). Food consisted of 5.0 mg/L of yeast and 6.8 mg/L of Chlamydomonas reinhardtii. This medium-diet pair was assessed as a standard to represent control conditions. Ft. Detrick also cultured daphnids for two generations in the supplemental MD medium-diet without EDTA to determine whether ETDA could be eliminated from the medium. This medium-diet was evaluated since the elimination of EDTA would be desirable when determining the toxicity of metals. The unchelated medium was described as yellow colored; however no solid particles were observed. Batches of each medium were prepared weekly. Total hardness, total alkalinity, pH, and specific conductance were measured with each new batch of medium.

Four criteria were established to assess the suitability of each medium-diet combination tested for culturing and testing D. magna. These criteria were:

1) Do D. magna cultured in the medium-diet combinations meet minimum criteria established by ASTM for survival (70%) and offspring/female (40) over 21 days?

2) Can the medium be prepared with minimum variability in water quality characteristics between batches?

3) Can consistent performance of D. magna be obtained when cultured in the medium-diet combination?

4) Is the medium and the diet economically and practically feasible?

Acclimation cultures

Daphnids were cultured in each medium-diet pair for 4 or 5 consecutive 21-day generations. Culture vessels were 1000-mL glass beakers, each containing 800 mL of medium. Three vessels were maintained for each medium-diet pair. The first generation was initiated by introducing ten daphnids ($\lesssim$ 24 hours old) obtained from stock cultures to each culture vessel. One daphnid was added to each vessel until all vessels contained ten organisms. Offspring obtained from 14-18 day old D. magna ($\sim$ 2nd-4th brood) of each generation were used to initiate the subsequent generation. Media and food additives were renewed three times weekly (Monday, Wednesday, Friday). Only parental daphnids were returned to renewed solutions. All offspring were counted and removed from the vessels on the day prior to initiating a new generation to ensure that the new generation was initiated with daphnids which were less than 24 hours old. Subsequent generations were initiated in the same manner as the first.

Survival of parental daphnids and the number of offspring produced were assessed at each renewal period. In addition, the temperature, dissolved oxygen concentration and pH of each old and new solution were measured in one replicate vessel at each renewal. After 21 days, the individual length of each surviving parental daphnid was determined and the percentage survival of adults and number of offspring produced per surviving female per reproductive day were calculated.

Media suitability test

The performance of daphnids cultured in each combination medium and diet was assessed through two 21-day generations. Test vessels were 100-mL glass beakers each containing 80 mL of the appropriate medium-diet combination. Daphnids (< 24 hours old) used to initiate this test were offspring from second generation acclimation organisms and were isolated in the same manner used to initiate subsequent generations of the acclimation cultures. On day 14 of the first generation, offspring (< 24 hours old) were used to initiate the second generation test.

Culture media and food additives were renewed three times weekly (Monday, Wednesday, Friday). Only parental daphnids were returned to the test vessels at each renewal. All offspring were removed, counted and discarded. Additional observations (Tuesday, Thursday) were made when required for determining time to first brood.

Survival of parental daphnids and number of offspring produced were assessed at each renewal period. In addition, the temperature, dissolved oxygen concentration and pH of each old and new solution were measured in one replicate vessel. Individual lengths of surviving daphnids were determined on day 21. The number of broods per female alive at day 21, the number of offspring per female per reproductive day for females alive at day 21, the number of days to first brood and lengths of female daphnids were subjected to analysis of variance. Significant ($p < 0.05$) differences between the response of daphnids reared in each

medium-diet combination were determined using Duncans Multiple Range Test (Steel and Torrie, 1960).[1]

RESULTS

A summary of the characterization of each batch of culture medium prepared for this study is presented in Table 1. Each medium was relatively comparable between the two laboratories although pH values were lower in Bionomics Modified MS and were more variable in Bionomics Supplemented MD. The pH of the modified MS medium used for the acclimation cultures ranged from 6.3 to 9.1 and 6.6 to 8.7 at Bionomics and Ft. Detrick respectively. These ranges exceeded the recommended pH range of 6.8-8.5, however they did not appear to affect the daphnids response. The media MD, Supplemental MD and BM were comparable in characterization except that supplemental MD had a consistently lower alkalinity. Eliminating EDTA from the Supplemented MD medium resulted in more variable total alkalinities and specific conductances. The solutions were also yellow colored, suggesting possible incomplete solubilization of some components.

Acclimation

A summary of the water quality characterization during the multigeneration acclimations of D. magna to the various media-diet combinations is presented in Table 2 for the Bionomics cultures and Table 3 for the Ft. Detrick cultures. The mean dissolved oxygen concentrations were significantly (Student's t,

[1] Steel, R.G.D. and J.H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill, New York: 481 pp.

$p = 0.05$) higher in solutions of MD, Supplemented MD and Modified MS media prepared at Ft. Detrick as compared to those prepared at Bionomics.

Survival of daphnids was normal when cultured for several generations in MD and Supplemented MD medium-diet combinations at both Bionomics and Ft. Detrick (Tables 4 and 5). Survival of daphnids cultured in Modified MS medium at Ft. Detrick was consistently lower as compared to survival in the other media-diet pairs. Survival of generations 2 and 3 were below 80%. The first three generations of daphnids cultured in the Modified MS medium-diet combination at Bionomics survived normally; however, only 13% of the fourth generation survived.

There were no trends in reproduction or growth among generations of daphnids cultured in the same medium-diet combination at Bionomics or Ft. Detrick. However, daphnids cultured in all combinations of medium and diet at Bionomics produced significantly more offspring than the daphnids cultured in the comparable medium and diet at Ft. Detrick. There were no significant differences in size among daphnids cultured at Bionomics and Ft. Detrick.

Normal survival was observed among daphnids cultured for two generations in Supplemented MD medium-diet without EDTA. Daphnids reared in this medium-diet were generally smaller and produced fewer offspring as compared to daphnids reared in all other medium-diet combinations. In addition, first generation daphnids

cultured in the Supplemented MD medium-diet were pale in appearance and had very little fat reserves. These results, in addition to the coloration of the solutions suggesting incomplete solubilization of some constituents, indicate the Supplemented MD medium-diet combination should not be used to culture and test D. magna.

Medium diet

Communications between the two laboratories revealed several differences existed between culture methods used at Bionomics and Ft. Detrick. The light intensity provided to the acclimation culture at Bionomics was lowered from ~120 foot candles to 65 foot candles on the fifth day of the first generation. The adjustment in light intensity was made to provide comparable conditions between Bionomics and Ft. Detrick. Comparison of the survival, growth and the reproduction of daphnids during the first generation of acclimation with that observed in subsequent generations at Bionomics suggests that the adjustment made in light intensity had no effect on the daphnids response. Culture procedure modifications made at Ft. Detrick to simulate conditions at Bionomics were (1) media storage temperature increased from 4° to 20° C, (2) light intensity increased from an average of 42 foot candles to 65 foot candles, (3) algal suspended in the appropriate algal culture medium versus daphnid culture medium and, (4) addition of micronutrients to Supplemental MD medium at the time of preparation

versus 24 hours after preparation of the medium. The above mentioned modifications were made by Ft. Detrick personnel after the fourth generation acclimation cultures were completed. Evaluation of the fifth generation acclimation cultures indicated that there were no apparent differences in the daphnids' response between the fifth generation and those previously maintained at Ft. Detrick. The only observed effect of the modification made at Ft. Detrick was the reduction in the number of observed daphnids floating on the media's surface.

Media suitability test

A summary of the water quality characterization of solutions during the medium-diet suitability tests is presented in Table 6 for Bionomics and Table 7 for Ft. Detrick. The pH values and temperatures were generally comparable between generations and between laboratories. Dissolved oxygen concentrations were consistently lower in Bionomics' solutions as compared to Ft. Detrick. The reason for this variance was discussed previously.

Survival of daphnids was normal when cultured for two generations in the various medium-diet pairs at Bionomics (Table 8). There were no significant differences among the number of broods per female cultured in the various combinations of media and diets for two generations. There were no significant differences in the time to first brood among first generation daphnids cultured in the different medium-diet pairs at Bionomics. The second genera-

tion daphnids cultured in the MD medium-diet produced their first brood statistically significantly sooner than daphnids cultured in the other medium-diet pairs. Second generation daphnids cultured in the Supplemented MD medium-diet produced their first brood significantly later than the other medium-diet pairs. Although statistical comparison of time to first brood revealed several significant differences, these differences were not considered biologically significant since the age of the daphnids at the initiation of the test can vary within 24 hours and observations made every 24 hours may not provide the sensitivity to accurately define this parameter.

The first generation daphnids cultured at Bionomics in the MD medium-diet produced significantly fewer offspring and were significantly smaller than daphnids cultured in the other three medium-diet pairs. First generation daphnids cultured in the Supplemented MD medium-diet produced significantly fewer offspring and were significantly smaller than daphnids cultured in the BM or the Modified MS medium-diet. The relative response in growth and reproduction by first generation daphnids was reproduced by second generation organisms. The lengths of second generation daphnids cultured at Bionomics in the BM and the Modified MS media-diets were comparable; however each was significantly greater than the lengths of daphnids cultured in the Supplemented MD and the MD media-diets. Daphnids cultured in the Supplemented MD and the MD media-diets were of comparable lengths.

Normal survival was observed among daphnids cultured at Ft. Detrick in the MD and the Supplemented MD media-diets for two generations (Table 9). Only 55% of the first generation daphnids cultured in the Modified MS medium-diet survived. Survival of second generation daphnids cultured in the Modified MS medium-diet was normal. There were no significant differences in the time to production of the first brood of eggs among first generation daphnids cultured at Ft. Detrick in MD, Supplemented MD and Modified MS media-diets. Second generation daphnids cultured in the Supplemented MD medium-diet at Ft. Detrick produced their first brood of eggs significantly sooner than daphnids cultured in MD and Modified MS media-diets. First generation daphnids cultured at Ft. Detrick in the MD medium-diet produced significantly fewer broods than daphnids cultured in Supplemented MD and Modified MS media-diets. Second generation daphnids cultured at Ft. Detrick in the Modified MS medium-diet produced significantly more broods than daphnids cultured in MD or Modified MD media-diets.

First generation daphnids cultured in the MD medium-diet at Ft. Detrick produced significantly more offspring than daphnids cultured in Supplemented MD or Modified MS media-diets. First generation daphnids cultured at Ft. Detrick in the Modified MS medium-diet were significantly smaller than daphnids cultured in MD or Supplemented MD media-diets. The lengths and number of offspring produced by second generation daphnids cultured at Ft. Detrick in the three combinations of media and diets were each significantly different from the others.

DISCUSSION

The results of the suitability test were used to quantitatively assess the medium-diet combinations according to the previously mentioned criteria and the acclimation results were used as a qualitative comparison of the test results.

Criteria 1

The MD and the Supplemented MD media-diets consistently met the minimum criteria for survival. Below acceptable survival occurred with the Modified MS medium-diet during the first generation test at Ft. Detrick. Below acceptable survival also occurred with this medium-diet combination during the fourth generation acclimation at Bionomics and second generation acclimation at Ft. Detrick. To meet the minimum reproductive requirement, an average of approximately 4 offspring/female/reproductive day would be necessary. Daphnids cultured in all media-diets during testing and acclimation met this requirement. Based on criteria 1, the MD or the Supplemented MD medium-diet would be acceptable for culturing and testing D. magna.

Although significant differences in the biological parameters measured existed between daphnid cultures in the MD and Supplemented MD media-diets, these differences were not consistent between laboratories. In addition, these differences always occurred above the considered minimum acceptable criterion levels. Based on these data, the differences in biological response of daphnids cultured in the MD and the Supplemented MD media-diets were not considered when evaluating each medium-diet combination for suitability for culturing and testing of D. magna.

Criteria 2

Coefficients of variation were calculated for total hardness, total alkalinity, specific conductance and pH of the batches of media prepared at both testing laboratories (Table 10). Analyses of these values by ANOVA and Duncans Multiple Range Test, using the values from each laboratory as replicates, indicated significant variability occurred with total hardness measurements of Modified MS medium, and total alkalinity of Supplemented MD and Modified MS medium. In addition, appreciable variation occurred with pH measurements at Bionomics and specific conductance measurements at Ft. Detrick of Supplemented MS medium. Based on criteria 2, MD medium would be the most suitable medium for culturing and testing D. magna.

Criteria 3

Coefficients of variation were calculated for the day to first brood, number of broods per female, offspring production, and lengths of D. magna during the suitability test of the different medium-diet combinations (Table 10). Analyses of these values as described for criteria 2 indicated no significant differences in variability existed between each medium-diet combination. Based on criteria 3, all three combinations of medium and diet were acceptable for the culturing of D. magna.

Criteria 4

The three combinations of medium and diet evaluated proved to be relatively practical for use in culturing and testing D. magna. Greater time was expended in preparing the Supplemented MD and Modified MS media-diets as compared to the MD medium-diet. It was estimated that it would cost 15 to 25% more to perform a static renewal toxicity test using Supplemented MD or Modified MS medium-diet, respectively, as compared to MD medium-diet. Based on criteria 4, MD medium-deit combination is the best choice for use in the culturing and the testing of D. magna.

Conclusion

The results of this study suggest that the medium-diet best suitable for the culturing and the testing of D. magna is the MD medium-diet.

Table 1. Water quality characterization of media used to culture D. magna at Ft. Detrick and Bionomics.

Lab	Medium	Total Hardness (mg/L $CaCO_3$)[a]	Total Alkalinity (mg/L $CaCO_3$)[a]	Specific Conductance (μmhos/cm)[a]	pH[b]
Bionomics	MD	163(5)	110(9)	500(0)	8.2-8.4
	Supplemented MD	162(4)	60(15)	500(0)	7.2-8.3
	Modified MS	34(5)	9.2(3.2)	288(23)	7.2-7.4
	BM	164(6)	121(2)	500(0)	7.9-8.3
Ft. Detrick	MD	174(4)	111(2)	517(29)	8.0-8.3
	Supplemented MD	167(7)	94(17)	638(95)	8.2-8.3
	Modified MS	40(5)	9.1(3.2)	340(32)	8.1-8.4
	Supplemented MD w/o EDTA	168(8)	79(27)	622(125)	8.2-8.3

[a]Mean (and standard deviation).

[b]Range

Table 2. Water quality measurements made during the acclimation culturing of Daphnia magna in different combinations of media and diets at Bionomics.

Medium	Generation	Dissolved Oxygen[a] (mg/L)	pH[b]	Temperature[a] (°C)
MD	1	8.2(0.1)	7.9-8.4	20(0)
	2	8.0(0.8)	7.5-8.3	20(0)
	3	8.0(0.6)	7.9-8.4	20(0)
	4	7.8(0.6)	7.9-8.3	20(0)
Supplemented MD	1	8.4(0.7)	7.5-8.5	20(0)
	2	7.9(0.6)	7.6-8.3	20(0)
	3	7.1(1.1)	7.5-8.3	20(0)
	4	7.7(0.6)	7.5-8.0	20(0)
Modified MS	1	8.3(1.4)	6.8-9.1	20(0)
	2	7.2(0.9)	6.6-7.6	20(0)
	3	7.1(1.1)	6.4-7.2	20(0)
	4	7.2(0.8)	6.3-7.2	20(0)
BM	1	8.0(0.9)	7.9-8.7	20(0)
	2	7.7(0.9)	7.7-8.3	20(0)
	3	7.4(0.8)	7.7-8.3	20(0)
	4	7.5(0.6)	7.9-8.3	20(0)

[a]Mean (and standard deviation)

[b]Range

Table 3. Water quality measurements made during the acclimation culturing of Dapnnia magna in different combinations of media and diets at Fort Detrick.

Medium	Generation	Dissolved Oxygen[a] (mg/L)	pH[b]	Temperature[a] (°C)
MD	1	8.3(0.7)	7.9-8.4	20(0)
	2	8.5(0.5)	8.0-8.4	20(0)
	3	8.4(0.5)	7.9-8.4	20(0)
	4	8.6(0.5)	7.8-8.3	20(0)
	5	8.1(0.6)	6.8-8.6	20(0)
Supplemented MD	1	8.5(0.5)	8.0-8.4	20(0)
	2	8.5(0.5)	8.0-8.4	20(0)
	3	8.5(0.5)	7.9-8.4	20(0)
	4	8.6(0.5)	7.8-8.3	20(0)
	5	8.2(0.4)	7.8-8.3	20(0)
Modified MS	1	8.6(0.4)	6.7-6.9	20(0)
	2	8.5(0.4)	6.7-7.2	20(0)
	3	8.5(0.5)	6.6-7.3	20(0)
	4	8.6(0.4)	6.6-7.2	20(0)
	5	8.1(0.4)	6.8-8.7	20(0)
Supplemented MD without EDTA	1	8.6(0.5)	7.8-8.3	20(0)
	2	8.2(0.3)	7.7-8.8	20(0)

[a]Mean (and standard deviation)

[b]Range

Table 4. Survival, growth and reproduction of D. magna cultured in several medium-diet combinations for four acclimation generations at Bionomics.

Generation	% Survival [a]	Offspring/ Daph./ Repro. Day [a]	Length [a] (mm)
MD			
1	100(0)	7.8(0.0)	4.3(0.1)
2	100(0)	9.6(0.5)	4.3(0.1)
3	93(5.8)	7.3(0.6)	4.0(0.1)
4	93(12)	6.0(1.1)	3.9(0.1)
Supplemented MD			
1	97(5.8)	10.6(0.7)	4.7(0.1)
2	93(5.8)	8.9(0.6)	4.2(0.1)
3	100(0)	8.0(0.5)	4.0(0.2)
4	97(6)	9.3(0.4)	4.3(0.1)
Modified MS			
1	97(5.8)	11.9(0.8)	4.8(0.1)
2	97(5.8)	11.9(1.2)	4.8(0.2)
3	97(5.8)	10.7(1.3)	4.4(0.1)
4	13(15)	20.0(12.6)	4.2(1.0)
BM			
1	93(5.8)	10.6(0.7)	4.8(0.1)
2	100(0)	8.8(1.3)	4.4(0.2)
3	100(0)	10.9(1.3)	4.6(0.1)
4	100(0)	12.3(0.9)	4.7(0.1)

[a]Mean (and standard deviation)

Table 5. Survival, growth, and reproduction of D. magna cultured in several medium-diet combinations for five acclimation generations at Fort Detrick.

Generation	% Survival	Offspring/ Daph./ Repro. Day	Length (mm)
MD			
1	93(12)[a]	6.90(0.22)	4.37(0.29)
2	83(15)	4.92(0.45)	4.18(0.18)
3	100(0)	4.42(0.52)	4.14(0.12)
4	93(5.6)	4.45(0.21)	4.32(0.13)
5	97(5.8)	5.31(0.18)	4.51(0.11)
Supplemented MD			
1	90(0)	7.06(0.04)	4.55(0.19)
2	87(15)	8.55(0.44)	4.62(0.20)
3	90(10)	4.96(1.40)	4.21(0.24)
4	93(5.8)	4.45(0.21)	4.32(0.13)
5	93(5.8)	4.96(0.88)	4.37(0.11)
Modified MS			
1	87(5.8)	5.49(0.94)	4.23(0.14)
2	60(10)	6.51(0.45)	4.48(0.15)
3	73(15)	4.44(0.30)	4.19(0.25)
4	87(15)	4.63(0.26)	4.17(0.12)
5	83(15)	5.05(0.35)	4.39(0.18)
Supplemented MD without EDTA			
1	97(5.8)	4.35(1.10)	3.80(0.26)
2	90(10)	4.03(0.38)	4.02(0.18)

[a]Mean (and standard deviation)

Table 6. Water quality measurements made during assessment of various medium-diet combinations for culturing and testing of D. magna. Assessment was performed at Bionomics.

Generation	Medium	Dissolved Oxygen[a] (mg/L)	pH[b]	Temperature[a] (°C)
1	MD	8.0(0.5)	8.0-8.3	20(0)
	Supplemented MD	8.0(0.4)	7.7-8.3	20(0)
	Modified MS	7.6(0.6)	6.5-7.2	20(0)
	BM	7.8(0.5)	8.0-8.3	20(0)
2	MD	8.0(0.4)	7.8-8.4	20(0)
	Supplemented MD	8.0(0.5)	7.7-8.0	20(0)
	Modified MS	7.5(0.8)	6.5-7.2	20(0)
	BM	7.8(0.6)	8.0-8.3	20(0)

[a]Mean (and standard deviation)

[b]Range

Table 7. Water quality measurements made during the suitability assessment of various medium-diet combinations for culturing and testing of D. magna. Assessment was performed at Fort Detrick.

Generation	Medium	Dissolved Oxygen[a] (mg/L)	pH[b]	Temperature[a] (°C)
1	MD	8.8(0.3)	8.0-8.4	20(0)
	Supplemented MD	8.8(0.3)	8.0-8.9	20(0)
	Modified MS	8.8(0.4)	6.6-7.1	20(0)
2	MD	8.8(0.2)	8.1-8.3	20(0)
	Supplemented MD	8.9(0.2)	8.1-8.2	20(0)
	Modified MS	8.8(0.3)	6.8-7.0	20(0)

[a]Mean (and standard deviation)

[b]Range

Table 8. Performance of D. magna during the suitability assessment of various culture media-diets tested at Bionomics.

Generation	Media	Days to First Brood[a]	% Survival	Broods/ Female[a]	Offspring/ Daph./ Repro. Day[a]	Length[a] (mm)
1	MD	10.0(0.8)	95	4.4(0.6)	6.8(1.1)	3.8(0.1)
	Supplemented MD	10.8(1.7)	95	4.0(0.4)	7.8(1.2)	4.0(0.1)
	Modified MS	10.4(1.0)	95	4.5(0.7)	10.9(1.5)	4.5(0.1)
	BM	10.4(0.9)	90	4.1(0.3)	10.7(1.2)	4.4(0.1)
2	MD	10.1(1.5)	95	4.3(0.7)	5.9(1.1)	3.9(0.1)
	Supplemented MD	12.0(0.0)	100	3.9(0.6)	7.4(1.2)	3.9(0.2)
	Modified MS	11.3(1.8)	90	4.3(0.5)	11.7(2.1)	4.5(0.1)
	BM	10.9(1.5)	100	4.1(0.5)	10.5(1.4)	4.4(0.1)

[a]Mean (and standard deviation)

Table 9. Performance of D. magna during the suitability assessment of various culture media-diets tested at Fort Detrick.

Generation	Media	Days to First Brood[a]	% Survival	# Broods/ Female[a]	Offspring/ Daph./ Repro. Day[a]	Length[a] (mm)
1	MD	8.9(0.9)	85	4.6(0.6)	6.3(1.3)	4.22(0.10)
	Supplemented MD	8.0(0.0)	100	5.0(0.4)	4.8(0.4)	4.16(0.10)
	Modified MS	7.2(0.4)	55	5.0(0.4)	4.2(0.6)	3.95(0.09)
2	MD	8.4(0.8)	95	4.2(0.4)	5.7(0.5)	4.22(0.05)
	Supplemented MD	9.6(1.1)	95	4.0(0.2)	4.7(0.5)	4.08(0.08)
	Modified MS	8.7(0.8)	90	4.5(0.5)	4.0(0.4)	4.01(0.10)

[a]Mean (and standard deviation)

Table 10. Variability in chemical and biological parameters during the suitability tests of various combinations of media and diets for use in the culturing and testing of *D. magna*.

Laboratory	Medium	Coefficient of Variation											
		Total hardness	Total alkalinity	Specific conductance	pH[a]	Day to 1st brood		# broods/female		Offspring		Length	
						Gen 1	Gen 2	Gen 1	Gen 2	Gen 1	Gen 2	Gen 1	Gen 2
Bionomics	MD	3.0	8.2	0.0	0.2	8.0	14.8	13.6	16.3	16.2	18.6	2.6	2.6
	Supplemented MD	2.5	25.0[b]	0.0	1.1	15.7	0.0	10.0	15.4	15.4	16.2	2.5	5.1
	Modified MS	14.7[b]	34.8[b]	8.0	0.2	9.6	15.9	15.6	11.6	13.8	17.9	2.2	2.2
Ft. Detrick	MD	2.3	1.8	5.6	0.3	10.1	9.5	13.0	9.5	20.6	8.8	2.4	1.2
	Supplemented MD	4.2	18.1[b]	14.9	0.1	0.0	11.4	8.0	5.0	8.3	10.6	2.4	2.0
	Modified MS	12.5[b]	35.2[b]	9.4	0.3	5.6	9.2	8.0	11.1	14.3	10.0	2.3	2.5

[a]Coefficient of variation presented as highest value minus the lowest value.

[b]Significantly (p=0.05) higher variation.

APPENDIX I

RECONSTITUTED HARD WATER PREPARATION

Materials needed:

1. 5 gallon glass container or plastic carboy
2. deionized distilled water
3. chemicals
   - $NaHCO_3$
   - $CaCO_3 \cdot 2H_2O$
   - $MgSO_4$
   - KCl
4. weighing pans and spatula
5. balance (accurate to 0.001 gram)
6. storage jars for salts (optional)

Methods:

1. Thoroughly rinse the 5-gallon container with a 10% solution of nitric acid. Slowly pour out acid solution into cold running water. Rinse carboy thoroughly with deionized distilled water at least 5 times. Accurately mark the 19-liter level in the container to facilitate preparation of water each time.
2. Weigh out stock chemicals one at a time in the following amounts: 3.65 g $NaHCO_3$
   2.28 g $CaCO_3 \cdot 2H_2O$
   2.28 g $MgSO_4$
   0.15 g KCl

Extra stock mixtures can be weighed out in advance for use in the next week if stored in tightly covered jars.

3. Add approximately 15 liters of deionized distilled water to the carboy. Add the chemicals in the order given, mixing thoroughly after each addition. Rinse storage jar with deionized distilled water and add rinse water to solution in carboy. Mix solution thoroughly. Add deionized distilled water to a total solution volume of 19 liters.
4. Using a magnetic stirrer, stir for 24 hours with the container lid off, but covered with a foam plug or glass wool, to assure complete mixing of chemicals and saturation of dissolved oxygen.
5. Measure hardness, alkalinity and dissolved oxygen. The hardness must be from 160-180 mg/ℓ $CaCO_3$ and the alkalinity 110-120 mg/ℓ $CaCO_3$. This will verify proper measurement and mixing of salts in preparing the reconstituted water. If the hardness, alkalinity and pH requirements are not met, the reconstituted water must be prepared again.

DAPHNIA TROUT FOOD PREPARATION

- Add 15 grams of trout food (No. 1 granules) to 800 mL of reconstituted hard water and blend for 15 minutes to liquify.
- Pour into a suitable container and add 200 mL of reconstituted hard water.
- Let stand for 15 minutes and then carefully decant the upper 800 mL and discard the remaining precipitate.
- Thoroughly mix the suspension and withdraw three 10-mL aliquots.
- Dry the aliquots at 104°C for 24 hours in preweighed tares.
- Weigh dry samples and subtract tare weight.
- Calculate average weight of a dry sample and the standard deviation.
- Calculate weight for one mL of dry solids. The final concentration must be 5 mg dry solids per mL of food, so the volume must be adjusted by adding water. The total volume of water (X) to add equals the number of mL in the sample after removal of the aliquots (770 mL) times the mg/mL of dry food weighed (Y) divided by the mg/mL of dry food desired (5 mg/mL) minus the number of mL in the sample after the removal of the aliquots.

  For example, if the dry food weighed 6.32 mg/mL (Y), the following equation will give X:

  $$X = \frac{(770)(Y)}{5} - 770 \text{ where } Y = \text{mg/mL dry weight}$$

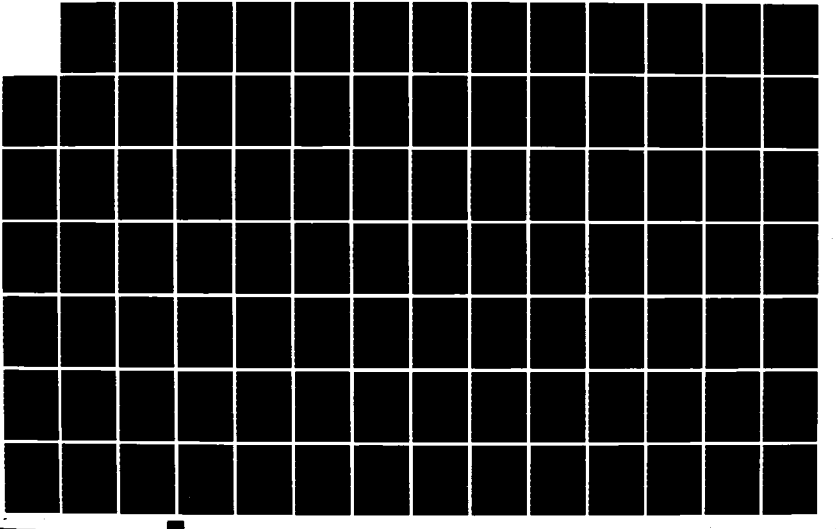
  $$X = \frac{(770)(6.32)}{5} - 770$$

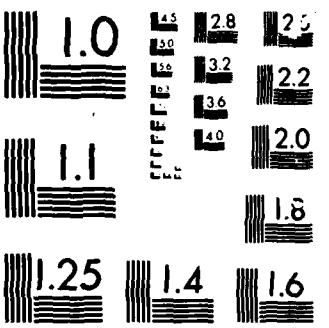
  X = 203 mL of water to add to 770 mL to give a concentration of 5 mg/mL of dry food.

- Store trout food in a refrigerator. This food may be used up to 14 days. The trout food must conform to the current U.S. Fish and Wildlife Service Specifications which can be obtained through livestock feed stores. The dry fish food should be stored in the dark at 4°C for not longer than one year. The current year's specifications follow.

AD-A166 957 COLLABORATIVE STUDY OF DAPHNIA MAGNA STATIC RENEWAL ASSAYS(U) SPRINGBORN BIONOMICS INC WAREHAM MA* R E BENTLEY ET AL. JAN 86 DAMD17-80-C-0011 2/4

UNCLASSIFIED F/G 13/2 NL





MICROCOPY CHART

Formulation Specifications for Starter Diet, SD9-30

(Starter, No. 1, and No. 2 granules)

1. Fish food mixture shall be composed of the following items. The final product to carry the following guaranteed analysis:

   Crude protein, not less than 50%
   Fish meal protein, not less than 33%
   Crude fat, not less than 17%
   Moisture, not more than 10.0%

2. Fish meal: stabilized, maximum fat 13%, maximum moisture 10%, stored at the manufacturer's no longer than 6 months as indicated by the bill of lading. Meal must be of fair average quality. Different meals may not be combined for use in the feed. Maximum allowable salt content of 5%.

3. Wheat feed flour: minimum protein 14%, maximum fiber 1.5%.

4. Soy flour: defatted, minimum protein 48.5%, maximum fat 1% (flour must be adequately toasted with a protein dispersibility index of less than or equal to 20).

5. Dried blood flour or ring dried blood meal: minimum protein 80%.

6. Trace mineral premix No. 1 (see Section 5 of specifications).

7. Vitamin premix No. 3 (see Sections 4 and 7 of specifications).

8. Choline chloride, 50%.

9. Ascorbic acid.

10. Fish oil: stabilized with 0.04% BHA-BHT (1:1) or 0.01% ethoxyquin, less than 3% free fatty acids and not alkaline reprocessed.

. Lignin sulphonate pellet binder (e.g. Ameribond, Orzan, or equivalent).

*Fish meal may be increased depending upon protein content but must provide not less than 33% fish protein. Quantity of added oil may be adjusted so that the finished feed shall contain not less than 17% crude fat. Wheat flour is to be adjusted to compensate for the above variations. Not less than 6% of the added oil is to be applied to the granules as a top dressing; the rest of the oil to be included in the feed mix.

APPENDIX III

ALGAL CULTURE AND STOCK SOLUTION PREPARATION

Algal Culturing Methods

1. Introduction - The method described below is based largely on conversations with Dr. Clyde Goulden and Ms. Linda Henry (Academy of Natural Sciences, Philadelphia) for Selenastrum culture in micronutrient supplemented MBL medium.

2. Glassware Cleaning - All glassware used for any aspect of algal culturing must be cleaned as follows: scrub with a 1% solution of Liquinox-non-phosphate detergent, rinse with tap water until sudsing has ceased, then rinse three more times with tap water. Rinse three times with distilled water, rinse once with 10% HNO_3, rinse once with acetone, and rinse three times with distilled water. A final rinse with the solution to be stored in the glassware is required.

3. Preparation of Culture Media

   3.1 Selenastrum capricornutum. (Algal source: American Type Culture Collection No. 22662).

   3.1.1 Macronutrient stocks. Prepare separate stocks (for Woods Hole MBL medium) of each of the following compounds by dissolving the specified weight into a total volume of one liter of glass distilled water.

Compound	Grams/Liter
$CaCl_2 \cdot 2H_2O$	36.76
$MgSO_4 \cdot 7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$	85.01
$Na_2SiO_3 \cdot 9H_2O$	28.42*

*Filter sterilize this stock solution and add 1 mL/L to culture medium after autoclaving, using sterile technique.

3.1.2 Micronutrient stocks. Prepare each stock solution shown below in a final volume of one liter of glass-distilled water. Mix until dissolved. For stock No. 3, add chemicals in the order shown.

Stock No.	Compound	Grams/Liter
1	Na_2EDTA	4.36*
2	$FeCl_3 \cdot 6H_2O$	1.575**
3	$CuSO_4 \cdot 5H_2O$	0.01
	$CoCl_2 \cdot 6H_2O$	0.01
	$ZnSO_4 \cdot 7H_2O$	0.022
	$MnCl_2 \cdot 4H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.006
	H_3BO_3	1.0

*Stock must be less than three months old.

**Use 2 mL/L of medium.

3.1.3 Record stock solution preparation information on Data form 1. All compounds used must be ACS Reagent grade (or other high purity grade if no ACS standard has been established for the compound used). Refrigerate all stocks. Stocks showing any evidence of precipitation or contamination must not be used. Precipitation of the sodium silicate may occur with time, but the stock can still be used.

3.1.4 For each liter of culture medium being prepared, include one milliliter of each macronutrient stock (3.1.1, except sodium silicate) and one milliliter of each micronutrient stock (3.1.2). Place one liter of medium in a 2 L Erlenmeyer flask, add a cleaned 50 mm (2 inch) Teflon stirring bar, and cap with a foam plug (Gaymar IDENTI-PLUGS are recommended - Miller et al, 1978) or with a cotton plug wrapped in cheesecloth. Cover the top with aluminum foil. Autoclave at 1.1 Kg/cm^2 (15 psi) and 121°C for 15 minutes. Allow to come to room temperature. Add 1 mL $Na_2SiO_3 \cdot 9H_2O$ stock using sterile technique.

3.1.4.1 Use similar procedures for preparing 1000 mL of media in a 2000 mL Erlenmeyer flask.

3.1.4.2 For agar slants and petri plates, prepare medium as above but, in addition, dissolve 1% (w/v) agar (DIFCO Bacto-Agar or equivalent) prior to autoclaving. Place agar solution into test tubes for slants; tilt after removal from autoclave but before the agar has jelled. Pour autoclaved solution into sterile petri plates using sterile technique.

4. Obtaining Uncontaminated Algal Cultures. If stock algal cultures become contaminated or if it is necessary to obtain new uncontaminated algal stocks, use the procedure described below.

4.1 Using a sterile pipette, transfer one drop of algae in algal medium to a sterile petri plate with the appropriate agar medium. Streak and allow colonies to grow.

4.2 Select a presumptive clean single cell isolate from the plate and transfer to a new plate. Streak again. Use the uncontaminated single cell isolates from this plate to start new agar slants.

5. Initiating and Growing Algal Cultures.

5.1 Obtain uncontaminated cells from isolates as described in 4. Prepare agar slants by transfer from clean agar slants. Sufficient agar slants should be prepared such that one is available every time a new algal inoculum must be prepared. Keep slants for three to six months, but discard after use in one set of transfers.

5.2 Make a new set of slants (as required) from an available slant, then inoculate 100 mL of medium with algae from the slant (3.1.4.1 and 3.1.4.2). Allow the algae to grow in the medium and use the inoculum prior to the stationary phase of growth. This may be determined by visual examination of the color of the medium once sufficient experience is gained with culturing. Otherwise, a sample must be withdrawn with a sterile pipette and counted with a hemacytometer to ensure that the cells are in log-phase

growth. (It is assumed that baseline data is available on the growth curve of the alga so that the cell concentration at the beginning of the stationary phase of growth is known).

5.2.1 Grow incoluation cultures of algae under cool-white fluorescent lights at 4300 lumens (400 ± 10% fc) at a temperature of 24 ± 1°C. Stir algae to increase growth rate. Place a piece of styrofoam between the stirrer and the flask to reduce heat build-up in the flask.

5.2.2 Grow algae under the conditions described in 5.2.1. Any algal cultures having a typical coloration or showing gross bacterial contamination must be discarded.

6. Harvesting Algae. (Either a batch or continuous-flow centrifuge may be used for harvesting).

6.1 Method 1. Check cell concentrations to confirm log-phase growth. Centrifuge the algae at a speed and time sufficient to remove the algae from the water column (700 xg for 15 minutes is suggested). Pour off the supernatant, leaving as little of the algal medium as possible behind. Resuspend the algae in a small amount of the same solution used for culturing the daphnids to be fed. Remove a small portion of the combined algal solutions and dilute as needed to perform a hemacytometer count. Count at least 100 cells per field; determine the original cell concentration per milliliter as follows:

Cells/mL = (cell count) (10,000) (25 ÷ the number of double lined fields counted) (dilution factor)

Dilute the combined algal solution with the appropriate daphnid culture medium so that one milliliter, when added to 800 mL of daphnid medium, will create the appropriate food concentration. Confirm the final cell concentration with a hemacytometer count. Record data on Data Form 3.

6.2 Method 2. Follows suggestions prescribed in Appendix D of the revised test protocol (January 1983, attached as Appendix B).

6.3 Harvested Selenastrum may be stored in the refrigerator for 7-12 days after harvest.

7. Reference.

Miller, W.E., J.C. Greene, and T. Shiroyama. 1978. The Selenastrum capricornutum Printz algal assay bottle test. EPA-600/9-78-018. U.S. Environmental Protection Agency, Environmental Research Laboratory. Corvallis, OR. p. 80.

APPENDIX IV

KEATING'S DAPHNID CULTURE MEDIUM

Separate stock solutions of macronutrients (salts) and micronutrients (metals) are prepared by adding ACS reagent grade (or other high purity grade) compounds in the following amounts to 1-liter of glass distilled water. These solutions are added (1 mL/L, except $FeCL_3$) to the culture medium and the pH is adjusted.

Compound	Stock solution concentration (g/L as whole compound	Medium concentration (mg/L as whole compound	Stock added to medium (mL/L)
Salts			
Na_2EDTA	5	5	1
KCl	10	10	1
$K_2HPO_4 \cdot 3H_2O$	10	10	1
KH_2PO_4	10	10	1
$CaCl_2 \cdot 2H_2O$	36.67	36.67	1
$MgSO_4 \cdot 7H_2O$	20	20	1
$NaNO_3$	50	50	1
$Na_2SiO_3 \cdot 9H_2O$	10	10	1
Metals			
$FeCl_3$	0.290[a]	0.4 (as metal)	4
$MnCl_2 \cdot 4H_2O$	0.720	0.2	1
$CoCl_2 \cdot 6H_2O$	0.020	0.005	1
$Na_2MoO_4 \cdot 2H_2O$	0.126	0.05	1
H_3BO_3	5.72	1.0	1
NaBr	0.064	0.05	1
$SrCl_2 \cdot 6H_2O$	0.304	0.10	1
RbCl	0.141	0.10	1
LiCl	0.611	0.10	1
KI	0.00654[b]	0.005	1
NH_4VO_3	0.00114[c]	0.0005	1
$CuCl_2 \cdot 2H_2O$	0.067	0.025	1
$ZnCl_2$	0.052	0.025	1
GeO_2	[illegible]	0.002	1

a
Add 25 mL concentrated HCl per liter: stir 24 hours to dissolve.

b
Make a 0.654 g/L stock and diluter 1:100.

c
Make a 0.114 g/L stock and dilute 1:100.

d
Atomic absorption standard is 1 mg/mL, dilute 1:500.

APPENDIX V

ALGAL CULTURE AND STOCK SOLUTION PREPARATION

Algal Culturing Methods

1. Introduction - The method described below for culturing Chlamydomonas sp. is based largely on conversations with Dr. Kathleen Keating (Rutgers University) and Dr. William van der Schalie (U.S. Army Medical Bioengineering Research and Development Laboratory).
2. Glassware Cleaning - All glassware used for any aspect of algal culturing must be cleaned as follows: scrub with a 1% solution of Liquinox-non-phosphate detergent, rinse with tap water until sudsing has ceased, then rinse three more times with tap water. Rinse three times with distilled water, rinse once with 10% HNO_3, rinse once with acetone, and rinse three times with distilled water. A final rinse with the solution to be stored in the glassware is required.
3. Preparation of Culture Media

   3.1 Chlamydomonas reinhardtii. (Algal source: Dr. Kathleen Keating, Rutgers University)

   3.1.1 Macronutrient (salt) stocks. Utilize stock solutions prepared for Keating's daphnid medium with the additions shown in Appendix A and Data Form 2.

   3.1.2 Micronutrient (metal) stocks. Utilize stock solutions prepared for Keating's daphnid medium (Table A).

   3.1.3 Record algal-unique stock solution preparation information on Data Form 2. All compounds used must be ACS reagent grade (or other high purity grade if no ACS standard has been established for the compound used). Refrigerate all stocks. Stocks showing any evidence of precipitation or contamination must not be used. Precipitation of the sodium silicate may occur with time, but the stock can still be used.

3.1.4. For each liter of culture medium being prepared, include one milliliter of the micronutrient stocks (3.2.2) and one milliliter of the macronutrient stocks (except ferric chloride and certain compounds described in 3.2.1). Adjust the pH to 8.3-8.5 with NaOH (and HCl if necessary). Reduce the amount of liquid added by first adding strong solutions (e.g., 10 N) a drop at a time and going to weaker solutions (1.0 N, 0.1 N) as the pH approaches the desired range. Distribute one liter of the medium into 10-250 mL Erlenmeyer flasks (100 mL per flask), cap as described in 3.1.4. Flasks were covered with aluminum foil at Bionomics. Flasks were uncovered at Ft. Detrick. Autoclave at 1.1 Kg/cm^2 (15 psi) and 121°C for 15 minutes. Allow to come to room temperature. Using sterile technique, remove a small volume from one flask and check the pH. It should be approximately 7.8. See 3.1.4.2 for information on agar preparations.

4. Obtaining Uncontaminated Algal Cultures. If stock algal cultures become contaminated or if it is necessary to obtain new uncontaminated algal stocks, use the procedure described below.

4.1 Using a sterile pipette, transfer one drop of algae in algal medium to a sterile petri plate with the appropriate agar medium. Streak and allow colonies to grow.

4.2 Select a presumptive clean single cell isolate from the plate and transfer to a new plate. Streak again. Use the uncontaminated single cell isolates from this plate to start new agar slants.

5. Initiating and Growing Algal Cultures.

5.1 Obtain uncontaminated cells from isolates as described in 4. Prepare agar slants by transfer from clean agar slants. Sufficient agar slants should be prepared such that one is available every time a new liquid inoculum must be prepared. Keep slants for three to six months, but discard after use in one set of transfers.

TABLE A
Keating's Culture Media

Compound	Daphnia mg/L	Stock Solution g/L	mL Stock/ L Medium	Algal mg/L	Chlamydomonas mL Stock/ L Medium
Salts	as whole compound				
KCl	10	10	1	10	1
$K_2HPO_4 \cdot 3H_2O$	10	10	1	10	1
KH_2PO_4	10	10	1	25	2.5
Glycylglycine	-	-	-	250	weigh 0.250 g
$CaCl_2 \cdot 2H_2O$	36.67	36.67	1	36.67	1
$MgSO_4 \cdot 7H_2O$	20	20	1	20	1
$NaNO_3$	50	50	1	150	3
$NaSiO_3 \cdot 9H_2O$	10	10	1	10	1
Vitamin B_{12}	-	0.00075	-	0.00075	1
Thiamine (HCl)	-	0.075	-	0.075	1
Biotin	-	0.00075	-	0.00075	1
Metals	as metal	as whole compound			
Na_2EDTA	-	5	1	5	1
$FeCl_3$	0.4	0.290[a]	4	0.4	4
$MnCl_2 \cdot 4H_2O$	0.2	0.720	1	0.2	1
$CoCl_2 \cdot 6H_2O$	0.005	0.020	1	0.005	1
$Na_2MoO_4 \cdot 2H_2O$	0.05	0.126	1	0.05	1
H_3BO_3	1.0	5.72	1	1.0	1
NaBr	0.05	0.064	1	0.05	1
$SrCl_2 \cdot 6H_2O$	0.10	0.304	1	0.10	1
RbCl	0.10	0.141	1	0.10	1
LiCl	0.10	0.611	1	0.10	1
KI	0.005	0.00654[b]	1	0.005	1
NH_4VO_3	0.0005	0.00114[c]	1	0.0005	1
$CuCl_2 \cdot 2H_2O$	0.025	0.067	1	0.025	1
$ZnCl_2$	0.025	0.052	1	0.025	1
SeO_2	0.002	-[d]	1	0.002	1

[a] Add 25 mL concentrated HCl per liter; stir 24 hours to dissolve.

[b] Make a 0.654 g/L stock and dilute 1:100.

[c] Make a 0.114 g/L stock and dilute 1:100.

[d] If the atomic absorption standard is 1 mg/mL, dilute 1:500.

5.2 Make a new set of slants (as required) from an available slant, then inoculate 100 mL of medium with algae from the slant (3.1.4.1 and 3.1.4.2). Allow the algae to grow in the medium and use the inoculum prior to the stationary phase of growth. This may be determined by visual examination of the color of the medium once sufficient experience is gained with culturing. Otherwise, a sample must be withdrawn with a sterile pipette and counted with a hemacytometer to ensure that the cells are in log-phase growth. (It is assumed that baseline data is available on the growth curve of the alga so that the cell concentration at the beginning of the stationary phase of growth is known).

5.2.1 Grow inoculation cultures of algae under cool-white fluorescent lights at 4300 lumens (400 ± 10% fc) at a temperature of 24 ± 1°C.

5.2.2 When adequate growth has been achieved, transfer (using sterile technique) number of milliliters to give an initial inoculum in a 250-mL flask which is sufficiently high to allow harvesting of the flask within a reasonable period of time. Shake each 250-mL flask once in the morning and once in the afternoon. Mixing may be accomplished by continuous aeration. So that fresh Chlamydomonas are available, start five new 250-mL flasks every Monday, Wednesday and Friday.

5.2.3 Grow algae under the conditions described in 5.2.1. Any algal cultures having atypical coloration or showing gross bacterial contamination, exhibiting low motility or clumped cells must be discarded.

6. Harvesting Algae. (Either a batch or continuous-flow centrifuge may be used for harvesting).

6.1 Method 1. Check cell concentrations to confirm log-phase growth. Centrifuge the algae at a speed and time sufficient to remove the algae from the water column (700 xg for 15 minutes is suggested). Pour off the supernatant, leaving as little of the algal medium as possible behind. Resuspend the algae in a small amount of the same solution used for

culturing the daphnids to be fed. Remove a small portion of the combined algal solutions and dilute as needed to perform a hemacytometer count.[1] Count at least 100 cells per field; determine the original cell concentration per milliliter as follows:

Cells/mL = (cell count) (10,000) (25/the number of double lined fields counted) (dilution factor)

Dilute the combined algal solution with the appropriate daphnid culture medium so that one milliliter, when added to 800 mL of daphnid medium, will create the appropriate food concentration. Confirm the final cell concentration with a hemacytometer count. Record data on Data Form 3.

6.2 Method 2. A drop of algae from well-mixed culture of algae is used to fill a haemacytometer counting cell. Enough sets (having 16 squares each) are counted so that between 100 and 200 algae cells are counted. A conversion of the number of cells counted into the number of cells per milliliter is made using the following formula:

$$\frac{\text{(no. of cells counted)} \times (4 \times 10^6)}{\text{No. of squares counted}} = \text{No. of cells/mL}$$

The number of mL needed to get 10^8 cells is determined by dividing 10^8 cells by the number of cells per mL in the culture. The volume (mL) thus determined is measured, placed in centrifuge tubes, and centrifuged at 2,200 RPMs (700 g) for 15 minutes. The algae medium is then carefully poured off, and ten milliliters of daphnid culture medium is added to resuspend the algae (e.g., 10 mL will then contain 10^8 cells of algae. The algae in the reconstituted water is then added to volumetric flasks containing approximately 950 mL of daphnid culture medium. The centrifuge tubes are rinsed twice to assure that all algae are removed, and the rinse water is then added to the test solution. The test solution is then made up to one liter and is ready for dispensing into the test or culture chambers.

[1] A small quantity of Lugol's solution may be added to the sample prior to hemacytometer counting to prevent motile algae cells (e.g., Chlamydomonas).

APPENDIX 2

Protocol for Evaluation of Waste Leachate Acute and Chronic Toxicity with *Daphnia magna*

INTERIM PROCEDURES FOR CONDUCTING THE DAPHNIA MAGNA TOXICITY ASSAY

CHANGES AND/OR DELETIONS - 22 MAY 1984

page iii - add - and survival - after 21 days)...
page 2 - length is not an optional endpoint for this study.
page 3 - pipet size should be ~ 1.5 times the size of the organisms being transferred.
page 4 - after ...large numbers of young - add (3 young per female per reproductive day).
page 5 - in water quality measurements section - change to: Hardness and alkalinity will be done once per batch. Dissolved oxygen and pH measurements must be made when solutions are prepared and again after the transfer of daphnids (on 2-3 day old solutions) enough times to characterize the medium (a minimum of 3 times on new solutions and 3 times on old solutions/28-day culture period).
page 6 - Methods section - pipet size should be ~1.5 times the size of the organism being transferred.
page 6 - Acetate controls section - delete ...and all concentr tions... before tested must... and insert ...other - before solvents.
page 8 - in Water Quality Measurements section - change to: Hardness and alkalinty will be determined in the high and low concentrations and control at 0-hour. pH and dissolved oxygen measurements must be made in the high middle and low concentrations and the control at 0 and 48 hours. Delete up to - Control concentrations... In the pH section - add - of the test water - after pH.
page 9 - #6 - for clearer explanation, see p. 13 randomization.
page 9 - #8 - add (optional) after dead.
page 9 - #9 - same changes as on page 8.
page 10 - delete from ..., one of these tests... to ...repeated.
page 12 - in the methods section - add after ...beakers must be distributed randomly - at the initiation of the test only.
page 12 - fire polished pipet size is ~1.5 times the size of the organisms.
page 14 - in Water Quality Measurements section - change to: Hardness and alkalinity will be measured on each new batch of water and test solutions on days 0, 7, 14 and 21 for the controls and the highest concentration (with survivors) tested. Measurements of pH and dissolved

oxygen will be made on day 0 (new solution), day 7 (old and new solutions), day 14 (old and new solutions) and day 21 (old solution) in the control and the high middle and low test concentrations.
page 15 - Leachate measurements (toxicant) will be as follows: Days 0, 7, 14 samples will be removed from all concentrations and controls prior to the addition of food and the division into replicates. On day 21, all replicate solutions from each existing concentration will be composited and sampled.
page 16 - point #6 - two stage ramdomization procedure.
page 16 - " #8 - we suggest that broods may be based on number of exoskeletons.
page 16 - point #9 - see previous comment for page 14.
page 18 - add - If before Control - delete will be and add is, and after (Finney, 1971), add please indicate.
page 22 - pipet is ~ 1.5 times the size of the organisms.
page 24 - after ...methods used for measuring... add pH and temperature, and at the end of the sentence, add and the results of these measurements.
page A-2 - add a point #6 - Reconstituted water will not be used for more than one month (4 weeks).
page B-1 - before - Freeze trout food..., insert Either...; after ...needed, add or refrigerate (if refrigerated, the food can be used for a maximum of one week).
page C-1 - after ...5000 ± 10%, insert lumens.
page C-3 - point #4 - delete the word include and replace it with add. After ...each micronutrient stock (3.1.2), add to about 900 mL deionized, distilled water.
page C-5 - point #4 - delete and vitamins.
page D-1 - The formula given should be:

$$\frac{\text{(No. of cells counted) x dilution x } 10^4}{\text{No. of squares counted}} = \text{No. of cells/ml}$$

page D-1 - last line - change 3 to 4 to 10 to 12.
page D-2 - last line - method 2, change 3-4 to 10-12.

INTERIM PROCEDURES FOR CONDUCTING THE *DAPHNIA MAGNA* TOXICITY ASSAY

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U.S. ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

This protocol describes static acute and static-renewal chronic tests which are designed to provide information on the acute (dead or immobile) and chronic (survival and reproduction) toxicity of solid waste leachates to Daphnia magna.

Acute test results are reported as a 48-hr EC50 with 95% confidence intervals. Chronic test results are reported as 21 day LC50 with 95% confidence limits, and the lowest concentration at which there was a significant (95% confidence interval) effect on reproduction (time to first brood, number of broods per female and number of young per reproductive day per female, for females surviving 21 days) of the Daphnia, and the highest concentration at which there was no significant effect.

TABLE OF CONTENTS

	Page
Acknowledgments	ii
Abstract	iii
List of Tables	vi
Definitions	vii
Summary	1
Culture and Testing Methods	3
General Culture Procedures for Brood Stocks	3
Acclimation of Culture Procedures	4
Organisms	4
Food and Feeding	4
Methods	4
Containers	5
Replication	5
Aeration	5
Cleaning	5
Light and Photoperiod	5
Temperature	5
Water Quality Measurements	5
pH	5
Acute Tests	5
Specific Procedures	5
Organisms	6
Food and Feeding	6
Methods	6
Containers	6
Leachates	6
Dilution Water	6
Controls	6
Acetate Controls	6
Test Concentrations	6
Randomization	7
Replication	7
Aeration	7
Cleaning	7
Light and Photoperiod	7
Temperature	8
Water Quality Measurements	8
pH	8
Leachate Measurements	8
Test Apparatus	8
General Acute Test Procedures	9
Statistical Evaluations	10

	Page
Chronic Static-Renewal Tests	12
Specific Procedures	12
Organisms	12
Food and Feeding	12
Methods	12
Containers	12
Leachate	12
Dilution Water	13
Controls	13
Acetate Controls	13
Test Concentrations	14
Randomization	14
Replication	13
Aeration	14
Cleaning	14
Light and Photoperiod	14
Temperature	14
Water Quality Measurements	14
pH	15
Leachate Measurements	15
Test Apparatus	15
General Chronic Test Procedures	15
Statistical Evaluations	17
Survival	18
Reproduction and Length	18
Confidence Intervals and After the Fact Power Calculations	21
Obtaining and Recording Data	22
Acute	22
Chronic	22
Data Reporting	24
Literature Cited	26
References	28
Appendices	
A. Reconstituted water preparation	A-1
B. *Daphnia* food preparation protocol	B-1
C. Culturing *Selenastrum capricornutum*	C-1
D. Preparation of algae for feeding *Daphnia*	D-1
E. Equipment	E-1
F. Data forms	F-1
G. Quality assurance	G-1
H. Statistical procedures	H-1

LIST OF TABLES

Page

Table 1. Acute toxicity test data sheet 18

Table 2. Chronic toxicity test data sheet 19

DEFINITIONS

Acute toxicity: a relatively short-term lethal or other (e.g., immobilization, equilibrium loss) effect, usually defined as occurring within 48 hours for Daphnia.

Chronic toxicity: full life-cycle effects (21 days for Daphnia), such as changes in growth, reproduction, mutations, or death.

LC50: a statistically estimated toxicant concentration killing 50% of exposed organisms at a specific time of observation, for example, 48-hr, 7-day, 14-day or 21-day LC50 for Daphnia.

EC50: toxicant concentration affecting a specific response (i.e., death or immobilization) of 50% of exposed organisms at a specific time of observation; for example, 48-hr EC50 immobilization.

Immobilization: no visible movement of appendages when gently prodded.

Static bioassay: test in which solutions and test organisms are placed in test chambers and kept there for the duration of the test (24 or 48 hours for Daphnia).

Renewal bioassay: a test with periodic exposure (Monday, Wednesday and Friday or a similar schedule) of test organisms to fresh test solutions of the same composition. This is accomplished by transferring test organisms into new test chambers containing the appropriate test solutions and food.

Trimmed Spearman-Karber Method: calculation method for median lethal or median effect concentrations and 95% confidence intervals for toxicity data.

Dunnett's test: a multiple comparison of treatment means against the control mean for analysis of variance.

Brood: young produced at one time from an adult Daphnia.

Time to first brood: the number of days elapsed before a given <24-hr-old female has her first brood of young.

Number of young: the total number of young in the test period for females alive at the end of a chronic test.

Number of young/reproductive day: the number of young from the first brood day to day 21 (for females alive at the end of the test period) divided by the number of days.

Number of broods: the total number of broods in the test period per female alive at the end of a chronic test.

Length: the total length (mm) of females (from the top of the head to base of the spine) alive at the end of a chronic test.

SUMMARY

Adult daphnids in cultures used for providing young for testing must be healthy and free of ephippia. Culture mortality of adult organisms must not exceed 10% during the fourteen days prior to testing. Culturing and testing are conducted at a constant temperature of 20 ± 2° C with a 16 hour photoperiod. Daphnids are cultured and tested in hard reconstituted water (American Public Health Association et al., 1980) and fed trout food and Selenastrum capricornutum.

A 48-hr screening test may be used as a range-finder prior to an acute test for samples in limited quantity or if nothing is known about the toxicity. The screening test is conducted with a control, 1, 10, and 100 percent leachate with 5 <24-hr-old Daphnia magna in 80 ml of solution in each 100-ml beaker. A 48-hr static acute test is started with <24-hr-old Daphnia. The daphnids in 48-hr tests are tested with 5 organisms per 80 ml of solution in a 100-ml beaker. Five or more concentrations and a control (plus an acetate or solvent control, if needed) are tested in quadruplicate. The daphnids are tested unfed. Immobilization or death is recorded at test termination, and a 48-hr EC50 concentration is calculated. The beakers in both tests are covered with glass to minimize evaporation.

The 21-day chronic test is conducted using 10 100-ml beakers per experimental condition started with one <24-hr-old Daphnia magna per 80 ml of solution. Waste leachate test concentrations are selected based on acute EC50 values. The solution is changed and endpoints are recorded three times weekly (M,W,F). Temperature is monitored continuously. Dissolved oxygen, pH, hardness, and alkalinity are measured initially and on 2- or 3-day-old samples when the solution is renewed. The daphnids are fed 3 mg/l of trout

food plus 10^8 cells/l (10^5 cells/ml) of Selenastrum capricornutum three times weekly (M,W,F). Four endpoints must be determined for each test:

(1) time to first brood;

(2) number of broods per female, for females alive at the end of the test period;

(3) number of young/reproductive day for females alive at the end of the test period; and

(4) survival.

An optional endpoint is the length of adult Daphnia alive at the end of the experiment.

CULTURE AND TESTING METHODS

Daphnia magna are recommended because of their sensitivity to toxic substances, large size, ease of identification, availability from laboratories and commercial services, ease of handling, and extensive use in toxicity testing. Daphnids must come from an established laboratory culture. Daphnia tested in any toxicant must not be retained for culturing or testing with other toxicants.

General Culture Procedure For Brood Stocks

Daphnia magna may be cultured in 2000-ml glass containers, each having 20 daphnids per 1600 ml of hard reconstituted (hardness 160-180 mg/l $CaCO_3$) water (American Public Health Association et al., 1980; also see Appendices A and F-1). The culture must be maintained at 20 $\pm$ 2°C in a constant temperature bath or room with a 16 hour photoperiod. The Daphnia must be transferred to fresh water weekly (minimum) and fed 5 mg/l of trout food (Appendices B, D and F-4) plus 10^8 cells/l (10^5 cells/ml) of Selenastrum capricornutum (Appendices C, D and F-2) each M,W,F (this number of cells will make about 1.8 mg dry weight of Selenastrum). At the time of transfer only the adults are transferred and the young are disposed of. The young from the 2nd to 6th broods of adults are used to start new cultures each week. When the adults are 4 weeks old they are disposed of. The cultures should be spaced one week apart in age for providing animals for acclimation culture. Maintaining cultures by this method minimizes overcrowding, male production, ephippia formation, and population "crashes". It also helps to control bacteria and fungi.

Use ~3-mm inside diameter pipettes for transferring adults, and ~5-mm inside diameter pipettes for transferring young. Care must be taken not to

bump or bruise the daphnids while transferring; they must be introduced into new media below the surface of the water to avoid trapping air under their carapaces.

Acclimation Culture Procedures

Organisms - Adult daphnids (brood stock) about to have their second to sixth broods are cultured under conditions similar to those for chronic tests. The brood stock must be healthy as indicated from: survival; absence of floaters; absence of ephippia; large size of adults; dark colored animals; absence of external parasites; and presence of large numbers of young. Young daphnids produced from these adults are then transferred into new media and reared for at least two weeks. These animals must be healthy as indicated by the criteria given above. Young from these daphnids are then used for both acute and chronic tests.

Food and Feeding - Animals must be fed 5 mg/l of trout food and 10^8 cells/l Selenastrum capricornutum three times each week when the media is changed.

Methods - Young daphnids <24 hrs old, from the parental generation set aside for acclimation, must be placed in culture chambers and subjected to test conditions for at least 14 days. Culture vessels for acclimation must provide 80 ml of water per animal and must be covered with glass to minimize evaporation. Daphnids must be transferred under the water surface with a ≥8 mm inside diameter fire polished pipette into clean containers every M,W,F when the medium is changed. Survival of the test animals must be noted each time the medium is changed. Mortality must not exceed 10 percent if the animals are to be used for producing young to start an experiment. Reproduction must be noted by counting the number of young when the media is

changed. Young used for starting experiment must come from the second to the sixth broods.

Containers - One to three liter glass containers containing sufficient water to provide 80 ml to each daphnid.

Replication - Sufficient replicates to assure that a sufficient number of young daphnids are available to begin a test.

Aeration - Must not be used.

Cleaning - All glassware must be scrubbed with a 1% solution of Liquinox or another non-phosphate detergent, rinsed with tap water until sudsing has ceased, then rinsed three more times with tap water. Then rinse three times with distilled water, rinse once with 10% HNO_3, rinse once with acetone, and rinse six times with distilled water.

Light and Photoperiod - Fluorescent light bulbs must provide a color rendering index $\geq$90 with a 16-hr photoperiod automatically controlled. A light intensity of 30 to 100 foot candles must be used.

Temperature - An instantaneous temperature of 20 $\pm$ 2°C must not be exceeded; the daily mean temperatures must be 20 $\pm$ 1°C. Temperature should be monitored continuously or measured with a maximum-minimum thermometer.

Water Quality Mesurements - Hardness, alkalinity, pH and dissolved oxygen measurements must be made when solutions are prepared and again after the transfer of daphnids (on 2-3 day-old solutions) enough times to characterize the medium.

pH - The pH must be between 6.8 and 8.5

Acute Tests

Specific Procedures

All data will be recorded using the form provided in Appendix F-5.

Organisms - Young daphnids used for testing must come from the second to sixth broods of laboratory reared animals from healthy cultures.

Food and Feeding - Do not feed for acute tests.

Methods - Place young Daphnia <24-hr-old in test chambers and subject to test conditions for 48 hours. Daphnia must be transferred with a firepolished pipette (5 mm inside diameter) into beakers which then must be covered with a pane of glass or a watch glass to minimize evaporation.

Containers - Use 100-ml borosilicate glass beakers containing 80 ml of test solution.

Leachates - Leachates (toxicants) must be stored at 4°C in the dark, but allowed to gradually come to 20 $\pm$ 1°C before adding daphnids. Leachate dilutions are made in volumetric flasks and then poured into the test beakers.

Dilution Water - Dilution water must be the same as the culture water.

Controls - Controls must be set up and treated identically with regard to experimental conditions as test containers, except that no leachate is added. No more than 10% mortality may occur in 48 hours among control daphnids for the test to be valid.

Acetate Controls - Acetate controls must be run in addition to water controls whenever acetate is used in generating the solid waste leachate to be tested. The acetate concentration in the control and all concentrations tested must be the same as that in the highest concentration. No more than 10% mortality may occur in 48 hours among acetate-control daphnids for the test to be valid. (If solvents are used the same procedure is applicable.)

Test Concentrations - At least five toxicant concentrations with a dilution factor of 0.5 (e.g., 100%, 50%, etc.) or greater (0.75, e.g., 100%, 75%, 56%, etc.) must be used for 48-hr tests. The highest concentration to test may be

determined by a 48-hr screening test using order of magnitude leachate dilutions (i.e., 100%, 10% and 1%), with five daphnids in 80 ml of solution for each concentration and control. The screening test solutions do not need to be duplicated but will aid in determining 48-hr acute test concentrations. For example, if all animals die at 100% of the leachate and no animals die at 10%, then the following concentrations should be tested for 48-hr acute tests: 100%, 50%, 25%, 12.5%, and 6.25%.

Randomization - Daphnids are assigned completely at random from the culture stock to the test beakers. A two-stage transfer procedure is needed. Daphnids from the culture stock are randomly transferred into beakers containing dilution water which corresponds to each test group. The order of assignment is determined from a table of random numbers or another method of random allocation. A second transfer is then made into beakers containing the appropriate experimental conditions. Beakers are then randomly placed in a water bath, or a controlled temperature incubator or room.

Replication - Four containers, each containing five daphnids (a total of 20 animals), are required for each experimental condition.

Aeration - Must not be used.

Cleaning - All glassware must be thoroughly washed with a laboratory detergent and rinsed with the tap water. Since most leachates are unknown mixtures,a 10% nitric acid rinse followed by distilled water and an acetone rinse followed by at least three distilled water rinses are required. Test containers and flasks must have an additional rinse with the dilution water to be used for testing just before a test is started.

Light and Photoperiod - Fluorescent light bulbs must provide a color rendering index [illegible]. With a 16-hr photoperiod automatically controlled. A light intensity of 50 to 100 foot candles must be used.

Temperature - An instantaneous temperature of 20 ± 2°C must not be exceeded; the daily mean temperature must be 20 ± 1°C. Temperature must be monitored continuously.

Water Quality Measurements - Hardness, alkalinity, pH, and dissolved oxygen measurements must be made when solutions are prepared and at the end of the test. These measurements must be made on controls and the highest concentration tested; if there is a difference between these, then measurements must be made on all intermediate concentrations. Control concentrations when the test is started for hardness, alkalinity, and pH for hard reconstituted water should be: 170 ± 10 mg/l $CaCO_3$; 115 ± 5 mg/l $CaCO_3$; and 7.6-8.5, respectively (American Public Health Association et al., 1980); dissolved oxygen must be from 90-100% saturated.

pH - The pH must be from 7.6 to 8.5. If the pH of the leachate is initially between 6.8 and 8.5, no adjustments are required. If not, the pH of the leachate must be adjusted by using sodium hydroxide to raise the pH to 6.8 or by using hydrochloric acid to lower the pH to 8.5. The pH of the leachate must be measured and adjusted just prior to beginning the acute test.

Leachate Measurements - Test solutions of leachates should be measured either directly or indirectly. If leachates have had preliminary chemical analyses, one of the dominant constituents (e.g., ammonia) may be measured to check dilutions; if not, either conductivity or total organic carbon may be used.

Test Apparatus - Test equipment should consist primarily of high grade borosilicate glass, and/or stainless steel. Fluorocarbons and high density polyethylene equipment is acceptable. Rubber and plasticized materials must be avoided.

General Acute Test Procedure

1. Transfer parent generation to new culture beakers containing food 24 hours prior to the start of the test to ensure that only <24-hr-old daphnids will be available for testing.
2. Prepare leachate by adjusting the temperature to 20 ± 2°C and adjusting the pH to 6.8 to 8.5 if needed.
3. Label all test beakers.
4. Prepare test solutions by making the appropriate dilutions.
5. Fill test beakers with appropriate test solutions. The test commences when the first animal is added, and so this time must be recorded.
6. Randomly add <24-hr-old daphnids into each beaker until each beaker contains 5 Daphnia. This should be accomplished in less than one hour.
7. Randomize control and test concentrations into rows, randomize beakers within each row and cover with glass.
8. At the end of 24 and 48 hours count and record the number of dead and immobilized Daphnia per beaker.
9. Measure dissolved oxygen, pH, hardness, and alkalinity of the control and the highest concentration, and of intermediate concentrations if the highest concentration is different from the control, at the beginning and at the end of the test.
10. Measure test concentrations of leachates either directly or indirectly at the beginning and at the end of the test.
11. Calculate the 48-hr EC50 and its 95% confidence limits unless 100% of the leachate is nontoxic.

Statistical Evaluations

An acceptable test will have at least two test concentrations with response rates bracketing 50 percent, one of these tests must have a partial kill. Otherwise the test must be repeated, unless there is less than 50 percent response in the 100 percent leachate. If the lowest test concentration results in excess of 50 percent response, the test must be repeated.

An EC50 estimate must be calculated unless there is less than a 50 percent response in 100 percent leachate.

The analysis of the data must include the following components:

(a) A preliminary scatterplot of the response rates observed in each test or control beaker versus group number, concentration, or logarithm of concentration to look for patterns of response and outlying beakers.

(b) EC50 estimates based on the responses in the treatment groups, unless they cannot be calculated for the reasons stated previously. EC50 estimates should be accompanied by estimates of their standard errors and 95 percent confidence intervals. In the event that the confidence intervals are very wide (e.g., if the concentration effect curve is very shallow) the highest concentration for the chronic test should be chosen below the EC50.

(c) The results of outlier tests to detect outlying beakers within a treatment or control group. The details of the suggested outlier test are shown in Appendix H-13.

(d) If the results from one or more beakers are determined to be outliers, then EC50 estimates, standard errors, and confidence intervals will be calculated both including and excluding these values.

The experimental records corresponding to suspected outliers will be examined. If these records are found to contain clerical or experimental errors leading to erroneous values, the erroneous values will be corrected or discarded and the analysis will proceed. If the outlying values are not obviously the result of any such errors, an outlier detection test (Miller, 1966, Barnett and Lewis, 1978) will be carried out. If the outlier test declares the value to be an outlier, then subsequent analyses will be carried out both with and without the response and both sets of estimates will be presented. If the outlier test does not declare the value to be an outlier, then all subsequent analyses will include the suspect value.

Acceptable methods of estimating the EC50 include the two parameter probit or logit methods (Finney, 1978) and the trimmed Spearman-Karber method (trimming proportion must be reported, Hamilton et al., 1977). The method of estimation used must be specified, along with any assumptions or discretionary adjustments that are used. Any other method of estimation must be justified, by citing generally acceptable references in which the estimation method is described and recommended.

Any computer program may be used to calculate the estimates, however the program must yield EC50 estimates within acceptable ranges for all of the benchmark data sets given in Appendix H-14. The computer programs used must be specified in the experimental documentation.

In addition to the above required analyses and displays, the investigator may, at his discretion, provide indications of the steepness of the concentration effect curve by presenting estimates of lower effect levels, such as the EC10, EC20, etc. The ratio of the EC50 to the EC20 might be compared to that for reference toxicants.

The format and organization of the data analysis should resemble that of the model analysis shown in Appendix H-15.

Chronic Static-Renewal Tests

Specific Procedures

All data will be recorded using the form in Appendix F-6.

Organisms - Test animals must come from a healthy culture and must be reared under controlled culture conditions for a minimum of 14 days prior to the start of a test. Parental organisms about to have their second to sixth broods must be transferred into new media <24-hr prior to starting a test.

Food and Feeding - Fish food (5 mg/l) plus Selenastrum capricornutum (10^8 cells/l) are required. Food must be added with the toxicant in the flask initially and when test solutions are renewed (three times each week).

Methods - Young daphnids <24-hrs-old must be placed in test chambers and subjected to test conditions for 21 days. Ten 100 ml beakers are used for each experimental group for each test. One daphnid is placed in each beaker containing 80 ml of test solution. The beakers must be distributed randomly. The beakers must be covered with a glass cover to minimize evaporation and keep out debris. Daphnids must be transferred under the water surface with a fire polished pipette (~8 mm inside diameter) into clean containers every M,W,F when the medium is changed. Survival of the test organisms must be noted each time the medium is changed. Reproduction must be noted by counting the number of live and dead young; the young must be counted and discarded each time the adults are transferred and at the end of each experiment.

Containers - 100-ml borosilicate glass beakers containing 80 ml of control or test solution.

Leachate - Leachate (toxicants) must be stored at 4°C in the dark, but allowed to come to 20 ± 1°C before adding daphnids. Leachate dilution and food mixing are best accomplished in volumetric flasks; the solutions can

then be poured into test containers. The solutions must be renewed three times each week; this is best accomplished by setting up clean beakers with food and toxicant added, and then transferring adult daphnids. Daphnids must be added within one hour after the solutions have been prepared.

Dilution Water - Dilution water must be the same as for culturing (e.g., hard reconstituted water).

Controls - Controls must be set up and treated identically with regard to experimental conditions as test containers, except that no leachate is added. Control animals must produce a minimum average of 40 young in 21 days for the experiment to be valid. Adult survival in the control water must be 80% or more after 3 weeks for the test to be valid.

Acetate Controls - Acetate controls must be run in addition to water controls whenever acetate is used in generating the solid waste leachate. The acetate concentration in the control and all concentrations tested must be the same as that in the highest leachate concentration. No more than 20% mortality may occur in 21 days among acetate-control daphnids for the test to be valid. (If solvents are used the same procedure is applicable.)

Concentrations - The number of concentrations to be tested should be at least 5 and be made up in a geometric progression with a dilution factor of 0.5 (e.g., 100%, 50%, 25%, etc.) or greater (100%, 75%, 56.25%, etc.). Initial concentrations tested should be designed to bracket previous results (i.e., above and below), or be based on results from acute tests with the highest test concentration equal to the 48-hr EC50.

Randomization - Daphnids are assigned completely at random from the culture stock to the test beakers. A two-stage transfer procedure is needed. Daphnids from the culture stock are randomly transferred into beakers containing dilution water which corresponds to each test group. The order of

assignment is determined from a table of random numbers or another method of random allocation. A second transfer is then made into beakers containing the appropriate experimental conditions. The control and test concentrations are then randomized into rows and the beakers are randomized in each row.

Replication - Ten containers, each containing one daphnid (a total of 10 animals), is required for each experimental condition.

Aeration - Must not be used.

Cleaning - All glassware must be cleaned as follows: scrub with a 1% solution of Liquinox-non-phosphate detergent, rinse with tap water until sudsing has ceased, then rinse three more times with tap water. Rinse three times with distilled water, rinse once with 10% HNO_3, rinse once with acetone, and rinse six times with distilled water.

Light and Photoperiod - Fluorescent light bulbs must provide a color rendering index $\geq$90 with a 16-hour photoperiod automatically controlled. A light intensity of 30 to 100 foot candles must be used.

Temperature - An instantaneous temperature of 20 $\pm$ 2°C must not be exceeded; the daily mean temperature must be 20 $\pm$ 1°C. Temperature must be monitored continouously.

Water Quality Measurements - Hardness, alkalinity, pH, and dissolved oxygen measurements must be made when solutions are prepared and again after transfer of daphnids on 2- to 3-day-old solutions. These measurements must be made on controls and the highest concentration tested; if there is a difference between these, then measurements must be made on intermediate concentrations. In addition to the above measurements, the dissolved oxygen must be measured the morning after solutions have been added before the lights come on; this should be accomplished by setting up an additional

control with food but no daphnid (i.e., set up additional controls once or twice during the experiment to be used for checking dissolved oxygen).

pH - The pH of the leachate to be used in testing will be adjusted by using sodium hydroxide to raise the pH to 6.8 or by using hydrochloric acid to lower the pH to 8.5. If the pH of the leachate is initially between 6.8 and 8.5, no adjustments are required. The pH of the leachate must be measured and adjusted prior to the beginning and just befor each renewal for chronic tests.

Leachate Measurements - Test solutions of leachates should be measured either directly or indirectly. If leachates have had preliminary chemical analyses, one of the dominant constituents (e.g., ammonia) may be measured to check dilutions; if not, either conductivity or total organic carbon may be used (see Appendix F-7).

Test Apparatus - Test equipment should consist primarily of high grade borosilicate glass and/or stainless steel. Fluorocarbons and high density polyethylene equipment are acceptable. Rubber and plasticized materials must be avoided.

General Chronic Test Procedures

1. Transfer parent generation to new culture beakers containing food 24 hours prior to the start of a test to ensure that only ≤24-hr-old young will be available for testing.
2. Prepare dilutions in volumetric flasks and add dilution water nearly up to the desired volume.
3. Add trout food plus Selenastrum to volumetric flasks, make up to the appropriate volume (usually 1 liter) with reconstituted water, and mix well.

4. Carefully label all beakers.

5. Fill test beakers with 80 ml of the appropriate test solutions (diluted leachate plus food).

6. Randomly add <24-hr-old daphnids into each beaker until all beakers contain one Daphnia noting the time when the first daphnid is added.

7. Randomize control and test concentration beakers into rows, randomize beakers within each row, cover with glass, and record the time.

8. Every M,W,F: Count number of adult mortalities

   Mix fresh test solutions containing food for each experimental condition.

   Pour test solutions into clean beakers and transfer daphnids.

   Count number of broods per female

   Count number of young per female

   Discard dead adults and all young

9. Measure dissolved oxygen and pH every set-up in both old and new solutions and hardness and alkalinity when experiment is set up and on 2- or 3-day-old samples for the controls and highest concentration tested and intermediate concentrations if the highest concentration and control differ substantially. Measure subsequent set-ups enough to characterize (a minimum of 3 times during an experiment).

10. Check for time to first brood by observing daphnids daily from the seventh day until all daphnids have released broods. Record the day young are born. If length is to be used as an endpoint, measure Daphnia (total length from the top of the head to the base of the spine) at the end of the experiment (21 days).

11. Record and evaluate adult mortality, young per female per reproductive day, and time to first brood and the number of broods for animals living 21 days, using appropriate statistical procedures. An optional measurement includes Daphnia length at the end of the experiment.

Statistical Evaluations

Statistical analysis of the chronic test results will be carried out for the mortality and reproduction responses. Statistical analyses of lengths may be presented, at the discretion of the investigator. Analyses of reproduction and length responses will be carried out only on those daphnids that survive to the end of the test.

For analysis of mortality results, a distinction will be made between toxicant-related and accidental mortality. The causes, if known, of all accidental related deaths will be documented. Accidental related deaths per treatment level must not be >20% of the daphnids tested. Final (21 day) mortality results will be adjusted for accidental related mortality by disregarding those deaths (e.g., those daphnids are excluded from both numerator and denominator when calculating the toxicant related mortality rates in each group).

Results of the statistical analyses on the mortality, reproduction and length responses will be presented in terms of a no-effect concentration (NOEC) and a statistically significant effect concentration. The no-effect concentration is the highest test concentration at and below which the average response does not differ significantly from the control group response. The statistically significant concentration is the next highest concentration.

Estimates of the LC50 and/or LC10 for toxicant-related mortality, along with associated standard errors and confidence intervals, will also be presented.

Survival - Preliminary scatterplots will be prepared of the toxicant-related mortality rates versus group number, concentration, or the logarithm of concentration.

The proportion of toxicant-related deaths within each group will be calculated by dividing the number of toxicant related deaths at 21 days by group size minus the number of accidental deaths. Each such proportion, $\hat{p}$, will be transformed by the arcsine *variance stabilizing transformation* to $(\arcsin \sqrt{r/n+1} + \arcsin \sqrt{(r+1)/(n+1)})$ for small sample sizes. The transformed proportions will be tested for equality by a one way analysis of variance. See Appendix H-25 for details. Multiple comparisons between each treatment group and the solvent or acetate control group will be carried out by Dunnett's many-one t procedure or the Bonferroni t procedure (Miller, 1966) to determine which treatment groups have significantly different mortality rates (at the 5 percent level) than the control group. Williams method (Williams, 1971, 1972) may be used if the mortality rates are believed to vary monotonically with increasing concentration.

The LC50 or LC10 concentrations and associated standard errors and confidence intervals may be estimated by any of the methods discussed for the acute test. The trimmed Spearman-Karber method and the moving average methods are appropriate only for estimation of the LC50. Control mortality in the solvent or acetate control group will be adjusted for by Abbott's correction (Finney, 1971).

Reproduction and Length - The statistical analyses of reproduction and length will be similar to one another. Analyses will be confined to 21-day

survivors. Reproduction will be reported as total number of offspring per female and the total numbers of offspring per reproductive day. Lengths will refer to 21-day lengths.

Preliminary scatterplots of individual responses versus group number, concentration, or the logarithm of concentration will be prepared. Group average responses will be included in these displays. These plots will be examined to determine the nature of the relation between concentration and average response, the relation between average response and standard deviation, and the presence of outliers.

The experimental records corresponding to suspected outliers will be examined. If these records are found to contain clerical or experimental errors leading to erroneous values, the erroneous values will be corrected or discarded and the analysis will proceed. If the outlying values are not obviously the result of any such errors, an outlier detection test (Miller, 1966, Barnett and Lewis, 1978) will be carried out. If the outlier test declares the value to be an outlier, then subsequent analyses will be carried out both with and without the response and both sets of estimates will be presented. If the outlier test does not declare the value to be an outlier, then all subsequent analyses will include the suspect value.

If the variability appears to vary from group to group in the preliminary scatterplots, tests of homogeneity of variance will be carried out. Formal tests of homogeneity of variance such as Bartlett's test or Hartley's test (Neter and Wasserman, 1974, pp. 509-515) or Levene's test (Brown and Forsythe, 1974) may be used. Alternatively the natural logarithm of the standard deviation in each group may be plotted against the group mean and the slope, $\hat{\beta}$, of an appropriate linear relation noted. An approximate

variance stabilizing transformation is $X^{1-\beta}$. Some special commonly occurring cases are:

$\beta = 0$ (constant standard deviation)	no transformation
$\beta = 1/2$ (variance proportional to mean)	square root transformation
$\beta = 1$ (standard deviation proportional to mean)	logarithmic transformation

The presence of heterogeneity of variability and the nature of the relation between variability and average level will be reported as part of the experimental documentation.

The original or transformed average values within each group will be tested for equality by a parametric or nonparametric one way analysis of variance.

Parametric or nonparametric multiple comparisons between each treatment group and the solvent or acetate control group will be carried out by Dunnett's many-one t procedure or the Bonferroni t procedure (Miller, 1966) or the Kruskal-Wallis rank sum based procedure (Hollander and Wolfe, 1973, p. 124) to determine which treatment groups have significantly different response rates (at the 5 percent level) than the control group. Williams method (Williams, 1971, 1972) may be used if the response rates are believed to vary monotonically with increasing concentration. For most leachates, the response is unknown therefore the Williams method should not be used.

Confidence Intervals and After the Fact Power Calculations

The determination of NOEC's and statistically significant concentrations does not impart information about the sensitivity of the inferences. Namely an insensitive test might not reveal statistically significant differences in

group average responses even when the differences are highly biologically significant.

After the fact power calculations will be carried out to determine how large a treatment group response must be before it has high probability of being declared statistically significantly different from the control group response. Power calculations for length and productivity responses will be based on the noncentral t distribution, adjusting for multiple comparisons by Bonferroni's method. See Appendix H-28 for details. Power calculations for mortality responses will be based on the power of Fisher's exact test (Bennett and Hsu, 1960, Haseman, 1978).

Confidence intervals (95 percent) on the differences between the average responses in the solvent or acetate control group and those at the NOEC or statistically significant concentration will be prepared. Confidence intervals for the reproduction and length responses will be based on the t-distribution, accounting for multiple comparisons and for possibly heterogeneous variances. See Appendix H-39 for details. Confidence intervals for mortality responses will be based on the Poisson approach (Feder, 1981, p. 354ff, Nelson, 1970), accounting for multiple comparisons. See Appendix H-30 for details.

OBTAINING AND RECORDING DATA

Acute

After 24 hours and at the completion of the acute test the number of dead and immobile daphnids in each beaker must be counted for determining an EC50. If calculating an optional LC50, the daphnids that are immobile must be carefully pipetted with a glass-pipette (~8 mm inside diameter) into a petri dish or watch glass. Using a 30X dissecting microscope, observe each daphnid individually for heartbeat or movement of the appendages. Absence of movement or heartbeat will constitute a dead daphnid and provide data for the determination of an LC50.

Chronic

The number of dead adult Daphnia are counted by observation only (no microscopic examination required).

The number of young are most easily counted by removing them with a pipette from the test beaker after the adult has been transferred, and then counting them. An automatic counter is not recommended as this will count food particles, etc., which may be of a similar size.

The time to first brood is determined by observing daphnids every day after the seventh day until all animals have their first brood. The number of young per female per reproductive day is determined by adding the total number of young from females alive at the end of the test and dividing by the number of reproductive days (e.g., 21 days minus the number of days to release of the first brood). Females that die during the test are not used for determining reproductive effects.

The number of broods per female is the total number produced during the test. If length measurements are to be used adult daphnids alive at the end

of the test are measured using a 30x compound microscope with a calibrated micrometer eyepiece insert.

The following endpoints must be reported: 21 day LC50, time to first brood, number of broods, and the number of young per female per reproductive day for females surviving 21 days. Any one of these mesurements may be the most sensitive; the lowest concentration for the one that is the most sensitive (95% confidence level) must be reported; this will constitute the toxic concentration. The next lower concentration will constitute the no-significant-effect (or no observed effect) concentration at the 95% confidence level.

DATA REPORTING

(adapted from Peltier 1978)

A report of the test results must include:

- The name of the test method, investigator and laboratory.
- A description of the leachate, including its source, and any physical and chemical properties known.
- A description about the extraction procedure used for preparing the leachate.
- The chemical characteristics of the dilution water.
- The scientific name and source of the test organism.
- A description of the test procedure.
- The methods used for measuring hardness, alkalinity, and dissolved oxygen.
- Direct or indirect measurements of leachates.
- Methods used for all chemical analyses.
- For acute test results:
  - A description of the endpoint used and the statistical analyses.
  - The percent of organisms that lived for each experimental solution.
  - An EC50 value and the 95 percent confidence limit unless all organisms lived in 100% of the leachate.
  - The methods used for statistical analyses of the data.
- For chronic test results:
  - A description of the endpoints used.
  - The number of mortalities and effects observed in controls.

- A nonsignificant and a significant effect concentration at the 95% confidence level for the number of young per female and the number of young per reproductive day unless there was no effect at 100% of the leachate.
- A 21-day LC50 with 95% confidence limits.
- Methods used for statistical analyses.
- Behavioral or other relevant information.

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APPENDIX A

RECONSTITUTED HARD WATER PREPARATION*

Materials needed:

1. 5 gallon carboy
2. deionized distilled water
3. chemicals
   - $NaHCO_3$
   - $CaSO_4 \cdot 2H_2O$
   - $MgSO_4$
   - KCl
4. weighing pans and spatula
5. balance (accurate to 0.001 gram)
6. storage jars for salts (optional)

Methods:

1. Thoroughly rinse the 5 gallon carboy with a 10% solution of nitric acid. Slowly pour out acid solution into cold running water. Rinse carboy thoroughly with deionized distilled water at least 5 times. Accurately mark the 19 liter level in the carboy to facilitate preparation of water each time.
2. Weigh out stock chemicals one at a time in the following amounts:

   3.65 g $NaHCO_3$

   2.28 g $CaSO_4 \cdot 2H_2O$

   2.28 g $MgSO_4$

   0.15 g KCl

Extra stock mixtures can be weighed out in advance for use in the next week if stored in tightly covered jars.

3. Add approximately 15 liters of deionized distilled water to the carboy. Add the chemicals in the order given, mixing thoroughly after each addition. Rinse storage jar with deionized distilled water and add rinse water to solution in carboy. Mix solution thoroughly. Add deionized distilled water to a total solution volume of 19 liters.
4. Using a magnetic stirrer, stir for 24 hours with the carboy lid off, but covered with a foam plug or glass wool, to assure complete mixing of chemicals and saturation of dissolved oxygen.
5. Measure hardness, alkalinity, dissolved oxygen, and pH. The hardness must be from 160-180 mg/l $CaCO_3$, the alkalinity 110-120 mg/l $CaCO_3$, and the pH from 7.6-8.5. This will verify proper measurement and mixing of salts in preparing the reconstituted water. If the hardness, alkalinity and pH requirements are not met, the reconstituted water must be prepared again.

*The 15th edition of Standard Methods (American Public Health Association et al., 1980, p. 627) has a table for hard reconstituted water.

APPENDIX B

DAPHNIA TROUT FOOD PREPARATION

- Add 15 grams of trout food (No. 1 granules) to 800 ml of reconstituted hard water and blend for 15 minutes to liquify.
- Pour into a suitable container and add 200 ml of reconstituted hard water.
- Let stand for 15 minutes and then carefully decant the upper 800 ml and discard the remaining precipitate.
- Thoroughly mix the suspension and withdraw three 10-ml aliquots.
- Dry the aliquots at 104°C for 24 hours in preweighed tares.
- Weigh dry samples and subtract tare weight.
- Calculate average weight of a dry sample and the standard deviation.
- Calculate weight for one ml of dry solids. The final concentration must be 5 mg dry solids per ml of food, so the volume must be adjusted by adding water. The total volume of water (X) to add equals the number of ml in the sample after removal of the aliquots (770 ml) times the mg/ml of dry food weighed (Y) divided by the mg/ml of dry food desired (5 mg/ml) minus the number of ml in the sample after the removal of the aliquots.

  For example, if the dry food weighed 6.32 mg/ml (Y), the following equation will give X:

$$X = \frac{(770)(Y)}{5} - 770 \text{ where } Y = \text{mg/ml dry weight}$$

$$X = \frac{(770)(6.32)}{5} - 770$$

  X = 203 ml of water to add to 770 ml to give a concentration of 5 mg/ml of dry food.

- Freeze trout food in aliquots sufficient for feeding test animals and culture for each day needed. Place frozen aliquot of food in a refrigerator to thaw one day before they are needed for feeding. The trout food must conform to the current U.S. Fish and Wildlife Service Specifications

which can be obtained through livestock feed stores. The dry fish food should be stored in the dark at 4°C for not longer than one year. The current year's specifications follow.

Formulation Specifications for Starter Diet, SDI-30

(Starter, No. 1, and No. 2 granules)

1. Fish food mixture shall be composed of the following items. The final product to carry the following guaranteed analysis:

   Crude protein, not less than 50%

   Fish meal protein, not less than 33%

   Crude fat, not less than 17%

   Moisture, not more than 10.0% at sack-off

		%
2.	Fish meal: stabilized, maximum fat 13%, maximum moisture 10%, stored at the manufacturer's no longer than 6 months as indicated by the bill of lading. Meal must be of fair average quality. Different meals may not be combined for use in the feed. Maximum allowable salt content of 5%.	Not less than 50
	a. Herring meal (minimum protein 67.5%)	
	b. Anchovy meal (minimum protein 65%)	
3.	Wheat feed flour: minimum protein 14%, maximum fiber 1.5%	10.3*
4.	Soy flour: defatted, minimum protein 48.5%, maximum fat 1% (flour must be adequately toasted with a protein dispersibility index of less than or equal to 20).	15
5.	Dried blood flour or ring dried blood meal: minimum protein 80%.	10
6.	Trace mineral premix No. 1 (see Section 5 of specifications).	1#/ton
7.	Vitamin premix No. 30 (see Sections 4 and 7 of specifications).	8#/ton
8.	Choline chloride, 50%.	4.5#/ton
9.	Ascorbic acid.	1.5#/ton
10.	Fish oil: stabilized with 0.04% BHA-BHT (1:1) or 0.01% ethoxyquin, less than 3% free fatty acids and not alkaline reprocessed.	12*
	. Lignin sulphonate pellet binder (e.g. Ameribond, Orzan, or equivalent).	2

* Fish meal may be increased depending upon protein content but must provide not less than 33% fish protein. Quantity of added oil may be adjusted so that the finished feed shall contain not less than 17% crude fat. Wheat flour is to be adjusted to compensate for the above variations. Not less than 6% of the added oil is to be applied to the granules as a top dressing, the rest of the oil to be included in the feed mix.

Specification for Vitamin Premix No. 30

Vitamin	Guaranteed potency per pound of premix (grams unless otherwise listed)
D calcium pantothenate	12.0
Pyridoxine (pyridoxine HCl)	3.5
Riboflavin	6.0
Niacinamide	25.0
Folic acid	1.0
Thiamine (thiamine mononitrate)	4.0
Biotin	40.0 mg
Vitamin B_{12}	2.5 mg
Menadione sodium bisulfite complex	1.25
Vitamin E (d or dl alpha tocopherol acetate)	40,000 i.u.
Vitamin D_3, stabilized	50,000 i.u.
Vitamin A (vitamin A palmitate or acetate), stabilized	750,000 USP

Choline chloride, ascorbic acid, and the vitamin premix No. 30 are to be stored separately and never mixed one with another before being added to the feed mixture.

The certified vitamin premix is to be supplied by a recognized manufacturer and must show the date of preparation. The vitamin premix is not to be stored for longer than 4 months after date of preparation.

The vitamin premix is to be made with a wheat or soybean by-product base. Rice hulls or oat feed are not acceptable.

Specification for Trace Mineral Premix No. 1

Mineral	Guaranteed Analysis of Element (g/lb mineral mix)
Zinc ($ZnSO_4$: 84 g/lb mineral mix)	34
Manganese ($MnSO_4$: 94 g/lb mineral mix)	34
Iron ($FeSO_4 \cdot 7\ H_2O$: 22.5 g/lb mineral mix)	4.5
Copper ($CuSO_4$: 1.75 g/lb mineral mix)	0.7
Iodine (KIO_3: 0.38 g/lb mineral mix)	0.23

An inert carrier can be used to make up the mixture to the pound.

The mineral mixture is to be added at 1.0 pound per ton SD9 feed and 2.0 pounds per ton for GR3 and GR4 feeds.

APPENDIX C

CULTURING *Selenastrum capricornutum*

Algae origin:

American Type Culture Collection
12301 Parklawn Drive
Rockville, MD 20852

OR

The Starr Collection
Department of Biology
University of Texas at Austin
Austin, TX 78712

Algae type:

1. *Selenastrum capricornutum* ATC #22662
2. *Selenastrum capricornutum* UTEX1648

Maintenance conditions:

1. Constant temperature from 18 ± 1°C to 24 ± 1°C
2. Lighting continuous "cool-white" fluorescent light from 4000 ± 10% to 5000 ± 10%; photoperiod from 14L:10D to continuous lighting.
3. The cultures must be maintained sterile in a chemostat (flow-through) system or have continuous aeration, stirred with a magnetic stirrer or shaken on a suitable shaker.

Glassware Cleaning - All glassware used for any aspect of algal culturing must be cleaned as follows: scrub with a 1% solution of Liquinox or other non-phosphate detergent, rinse with tap water until sudsing has ceased, then rinse three more times with tap water. Rinse three times with distilled water, rinse once with 10% HNO_3, rinse once with acetone, and rinse six times with distilled water. Autoclave all glassware to be used for all phases of algae culture.

Synthetic algal media stock preparation[a]

1. Macronutrient stocks. Prepare separate stocks (for Woods Hole MBL medium) of each of the following compounds by dissolving the specified weight into a total volume of one liter of glass distilled water.

Compound	Grams/Liter
$CaCl_2 \cdot 2H_2O$	36.76
$MgSO_4 \cdot 7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$	85.01
$Na_2SiO_3 \cdot 9H_2O$	28.42*

*Filter sterilize this stock solution and add 1 ml to the culture medium after autoclaving, using sterile technique.

2. Micronutrient stocks. Prepare each stock solution shown below in a final volume of one liter of glass-distilled water. Mix until dissolved. For stock No. 3, add chemicals in the order shown.

Stock No.	Compound	Grams/Liter
1	Na_2EDTA	4.36*
2	$FeCl_3 \cdot 6H_2O$	1.575**
3	$CuSO_4 \cdot 5H_2O$	0.01
	$CoCl_2 \cdot 6H_2O$	0.01
	$ZnSO_4 \cdot 7H_2O$	0.022
	$MnCl_2 \cdot 4H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.006
	H_3BO_3	1.0

* Stock must be less than three months old.

**Use 2 ml/l of medium.

---

[a] The method is based largely on conversations with Dr. Clyde Goulden and Ms. Linda Henry (Academy of Natural Sciences, Philadelphia) for Selenastrum culture in micronutrient supplemented MBL medium.

3. Record stock solution preparation information. All compounds used must be ACS Reagent grade (or other high purity grade if no ACS standard has been established for the compound used). Refrigerate all stocks. Stocks showing any evidence of precipitation or contamination must not be used. Precipitation of the sodium silicate may occur with time, but the stock can still be used.
4. For each liter of culture medium being prepared, include one milliliter of each macronutrient stock (3.1.1, except sodium silicate) and one milliliter of each micronutrient stock (3.1.2). Place one liter of medium in a 2 l Erlenmeyer flask, add a cleaned 50 mm (2 inch) Teflon stirring bar, and cap with a foam plug (Gaymar IDENTI-PLUGS are recommended - Miller et al., 1978) or with a cotton plug wrapped in cheesecloth. Cover the top with aluminum foil. Autoclave at 1.1 kg/cm^2 (15 psi) and 121°C for 15 minutes. Allow to come to room temperature. Add 1 ml $Na_2SiO_3 \cdot 9H_2O$ stock using sterile technique.
5. Use similar procedures for preparing 1000 ml of media in a 2000 ml Erlenmeyer flask.
6. For agar slants and petri plates, prepare medium as above but, in addition, dissolve 1% (w/v) agar (DIFCO Bacto-Agar or equivalent) prior to autoclaving. Place agar solution into test tubes for slants; tilt after removal from autoclave but before the agar has jelled. Pour autoclaved solution into sterile petri plates using sterile technique.

Obtaining Uncontaminated Algal Cultures. If stock algal cultures become contaminated or if it is necessary to obtain new uncontaminated algal stocks, use the procedure described below.

1. Using a sterile pipette, transfer one drop of algae in algal medium to a sterile petri plate with the appropriate agar medium. Streak and allow colonies to grow.
2. Select a presumptive clean single cell isolate from the plate and transfer to a new plate. Streak again. Use the uncontaminated single cell isolates from this plate to start new agar slants.

Initiating and Growing Algal Cultures

1. Obtain uncontaminated cells from isolates as described above. Prepare agar slants by transfer from clean agar slants. Sufficient agar slants should be prepared such that one is available every time a new algal inoculum must be prepared. Keep slants for three to six months, but discard after use in one set of transfers.
2. Make a new set of slants (as required) from an available slant, then inoculate 100 ml of medium with algae from the slant. Allow the algae to grow in the medium and use the inoculum prior to the stationary phase of growth. This may be determined by visual examination of the color of the medium once sufficient experience is gained with culturing. Otherwise, a sample must be withdrawn with a sterile pipette and counted with a haemacytometer to ensure that the cells are in log-phase growth. (It is assumed that baseline data is available on the growth curve of the alga so that the cell concentration at the beginning of the stationary phase of growth is known.)
3. Static cultures are prepared by inoculating a vessel of MBL with a batch culture. Each vessel should be covered with a cotton stopper, and continuously aerated and stirred with a magnetic stir-bar or placed on a shaker table. If this system is used in an on-going feeding program new

vessels must be inoculated on a careful schedule to insure that adequate supplies of algae are available at all times.

4. The semi-continuous culture system is prepared by hooking a 4 or 9 liter reservoir of the culture medium to a 4 liter aspirator bottle with a silicone rubber siphon. The aspirator is first inoculated with a batch culture of algae and culture media is then siphoned from the reservoir placed above the aspirator bottle. When the culture is ready for harvesting, algae may be removed for use and replaced with fresh media and vitamins as needed. Semi-continuous cultures should not be used for more than one month. A similar but more complex system for semi-continuous culturing is described in chapter 15 of Stein's (1973) Phycological Methods. Air lines should have a cotton filled trap to absorb oil or toxic liquids.

APPENDIX D

PREPARATION OF ALGAE FOR FEEDING DAPHNIDS

Method 1

A drop of algae from a well-mixed culture of Selenastrum is used to fill a haemacytometer counting cell. Enough sets (having 16 squares each) are counted so that between 100 and 200 algae cells are counted. A conversion of the number of cells counted into the number of cells per milliliter is made using the following formula:

$$\frac{(\text{No. of cells counted}) \times (4 \times 10^6)}{\text{No. of squares counted}} = \text{No. of cells/ml}$$

The number of ml needed to get 10^8 cells is determined by dividing 10^8 cells by the number of cells per ml in the culture. The volume (ml) thus determined is measured, placed in centrifuge tubes, and centrifuged at 2,200 RPMs (700 g) for 15 minutes. The algae media is then carefully poured off, and ten milliliters of reconstituted water is added to resuspend the algae (e.g., 10 ml will then contain 10^8 Selenastrum). Selenastrum in the reconstituted water is then added to volumetric flasks containing approximately 950 ml of leachate, fish food, and reconstituted water. The centrifuge tubes are rinsed twice to assure that all algae are removed, and the rinse water is then added to the test solution. The test solution is then made up to one liter and is ready for dispensing into the test chambers. Algae in centrifuge cells may be stored in the dark at 4°C for 3 to 4 days for subsequent feeding to daphnids.

Method 2

Check cell concentrations to confirm log-phase growth. Centrifuge the algae at a speed and time sufficient to remove the algae from the water column (700 xg for 15 minutes is suggested). Pour off the supernatant, leaving as little of the algal medium as possible behind. Resuspend the algae in a small amount of the same solution used for culturing the daphnids to be fed. Remove a small portion of the combined algal solutions and dilute as needed to perform a hemacytometer count. Count at least 100 cells per field; determine the original cell concentration per milliliter as follows:

Cells/ml = (cell count) (10,000) (25/the number of double lined fields counted) (dilution factor)

Dilute the combined algal solution with the appropriate daphnid culture medium so that one milliliter, when added to 800 ml of daphnid medium, will create the appropriate food concentration. Confirm the final cell concentration with a hemacytometer count. Harvested Selenastrum may be stored in the refrigerator for 3-4 days after harvest.

Method 3

A particle counter may be used for counting algae cells.

Note: If the algae appears yellowish, brownish, clumps heavily on the sides of the culture vessels, or does not appear in the microscope as intact cells something is wrong with either the algae stocks or your culture technique. Common problems include errors in media preparation or heavy contamination with some other organism such as bacteria. If the above problems occur, the algae cultures should be replaced. If they persist the media preparations should be replaced and new slants ordered from the collections mentioned earlier.

References

Stein, J. 1973. Handbook of Phycological Methods. Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, England.

APPENDIX E

EQUIPMENT

Equipment	Model - Specifications	Manufacturer*
Pipettes (daphnids)	5-mm and 8-mm	-
Pipettes (algae)	1-ml x 1/100 Polystyrene plugged sterile disposable	-
Suction bulbs	rubber, 1/2 ounce	-
Culture beakers (daphnids)	2000-ml glass containers	-
Test beakers (daphnids)	100-ml Pyrex or Kimax	-
Erlenmeyer flasks (algae)	1000- and 2000-ml Pyrex or Kimax	-
Foam plugs (algae)	nontoxic foam plug 35-45 mm	Scientific Products diSPO #T1387
Carboys	5 gallon plastic w/spigot	Sybron/Nalgene
Fluorescent lights (algae and daphnid maintenance)	"cool-white" for algae "Grow-Lux" and "Vita-Life" for daphnids	Sylvania
Light table	Model GB 11-17 30 watts "Glow Box"	Instruments for Research and Industry
Light meter	Model #200	PhotoVolt Corp.
Dissolved oxygen meter	Model 0260 Oxygen Analyzer	Beckman
pH meter	0-14 pH units $\pm$ 1/10 pH	Beckman "Altex"
Compound microscope	-	Leitz-Wetzler Co. "Ortholux"
Dissecting microscope	15 x W.F., Cat. 147	American Optical "Spencer"

APPENDIX E (continued)

Equipment	Model - Specifications	Manufacturer*
Micrometer	0.01 or 0.001 inches at 4X	American Optical
Hemacytometer		
Centrifuge	Model Pr-2 1000 x g force	International Portable Refrigerated Centrifuge
Membrane filter apparatus		
Autoclave or pressure cooker		
Drying oven	Temperature capability 120° C	Precision Scientific Co.
Dishwasher	L/A-7537 glassware washer	Forma Fury
Balance	Accurate to 0.0001 gram	Mettler

* Or equivalent.

APPENDIX F

DATA FORMS

DATA FORM #1
F

PREPARATION OF RECONSTITUTED HARD WATER

BATCH # _____ TOTAL VOLUME _____

DATE PREPARED _____ DATA BY _____

SALTS	AMOUNT ADDED TO FINAL VOLUME _____ L	NOTES
$NaHCO_3$		
$CaSO_4 \cdot 2H_2O$		
$MgSO_4$		
KCl		

RECONSTITUTED HARD WATER - WATER QUALITY

pH	
SPECIFIC CONDUCTANCE	
TOTAL HARDNESS	

SAMPLE VOL.	DILUTED TO	mL TITRANT USED	mg/L $CaCO_3$
_____	_____	_____	_____

TOTAL ALKALINITY	

SAMPLE VOL.	DILUTED TO	mL TITRANT USED	mg/L $CaCO_3$
_____	_____	_____	_____

DATE: _____

DATA FORM 2. WOODS HOLE MBL STOCK SOLUTION PREPARATION

SIGNATURE _____

Compound	Grams/ Liter	No. Liters Prepared	Grams Added	mL Stock per L Culture medium	Comments
$CaCl_2 \cdot 2H_2O$	36.76			1	
$MgSO_4 \cdot 7H_2O$	36.97			1	
$NaHCO_3$	12.60			1	
K_2HPO_4	8.71			1	
$NaNO_3$	85.01			1	
$Na_2SiO_3 \cdot 9H_2O$	28.42			1[a]	
Na_2EDTA	4.36			1[b]	
$FeCL_3 \cdot 6H_2O$	1.575			2	

Combine the remaining compounds into one stock solution:

$CuSO_4 \cdot 5H_2O$	0.01			1	
$CoCl_2 \cdot 6H_2O$	0.01			-	
$ZnSO_4 \cdot 7H_2O$	0.022			-	
$MnCl_2 \cdot 4H_2O$	0.18			-	
$Na_2MoO_4 \cdot 2H_2O$	0.006			-	
H_3BO_3	1.0			-	

[a]Filter sterilize stock; add after autoclaving.

[b]Make new stock at least every 3 months.

DATA FORM 3. Daphnid Food Preparation

Species _____

(See protocol Appendix D-1 for method)

Medium _____

Date Prepared	Batch No.	Hemacytometer cell count at harvest (cells/mL) A	Hemacytometer count (cells/mL) of concentrate[a] B	Undiluted Volume (mL) = C	Desired[b] Conc. (Cells/mL) = D	Diluted to Total Vol. (mL) (BXC)/D	Diluted Algae Conc.	Comments	Initials

[a] Obtained by concentrating algae described in column A.

[b] Cells/mL required to give desired food concentration in 800 mL daphnid medium.

DATA FORM 4 Page _____

DAPHNID FOOD PREPARATION - TROUT FOOD SUSPENSION

(See protocol Appendix B-1 for method of preparation)

Date: _____ Prepared By: _____

Stock A Preparation:

Approx. _____ g of trout food/ _____(Other)

added to _____ mL diluent water. Mixed and diluted to _____ L.

Stock A Dry Weight Determination:

	Gross Dry Weight	Tare Weight	Net Dry Weight
Rep. A			
Rep. B			
Rep. C			

$\bar{x}$ concentration _____mg/mL

Stock B Concentration Calculation:

_____ mL stock A diluted to _____ mL = _____mg/mL

Used for feeding test and/or culture daphnids: _____(Dates)

_____

DATA FORM 5. STATIC ACUTE TOXICITY TEST (SIDE A)

TEST MATERIAL _____

DATE		
TIME		
CONTROL TEMP		
DATA BY		

REP	CONC	DO	pH	HRD	ALK	DO	pH	HRD	ALK

STOCK CONCENTRATION _____ SOLVENT _____

PREPARED BY _____ DATE PREPARED _____

TEST CONCENTRATIONS HIGH → LOW

mg/ℓ μg/ℓ	CONT.	S.CONT.							
AMOUNT OF STOCK ADDED									
AMOUNT OF DILUTION H_2O ADDED									

DATE	0-HOUR	24-HOUR	48-HOUR
TIME			
DATA BY			

REP.	A	B	C	D	A		B		C		D		CUM. NO. DEAD	A		B		C		D		CUM. NO. DEAD
CONC. mg/ℓ μg/ℓ	OBSER.	OBSER.	OBSER.	OBSER.	OBSER.	# DEAD	OBSER.	# DEAD	OBSER	# D	OBSER.	# D		OBSER.	# DEAD	OBSER.	# D	OBSER	# D	OBSER.	# D	

DATA FORM 5. STATIC ACUTE TOXICITY TEST (SIDE B)

TEST MATERIAL _____

SAMPLE LOT # _____

PRINCIPAL INVESTIGATOR(S) _____

TIME ADDED TEST MATERIAL/DAPHNIDS	NO. OF DAPHNIDS PER VESSEL	NO. OF REPLICATES PER TREATMENT LEVEL	TYPE TEST VESSEL	TEST SYSTEM USED
				OPEN CLOSED

TEST CHAMBER VOLUME	TOTAL SOLUTION VOLUME	TEST ORGANISM (SPECIES)	SOLUTION VOLUME PER REPLICATE TEST VESSEL	AGE OF DAPHNID AT TEST INITIATION (HOURS)

WATER QUALITY OF DILUTION WATER

DATA TRANSCRIBED NOTEBOOK _____	SOURCE _____	TOTAL ALKALINITY _____
PAGE NO.	BATCH # _____	TOTAL HARDNESS _____
LOCATION	pH _____	CONDUCTIVITY _____

COMMENTS _____

NO DISCERNIBLE EFFECT LEVEL THROUGH 48 HOURS _____

OBSERVATION KEY

OS - ON SURFACE	CO - CAUGHT ON
OB - ON BOTTOM	CLDY - CLOUDY
LETH - LETHARGIC	PRE - PRECIPITATE
ERR - ERRATIC SWIMMING	UM - UNDISSOLVED MATERIAL
FC - FLARED CARAPACE	PM - PARTICULATE MATTER
SC - SWIMMING, CARRYING	F - FILM

SIGNATURE INITIALS

AGE OF D. magna ______

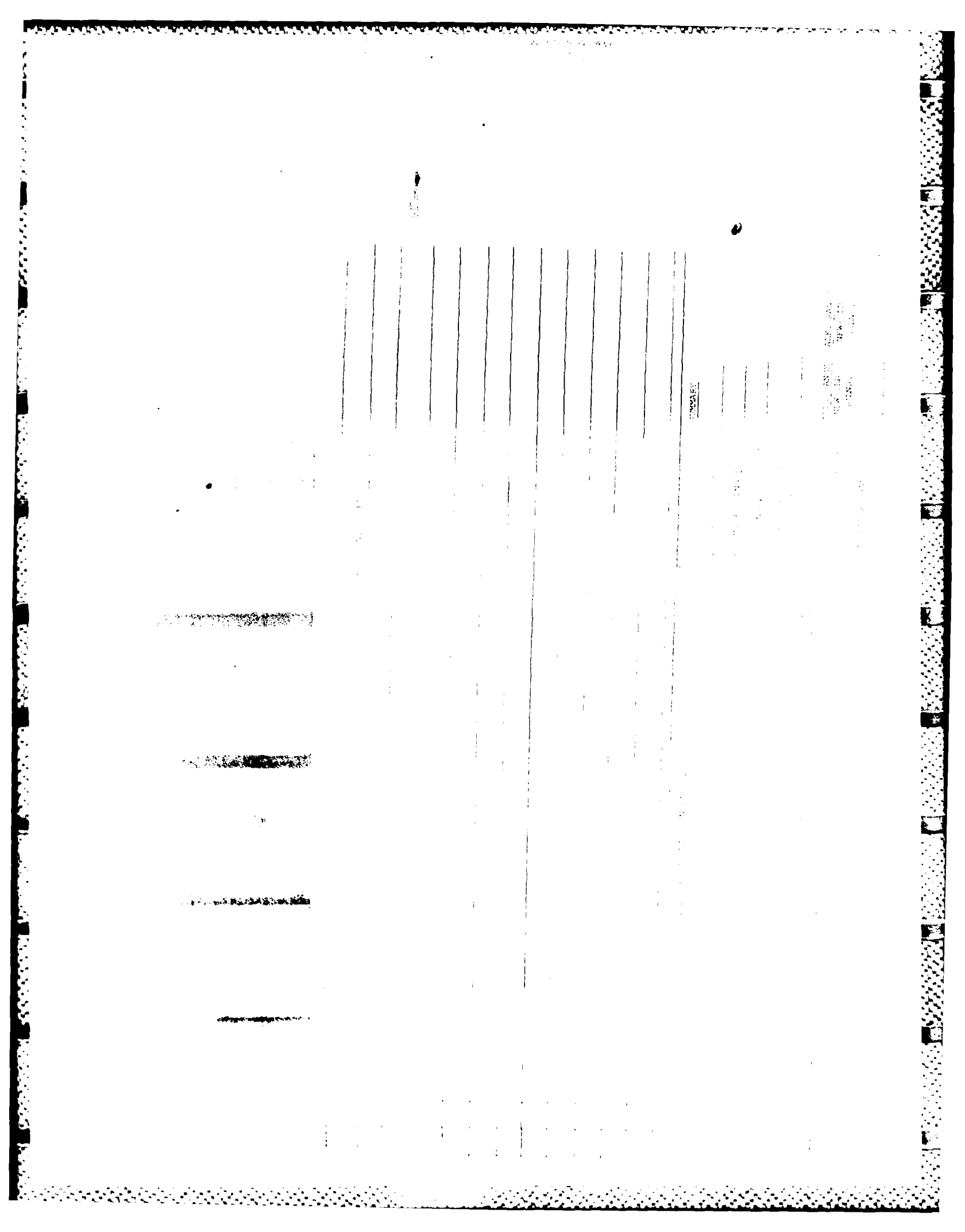
BROOD # REMOVE FROM ______

SAMPLE ______ TOTAL COUNT ______

PRINCIPAL INVESTIGATOR(S) ______

PHOTOPERIOD/LIGHT INTENSITY ______

		# ALIVE/# MOLTS/# OFFSPRING										SOLN. RENEW. USING DILU. WTR. BATCH#	pH NEW/OLD SOLN.	DO (mg/) NEW/OLD SOLN.
		A	B	C	D	E	F	G	H	I	J			



DATA FORM 7. SAMPLE SUBMISSION (SIDE A)

SUBJECT: SUBMISSION OF SAMPLES FOR CHEMICAL ANALYSIS

TO BE FILLED OUT BY TRANSMITTER

CLIENT: _____ TEST MATERIAL: _____ PROJECT NO. _____

DESCRIPTION OF TEST: _____ SPECIES: _____

TYPE SAMPLE ☐ WATER

SAMPLING CONTAINER ☐ GLASS CLEAR ☐ GLASS AMBER ☐ PLASTIC ☐ OTHER _____ ☐ CAP LINERS _____

SAMPLE PRESERVATION TYPE _____ AMOUNT ADDED _____

DATE/TIME SAMPLED: _____ SAMPLED BY: _____ TEST DAY: _____

SAMPLE STORAGE REQUIREMENTS: _____

SAMPLES SUBMITTED TO: CONTACT: _____

SAMPLE ID NUMBER	NOMINAL CONCENRATION (DEFINE UNITS)	TANK REP	FURTHER DESCRIPTION OF SAMPLE	VOLUME WEIGHT SAMPLED	*ANALYTICAL RESULTS (DEFINE UNITS)	*REMARKS

* FILLED OUT BY RECEIVER NOT RAW DATA TRANSCRIBED FROM CHEMISTRY DATA SHEET.

SEE BACK

DATA FORM 7. SAMPLE SUBMISSION (SIDE B)

TO BE FILLED OUT BY TRANSMITTER (FOR SAMPLES SHIPPED)

SAMPLES PACKED BY: _____ DATE: _____

INSPECTED BY: _____ DATE: _____

SHIPPED BY: _____ DATE: _____

SHIPPING CONDITIONS: ☐ DRY ICE ☐ AMBIENT TEMP. ☐ OTHER _____

TO BE FILLED OUT BY RECEIVER (UPON RECEIPT)

RECEIVED BY: _____ DATE: _____

ARRIVAL CONDITION OF SAMPLES: ☐ FROZEN ☐ AMBIENT ☐ OTHER _____

DOCUMENT MISSING SAMPLES, BROKEN CONTAINERS, ETC. (IDENTIFY BY NUMBER):

_____

_____

_____

_____

DISPOSITION OF SAMPLES: ☐ STORED LOCATION: _____

☐ ANALYZED

☐

SIGNATURE OF SUPERVISOR NOTIFIED: _____

SAMPLES ANALYZED BY: _____ DATE: _____

TO BE FILLED OUT BY REPORTER OF RESULTS

RESULTS APPROVED BY: _____ DATE: _____

RESULTS REPORTED BY: _____ DATE: _____

DISPOSITION OF SAMPLES AFTER ANALYSIS: ☐ DISCARDED

☐ RETAINED LOCATION: _____

COPIES OF RAW DATA INCLUDED ☐ YES

☐ NO

APPENDIX G

In order to ensure that all studies conducted utilizing this Protocol produce data which are equally comparable and meet general industry standards, all work conducted utilizing this Protocol will be accomplished under the provisions of the Environmental Protection Agency's Good Laboratory Practice Regulations published in November 1983, or subsequent revisions.

APPENDIX H

STATISTICAL PROCEDURES

This appendix provides descriptions of and references to statistical procedures that are required and/or recommended in the body of the protocol for the statistical analyses of acute and chronic Daphnia toxicity testing data. References to more detailed discussions of these procedures and to computer programs to carry them out are also given. An example of the use of these procedures and the reporting of analysis results is provided, based on hypothetical data, randomly generated to conform to the experimental design described in the protocol.

The following are included:

	Page
Acute Test	H-2
Acute Test Example	H-15
Chronic Test Mortality Data	H-25
Chronic Test Mortality Date Example	H-32
Chronic Test Reproduction and Length Data	H-38
Chronic Test Length Data Example	H-45

I. ACUTE TEST

A. Experimental Design - See "Acute Tests" section in body of protocol for details of experimental layout. An acceptable test will have no more than 10 percent mortality in any of the water control, acetate control, or solvent control groups. Estimates of the 48-hour EC50 will not be adjusted for control mortality; therefore, the control groups will not be included in the discussion of statistical procedures for acute test results.

B. Notation - An acute toxicity test will result in the following values:

1. $C_1, C_2, \ldots, C_k$ — the k test concentrations of toxicant ~~and~~ arranged in increasing order so that $C_1 < C_2 < \ldots < C_k$;
2. $x_i = \log C_i, i = 1, \ldots, k$ — natural logarithms of the k concentrations;
3. $n_1, n_2, \ldots, n_k$ — the numbers of daphnids exposed to the k concentrations, respectively;
4. $r_1, r_2, \ldots, r_k$ — the numbers of daphnids that die or are immobilized within 48 hours of exposure to the k concentrations, respectively;
5. $p_1, p_2, \ldots, p_k$ — the observed mortality proportions for the k concentrations, respectively; $p_1 = r_1/n_1$, $p_2 = r_2/n_2, \ldots, p_k = r_k/n_k$.

When it is necessary to refer to individual beakers, the following notation will be used: n_{i1}, n_{i2}, n_{i3}, and n_{i4} will denote the numbers of daphnids in the 4 beakers at concentration C_i; r_{i1}, r_{i2}, r_{i3}, and r_{i4} will denote the numbers of daphnids in the 4 beakers at concentration C_i that die or are immobilized within 48 hours; p_{i1}, p_{i2}, p_{i3}, and p_{i4} will denote the observed mortality proportions in the 4 beakers at concentration C_i. Thus $n_i = n_{i1} + n_{i2} + n_{i3} + n_{i4}$ and $r_i = r_{i1} + r_{i2} + r_{i3} + r_{i4}$.

P(x) will denote the true, unknown proportion of daphnids in the entire reference population that would die or become immobilized within 48 hours if exposed to the concentration C whose logarithm is x. Throughout the appendix, logarithms will always refer to natural logarithms unless there is an explicit statement to the contrary.

The median effective concentration (EC50) will be denoted by μ and estimates of μ will be denoted by $\hat{\mu}$. The asymptotic standard error of $\log(\hat{\mu})$ will be denoted by σ and estimates of σ will be denoted by $\hat{\sigma}$.

C. Preliminary Scatterplot - A preliminary scatterplot of the responses versus log-concentration will be formed. The observed individual beaker mortality proportions $\{p_{ij}$'s$\}$ will be plotted versus log-concentration $\{x_i$'s$\}$; the observed average mortality proportions $\{p_i$'s$\}$ will be included in this plot using a different plotting symbol.

D. Point and Confidence Interval Estimation of the EC50 Using the Two-Parameter Probit Model - This method assumes that

$$P(x) = \Phi(\alpha+\beta x)$$

or that

$$\Phi^{-1}[P(x)] = \alpha+\beta x$$

where Φ is the standard normal distribution function and Φ^{-1} is the inverse of the standard normal distribution function. This method requires at least two partial kills in order to estimate the EC50. Point and confidence interval estimates may be obtained directly by using one of a number of computer programs designed to perform a probit analysis. Among these programs are the Statistical Analysis System (SAS) [1] PROC PROBIT and a program developed by Charles Stephan and others [2] that is based on a paper by Stephan [2] on the methodology for calculating an EC50. A listing of the Stephan program is contained in Appendix A. It should be noted that some programs, including SAS PROC PROBIT, actually fit the model

$$P(x) = \Phi(\alpha-5+\beta x)$$

The program documentation should be checked to determine which model is being fitted.

If a probit analysis program is not available, a general purpose nonlinear regression program, such as BMDPAR [4], that produces estimates of the variances and covariances of the parameter estimates can be used to carry out the calculations. The nonlinear regression model

$$P_i = \Phi(\alpha+\beta x_i) + \epsilon_i \qquad i=1,\ldots,k$$

is iteratively fitted to the data using a weighted least squares analysis. The ith data point is given weight

$$w_i = \frac{n_i}{\Phi(\hat{\alpha}+\hat{\beta}x_i)[1-\Phi(\hat{\alpha}+\hat{\beta}x_i)]}$$

The residual variance in the regression analysis is specified to be 1.0 and this value is used in the calculation of residual variances and covariances. See the example for details. The estimation procedure results in $\hat{\alpha}$ and $\hat{\beta}$, estimates of α and β. A point estimate of the EC50 is

$$\hat{\mu} = \exp(-\hat{\alpha}/\hat{\beta})$$

Let V_1^2 and V_2^2 denote the estimates of the variances of $\hat{\alpha}$ and β, respectively, and V_{12} denote the estimate of the covariance of $\hat{\alpha}$ and β. Then

$$\hat{\sigma} = \frac{1}{\hat{\beta}}\left[V_1^2 + 2(\log\hat{\mu})V_{12} + (\log\hat{\mu})^2 V_2^2\right]^{1/2}$$

is an estimate of the standard error of $\log(\hat{\mu})$.

$$(\hat{\mu}\cdot\exp(-1.96\hat{\sigma}),\ \hat{\mu}\cdot\exp(1.96\hat{\sigma}))$$

is an approximate 95 percent confidence interval for the EC50. Note that some programs calculate ρ_{12}, the correlation coefficient between $\hat{\alpha}$ and β, instead of V_{12}. In this case calculate V_{12} by the formula $V_{12} = \rho_{12}V_1V_2$.

An alternative method to that above for calculating a confidence interval for the EC50 is based on Fieller's Theorem. Many probit analysis programs such as SAS PROC PROBIT and the Stephan program use this method. For more information on the two-parameter probit model and Fieller's theorem, refer to Finney ([5], 1971; pp. 78-80).

E. Point and Confidence Interval Estimation of the EC50 Using the Two-Parameter Logit Model - This method assumes that

$$P(x) = \frac{1}{1 + \exp[-(\alpha+\beta x)]}$$

or that

$$\log\left[\frac{P(x)}{1-P(x)}\right] = \alpha + \beta x$$

and requires at least two partial kills in order to produce both point and interval estimates of the EC50. This model is directly analogous to the two-parameter probit model. A general purpose logistic regression program, such as BMDPLR [14] (UC 1981), or a general purpose nonlinear regression program, such as SAS PROC NLIN [17] SAS I 1982 or BMDPAR [16] (UC 1981), will produce the necessary output to compute point and confidence interval estimates of the EC50. If a nonlinear regression program is employed, the nonlinear model

$$P_i = \frac{1}{1+\exp[-(\alpha+\beta x_i)]} + \epsilon_i \qquad i = 1, \ldots, k$$

is iteratively fitted to the data using a weighted least squares analysis. The ith data point given is weight

$$w_i = \frac{n_i\left\{1+\exp[-(\hat{\alpha}+\hat{\beta}x_i)]\right\}^2}{\exp[-(\hat{\alpha}+\hat{\beta}x_i)]}$$

See the example for details. The estimation procedure results in $\hat{\alpha}$ and $\hat{\beta}$, estimates of α and β. A point estimate of the EC50 is then

$$\hat{\mu} = \exp(-\hat{\alpha}/\hat{\beta})$$

Expressions for the standard error, $\hat{\sigma}$, of $\log(\hat{\mu})$ and an approximate 95 percent confidence interval for the EC50 are the same as those shown for the two-parameter probit model.

For more information on the two-parameter logit model, refer to Hamilton (1979) [6].

F. Smoothing the Observed Mortality Proportions - It is known that $P(x_1) \leq P(x_2) \leq \ldots \leq P(x_k)$. Because of random variation, the observed mortality proportions $p_1, p_2, \ldots p_k$ may not show this monotone behavior. When this is the case, several methods to be discussed subsequently in this appendix require as a first step the smoothing of the observed mortality proportions to monotone nondecreasing order. New mortality proportions $p_1, p_2, \ldots, p_k$ are calculated by combining the mortalities (r_i's) and numbers of daphnids (n_i's) of any adjacent p_i's which are not in the proper monotone order to obtain a single average mortality proportion p for the two groups. That is, suppose $p_3 > p_4$. Then p_3 and p_4 are each replaced by $p_3 = p_4 = (r_3 + r_4)/(n_3 + n_4)$. This process is continued until $p_1 \leq p_2 \leq \ldots \leq p_k$. Note that once two adjacent groups are combined, they remain combined throughout the averaging process.

G. Point and Confidence Interval Estimation of the EC50 Using the Conventional and the Trimmed Spearman-Karber Methods - These methods assume only that the population mortality rate P(x) is symmetric about the log EC50 in such a way that

$$P(\log EC50 + x) = 1 - P(\log EC50 - x)$$

for all x; they do not assume a specific functional form for P(x). The conventional Spearman-Karber method requires that at least one low concentration yield no mortalities and that at least one high concentration yield 100 percent mortalities.

Let x_1 be the highest log-concentration producing 0 percent mortality such that all lower concentrations also produce 0 percent mortality. Let x_k be the lowest log-concentration producing 100 percent mortality such that all higher concentrations also produce 100 percent mortality. All log concentrations below x_1 or above x_k are excluded from the analysis. The first step is to smooth $p_1, p_2, \ldots, p_k$ as outlined above to obtain the monotone nondecreasing values. The conventional Spearman-Karber estimate of the EC50 is

$$\hat{\mu}_0 = \exp\left[\sum_{i=1}^{k-1} (p_{i+1} - p_i) \left(\frac{x_i + x_{i+1}}{2} \right) \right]$$

and an estimate of the standard error of $\log(\hat{\mu})$ is given by

$$\hat{\sigma}_0 = \frac{1}{2} \left[\sum_{i=2}^{k-1} \frac{(x_{i+1} - x_{i-1})^2 p_i (1 - p_i)}{n_i} \right]^{1/2}$$

The conventional Spearman-Karber estimate can be thought of as the 0 percent trimmed Spearman-Karber estimate.

In order to obtain an α trimmed Spearman-Karber estimate ($0 < \alpha < 0.5$), the upper 100α percent and lower 100α percent of the estimated tolerance distribution is trimmed off. Let $L = \max \{ i : p_j \leq \alpha \}$ and $U = \min \{ i = p_j \geq 1 - \alpha \}$ and define new log-concentration values

$$x_L^* = x_L + \left(\frac{\alpha - p_L}{p_{L+1} - p_L} \right) (x_{L+1} - x_L)$$

and

$$x_U^* = x_{U-1} + \left(\frac{1 - \alpha - p_{U-1}}{p_U - p_{U-1}} \right) (x_U - x_{U-1})$$

and new proportion mortalities $p_L^* = 0$ and $p_U^* = 1$. In addition, let

$$x_i^* = x_i \quad \text{and} \quad p_i^* = \frac{p_i - \alpha}{1 - 2\alpha}$$

for i = L+1, L+2,..., U-1. Then the α-trimmed Spearman-Karber estimate is

$$\hat{\mu}_\alpha = \exp\left[\sum_{i=L}^{U-1} (p_{i+1}^* - p_i^*)\left(\frac{x_i^* + x_{i+1}^*}{2}\right)\right]$$

The formula for the estimated standard error of $\hat{\mu}_\alpha$ is rather lengthy. Hamilton, et al (1977; page 715) ~~Russo, and Thurston [7,page 718]~~ discuss this formula in some detail in the appendix to their paper. ~~See [7]~~ Hamilton et al (1977) for details.

An approximate 95 percent confidence interval for the EC50 is then

$$(\hat{\mu} \cdot \exp(-1.96\hat{\tau}), \hat{\mu} \cdot \exp(1.96\hat{\tau}))$$

If $p_1 \neq 0$ or if $p_k \neq 1$, the conventional Spearman-Karber estimate cannot be calculated. It is sometimes suggested that if the log-concentrations are equally spaced, the next log-concentration below or above the series used should be assumed to have given the desired result of p = 0 or p = 1, respectively. The estimation is then completed for the augmented series of log-concentrations. This fabrication of data could be seriously misleading unless p_1 and p_k are very close to 0 and 1, respectively. If $p_1 > \alpha$ or if $p_k < 1-\alpha$, the α-trimmed Spearman-Karber estimate cannot be calculated. Again, fabrication of data to allow calculation of an estimate could be very misleading unless p_1 and p_k are very close to α and 1-α.

~~We will adopt~~ the convention adopted here that the α-trimmed Spearman-Karber estimate will be calculated only if $p_1 < \alpha + 0.10$ and $p_k > 1 - \alpha - 0.10$. Hamilton, et al (1977) ~~Russo, and Thurston [7]~~ recommend a choice of α = 0.10 for an experiment where the lowest concentrations cause approximately 5 percent mortality or less, and/or the highest concentrations cause approximately 95 percent mortality or more. For more information on the conventional and the α-trimmed Spearman-Karber methods, refer to Finney (1964) ~~[8]~~ and Hamilton, et al (1977), ~~Russo, and Thurston [7].~~

Hamilton has developed a set of FORTRAN subroutines to calculate the Spearman-Karber estimate of the LC50 and the associated 95 percent confidence interval, as described in Hamilton, et al (1977) ~~Russo, and Thurston [7]. A listing of these subroutines is contained in Appendix A.~~

H. Point and Confidence Interval Estimation of the EC50 Using the Moving Average Method - The moving average method assumes that P(x) is symmetric about the log EC50 and in the case of unequally spaced log-concentrations (x's) further assumes that P(x) is linear in x (at least in a neighborhood of the EC50). Like the Spearman-Karber method, it does not assume a specific functional form for P(x). The first step is to smooth $p_1,\ldots,p_k$ to obtain monotone nondecreasing values as outlined previously. After selecting a span K for the moving average where $2 \leq K \leq k-1$, the following quantities are calculated:

$$p_j^* = \frac{1}{K}\sum_{i=j}^{j+K-1} p_i \qquad \text{for } j=1,\ldots,k-K+1$$

$$x_j^* = \frac{1}{K}\sum_{i=j}^{j+K-1} x_i \qquad \text{for } j=1,\ldots,k-K+1$$

$$L = \max\{i : p_i^* \leq 1/2\}$$

$$U = \min\{i : p_i^* \geq 1/2\}$$

The moving average estimate of the EC50 with span K is

$$\hat{\mu}_K = \exp\left[x_L^* + f(x_U^* - x_L^*)\right]$$

where

$$f = \begin{cases} \dfrac{0.5 - p_L^*}{p_U^* - p_L^*} & \text{if } p_U^* > p_L^* \\ -1/2 & \text{if } p_U^* = p_L^* \end{cases}$$

An estimate of the standard error of $\log(\hat{\mu}_K)$ is

$$\hat{\sigma}_k = \frac{(x_U^* - x_L^*)}{K(p_U^* - p_L^*)} \left[\frac{(1-f)^2 p_L (1-p_L)}{n_L} + \sum_{i=L+1}^{L+K-1} \frac{p_i(1-p_i)}{n_i} + \frac{f^2 p_{L+K}(1-p_{L+K})}{n_{L+K}} \right]^{1/2}$$

An approximate 95 percent confidence interval for the EC50 is given by

$$\left(\hat{\mu}_K \cdot \exp(-1.96\hat{\sigma}_K), \hat{\mu}_K \cdot \exp(1.96\hat{\sigma}_K)\right)$$

Stephen (1977) recommends that the moving average method not be used unless p_L^* and p_U^* are each based on at least two mortality proportions strictly between 0 and 1. It is also recommended that the span K be chosen as large as possible for each given data set while still allowing use of the method according to the previous recommendation. For more information on the moving average method, refer to Finney (1964, pp. 537-40).

In some applications of the moving average method, a variance stablizing transformation is applied to the mortality proportions prior to application of the method and/or Fieller's theorem is used to obtain the confidence interval for the EC50. For more information on variance stabilizing transformations and Fieller's theorem, refer to Harris (1954). The Stephan program, described earlier in conjunction with the two-parameter probit method, may be used to carry out the moving average method. The program employs both a variance stabilizing transformation and Fieller's theorem.

I. Point and Confidence Interval Estimation of the EC50 Using the Minimum Logit Chi-Square Method - Like the two-parameter logit method, this method assumes that

$$P(x) = \frac{1}{1 + \exp[-(\alpha + \beta x)]}$$

or that

$$\log\left[\frac{P(x)}{1-P(x)}\right] = \alpha + \beta x$$

and requires at least two partial kills in order to produce both point and interval estimates of the EC50. Define the empirical logit

$$L_i = \log\left[\frac{p_i + 1/2n_i}{1 - p_i + 1/2n_i}\right]$$

and the empirical weight

$$w_i = n_i\left[\frac{1}{p_i + 1/2n_i} + \frac{1}{1-p_i+1/2n_i}\right]^{-1}$$

for i=1,...,k. The weight W_i is set to zero if either $p_1=p_2=\ldots=p_{i+1}=0$ or if $p_{i-1}=p_i=\ldots=p_k=1.0$.

The minimum logit chi-square estimates of α and β are obtained by fitting the simple linear regression model

$$L_i = \alpha + \beta x_i + \epsilon_i \qquad i = 1, \ldots, k$$

using weighted least squares with weights W_i. Any linear regression analysis program that permits the use of case weights is acceptable. Several such programs are PROC GLM in the SAS statistical computing system (SAS I, 1982) [1] or BMDP1R (1981), in the BMDP statistical computing system [4].

Let $\hat{\alpha}$, $\hat{\beta}$ denote the least squares estimates of α, β. Let $\hat{\sigma}^2_{RES}$ denote the observed residual mean square from the regression fit. Since the theoretical value of the residual mean square is 1, all variances and covariances displayed in the regression output need to be adjusted by dividing by the observed residual mean square $\hat{\sigma}^2_{RES}$ prior to being used in variance formulas. Let V_1, V_2, and V_{12} denote the estimated variances of $\hat{\alpha}$ and $\hat{\beta}$ and the estimated covariance of $\hat{\alpha}$, $\hat{\beta}$ after adjustment by dividing by $\hat{\sigma}^2_{RES}$.

The estimate of the EC50 is

$$\hat{\mu} = \exp(-\hat{\alpha}/\hat{\beta})$$

The estimated standard error of $\log(\hat{\mu})$ is

$$\hat{\sigma} = \frac{1}{\hat{\beta}}\left[V_1^2 + 2(\log\hat{\mu})V_{12} + (\log\hat{\mu})^2 V_2^2\right]^{1/2}$$

An approximate 95 percent confidence interval for the EC50 is given by

$$\left(\hat{\mu}\cdot\exp(-1.96\hat{\sigma}),\ \hat{\mu}\cdot\exp(1.96\hat{\sigma})\right)$$

For more information on the minimum logit chi-square method and for formulas that can be used for hand calculations refer to Hamilton (1977) [6].

J. Detection of Outlying Beakers Within a Concentration Group - Consider the ith concentration C_i and recall that (n_{i1}, r_{i1}, p_{i1}), (n_{i2}, r_{i2}, p_{i2}), (n_{i3}, r_{i3}, p_{i3}), and (n_{i4}, r_{i4}, p_{i4}) denote the number of daphnids, the number of mortalities, and the observed mortality proportions for the 4 beakers, respectively, at concentration C_i. Calculate Z_{i1}, Z_{i2}, Z_{i3}, and Z_{i4} for i=1,...,k using the formula

$$Z_{ij} = \frac{2n_{ij}^{1/2}}{(1-n_{ij}/n_i)^{1/2}}\left[\arcsin(p_{ij}^{1/2}) - \arcsin(p_i^{1/2})\right]$$

Let $N=n_1+n_2+\ldots+n_k$. Rank the Z_{ij}'s from least to greatest over all i and j and let R_{ij} denote the rank of Z_{ij}. The normal score associated with Z_{ij} is given by the expression

$$\Phi^{-1}\left((R_{ij}-3/8)/(N+1/4)\right)$$

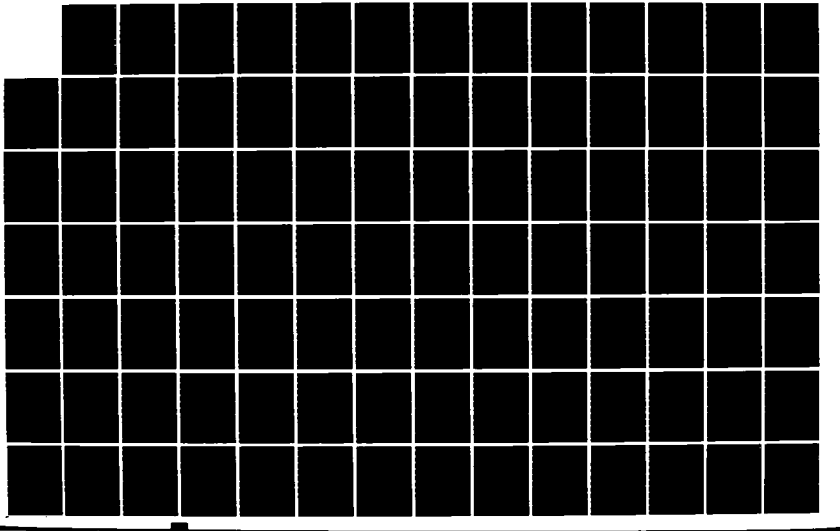
A normal probability plot of the Z_{ij}'s is formed by plotting Z_{ij} versus its normal score for i=1,...,k j=1,2,3,4. Theoretically, for large n_{ij} values, these points should fall approximately on the line which passes through the points (0,0) and (1,1). This line may be drawn in on the plot for reference. Beakers which correspond to extreme points which depart from a general smooth pattern established by the remainder of the plotted points should be identified as potential outlying beakers.

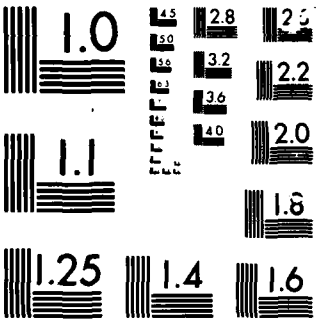
Once a beaker has been identified as a potential outlier, Fisher's exact test should be performed to compare the potentially outlying beaker with the combined results in the other 3 beakers in the same concentration group. Suppose that the jth beaker in the ith concentration group has been identified as a potential outlier. Let $L = \max(0, n_{ij} + r_i - n_i)$ and $U = \min(r_i, n_{ij})$. Calculate the two probabilities

$$P_L = \sum_{x=L}^{r_{ij}} \frac{\binom{r_i}{x}\binom{n_i-r_i}{n_{ij}-x}}{\binom{n_i}{n_{ij}}}$$

AD-A166 957 COLLABORATIVE STUDY OF DAPHNIA MAGNA STATIC RENEWAL ASSAYS(U) SPRINGBORN BIONOMICS INC WAREHAM MA* R E BENTLEY ET AL. JAN 86 DAMD17-80-C-0011 3/4

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and

$$P_U = \sum_{x=r_{ij}}^{U} \frac{\binom{r_i}{x}\binom{n_i - r_i}{n_{ij} - x}}{\binom{n_i}{n_{ij}}}$$

Then the observed two-tailed significance level of the test is

$$\underline{\alpha} = 2\min(P_L, P_U)$$

The potential outlier should be declared an outlying beaker if $4k\underline{\alpha} < 0.05$. Multiplying $\underline{\alpha}$ by 4k takes into account the fact that the jth beaker in the ith concentration group has been selected as an extreme beaker among the 4k beakers used in the study.

K. Benchmark Data Sets - The Committee on Methods for Toxicity Tests with Aquatic Organisms (EPA 1975) published hypothetical test data and "acceptable ranges" for the associated EC50 estimates and their 95 percent confidence limits to help scientists evaluate estimation procedures. These data sets and acceptable ranges are reproduced and discussed by Hamilton, et al (1977); Russo, and Thurston [7, Tables V and VI]. Any computer programs that are used to carry out the statistical procedures described in this appendix should be evaluated using these benchmark data sets and acceptable ranges.

II. ACUTE TEST EXAMPLE

A. Data Set - The following synthetic data set (Table 1) was randomly generated to conform to the acute test experimental design described in the protocol. The control group values are not included, since they are not used in the analyses of the acute data.

TABLE 1. SYNTHETIC ACUTE TEST DATA SET

Concentration	Log Concentration	Beaker Mortality Proportions (r_{ij}/n_{ij})				Mortality Proportions (r_i/n_i)
		1	2	3	4	
25	3.219	0/5	1/5	0/5	0/5	1/20
50	3.912	2/5	0/5	1/5	2/5	5/20
100	4.605	1/5	2/5	4/5	2/5	9/20
200	5.298	2/5	4/5	3/5	2/5	11/20
400	5.991	5/5	5/5	3/5	4/5	17/20

Thus, $k=5$, all n_{ij} values are 5, and n_i values are 20.

B. Preliminary Scatterplot - A preliminary scatterplot of the responses versus log-concentration is given in Figure 1. The individual beaker mortalities are plotted with the symbol "A" corresponding to a single point and with a number corresponding to multiple points in the same print position. The average mortality proportion for each concentration group is plotted with the symbol "B".

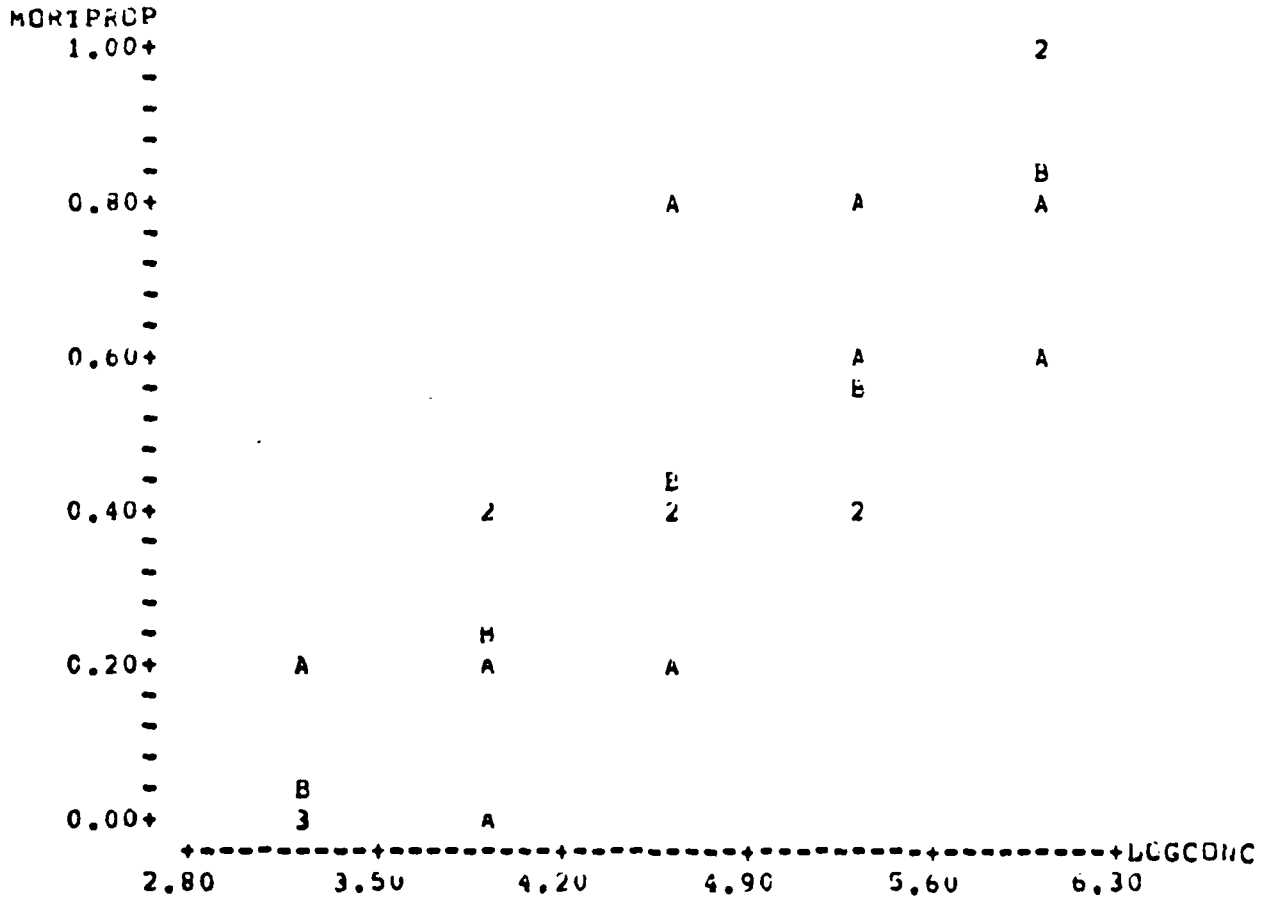


FIGURE 1. SCATTERPLOT OF INDIVIDUAL BEAKER AND AVERAGE MORTALITY PROPORTIONS VERSUS LOG-CONCENTRATIONS

C. Point and Confidence Interval Estimation of the EC50 Using the Two-Parameter Probit Model - The two-parameter probit method will be illustrated using the computer program BMDPAR (UC 1401) to carry out the calculations. Figure 2 contains a listing of the FORTRAN subroutine FUN in which the nonlinear regression function and the weights are specified. Figure 3 contains the BMDPAR program commands needed to generate the fit.

```
      SUBROUTINE FUN(F,P,X,N,KASE,NVAR,NPAR,IPASS,XLOSS)
      REAL F,P,X,XLOSS
      DIMENSION P(NPAR),X(NVAR)
      ARG=P(1)+P(2)*X(7)
      F=(1+ERF(ARG/1.4142))/2.0
      FF=F
      IF(F.LE.0.001)FF=0.001
      IF(F.GE.0.999)FF=0.999
      X(6)=X(3)/(FF*(1.0-FF))
      RETURN
      END
```

FIGURE 2. LISTING OF FORTRAN SUBROUTINE FUN

```
/PROBLEM TITLE IS 'TWO-PARAMETER PROBIT FIT USING BMDPAR NONLINEAR
                  REGRESSION PROGRAM'.
/INPUT
          VARIABLE=4.
          FORMAT='(4F10.0)'.
          UNIT=9.
/VARIABLE
          NAME=GROUP,CONC,NTEST,NDEAD,P,CASEWT,LCONC.
          ADD=3.
/TRANSFORM
          P=NDEAD/NTEST.
          CASEWT=1.0.
          LCONC=LN(CONC).
/REGRESS
          DEPENDENT=P.
          PARAMETERS=2.
          WEIGHT=CASEWT.
          HALVING=0.
          MEANSQUARE=1.0.
/PARAMETER
          INITIAL=-4.774,0.9706.
          NAME=INTCPT,SLOPE.
/PLOT
          VARIABLE=LCONC,CONC.
          SIZE=110,50.
/END
```

FIGURE 3. BMDPAR PROGRAM COMMANDS

The initial parameter values for the fit -4.774, 0.9706, can be obtained from the preliminary scatterplot. From the program output (not shown), the following information is obtained:

1. α = -4.118
2. β = 0.8421
3. ρ_{12} = -0.9829
4. V_1 = 0.7686
5. V_2 = 0.1598

V_{12} is calculated as $\rho_{12}V_1V_2$. Using these values and the formulas stated in Subsection I.D, the following values are obtained.

$V_1^2 = 0.5907$ $\quad V_2^2 = 0.02554$ $\quad V_{12} = -0.1207$

$\log(\hat{\rho}) = 4.890$ $\quad \hat{\rho} = 133.0$ $\quad \hat{\alpha} = 0.1720$

The approximate 95 percent confidence interval for the EC50 is (94.94, 186.3).

D. Point and Confidence Interval Estimation of the EC50 Using the Two-Parameter Logit Model - The two-parameter logit method will be illustrated using the computer program BMDPAR (UCI 1981) to carry out the calculations. Figure 4 contains a listing of the FORTRAN subroutine FUN in which the nonlinear regression function and the weights are specified. Figure 5 contains the BMDPAR program commands needed to generate the fit.

```
      SUBROUTINE FUN(F,P,X,N,KASE,NVAR,NPAR,IPASS,XLOSS)
      REAL F,P,X,XLOSS
      DIMENSION P(NPAR),X(NVAR)
      F=1.0/(1.0+EXP((-1.0)*(P(1)+P(2)*X(7))))
      FF=F
      IF(F.LE.0.001)FF=0.001
      IF(F.GE.0.999)FF=0.999
      X(6)=X(3)/(FF*(1.0-FF))
      RETURN
      END
```

FIGURE 4. FUN SUBROUTINE FOR BMDPAR TWO-PARAMETER LOGIT MODEL

```
/PROBLEM TITLE IS 'TWO-PARAMETER LOGIT FIT USING BMDPAR NONLINEAR
                  REGRESSION PROGRAM'.
/INPUT
          VARIABLE=4.
          FORMAT='(4F10.0)'.
          UNIT=9.
/VARIABLE
          NAME=GROUP,CONC,NTEST,NDEAD,P,CASEWT,LCONC.
          ADD=3.
/TRANSFORM
          P=NDEAD/NTEST.
          CASEWT=1.0.
          LCONC=LN(CONC).
/REGRESS
          DEPENDENT=P.
          PARAMETERS=2.
          WEIGHT=CASEWT.
          HALVING=0.
          MEANSQUARE=1.0.
/PARAMETER
          INITIAL=-9.548,1.94.
          NAME=INTCPT,SLOPE.
/PLOT
          VARIABLE=LCONC,CONC.
          SIZE=110,50.
/END
```

FIGURE 5. BMDPAR PROGRAM COMMANDS FOR TWO-PARAMETER LOGIT MODEL

Initial parameter values for the fit can be obtained from the preliminary scatterplot. From the program output (not shown), the following information is obtained:

1. α = -6.814
2. β = 1.392
3. ρ_{12} = -0.9849
4. V_1 = 1.394
5. V_2 = 0.2880

V_{12} is calculated as $\rho_{12}V_1V_2$. Using these values and the expressions stated in Subsection I.E, the following values are obtained.

$\hat{V}_1^2 = 1.943$ $\hat{V}_2^2 = 0.08294$ $\hat{V}_{12} = -0.3954$

$\log(\hat{\mu}) = 4.895$ $\hat{\mu} = 133.6$ $\hat{\sigma} = 0.1750$

The approximate 95 percent confidence interval for the EC50 is (94.81, 188.3).

E. Point and Confidence Interval Estimation of the EC50 Using the Trimmed Spearman-Karber Method - The calculation of the 20 percent-trimmed Spearman-Karber estimate is illustrated here. The observed mortality proportions and log-concentrations for the concentration groups are

TABLE 2. MORTALITY PROPORTIONS AND LOG-CONCENTRATIONS FOR THE ACUTE TEST DATA

i	1	2	3	4	5
x_i	3.219	3.912	4.605	5.298	5.991
p_i	0.05	0.25	0.45	0.55	0.85

Since $p_1 \leq p_2 \leq p_3 \leq p_4 \leq p_5$, there is no need to smooth the p-values. The following calculations are performed:

$L = \max \{ i: p_i \leq 0.20\} = 1$
$U = \min \{ i: p_i \geq 0.80\} = 5$

Using the above values of x_i's, p_i's, L, and U and the formulas stated in Subsection I.G., the following values are obtained.

$x_1^* = 3.739,\ x_5^* = 5.875,\ x_2^* = x_2,\ x_3^* = x_3,\ x_4^* = x_4$

$p_1^* = 0,\ p_5^* = 1,\ p_2^* = 0.08333,\ p_3^* = 0.4167,\ p_4^* = 0.5833$

$\hat{\mu}_{.20} = 130.3$

Using the expression for the estimated standard error of $\log(\hat{\mu}_{.20})$ given in the appendix to Hamilton, ~~Russo, and Thurston [7~~ et al (1977); page 718), with L = 1 and U = 5, the estimate $\hat{\theta}$ = 0.2001 is obtained. The approximate 95 percent confidence interval for the EC50 is (88.03, 192.9).

F. Point and Confidence Interval Estimation of the EC50 Using the Moving Average Method - Refer to Table 2, Section E for the mortality proportions and log-concentrations used in this example. Since $p_1 \leq p_2 \leq p_3 \leq p_4 \leq p_5$, it is not necessary to smooth the p-values. The following table contains the p* values for K = 1, 2, 3, 4.

TABLE 3. INTERMEDIATE p*-VALUES FOR MOVING AVERAGE CALCULATIONS

Span \ i	1	2	3	4	5
1	0.05	0.25	0.45	0.55	0.85
2	0.15	0.35	0.50	0.70	
3	0.25	0.4167	0.6167		
4	0.325	0.525			

Since K = 4 gives two p* values that surround 0.50 and each of these p* values is based on at least two mortality proportions strictly between 0 and 1, K is taken to be 4 (the largest possible value). Then L = 1, U = 2. The intermediate values necessary to calculate Ω_4 with K = 4 are obtained using the expressions stated in Subsection I.H. Namely

$x_1^* = 4.259$ $\quad x_2^* = 4.952$

$p_1^* = 0.325$ $\quad p_2^* = 0.525$

$f = 0.875$ $\quad \Omega_4 = 129.7$ $\quad \theta_4 = 0.1712$

The approximate 95 percent confidence interval for the EC50 is (92.73, 181.4).

G. Point and Confidence Interval Estimation of the EC50 Using the Minimum Logit Chi-Square Method - The empirical logits and the empirical weights are given in the following table, based on the expressions shown in Subsection I.I.

TABLE 4. EMPIRICAL WEIGHTS AND LOGITS FOR MINIMUM LOGIT CHI-SQUARE CALCULATIONS

i	1	2	3	4	5
L_i	-2.565	-1.036	-0.191	0.191	1.609
W_i	1.393	4.060	5.202	5.202	2.917

Based on fitting the simple linear regression model using weighted least squares with the weight W_i above, the following values are obtained.

$\hat{\alpha} = -6.219$
$\beta = 1.272$
$V_1^2 = 1.908$
$V_2^2 = 0.0818$
$V_{12} = -0.3896$

Recall that the values of $Var(\hat{\alpha})$, $Var(\beta)$, $Cov(\hat{\alpha},\beta)$ must be divided by $\hat{\sigma}^2_{RES}$, the residual mean square from the regression fit, to obtain V_1^2, V_2^2, V_{12}.

Using the expression stated in Subsection I.I, the following values are obtained.

$\log(\Omega) = 4.889$ $\Omega = 132.8$ $\hat{\sigma} = 0.1838$

The approximate 95 percent confidence interval for the EC50 is (92.63, 190.4).

H. Detecting Outlying Beakers Within a Log-Concentration Group - The Z_{ij}-values and associated normal scores for detecting outlying beakers are given

in Table 5. These values are obtained from the expressions shown in Subsection I.J.

TABLE 5. Z-VALUES AND NORMAL SCORES (IN PARENTHESES) FOR OUTLIER DETECTION PROBABLILITY PLOT

Concentration(i)	Beaker(j) 1	2	3	4
25	-1.16 (-0.74)	1.23 (0.74)	-1.16 (-0.74)	-1.16 (-0.74)
50	0.83 (0.52)	-2.70 (-1.87)	-0.31 (-0.06)	-0.83 (0.52)
100	-1.40 (-1.13)	-0.26 (0.12)	1.92 (1.13)	-0.26 (0.12)
200	-0.78 (-0.38)	1.40 (0.92)	0.26 (0.31)	-0.78 (-0.38)
400	2.05 (1.59)	2.05 (1.59)	-1.48 (-1.40)	-0.34 (-0.19)

The normal probability plot of the values in Table 5 is given in Figure 6. The theoretical N(0,1) line is drawn in for reference. Based on this plot, the lowest point appears as if it may be separated from the others and so is identified as a potential outlier. Table 5 shows that this point corresponds to $i = 2$, $j = 2$.

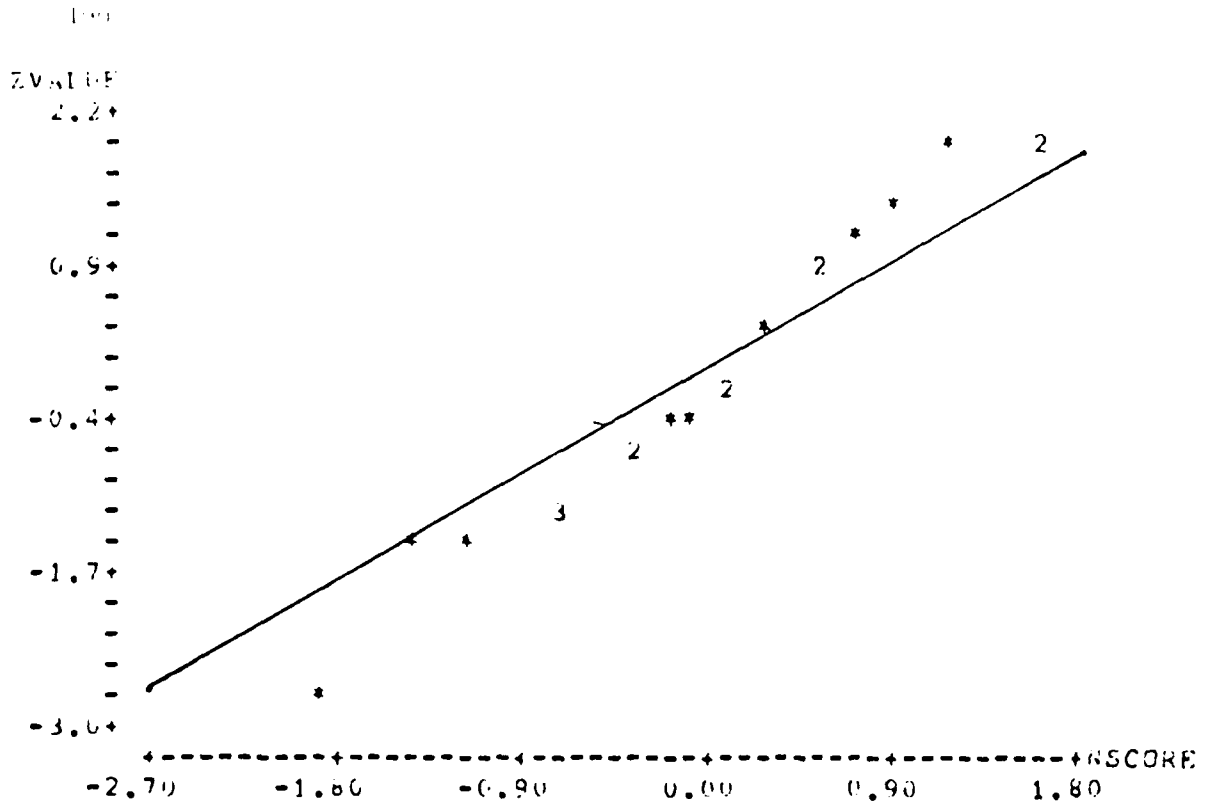


FIGURE 6. NORMAL PROBABILITY PLOT OF THE VALUES IN TABLE 5.

Fisher's exact test is performed, as discussed in Subsection I.J, to compare the potentially outlying beaker with the combined results in the other three beakers in its group. Namely

	Beaker 2	Beakers 1,3,4	
Dead	0	5	5
Live	5	10	15
Total	5	15	

For this table $r_{ij} = 0$, $n_{ij} = 5$, $r_i = 5$, $n_i = 20$, $L = 0$, $U = 5$.

$$P_L = \frac{\binom{5}{0}\binom{15}{5}}{\binom{20}{5}} = 0.194 \qquad P_U = 1 > P_L$$

$$\alpha = 2P_L = 0.387 > 0.05$$

Thus there is no statistical evidence that this potential outlier is in fact an outlier.

III. CHRONIC TEST MORTALITY DATA

A. Experimental Design - See "Chronic Static-Renewal Tests" section in body of protocol for details of experimental layout. An acceptable test will have no more than 20 percent mortality in any of the water control, acetate control, or solvent control groups. Estimates of the EC50 will be adjusted for control mortality. See the body of the protocol (Statistical Evaluations section) for selecting the appropriate control group or combination of control groups to be used in the analysis. The control group referred to in this appendix is the appropriate control group or combination of control groups selected by these procedures.

B. Notation - The notation used in this section is the same as that used in Section I, for the acute test mortality data. See Subsection I.B for details. The notations n_0, r_0, and p_0 denote the number of daphnids, the number of deaths or immobilizations, and the observed proportion of deaths or immobilizations, respectively, in the control group. The symbol θ will denote the control population mortality proportion at 21 days.

C. Preliminary Scatterplots - A preliminary scatterplot of the data is formed by plotting the observed mortality proportions $\{p_i\}$ versus the log-concentration $\{x_i\}$ for $i = 1,2,\ldots,k$. The control group mortality proportion is plotted versus a number smaller than x_1, which is chosen to separate the control point from the others.

D. Variance Stabilizing Transformation - Prior to performing an analysis of variance or multiple comparisons, a variance stabilizing transformation should be applied to the observed mortality proportions. The protocol suggests the transformation

$$Y_i = \arcsin\sqrt{\frac{r_i}{n_i+1}} + \arcsin\sqrt{\frac{r_i+1}{n_i+1}} \qquad i = 0,1,\ldots,k$$

after which Y_i is approximately normally distributed with mean $\arcsin(P(x_i)^{1/2})$ and variance $1/4n_i$ for $i=1,\ldots,k$ and Y_0 is approximately normally distributed with mean $\arcsin(\theta^{1/2})$ and variance $1/4n_0$.

E. Analysis of Variance - The following analysis of variance test procedure tests the null hypothesis $H_0: \theta = P(x_1) = \ldots = P(x_k)$. Use the variance stabilizing transformation outlined above to obtain $Y_0, Y_1 \ldots, Y_k$. Calculate

$$Q = \left[\sum_{i=0}^{k} n_i Y_i^2 - \left(\sum_{i=0}^{k} n_i Y_i \right)^2 \Big/ \left(\sum_{i=0}^{k} n_i \right) \right]$$

Let $\chi^2_{.95,k}$ denote the 95th percentile of the chi-square distribution with k degrees of freedom. If $Q > \chi^2_{.95,k}$ then reject $H_0: \theta = P(X_1) = \ldots = P(X_k)$; otherwise, fail to reject H_0.

F. Multiple Comparison Procedures - One of the following multiple comparison procedures should be performed to determine which treatment groups, if any, differ significantly from the control group.

Use the variance stablizing transformation outlined above to obtain $Y_0, Y_1 \ldots, Y_k$.

Dunnett's Procedure - Calculate

$$T_i = \frac{(Y_i - Y_0)}{[1/n_0 + 1/n_i]^{1/2}} \qquad i = 1, \ldots, k$$

and conclude that log-concentration x_i had a statistically significant detrimental effect on mortality if $T_i > t(0.05; k, \infty)$ where $t(.05; k, \infty)$ is the one-tailed critical point for Dunnett's multiple comparison procedure corresponding to $\alpha = .05$, k treatment groups and infinite degrees of freedom. Tables of these critical values may be found in Chew (1977) [~~11~~] or Dunnett (1955) [~~12~~].

Bonferroni Procedure - Calculate $T_1, T_2, \ldots, T_k$ as for Dunnett's procedure but conclude that log-concentration x_i had a statistically significant detrimental effect on mortality if $T_i > Z(.05/k)$ where $Z(.05/k)$ is the upper $.05/k$ percentile point of the standard normal distribution.

Williams' Method - Smooth the observed mortality proportions to nondecreasing order to obtain $p_0, p_1, \ldots, p_k$ by the process described in Subsection I.F. Apply the arcsin-square root variance stabilizing transformation to obtain

$$Y_i = \arcsin\sqrt{\frac{r_i}{n_i+1}} + \arcsin\sqrt{\frac{r_i+1}{n_i+1}} \qquad i = 0, 1, \ldots, k$$

and $Y_0 = \arcsin(p_0^{1/2})$. Note that Y_0 is calculated using the unadjusted mortality rate in the control group. Calculate

$$T_i = \frac{(Y_i - Y_0)}{[1/n_0 + 1/n_i]^{1/2}} \qquad i = 1, \ldots, k$$

and conclude that log-concentration x_i had a statistically significant detrimental effect on mortality if $T_i > t(.05; k, \infty)$ where $t(.05; k, \infty)$ is the one-tailed critical point for Williams' method corresponding to $\alpha = .05$, k treatment groups, and infinite degrees of freedom. Tables of these critical values may be found in Williams (1971) or Chew (1977)

If the assumption that the true mortality proportion function P(x) is nondecreasing is reasonable, then Williams' procedure should be used due to its superior power in detecting a true detrimental difference between a treatment group and the control group.

The smallest concentration declared to have had a statistically significant effect on mortality will constitute the toxic concentration. The next lower concentration will constitute the no-significant-effect concentration.

G. **After-the-Fact Power Calculations for the Bonferroni Multiple Comparison Procedure** - Consider the ith concentration group. The probability that the ith concentration group will be declared to be statistically significantly different from the control group is given by the equation

$$\text{Power} = \Phi\left[\frac{Y_i - Y_\theta}{(1/n_0 + 1/n_i)^{1/2}} - Z(0.05/k)\right]$$

where $$Y_\theta = \arcsin\sqrt{\frac{n_0\theta}{n_0+1}} + \arcsin\sqrt{\frac{n_0\theta+1}{n_0+1}}$$

where Φ is the standard normal distribution function and $Z(\alpha)$ is the upper α percentile point of the standard normal distribution.

This calculation provides an indication of the sensitivity of the multiple comparison procedures discussed in Subsection III.F (particularly, Bonferroni's procedure). It is of importance to determine how great the mortality level must be before it is very likely to be declared statistically significantly different from the control group mortality.

H. **Point and Confidence Interval Estimation of the EC50 Using the Three-Parameter Probit Model With Abbot's Correction** - This method assumes that

t

$$P(x) = \theta + (1-\theta)\Phi(\alpha+\beta x)$$

or that

$$\Phi^{-1}\left[\frac{P(x)-\theta}{1-\theta}\right] = \alpha+\beta x$$

where Φ is the standard normal distribution function and Φ^{-1} is the inverse of the standard normal distribution function. It requires at least two partial kills in order to estimate the EC50 or EC10. The form of the expression for background mortality is known as Abbott's correction. Point and interval estimates may be obtained directly using a computer program designed to perform a three-parameter probit analysis such as SAS PROC PROBIT[1]. It should be noted that some programs, including SAS PROC PROBIT, actually fit the model

$$P(x) = \theta + (1-\theta)\Phi(\alpha - 5 + \beta x)$$

The program documentation should be checked to determine which model is being fit. If the latter model is being fit, then α should be replaced by $\alpha - 5$ in all the formulas that follow in this section.

If a probit analysis program is not available, a general purpose nonlinear regression program, such as BMDPAR (UC, 1981), that produces estimates of the variances and covariances of the parameter estimates can be used to carry out the calculations. The nonlinear regression model

$$p_i = \theta + (1-\theta)\Phi(\alpha + \beta x_i) \qquad i = 0, \ldots, k$$

is iteratively fitted to the data using a weighted least squares type of analysis. The ith data point is given weight

$$W_i = \frac{n_i}{[\hat{\theta} + (1-\hat{\theta})\Phi(\hat{\alpha} + \hat{\beta} x_i)][(1-\hat{\theta})(1-\Phi(\hat{\alpha} + \hat{\beta} x_i)]}$$

See the example for details. The estimation procedure results in $\hat{\theta}$, $\hat{\alpha}$ and $\hat{\beta}$ which are estimates of θ, α and β, respectively.

Expressions for $\hat{\mu}$ (the estimated EC50 adjusted for background mortality by Abbott's correction), $\hat{\sigma}$, and an approximate 95 percent confidence interval for the EC50 are the same as those shown in Subsection I.D corresponding to the two-parameter probit model. An estimate of the EC10 is given by the formula

$$\hat{\mu}_{.10} = \exp\left[-(\hat{\alpha}+1.282)/\hat{\beta}\right]$$

and an estimate, $\hat{\sigma}_{.10}$, of the standard error of $\hat{\mu}_{.10}$ is obtained by substituting $\hat{\mu}_{.10}$ for $\hat{\mu}$ in the formula for $\hat{\sigma}$. The 95 percent confidence interval for the EC10 is

$$\left(\hat{\mu}_{.10}\exp(-1.96\hat{\sigma}_{.10}),\ \hat{\mu}_{.10}\exp(1.96\hat{\sigma}_{.10})\right)$$

I. **Confidence Intervals for Treatment-Control Mortality Rate Ratios** - The following procedure may be used to construct confidence intervals on the ratios $R_1=P(x_1)/\theta$, $R_2=P(x_2)/\theta,\ldots,$ $R_k=P(x_k)/\theta$ of treatment group mortality proportions to the control mortality proportion. If p_0, the observed proportion mortality in the control group, and p_i, the observed proportion mortality at log-concentration x_i, are both greater than zero, the lower and upper 95 percent confidence bounds, respectively, for R_i are

$$L_i = \exp\left[\log(p_i/p_0) - 1.96\left(\frac{1-p_0}{n_0 p_0} + \frac{1-p_i}{n_i p_i}\right)^{1/2}\right]$$

and

$$U_i = \exp\left[\log(p_i/p_0) + 1.96\left(\frac{1-p_0}{n_0 p_0} + \frac{1-p_i}{n_i p_i}\right)^{1/2}\right]$$

If $p_0=0$, the upper confidence bound for R_i is infinity and a 95 percent lower confidence bound for R_i is (Feder and Collins, [illegible])

$$L_i = n_0 p_i F(2r_i, 2; .025)$$

where $F(\nu_1,\nu_2;\alpha)$ represents the upper alpha point of the F-distribution with degrees of freedom ν_1 and ν_2. If $p_i=0$, the lower confidence bound for R_i is zero and a 95 percent upper confidence bound for R_i is

$$U_i = \frac{1}{n_i p_\bullet} F(2,2r_\bullet;0.975)$$

IV. CHRONIC TEST MORTALITY DATA EXAMPLE

A. Data Set - The following synthetic mortality data set (Table 6) was randomly generated to conform to the chronic test experimental design described in the protocol.

TABLE 6. SYNTHETIC CHRONIC MORTALITY DATA SET

Concentration	Log Concentration	Mortality Proportion (r_i/n_i)
Control	-	1/10
8.75	2.169	2/10
17.50	2.862	3/10
35.00	3.555	4/10
70.00	4.249	7/10
140.00	4.942	8/10

Thus $k=5$ and all the n_i values are 10, including n_0.

B. Preliminary Scatterplot - A preliminary scatterplot of the observed mortality proportions versus log-concentration is given in Figure 7.

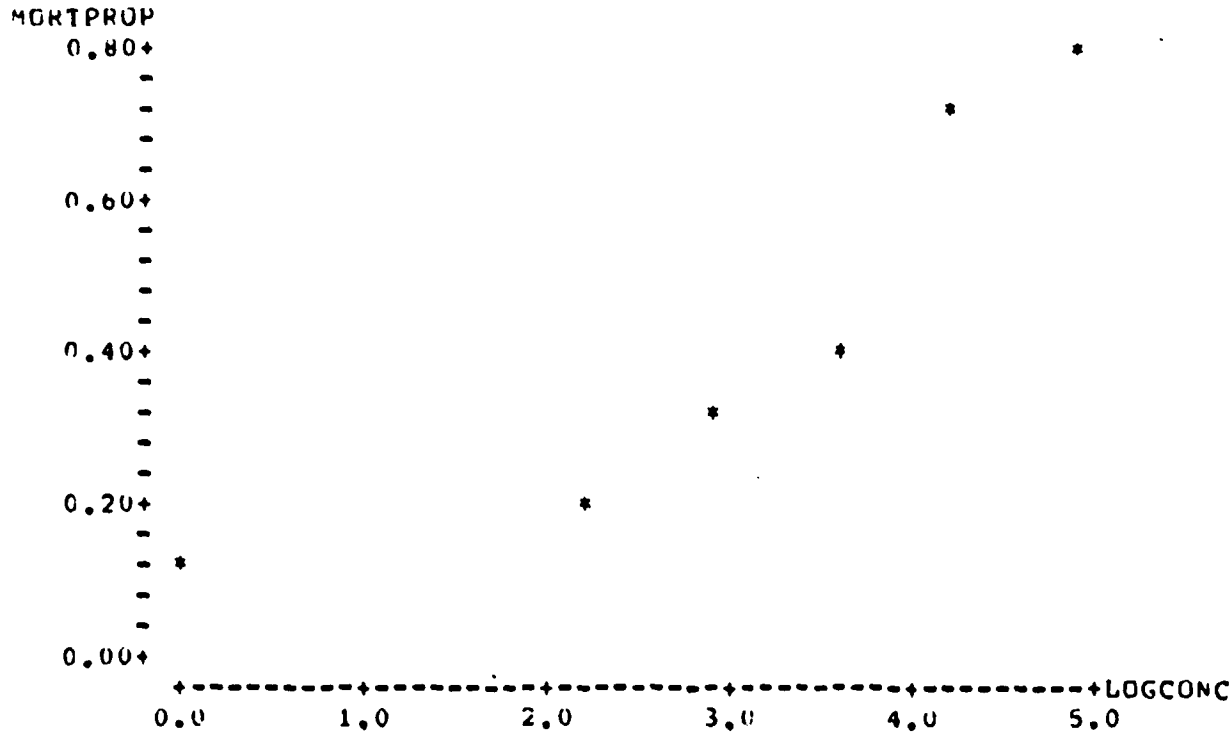


FIGURE 7. SCATTERPLOT OF MORTALITY PROPORTIONS VERSUS LOG-CONCENTRATION

The control mortality is plotted versus a log-concentration value that separates it from the remaining points.

C. Variance Stablizing Transformation - The following Y-values are obtained by applying the arcsin-square root transformation to the p_i's, using the expression in Subsection III.D.

Y_0=.7468

Y_1=.9900

Y_2=1.1968

Y_3=1.3872

Y_4=1.9448

Y_5=2.1516

D. Analysis of Variance - Some intermediate quantities and the test statistic are:

$$\sum_{i=0}^{5} n_i = 60$$

$$\sum_{i=0}^{5} n_i Y_i = 84.172$$

$$\sum_{i=0}^{5} n_i Y_i^2 = 133.661$$

$$Q = \left[133.06 - (84.172)^2/60\right] = 14.98$$

Since ~~18.55~~ 14.98 > $X^2_{.95,5}$ = 11.1, the hypothesis H_0:θ=P(2.169)=P(2.862)=P(3.555)=P(4.249)=P(4.842) is rejected at the 0.05 significance level.

E. Multiple Comparison Procedures - All three multiple comparison procedures are illustrated.

Dunnett's Procedure - The T-values are T_1= .544 T_2=1.006 T_3=1.432, T_4=2.679, and T_5=3.141. The one-tailed critical point is t(0.05;5,∞)=2.23. Thus concentrations 70 and 140 had a statistically significant detrimental effect on mortality. The toxic concentration is 70 and the no-significant effect concentration is 35.

Bonferroni Procedure - The T-values are as calculated for Dunnett's procedure but the critical point is Z(0.05/5)=Z(0.01)=2.33. Thus the conclusions are the same as for Dunnett's procedure.

Williams' Method - Since the mortality proportions are in nondecreasing order, it is not necessary to smooth them and the T-values are calculated as for Dunnett's procedure. The critical point is t(0.05;5,∞)=1.756. Thus the conclusions are the same as for Dunnett's procedure.

F. After-the-Fact Power Calculations for the Bonferroni Multiple Comparisons Procedure - Suppose that $P(x_i)$ and θ, the true mortality proportion for the ith group and the true background mortality proportion, are 0.5 and 0.1, respectively. Then the probability that the ith group will be declared to be statistically significantly different from the control group is

$$Q = \left[133.06 - (81.172)^2/60\right] = 14.98$$

$$\text{For } \Theta = .1 \quad Y_\theta = \arcsin\sqrt{\frac{(.1)(10)}{11}} + \arcsin\sqrt{\frac{(.1)10+1}{11}} = .7468$$

$$\text{Power} = \Phi\left[\frac{(1.571 - .7468)}{(0.1+0.1)^{1/2}} - 2.33\right]$$

$$= \Phi(-.487) = .312$$

This calculation says that the chances are just 3 in 10 that a true mortality level of 0.50 can be distinguished from a background level of 0.10 with the experimental design specified for the chronic test and with Bonferroni's procedure. If $P(x_i)$=0.7, the power increases to 0.64. Thus this test is sensitive only to very large changes in mortality levels.

G. Point and Confidence Interval Estimation of the EC50 Using the Three-Parameter Probit Model with Abbott's Correction - The three-parameter probit method will be illustrated using the computer program BMDPAR [4] to carry out the calculations. Figure 8 contains the FORTRAN subroutine FUN in which the nonlinear regression function and the weights are specified. Figure 9 contains the BMDPAR program commands needed to generate the fit.

Note that the control group is associated with a very small (but positive) concentration in this program so that when the logarithm of concentration is taken, an overflow does not occur. The control group is then treated as a special case in the fourth line of the FUN subroutine.

```
      SUBROUTINE FUN(F,P,X,N,KASE,NVAR,NPAR,IPASS,XLOSS)
      REAL F,P,X,XLOSS
      DIMENSION P(NPAR),X(NVAR)
      IF(X(2).LE.0.00001)F=P(3)
      IF(X(2).LE.0.00001)GO TO 10
      ARG=P(1)+P(2)*X(7)
      F=(1+ERF(ARG/1.4142))/2.0
      F=P(3)+(1.0-P(3))*F
   10 CONTINUE
      FF=F
      IF(F.LE.0.001)FF=0.001
      IF(F.GE.0.999)FF=0.999
      X(6)=X(3)/(FF*(1.0-FF))
      RETURN
      END
```

FIGURE 8. FUN SUBROUTINE TO CARRY OUT THREE-PARAMETER PROBIT FIT USING BMDPAR

```
/PROBLEM TITLE IS 'THREE-PARAMETER PROBIT FIT USING BMDPAR NONLINEAR
                  REGRESSION PROGRAM'.
/INPUT
          VARIABLE=4.
          FORMAT='(4F10.0)'.
          UNIT=9.
/VARIABLE
          NAME=GROUP,CONC,NTEST,NDEAD,P,CASEWT,LCONC.
          ADD=3.
/TRANSFORM
          P=NDEAD/NTEST.
          CASEWT=1.0.
          LCONC=LN(CONC).
/REGRESS
          DEPENDENT=P.
          PARAMETERS=3.
          WEIGHT=CASEWT.
          HALVING=0.
          MEANSQUARE=1.0.
/PARAMETER
          INITIAL=-1.92,0.54,0.1.
          NAME=INTCPT,SLOPE,THRESH.
/PLOT
          VARIABLE=LCONC,CONC.
          SIZE=110,50.
/END
```

FIGURE 9. BMDPAR PROGRAM COMMANDS TO CARRY OUT THREE-PARAMETER PROBIT FIT

From the program output, the following information is obtained:

1. $\hat{\alpha}$ = 3.021
2. $\hat{\beta}$ = 0.7748
3. $\hat{\theta}$ = 0.1044
4. V_1 = 1.289
5. V_2 = 0.3059
6. ρ_{12} = -0.9762

Using the formulas in Subsections I.D and III.H, the following are point and interval estimates of the EC50 and EC10.

$\hat{\mu}$ = 49.36 $\hat{\sigma}$ = 0.3706
(34.07,71.50) is a 95 percent confidence interval for the EC50

$\hat{\mu}_{.10}$ = 9.435 $\hat{\sigma}$ = 0.8214
(4.15,21.45) is a 95 percent confidence interval for the EC10

H. Confidence Intervals for Treatment-Control Mortality Rate Ratios - Since none of the mortality proportions are zero, the 95 percent confidence intervals are:

(0.21,18.69) for $R_1 = P(2.169)/\theta$
(0.37,24.17) for $R_2 = P(2.862)/\theta$
(0.54,29.81) for $R_3 = P(3.555)/\theta$
(1.04,46.95) for $R_4 = P(4.249)/\theta$
(1.21,52.69) for $R_5 = P(4.942)/\theta$

These confidence intervals indicate that the ratios of the chronic mortality rates in the treatment groups to that in the control group are not determined very precisely with this design and with the assumed background mortality rate of 0.1.

V. CHRONIC TEST REPRODUCTION AND LENGTH DATA

A chronic toxicity bioassay will yield the following endpoints for each female surviving 21 days: number of broods, total number of young produced, and (optionally) a length measurement.

A. Notation:

$n_0, n_1, \ldots, n_k$	numbers of daphnids surviving to 21 days in the control group and k test concentration groups, respectively[1].
$N = \sum_{i=0}^{k} n_i$	
$Y_{i1}, \ldots, Y_{in_i}$	endpoint (productivity or length) values in ith group, i=0,1,...,k or appropriate transformed values
$\bar{Y}_{0.}, \ldots, \bar{Y}_{k.}$	average (transformed) reponses in the test groups
$S_0, \ldots, S_k$	sample standard deviations in the control and test groups
$C_1, \ldots, C_k$	test concentrations in the k test groups
$x_1, \ldots, x_k$	logarithms (natural) of test concentrations in the k test groups
$\mu(x)$	population mean response at log-concentration x.
$\mu_0, \mu_1, \ldots, \mu_k$	population mean responses in groups 0,1,...,k.
σ	standard deviation of responses

B. Preliminary Scatterplot - A preliminary scatterplot of the responses versus the logarithm of concentration will be prepared. The observed individual data points $\{Y_{ij}\}$ will be plotted versus log-concentration $\{x_i\}$; the observed average data points $\{\bar{Y}_{i.}\}$ will be included in this plot with a different plotting symbol. The data points for the control group are plotted versus a small number less than x_1 chosen to separate the control points from the others.

---

[1] Note that $n_0, n_1, \ldots, n_k$ correspond to $r_0, r_1, \ldots, r_k$ of Section III.

C. Tests of Homogeneity of Variance - An underlying assumption of the multiple comparison procedures to be described in the following section is that the variances of the measurements be the same for the control group and for each of the treatment groups. Prior to performing the multiple comparisons, one of the following tests of homogeneity of variance should be carried out.

Bartlett's Test - Calculate

$$MSE = \frac{1}{N-k-1}\sum_{i=0}^{k}(n_i-1)S_i^2$$

$$C = 1+\frac{1}{3k}\left[\sum_{i=0}^{k}\frac{1}{n_i-1}-\frac{1}{N-k-1}\right]$$

and

$$B = \frac{1}{C}\left[(N-k-1)\log(MSE)-\sum_{i=0}^{k}(n_i-1)\log(S_i^2)\right]$$

If $B > \chi^2(0.95,k)$, where $\chi^2(0.95,k)$ is the 95th percentile of the chi-square distribution with k degrees of freedom, conclude that the variances are not equal. Otherwise, fail to reject the hypothesis that the variances are equal. This test is included in the computer program BMDP9D in the BMDP statistical computing system [4].

Hartley's Test - This procedure assumes that the individual sample sizes are equal, i.e. $n_0=n_1=\ldots=n_k=n$. Let max (S_i^2) denote the largest of the k+1 sample variances and min (S_i^2) denote the smallest of the k+1 sample variances. Calculate

$$H = \max(S_i^2)/\min(S_i^2)$$

and conclude that the variances are not equal if $H \geq H(.95;k+1,n)$ where $H(.95;k+1,n)$ is the 95th percentile of the null distribution of H corresponding to k+1 samples and individual sample size n. Tabled values of $H(.95;k+1,n)$ may be found in Neter and Wasserman [15] (1974).

Both Bartlett's test and Hartley's test assume that the observations are normally distributed. The following test of homogeniety of variance is less sensitive to departures from the normality assumption than are the Bartlett test and the Hartley test.

Levene's Test - Calculate

$$D_{ij} = \left| Y_{ij} - \bar{Y}_{i\cdot} \right|$$

for $i=0,1,\ldots,k$ and $j=1,\ldots,n_i$. Compute the one-way ANOVA F-statistic for testing the equality of the means of the k+1 samples $(D_{01},D_{02},\ldots,D_{0n_0})$, $(D_{11},D_{12},\ldots,D_{1n_1})$,..., $(D_{k1},D_{k2},\ldots,D_{kn_k})$. A computational formula for the ANOVA F-statistic is

$$F = \frac{(N-k-1)\left[\sum_{i=0}^{k} \frac{1}{n_i}\left(\sum_{j=1}^{n_i} D_{ij}\right)^2 - \frac{1}{N}\left(\sum_{i=0}^{k}\sum_{j=1}^{n_i} D_{ij}\right)^2\right]}{k\left[\sum_{i=0}^{k}\sum_{j=1}^{n_i} D_{ij}^2 - \sum_{i=0}^{k}\frac{1}{n_i}\left(\sum_{j=1}^{n_i} D_{ij}\right)^2\right]}$$

If $F > F(.95;k,N-k-1)$, where $F(.95;k,N-k-1)$ is the 95th percentile of the F distribution with k and N-k-1 degrees of freedom, conclude that the variances are not equal. Otherwise, fail to reject the hypothesis that the variances are equal. This test is included in the computer programs BMDP3D and BMDP7D in the BMDP statistical computing system [4] (UC 1981).

D. Variance Stabilizing Transformation - If the conclusion of the test of homogeneity of variance is that the variances are not equal, a variance stabilizing transformation may alleviate the homogeneity problem. Several suggested variance stabilizing transformations are listed in the protocol under Culture and Testing Methods - Statistical Evaluations.

Plot log $\bar{Y}_{i\cdot}$ versus log S_i for i=0,1,...,k. If the plotted points fall approximately on a straight line with slope b, then the variance stablizing transformation $Z=Y^{1-\beta}$ is called for. Important special cases are $\beta=1/2$ (square root transformation) and $\beta=1$ (logarithmic transformation).

E. Outlier Detection Tests - If the preliminary scatterplot indicates that the standard deviation in each group is related to the mean, then outlier detection tests should be carried out subsequent to any variance stabilizing transformations. If the standard deviations are unrelated to the means then the outlier detection tests should be carried out on the original responses. Calculate

$$D_{ij} = Y_{ij} - \bar{Y}_{i\cdot} \qquad i=0,\ldots,k \;,\; j=1,\ldots,n_i$$

Order the D_{ij}'s and plot the ordered values versus the normal scores of their ranks, as described in Subsection I.J for the acute mortality data. That is, if R_{ij} is the rank of D_{ij} then plot D_{ij} versus $\Phi^{-1}\left[(R_{ij}-3/8)/(N+1/4)\right]$, i=0,...k, j=1,...$n_i$. This produces a normal probability plot. If one or more of the extreme residuals lie apart from a straight line fitted by eye to the remaining residuals, they are considered potential outliers.

To determine whether there is any statistical evidence that these extreme residuals are in fact outliers, we compare them with what would be expected from the most extreme of N-(k+1) observations from a normal distribution with mean 0 and standard deviation σ. The value of σ is estimated by

$$\hat{\sigma} = \left[\frac{1}{N-(k+1)} \sum_{i=0}^{k} \sum_{j=1}^{n_i} D_{ij}^2\right]^{1/2}$$

Let D denote the most extreme of the D_{ij}'s. Calculate

$$1 - \left|2\Phi(D/\hat{\sigma})-1\right|^{N-(k+1)}$$

If this value is less than 0.10 then there is at least marginal statistical evidence that D is an outlier. Subsequent analyses might be carried out with and without this value to determine its influence on the conclusions.

F. Multiple Comparison Procedures - One of the following multiple comparison procedures should be performed to determine which treatment groups, if any, differ from the control group. These procedures should be carried out subsequent to any outlier detection tests or variance stabilizing transformations. The symbol MSE refers to the mean square error estimate of variance obtained from performing a one-way analysis of variance. A computational formula for MSE is

$$MSE = \frac{1}{(N-k-1)}\left[\sum_{i=0}^{k}\sum_{j=1}^{n_i} Y_{ij}^2 - \sum_{i=0}^{k}\frac{1}{n_i}\left(\sum_{j=1}^{n_i} Y_{ij}\right)^2\right]$$

MSE can be obtained as the residual mean square in a one-way analysis of variance. Calculate

$$T_i = \frac{\bar{Y}_0 - \bar{Y}_i}{\left[MSE\left(1/n_0 + 1/n_i\right)\right]^{1/2}} \qquad i = 1, \ldots, k$$

Dunnett's procedure, Bonferroni's procedure, and William's procedure are all based on comparing the T_i's to appropriate critical values. This is directly analogous to the procedures described for the chronic mortality data. Follow the procedures described in Subsection III.F, but defining the T_i's as described above.

Williams' method is based on smoothed versions of $\bar{Y}_{i\cdot}$ to produce monotone nonincreasing values $\bar{Y}_{1\cdot} > \bar{Y}_{2\cdot} > \ldots > \bar{Y}_{k\cdot}$. Follow the procedure described in Subsection I.F to smooth the $Y_{i\cdot}$'s; however the order relations must be interchanged.

See Miller [1966, 16, pp. 143-153] for a nonparametric multiple comparison procedure that is analogous to Dunnett's procedure.

If the assumption that the mean response function $\mu(x)$ is nonincreasing is reasonable, then Williams' procedure should be used due to its superior power in detecting a true detrimental difference between a treatment group and the control group.

The lowest concentration declared to have had a statistically significant detrimental effect is the toxic concentration. The next lower concentration is the no-significant-effect concentration.

G. After-the-Fact Power Calculations for the Bonferroni Multiple Comparisons Procedure - An approximation to the probability that the average response in the ith concentration group will be declared statistically significantly different from the control group may be found as follows. Calculate the noncentrality parameter

$$\delta = \frac{\mu_0 - \mu(x_i)}{\left[MSE\left(1/n_0 + 1/n_i\right)\right]^{1/2}}$$

Enter tables or graphs of the operating characteristics of the one-sided t-test for $\alpha = 0.05/k$ at noncentrality parameter δ and degrees of freedom N-k-1. The approximate power to detect a shift of the size $\mu_0 - \mu(x_i)$ can then be read from the table. When k=5, α = 0.01.

If N is greater than 20, the following approximate formula may be used.

$$Power = \Phi\left[\delta - Z(0.05/k)\right]$$

Power in excess of 0.80 is usually regarded as good sensitivity and power less than 0.60 is generally regarded as poor sensitivity for detecting a shift of a particular size.

H. Confidence Intervals for Differences Between Control Group and Treatment Group Mean Responses - The following procedure may be used to construct confidence intervals on the differences $D_1=\mu_0-\mu(x_1), D_2=\mu_0-\mu(x_2), \ldots D_i=\mu_0-\mu(x_i), \ldots D_k=\mu_0-\mu(x_k)$ between the control group mean response and the treatment group mean responses. Calculate

$$\hat{D}_i = \bar{Y}_{0.} - \bar{Y}_{i.} \qquad i=1,\ldots,k$$

$$\hat{\sigma}_i = \left[MSE(1/n_0 + 1/n_i)\right]$$

and obtain

$$T = t^*(0.05; k, N-k-1)$$

from tables of the two-sided Dunnett multiple comparison procedure [11,12]. Then

$$(\hat{D}_i - T\hat{\sigma}_i, \hat{D}_i + T\hat{\sigma}_i) \qquad i=1,\ldots,k$$

is a set of simultaneous 95 percent confidence intervals for $D_1, D_2, \ldots, D_k$ respectively.

VI. CHRONIC TEST LENGTH DATA EXAMPLE

A. Data Set - The following synthetic data set (Table 7) that simulates lengths of surviving daphnids was randomly generated to conform to the chronic test experimental design described in the protocol. A length measurement is given for each daphnid that survived for 21 days.

TABLE 7. SYNTHETIC CHRONIC TEST LENGTH DATA SET

Concentration	Lengths
Control	4.5,4.4,4.4,4.3,4.9 4.0,4.6,3.9,4.5
8.75	4.0,4.1,4.2,4.1,4.2 4.3,4.1,4.3
17.5	4.0,4.3,4.2,3.9,4.4 4.4,4.1
35	3.9,3.8,4.1,3.8,3.5 4.1
70	4.1,3.8,3.7
140	3.5,3.2

The number of survivors, mean and standard deviation are listed by group in Table 8.

TABLE 8. DESCRIPTIVE STATISTICS FOR THE CHRONIC TEST LENGTH DATA

Concentration	Log Concentration	Number of Survivors	Average Length	Standard Deviation
Control	-	9	4.389	.302
8.75	2.169	8	4.163	.106
17.5	2.862	7	4.186	.195
35	3.555	6	3.867	.225
70	4.249	3	3.867	.208
140	4.942	2	3.350	.212

B. Preliminary Scatterplot - The individual length measurements are plotted versus log-concentration in Figure 10 with a number indicating multiple points in the same plotting position. Average lengths are also plotted using the symbol "M".

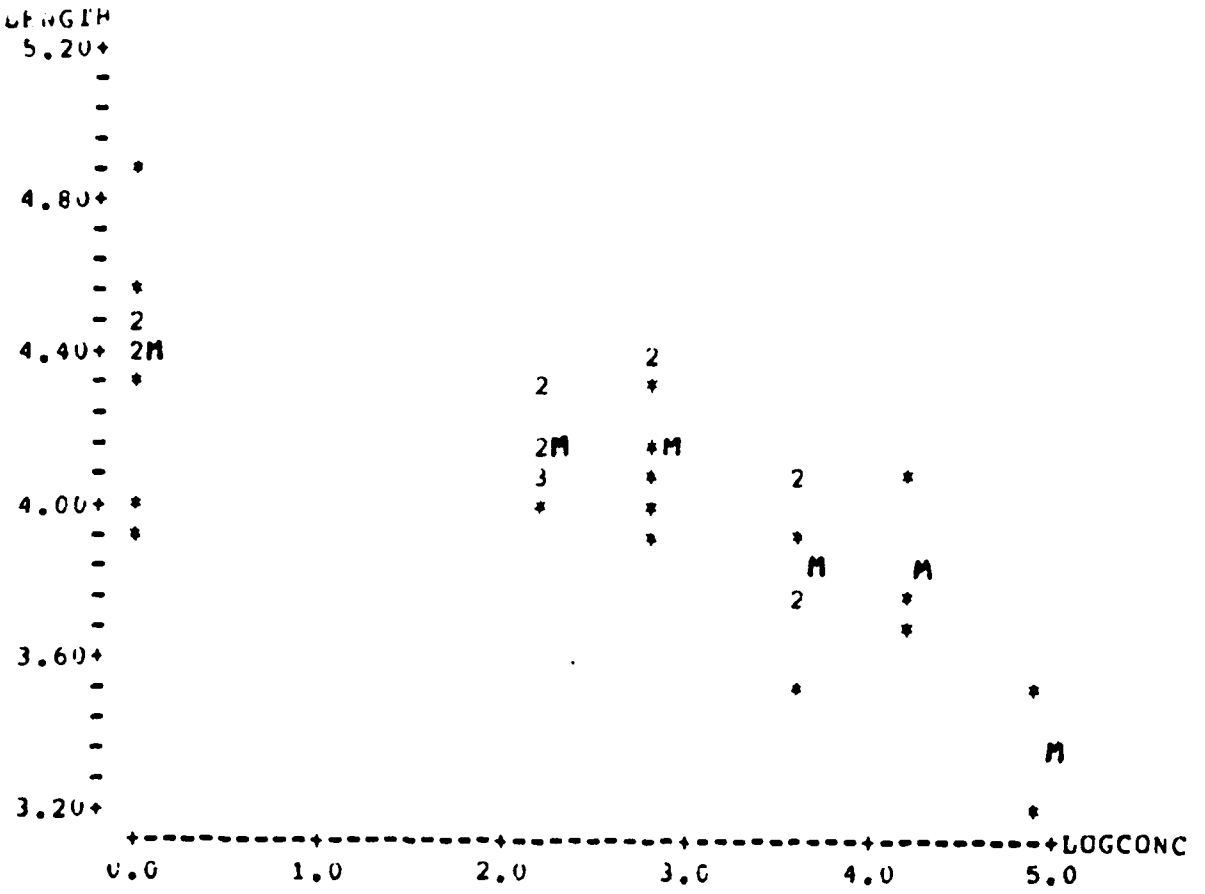


FIGURE 10. PLOT OF LENGTH MEASUREMENTS AND AVERAGE LENGTH VERSUS LOG-CONCENTRATION

C. Tests of Homogeneity of Variance - Hartley's test is not appropriate here since the sample sizes are not equal. The results for Bartlett's Test and Levene's Test are listed below.

Bartlett's Test

MSE = 0.049 C = 1.140

B = 6.17 $\chi^2(0.95,5)=11.1$

Since 6.17 < 11.1, do not reject the hypothesis of homogenous variances.

Levene's Test

$$\sum_{i=0}^{5} \frac{1}{n_i} \left(\sum_{j=1}^{n_i} D_{ij} \right)^2 = 0.93821$$

$$\sum_{i=0}^{5} \sum_{j=1}^{n_i} D_{ij} = 5.5143$$

$$\sum_{i=0}^{5} \sum_{j=1}^{n_i} D_{ij}^2 = 1.4212$$

F = 0.83 F(0.95;5,29)=2.55

Since 0.83 < 2.55, do not reject the hypothesis of homogeneous variances.

D. Outlier Detection Tests - A plot of the D_{ij} values versus their normal scores is given in Figure 11.

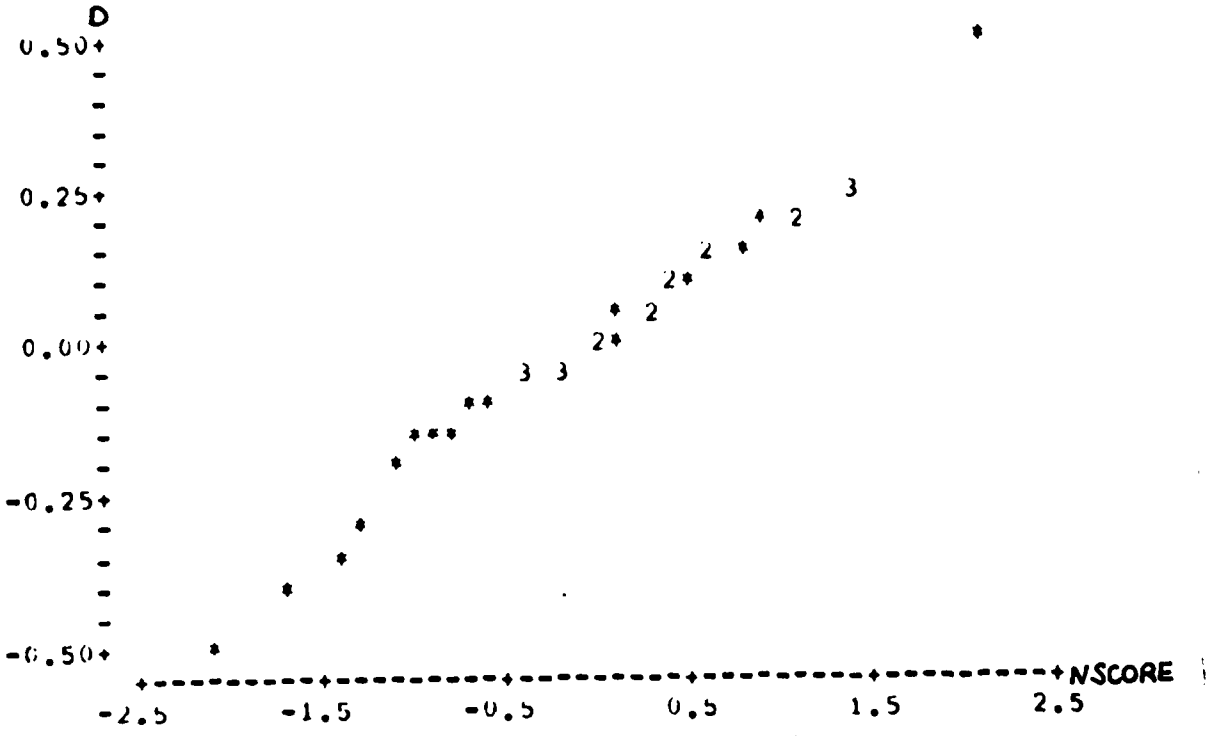


FIGURE 11. NORMAL PROBABILITY PLOT OF LENGTH MEASUREMENTS

Since all the plotted points fall approximately on a straight line, no observations are identified as potential outliers.

E. Multiple Comparison Procedures - All three multiple comparison procedures are illustrated. The value of MSE is 0.049.

Dunnett's Procedure - The T-values are T_1=2.10, T_2=1.82, T_3=4.48, T_4=3.54, and T_5=6.00. The one-tailed critical point is $t(0.05,5,29) \cong t(0.05,5,30)$=2.33. Thus concentrations 35, 70, and 140 had a statistically significant detrimental effect on length.

Bonferroni's Procedure - The T-values are calculated as for Dunnett's procedure but the critical point is $t(0.05/5;29)=t(0.01;29)$=2.462. Thus the conclusions are the same as for Dunnett's procedure.

Williams' Method - The average lengths $\bar{Y}_{1.}$ and $\bar{Y}_{2.}$ are replaced by their weighted average (4.173) so that the smoothed average lengths are monotone decreasing. The T-values are T_1=2.00, T_2=1.93, T_3=4.48, T_4=3.54, and T_5=6.00. The critical point is t(0.05;5,29)= t(0.05;5,28)=1.83. Thus by Williams' method, all 5 concentrations had a statistically significant detrimental effect on length.

F. After-the-Fact Power Calculation for the Bonferroni Multiple Comparison Procedure - Suppose that $\mu(x_i)$ and μ_0, the true average lengths for the ith treatment group and the control group, are 4.0 and 4.5, respectively, and n_0=9, n_i=8. The noncentrality parameter δ is

$$\delta = \frac{4.5-4.0}{[0.049(1/9+1/8)]^{1/2}} = 4.65$$

and the probability that the ith group will be declared statistically significantly different from the control group is Φ [4.65 - 2.33] = 0.99

This calculation says that a true average length of 4.0 mm is almost certain to be distinguished from the control group average length of 4.5 mm, if sample sizes n_0=9 and n_1=8 are achieved.

G. Confidence Intervals for Differences Between Control Group and Treatment Group Mean Responses - The critical point for the confidence intervals is T=t*(0.05;5,29)=2.66. The 95 percent confidence intervals are:

(-0.07, 0.50)	for	$D_1 = \mu_0 - \mu_1$
(-0.08, 0.51)	for	$D_2 = \mu_0 - \mu_2$
(0.21, 0.83)	for	$D_3 = \mu_0 - \mu_3$
(0.13, 0.91)	for	$D_4 = \mu_0 - \mu_4$
(0.58, 1.50)	for	$D_5 = \mu_0 - \mu_5$

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Note: A Fortran program for the Trimmed Spearman-Karber estimates is documented in Hamilton, et al. 1977.

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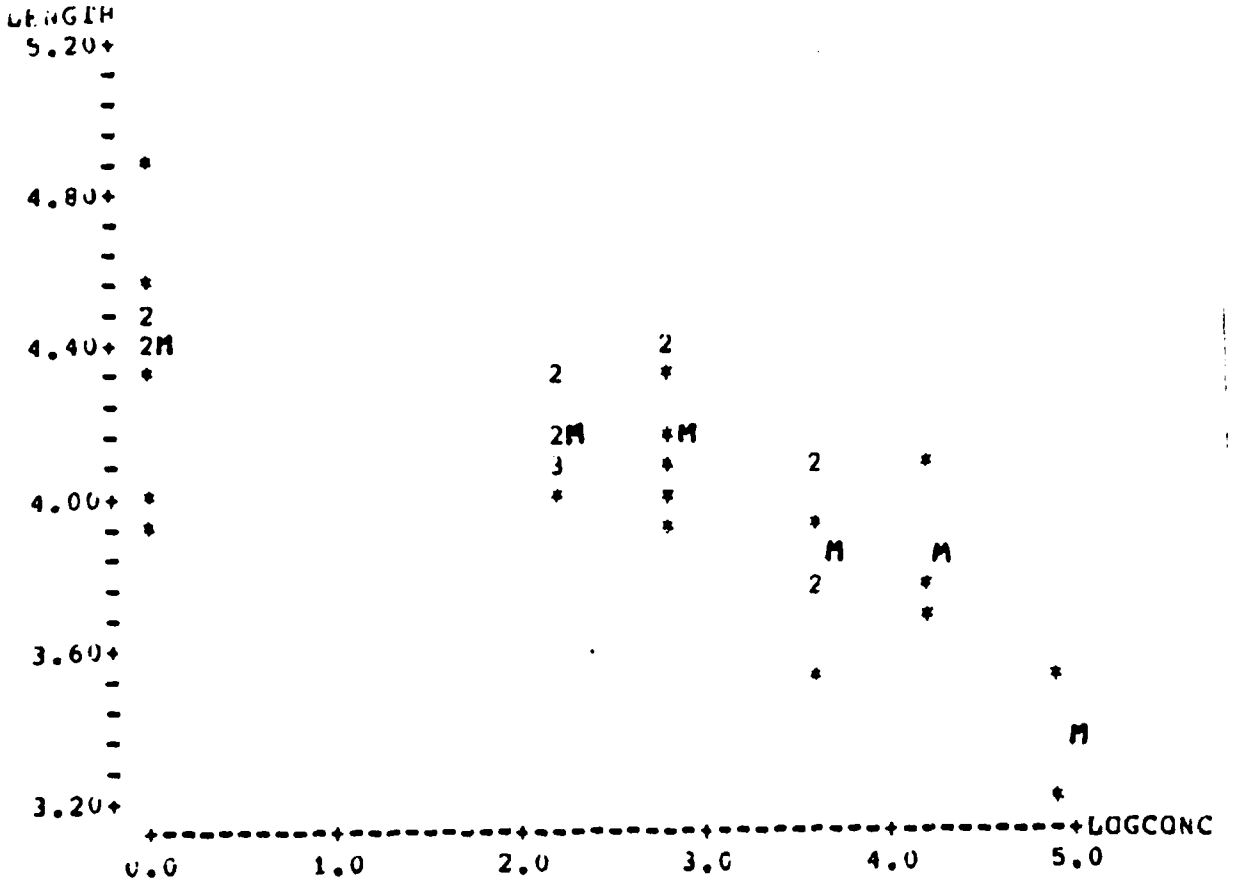


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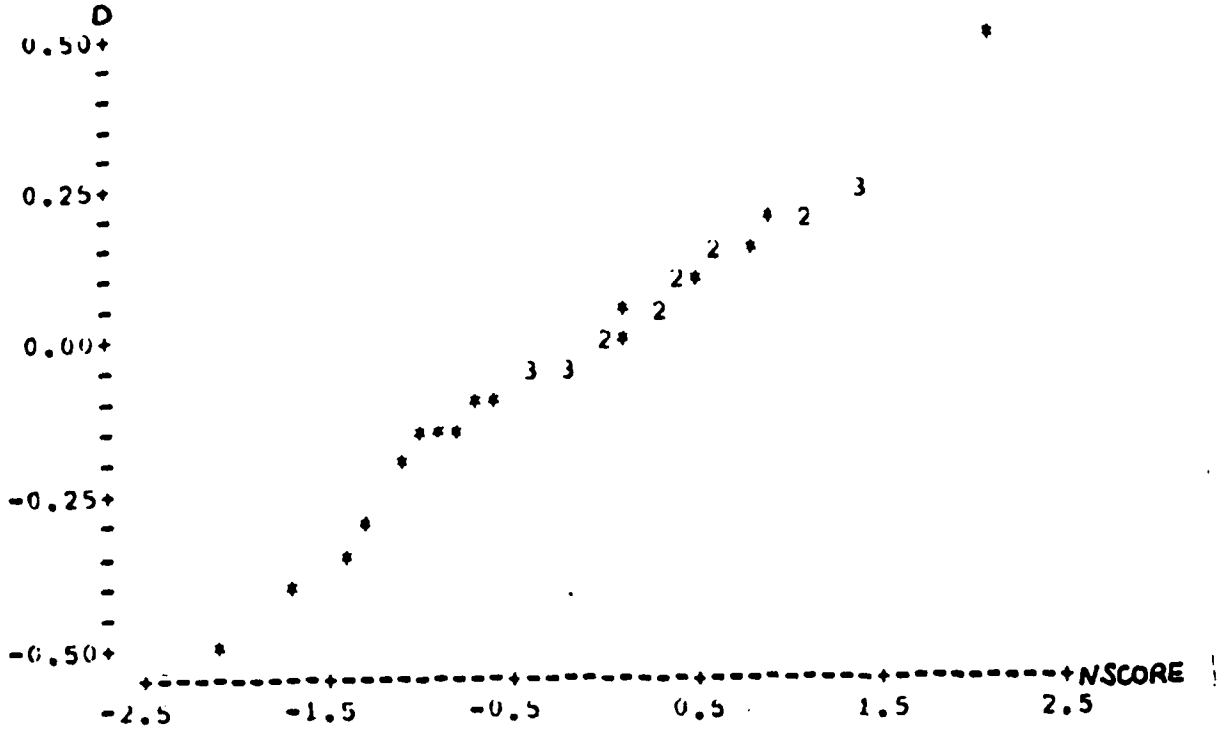


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(0.58, 1.50)	for	$D_5 = \mu_0 - \mu_5$

APPENDIX 3

Results of Precision and Accuracy Analyses

Conducted at

Springborn Bionomics, Inc.

Table 1. Analytical results for the accuracy of the pentachlorophenol analysis.

Nominal concentration (µg/mL)		Analytical result (µg/mL)	% recovery
control	A	<0.0030	N/A
	B	<0.0030	N/A
	C	<0.0030	N/A
	D	<0.0030	N/A
	E	<0.0030	N/A
0.0050	A	0.0060	120
	B	0.0058	116
	C	0.0078	156
	D	0.0052	104
	E	0.0050	100
0.025	A	0.025	100
	B	0.025	100
	C	0.026	102
	D	0.027	109
	E	0.026	102
0.10	A	0.096	96
	B	0.10	100
	C	0.10	100
	D	0.099	99
	E	0.098	98
0.42	A	0.39	93
	B	0.40	95
	C	0.40	95
	D	0.40	95
	E	0.40	95

Average recovery = 104 ± 14.

Table 2. Analytical results for the precision of the pentachlorophenol analysis.

Nominal concentration (µg/mL)	Day 1		Day 2		Day 3	
	Analytical result (µg/mL)	% recovery	Analytical result (µg/mL)	% recovery	Analytical result (µg/mL)	% recovery
0.025 A	0.024	96	0.026	104	0.024	96
0.025 A	0.026	104	0.026	104	0.026	104
0.025 A	0.025	100	0.024	96	0.027	108
0.025 A	0.025	100	0.024	96	0.027	108
0.025 A	0.025	100	0.024	96	0.027	108
Mean (Std. Dev.)		100(2.8)		99(4.4)		105(5.2)
0.42 A	0.38	90	0.40	95	0.40	95
0.42 A	0.38	90	0.41	98	0.39	93
0.42 A	0.38	90	0.39	93	0.40	95
0.42 A	0.38	90	0.38	90	0.40	95
0.42 A	0.38	90	0.40	95	0.39	93
Mean (Std. Dev.)		90(0)		94(2.9)		94(1.1)

Mean recovery for 3 days = 97 ± 5.7.

Table 3. Results of accuracy and precision studies conducted with the sodium pentachlorophenate/glacial acetic acid mixture (unknown 658) and copper chloride (unknown 852).

NaPCP/GAA - ACCURACY

	Nominal concentration (mg/L)			
	1.0	3.0	6.0	10
Measured concentration (mg/L)	1.1	2.8	5.5	10
	1.0	2.8	5.8	10
	1.1	2.7	5.8	11
	1.1	2.7	6.3	10
	1.1	2.7	6.4	11
$\bar{x}$ ± S.D. (meas. as % of nominal)	1.08 0.04 (108)	2.74 0.05 (91)	5.96 0.34 (99)	10.4 0.49 (104)

PRECISION

	Nominal concentration (mg/L)	
	3.0	10
Day 1	2.7	9.9
Measured concentration (mg/L)	2.6	9.8
	2.7	9.9
	2.7	9.9
	2.7	9.8
	2.7	10.1
	2.7	10.1
	2.7	10.9
	2.7	10.9
	2.6	10.9
$\bar{x}$ S.D. (meas. as % of nominal)	2.68±0.04 (99)	10.2±0.48 (102)

Table 3 continued

NaPCP/GAA - PRECISION (cont.)

	Nominal concentration (mg/L)	
	3.0	10
Day 2	2.6	10.2
	2.6	10.4
Measured concentration (mg/L)	2.6	10.4
	2.6	10.1
	2.6	10.2
	2.6	—[a]
	2.6	10.2
	2.6	—[a]
	2.6	10.5
	2.6	10.4
$\bar{x}$ ± S.D. (meas. as % of nominal)	2.6±0 (87)	10.3±0.14 (103)
Day 3	2.7	10.5
	2.6	10.5
Measured concentration (mg/L)	2.6	10.5
	2.6	10.5
	2.6	10.2
	2.6	10.7
	2.6	10.7
	2.6	10.9
	2.7	10.5
	2.7	10.7
$\bar{x}$ ± S.D. (meas. as % of nominal)	2.63±0.05 (88)	10.6±0.19 (106)

Table 3 continued

$CuCl_2$ - ACCURACY

	Nominal concentration (mg/L)			
	10	30	50	100
Measured concentration (mg/L)	9.1	28	45	94
	10	28	47	88
	9.1	30	49	70
	9.1	29	49	70
	9.1	29	49	70
$\bar{x}$ ± S.D. (meas. as % of nominal	9.28±0.40 (93)	28.8±0.84 (96)	47.8±1.8 (96)	78.4±11.7 (78)

PRECISION

	Nominal concentration (mg/L)	
	30	50
Day 1	26	46
	26	47
Measured concentration (mg/L)	26	46
	26	46
	26	48
	26	48
	25	48
	28	48
	27	48
	27	48
$\bar{x}$ ± S.D. (meas. as % of nominal)	26.3±0.82 (88)	47.3±0.95 (95)

Table 3 continued

$CuCl_2$ - PRECISION (cont.)

	Nominal concentration (mg/L)	
	30	50
Day 2	30	50
Measured concentration (mg/L)	30	52
	30	52
	30	52
	30	51
	30	51
	30	51
	31	51
	30	51
	31	51
$\bar{x}$ ± S.D. (meas. as % of nominal)	30.2±0.42 (101)	51.2±0.63 (102)
Day 3	30	49
Measured concentration (mg/L)	30	51
	30	52
	30	51
	30	52
	28	52
	30	54
	31	53
	30	53
	30	54
$\bar{x}$ ± S.D. (meas. as % of nominal)	29.9±0.74 (100)	52.1±1.5 (104)

[a]Sample lost.

Table 4. Results of accuracy and precision analyses of the $CuCl_2$: GAA mixture (unknown #124).

ACCURACY

	Nominal Concentration (µg/L)			
	5.0	20	50	100
Measured concentration (µg/L)	6.0	17	48	120
	5.0	17	51	100
	5.0	20	51	80
	5.0	19	52	80
	5.0	19	51	100
x±S.D. (mean as % of nominal)	5.2±0.45 (104)	18±1.2 (90)	51±1.5 (102)	96±17 (96)

	Nominal Concentration (µg/L)	
	5.0	50
DAY 1	6.0	47
Measured concentration (µg/L)	5.0	51
	5.0	52
	4.0	54
	5.0	52
	5.0	51
	5.0	50
	6.0	51
	5.0	51
	5.0	51
$\bar{x}$±S.D. (mean as % of nominal)	5.1±0.57 (102)	51±1.8 (102)

Table 4 continued.

	Nominal Concentration (µg/L)	
	5.0	50
DAY 2	7.0	50
Measured concentration (µg/L)	6.0	52
	4.0	52
	4.0	53
	4.0	53
	4.0	52
	4.0	53
	6.0	53
	4.0	52
	4.0	53
$\bar{x}$±S.D. (mean as % of nominal)	4.7±1.2 (94)	52±0.95 (104)

	Nominal Concentration (µg/L)	
	5.0	50
DAY 3	5.0	50
Measured concentration (µg/L)	4.0	53
	3.0	53
	3.0	53
	2.0	53
	3.0	52
	3.0	52
	7.0	52
	5.0	52
	5.0	52
$\bar{x}$·S.D. (mean as % of nominal)	4.0·1.5 (80)	52·0.92 (104)

APPENDIX 4

Results of Sample Stability Analyses

Conducted at

Springborn Bionomics, Inc.

TABLE 1. Results of the sample stability analyses conducted over a 120-day holding period.

NaPCP Day	Expected Concentration (mg/L)	Measured Concentration (mg/L)	% Recovery
0	1.0	1.09	109
	1.0	1.09	109
	2.0	2.09	104
	2.0	2.09	104
30	1.0	1.33	133
	1.0	1.36	136
	2.0	2.59	130
	2.0	2.57	128
60	1.0	1.10	110
	1.0	1.10	110
	2.0	2.12	106
	2.0	2.12	106
90	1.0	1.10	110
	1.0	1.10	110
	2.0	2.12	106
	2.0	2.14	107
120	1.0	1.11	111
	1.0	1.11	111
	2.0	2.12	106
	2.0	2.15	108

TABLE 1. (Cont.)

NaPCP/GAA (Compound #658)	Expected Concentration (mg/L)	Measured Concentration (mg/L)	% Recovery
0	1.0	1.11	111
	1.0	1.11	111
	2.0	2.21	110
	2.0	2.21	110
30	1.0	1.22	122
	1.0	1.22	122
	2.0	2.36	118
	2.0	2.36	118
60	1.0	1.12	112
	1.0	1.12	112
	2.0	2.20	110
	2.0	2.20	110
90	1.0	1.15	115
	1.0	1.15	115
	2.0	2.23	112
	2.0	2.20	110
120	1.0	1.18	118
	1.0	1.20	120
	2.0	2.28	114
	2.0	2.30	115

TABLE 1. (Cont.)

$CuCl_2$ (Coumpound #852) Day	Expected Concentration (mg/L)	Measured Concentration (mg/L)	% Recovery
0	1.0	1.09	109
	1.0	1.09	109
	2.0	2.10	105
	2.0	2.10	105
30	1.0	1.23	123
	1.0	1.25	125
	2.0	2.29	114
	2.0	2.29	114
60	1.0	1.10	110
	1.0	1.10	110
	2.0	2.19	110
	2.0	2.19	110
90	1.0	1.10	110
	1.0	1.10	110
	2.0	2.12	106
	2.0	2.12	106
120	1.0	1.12	112
	1.0	1.12	112
	2.0	2.15	108
	2.0	2.17	108

TABLE 1. (Cont.)

$CuCl_2$/GAA (Compound #124) Day	Expected Concentration (mg/L)	Measured Concentration (mg/L)	% Recovery
0	1.0	1.06	106
	1.0	1.04	104
	2.0	2.09	104
	2.0	2.11	106
30	1.0	1.11	111
	1.0	1.11	111
	2.0	2.13	106
	2.0	2.17	108
60	1.0	1.10	110
	1.0	1.10	110
	2.0	2.19	110
	2.0	2.19	110
90	1.0	1.10	110
	1.0	1.10	110
	2.0	2.14	107
	2.0	2.16	108
120	1.0	1.11	111
	1.0	1.13	113
	2.0	2.21	110
	2.0	2.21	110

APPENDIX 5

Results of the Analysis of Concentrations Measured
During Testing at the Various Collaborative Laboratories.
All Analyses were Performed at
Springborn Bionomics, Inc.

TABLE 1. Concentration of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #1 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)						
Nominal concentration (ug/L)	Day 0	Day 3	Day 7	Day 14	Day 21	Mean (s.d.)	n
800	1050	950	950	--	--	983 (58)	3
400	495	485	480	445	440	469 (25)	5
200	240	230	240	180	185	215 (30)	5
100	105	105	110	105	105	106 (2.2)	5
50	35	35	45	55	50	44 (8.9)	5
control	<11	< 11	<11	<10	< 10	< 11 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 2. Concentrations of sodium pentachlorophanate (NaPCP) measured in the test solutions submitted by Laboratory #2 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1600	1500	--	--	--	1500	1
800	900	895	--	--	898	2
400	470	435	410	570	471 (70)	4
200	240	205	215	200	215 (18)	4
100	110	110	110	110	110 (0)	4
control	< 11	< 5	<5	< 5	<11[a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 3. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #3 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
[illegible]	900	900	910	790	875 (57)	4
[illegible]	460	490	450	300	425 (85)	4
[illegible]	200	230	215	118	191 (50)	4
100	110	115	98	47	93 (81)	4
50	--	60	52	14	42 (25)	3
25	27	30	23	< 6.0	27 (4)	3
control	< 11	< 11	< 6.0	< 6.0	< 11[a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 4. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #4 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluoresdein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1000	1200	1200	--	--	1200	2
500	600	555	570	595	580 (21)	4
250	--[a]	320	310	320	317 (6.0)	3
125	135	140	135	130	135 (4.0)	4
62.5	75	59	80	75	72 (9.0)	4
control	< 11	< 9	< 9	< 9	< 11[b]	

[a] Sample broken in transit

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 5. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #5 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
700	850	765	810	--	808 (43)	3
350	410	390	400	360	390 (22)	4
175	172	--[a]	193	172	179 (12)	4
87.5	92	94	59	84	82 (16)	4
43.75	49	48	49	40	47 (4.0)	4
control	<10	< 10	< 10	< 10	< 10 [b]	

[a] Sample lost

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 6. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #6 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration	Measured Concentration					
	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
900	1050	--	--	--	1050	1
450	505	500	510	510	506 (5)	4
225	240	230	265	270	251 (19)	4
113	140	115	125	120	125 (11)	4
56	80	65	70	65	70 (7)	4
control	<11	<10	< 10	<10	< 11[a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 7. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #7 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

CHRONIC #2

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
800	1450	900	--	--	1175	2
400	425	460	485	500	467 (33)	4
200	320	200	230	220	243 (53)	4
100	210	110	110	110	135 (50)	4
50	110	55	60	55	70 (27)	4
control	<11	< 10	< 10	< 11	< 11 [a]	

[a]Value reported as less than the detection limit measured in any sampling interval.

TABLE 8. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #7 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

CHRONIC #1

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
800	1450	--	--	--	1450	1
400	750	600	430	525	576 (135)	4
200	310	250	190	--[a]	250 (60)	3
100	210	115	115	--[a]	147 (55)	4
50	115	60	60	60	74 (27)	4
control	<10	< 11	<11	< 11	< 11 [b]	

[a] Sample lost.

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 9. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #8 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0 [a]	Day 7	Day 14	Day 21	Mean (s.d.)	n
1000	< 5.0	1330	1460	--	1395	2
500	< 5.0	655	735	690	693 (40)	3
250	< 5.0	335	367	343	348 (17)	3
125	< 5.0	166	182	161	170 (11)	3
60	< 5.0	81	89	78	83 (5.7)	3
control	< 5.0	< 5.0	<5.0	<5.0	< 5.0 [b]	

[a]Samples received on this day contained no fluorescein. It was determined that a stock containing no fluorescein was used at this interval.

[b]Values reported as less than the detection limit measured at any sampling interval.

TABLE 10. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #9 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
800	880	1020	--	--	950	2
400	440	440	455	495	458 (26)	4
200	215	220	220	220	219 (2.5)	4
100	115	115	115	115	115 (0)	4
50	54	56	58	51	55 (3.0)	4
control	< 5	< 5	< 5	< 5	< 5[a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 11. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #10 during a 21-day static renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1500	1360	--	--	--	1360	1
750	700	840	910	840	822 (88)	4
375	375	440	470	450	444 (41)	4
188	170	217	--[a]	--[b]	194 (33)	3
94	89	107	98	105	100 (8.1)	4
control	5.0	5.0	5.0	5.0	5.0[c]	

[a]Sample broken in transit.
[b]Not shipped.
[c]Value reported as less than the detection limit measured at any sampling interval.

TABLE 12. Concentrations of sodium pentachlorophenate (NaPCP) measured in the test solutions submitted by Laboratory #11 during a 21-day static renewal chronic toxicity test with Daphnia magna. *Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.*

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
900	910	1050	--	--	980	2
450	472	520	520	425	484 (46)	4
225	253	220	233	214	230 (17)	4
112	124	126	110	101	115 (12)	4
56	59	63	59	42	56 (9.4)	4
control	<10	<10	< 10	<10	< 10 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 13. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #1 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1600	1850	---	--	--	1850	1
800	910	925	--	--	917	2
400	462	448	475	472	464 (12)	4
200	225	224	230	230	228 (4.2)	4
100	115	114	116	112	114 (1.7)	4
control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0[a]	
solvent control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0[a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 14. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #2 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1400	1580	--	--	--	1580	1
700	770	775	695	710	737 (41)	4
350	395	382	388	378	386 (7.4)	4
175	202	166	170	188	181 (17)	4
87.5	110	96.0	95.0	92.0	98 (8.0)	4
solvent	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	
solvent control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured in any sampling interval.

TABLE 15. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #3 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration	Measured Concentration					
	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
1500	1610	1660	1520	--	1597 (71)	3
750	815	815	785	755	793 (29)	4
375	413	413	405	358	397 (26)	4
187.5	206	201	201	167	194 (18)	4
93.75	105	102	102	75	96 (14)	4
control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 16. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #4 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
1000	1100	998	--	--	1049	2
500	552	530	543	517	535 (15)	4
250	272	259	264	252	262 (8.4)	4
125	132	121	134	130	129 (5.7)	4
62	69	71	74	67	70 (3.0)	4
control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	
solvent control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 17. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA0 (Unknown #658) measured in the test solutions submitted by Laboratory #5 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
1500	1640	--	--	--	1640	1
750	820	825	860	--	835 (22)	3
375	410	417	455	425	427 (20)	4
188	204	204	217	206	208 (6.2)	4
94	105	104	111	104	106 (3.4)	4
control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	
solvent control	<5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 18. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #6 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
700	795	800	--	--	797	2
350	407	406	412	475	425 (33)	4
175	199	202	202	201	201 (1.4)	4
88	101	102	97	99	100 (2.2)	4
44	51	51	47	50	50 (1.9)	4
control	< 5.0	<5.0	< 5.0	< 5.0	< 5.0 [a]	
solvent control	< 5.0	<5.0	b	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

[b] Sample broken in transit.

TABLE 19. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #7 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
800	470	850	800	850	743 (183)	4
400	390	425	380	430	406 (25)	4
200	750	160	175	195	320 (287)	4
100	500	100	100	90	197 (202)	4
50	280	49	55	46	107 (115)	4
GAA control	<40	<11	< 10	< 10	<40	
control	< 40	<11	< 10	< 10	<40	

TABLE 20. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #8 during a 21-day static-rewewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)						
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 20/21		Mean (s.d.)	n
900	1020	1000	--	--	--	1010	2
450	520	500	515	495	480	502 (16)	5
220	250	245	247	239	230	242 (7.9)	5
110	124	124	126	120	112	121 (5.6)	5
50	74	63	67	62	58	65 (6.1)	5
control	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	
solvent control	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 21. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #9 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
800	865	853	--	--	859	1
400	440	440	472	435	447 (17)	4
200	220	215	234	216	221 (8.8)	4
100	113	116	116	109	113 (3.3)	4
50	58	59	59	55	58 (1.9)	4
control	< 5.0	<5.0	< 5.0	< 5.0	< 5.0 [a]	
solvent control	< 5.0	<5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #10 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
[illegible]	2050	--	--	--	2050	1
[illegible]	1035	[illegible]5	850	--	937 (93)	3
500	480	475	450	--	468 (16)	3
250	234	240	243	214	233 (13)	4
125	109	114	124	127	119 (8.4)	4
control	< 5.0	<5.0	< 5.0	<5.0	<5.0[a]	
solvent control	< 5.0	<5.0	< 5.0	< 5.0	<5.0[a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 23. Concentration of sodium pentachlorophenate/glacial acetic acid (NaPCP/GAA) (Unknown #658) measured in the test solutions submitted by Laboratory #11 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescrin in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 8	Day 14	Day 21 [a]	Mean (s.d.)	n
875	945	1000	-		973	1
437.5	455	500	493		483 (24)	3
218.8	221	242	238		234 (11)	3
109.4	115	121	118		118 (3.0)	3
54.7	61	63	65		63 (2.0)	3
control	< 5.0	< 5.0	< 5.0		<5.0 [b]	
solvent control	< 5.0	< 5.0	< 5.0		<5.0 [b]	

[a] No samples shipped.

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 14. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #1 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the testing solutions.

Nominal Concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 8	Day 15	Day 21	Mean (s.d.)	n
100	107	112	--	--	110	2
50	55	58	56	56	56 (1.3)	4
25	25	27	26	26	26 (0.82)	4
12.5	9	11	11	10	10 (0.96)	4
6.5	< 5.0	<5.0	< 5.0	<5.0	<5.0 [a]	
Control	< 5.0	<5.0	< 5.0	<5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 25. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #2 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 5	Day 12	Day 21	Mean (s.d.)	n
400	430	--	--	--	430	1
200	212	--	--	--	212	1
100	109	232	--	--	171	2
50	55	122	52	53	71 (34)	4
25	26	35	23	29	28 (5.1)	4
control	< 5.0	< 5.0	<5.0	< 5.0	<5.0[a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 26. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #3 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
500	542	--	--	--	542	1
250	261	--	--	--	261	1
125	126	--	--	--	126	1
61	66	70	61	49	61 (9.1)	4
31	30	33	23	16	25 (7.6)	4
control	< 5.0	< 5.0	<5.0	<5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 27. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #4 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 6	Day 13	Day 21[a]	Mean (s.d.)	n
150	160	160	--		160	2
75	92	79	81		84 (7.0)	3
38	49	41	43		44 (4.2)	3
19	27	19	21		22 (4.2)	3
10	13	8	9.3		10 (2.6)	3
control	<5.0	<5.0	< 5.0		< 5.0 [b]	

[a] No samples shipped.

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 28. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #5 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
125	126	124	133	131	129 (42)	4
62.5	65.5	65.0	71.0	74.0	69 (4.5)	4
31.3	30.0	31.0	35.0	48.0	36 (8.3)	4
15.6	12.0	15	15	30.0	18 (8.2)	4
7.8	<5.0	<5.0	< 5.0	21.0	9.0 (8.0)	4
control	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 29. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #6 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
200	208	--	--	--	208	1
100	102	112	104	109	107 (4.6)	4
50	53	58	75	48	59 (12)	4
25	25	28	23	23	25 (2.4)	4
12.5	9.0	13.0	8.0	9.0	9.7 (22)	4
control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 30. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #7 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration	Measured Concentration Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
250	206	--	--	--	206	1
125	80	120	150	--	117 (35)	3
62	70	70	90	74	76 (10)	4
31	21	30	4	37	33 (10)	4
16	8.0	17	26	19	18 (7.4)	4
control	<3.0	< 10	< 11	< 11	< 11 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 31. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #8 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 15	Day 20-21	Mean (s.d.)	n
280	315	--	--	--	315	1
140	149	142	149	151 142	147 (4.3)	5
70	79	75	77	76 69	75 (3.8)	5
35	37	37	37	35 31	35 (2.6)	5
17	18	17	15	15 13	16 (1.9)	5
control	<5.0	<5.0	< 5.0	< 5.0 <5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 32. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #9 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
200	213	210	--	--	212	
100	108	106	109	107	108 (1.3)	4
50	67	55	57	59	60 (53)	4
25	25	25	28	28	27 (1.7)	4
12	8.5	10	11	13	11 (1.9)	4
control	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 33. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #10 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

Nominal concnetration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
300	313	--	--	--	313	1
150	148	132	146	--	142 (8.7)	3
75	83	85	73	79	80 (5.3)	4
37.5	44	37	37	35	38 (3.9)	4
18.8	19	23	14	13	17 (4.7)	4
control	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 34. Concentrations of copper (as $CuCl_2$) (Unknown #852) measured in the test solutions submitted by Laboratory #11 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based upon a known amount of the tracer fluorescein in the test solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21[a]	Mean (s.d.)	n
335	400	--	--		400	1
167.5	196	177	--		187	2
84	101	88	90		93 (7.0)	3
42	53	45	46		48 (4.4)	3
21	25	20	21		22 (2.7)	3
control	< 5.0	<5.0	< 5.0		< 5.0 [b]	

[a] No samples provided.

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 35. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #1 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
200	253	--	--	--	253	1
100	128	136	133	124	130 (5.3)	4
50	68	69	69	67	68 (0.96)	4
25	33	33	33	29	32 (2.0)	4
13	14	14	14	13	14 (0.50)	4
control	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	
solvent control	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 36. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #2 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 8	Day 14	Day 21	Mean (s.d.)	n
200	260	260	--	--	260	2
100	132	133	128	--	131 (2.7)	3
50	71	63	62	67	66 (4.1)	4
25	36	34	34	39	36 (2.4)	4
12.5	17	17	17	17	17 (0)	4
solvent control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	
control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

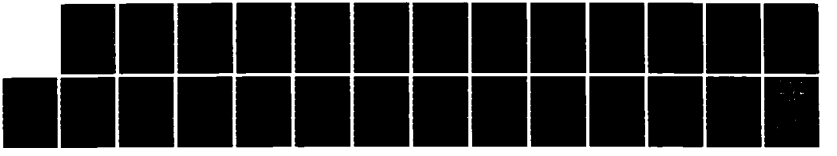
TABLE 37. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #3 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal Concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 6	Day 13	Day 21	Mean (s.d.)	n
200	250	--	--	--	250	
100	122	120	120	--	121	3
50	64	60	61	28	53 (17)	4
25	36	36	30	13	29 (11)	4
12.5	14	14	16	<5.0	12 (4.9)	4
solvent control	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	
control	<5.0	<5.0	<5.0	<5.0	<5.0 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

AD-A166 957 COLLABORATIVE STUDY OF DAPHNIA MAGNA STATIC RENEWAL ASSAYS(U) SPRINGBORN BIONOMICS INC WAREHAM MA* R E BENTLEY ET AL. JAN 86 DAMD17-80-C-0011 4/4

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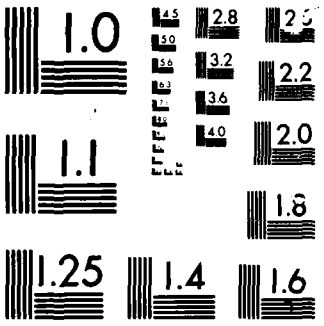

1.0
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MICROCOPY
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TABLE 38. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #4 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
160	190	--	--	--	190	1
80	104	108	92	--	101 (8.3)	3
40	56	54	54	42	52 (6.4)	4
20	28	26	26	20	25 (3.5)	4
10	13	12	13	10	12 (1.4)	4
control	< 5.0	< 5.0	<5.0	<5.0	<5.0 [a]	
solvent control	< 5.0	< 5.0	<5.0	<5.0	<5.0 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 39. Concentrations of copper/glacial acetic acid ($CuCl_2$/Gaa) (Unknown #124) measured in the test solutions submitted by Laboratory #5 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
220	252	--	--		252	1
110	134	121	122	115	123 (8.0)	4
55	69	60	65	57	63 (5.3)	4
28	43	29	29	19	30 (9.9)	4
14	27	11	13	12	16 (7.5)	4
Solvent control	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	
control	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 40. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #6 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
120	125	127	-- [a]	--	126	2
60	71	67	-- a	75	71 (4.0)	3
30	33	33	37	34	34 (1.7)	4
15	17	11	-- [a]	15	14 (3.1)	3
7.5	< 5.0	6.0	8.0	6.0	6.7 (1.2)	4
solvent control	< 5.0	<5.0	< 5.0	<5.0	< 5.0 [b]	4
control	< 5.0	<5.0	< 5.0	<5.0	<5.0 [b]	4

[a] Sample bottle broken in transit.

[b] Value reported as less than the detection limit measured at any sampling interval.

TABLE 41. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #7 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
250	250	224	--	--	237	2
125	104	104	95	--	101 (5.2)	3
62	75	66	77	90	77 (9.9)	4
31	40	25	32	27	31 (5.7)	4
16	17	16	12	10	14 (3.3)	4
control	<5.0	<5.0	< 5.0	< 5.0	< 5.0 [a]	
solvent control	[b]	[b]	< 5.0	< 5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

[b] Extraneous interference in sample.

TABLE 42. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #8 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
160	175	188	--	--	182	2
80	86	92	92	85	89 (3.8)	4
40	45	46	46	44	45 (0.96)	4
20	19	23	22	20	21 (1.8)	4
10	6.5	8.5	10	14	9.8 (3.2)	4
Solvent control	< 5.0	< 5.0	< 5.0	< 5.0[a]	< 5.0 [a]	
control	< 5.0	< 5.0	< 5.0	< 5.0[a]	< 5.0 [a]	

[a]Value reported as less than the detection limit measured at any sampling interval.

TABLE 43. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #9 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentration are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
150	166	166	--	--	166	2
75	85	87	87	88	87 (1.3)	4
38	44	43	46	41	43 (2.1)	4
19	19	19	16	18	18 (1.4)	4
9.5	6.5	6.5	7.0	6.0	6.5 (0.41)	4
Solvent control	< 5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	
control	< 5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

TABLE 44. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA) (Unknown #124) measured in the test solutions submitted by Laboratory #10 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

	Measured Concentration (ug/L)					
Nominal concentration (ug/L)	Day 0	Day 7	Day 14	Day 21	Mean (s.d.)	n
300	280	--	--	--	280	1
150	150	195	--	--	173	2
75	67	95	73	78	78 (12)	4
37.5	37	50	37	38	41 (6.3)	4
19	18	24	15	17	19 (3.9)	4
control	<5.0	<5.0	< 5.0	<5.0	< 5.0 [a]	
solvent control	--[b]	<5.0	< 5.0	<5.0	< 5.0 [a]	

[a] Value reported as less than the detection limit measured at any sampling interval.

[b] No sample shipped.

TABLE 45. Concentrations of copper/glacial acetic acid ($CuCl_2$/GAA (Unknown #124 measured in the test solutions submitted by Laboratory #11 during a 21-day static-renewal chronic toxicity test with Daphnia magna. Concentrations are based on the known amount of the tracer fluorescein in the testing solutions.

Nominal concentration (ug/L)	Measured Concentration (ug/L) Day 0	Day 7	Day 14	Day 21[a]	Mean (s.d.)	n
200	221	210	--		215	2
100	108	106	107		107 (1.0)	3
50	55	57	55		56 (1.1)	3
25	27	27	27		27 (0)	3
12.5	12	11	11		11 (0.6)	3
control	< 5.0	< 5.0	<5.0		<5.0 [b]	
solvent control	< 5.0	< 5.0	<5.0		<5.0 [b]	

[a] No Samples shipped.

[b] Value reported as less than the detection limit measured at any sampling interval.

APPENDIX 6

Results of Fortified Quality Assurance

Blind samples

Conducted at

Springborn Bionomics, Inc.

TABLE 1. Results of NaPCP fortified quality assurance blind samples analyzed concurrently with each laboratory's samples. Results are presented as mg/L of the tracer fluorescein.

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #1			
0	--[a]	--	--
7	--	--	--
14	--	--	--
21	--	--	--
Laboratory #2			
0	1.00	0.95	95
7	1.00	1.14	114
14	1.00	1.10	110
21	1.00	1.11	111
21	1.00	1.12	112
Laboratory #3			
0	1.0	0.70	70
7	1.0	0.78	78
14	1.0	0.98	98
21	1.0	1.00	100
Laboratory #4			
0	1.00	0.65	65
7	1.00	1.08	108
14	1.00	1.10	110
21	1.00	1.08	108

TABLE 1. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #5			
0	1.00	0.50	50
7	1.00	void (air bubble)	
14	1.00	1.10	110
21	1.00	1.11	111
21	1.00	1.23	123
Laboratory #6			
0	1.00	0.68	68
7	1.00	0.58	58
14	1.00	0.50	50
21	1.00	0.50	50
Laboratory #7 Test #1			
0	2.0	2.8	140
7	0.8	0.55	69
14	0.8	0.65	81
21	1.0	0.87	87
Test #2			
0	10	18	180[b]
7	1.0	0.71	71
14	1.0	0.76	76
21	1.0	0.84	84

TABLE 1. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #8			
0	1.00	0.54	54
7	1.00	0.53	53
14	1.00	1.06	106
21	1.00	1.04	104
21	1.00	1.04	104
Laboratory #9			
0	1.00	1.09	109
7	1.00	1.07	107
7	1.00	1.04	104
14	1.00	1.09	109
21	1.00	1.04	104
21	1.00	1.07	107
Laboratory #10			
0	0.50	0.51	102
7	1.50	1.60	107
14	0.50	0.50	100
14	1.50	1.60	107
21	0.50	0.50	100
21	1.50	1.58	105

TABLE 1. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #11			
0	0.50	0.50	100
7	1.50	1.50	100
14	1.50	1.47	98
14	1.50	1.34	89
21	0.50	0.46	92
21	0.50	0.43	86
21	1.50	1.61	107

[a]No QA samples were analyzed with this laboratory's samples.

[b]Based upon Chauvenet's criterion for rejection of outliers, this sample was rejected, and not used in the calculation of the mean.

Mean and standard deviation = 94±21%
(n = 53)

TABLE 2. Results of NaPCP/GAA (unknown #658) fortified quality assurance blind samples analyzed concurrently with each laboratory's samples. Results are presented as mg/L of the tracer fluorescein.

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #1			
0	0.50	0.54	108
0	1.50	1.69	113
7	0.50	0.53	106
14	1.50	1.68	112
21	0.50	0.53	106
Laboratory #2			
0 of 1	0.50	0.47	94
0	1.50	1.48	99
7	1.50	1.64	109
14	0.50	0.49	98
21	0.50	0.50	100
Laboratory #3			
0	0.50	0.50	100
0	1.50	1.60	107
7	0.50	0.49	98
7	1.50	1.62	108
14	0.50	0.50	100
21	0.50	0.43	86
21	1.50	1.48	98

TABLE 2. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #4			
0	0.50	0.42	84
0	1.50	1.50	100
8	0.50	0.50	100
8	1.50	1.56	104
15	0.50	0.51	102
15	1.50	1.55	103
21	1.50	1.57	105
Laboratory #5			
0	0.50	0.49	98
0	1.50	1.58	105
7	0.50	0.47	94
7	1.50	1.52	101
14	0.50	0.44	88
14	1.50	1.50	100
21	0.50	0.53	106
21	1.50	1.68	112
Laboratory #6			
0	0.50	0.51	102
0	1.50	1.62	108
7	0.50	0.46	92
7	1.50	1.59	106
14	1.50	1.64	109
14	0.50	0.44	88
21	0.50	0.44	88
21	1.50	1.48	98

TABLE 2. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #7			
0	1.00	1.10	110
0	1.00	1.00	100
8	1.00	1.06	106
8	1.00	1.06	106
14	1.00	1.10	110
14	1.00	0.96	96
21	1.00	0.94	94
21	1.00	1.10	110
Laboratory #8			
0	0.50	0.46	92
0	0.50	0.53	106
8	0.50	0.42	84
8	1.50	1.47	98
15	0.50	0.42	84
15	1.50	1.47	97
20	1.50	1.59	106
21	0.50	0.48	96
21	1.50	1.50	107
Laboratory #9			
0	0.50	0.50	100
0	1.50	1.59	106
7	0.50	0.47	94
14	1.50	1.51	101
21	0.50	0.48	96
21	1.50	1.56	104

TABLE 2. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #10			
0	0.50	0.55	110
0	1.50	1.68	112
7	0.50	0.49	98
7	1.50	1.47	98
14	0.50	0.49	98
14	1.50	1.57	105
21	0.50	9.49	98
21	1.50	1.58	105
Laboratory #11			
0	0.50	0.49	98
0	0.50	0.48	96
0	1.50	1.61	107
7	0.50	0.47	94
7	0.50	0.47	94
7	1.50	1.51	101
14	0.50	0.49	98
21	--[a]	--	--

[a]No samples submitted by this laboratory on this day.

Mean and standard deviation - 101±7%

(n=78)

TABLE 3. Results of $CuCl_2$ (unknown #852) fortified quality assurance blind samples analyzed concurrently with each laboratory's samples. Results are presented as mg/ℓ of the tracer fluorescein.

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #1			
0	0.50	0.41	82
0	1.50	1.44	96
8	0.50	0.39	78
8	1.50	1.43	95
15	0.50	0.41	82
15	1.50	1.45	97
21	0.50	0.41	82
Laboratory #2			
0	0.50	0.47	94
0	0.50	0.47	94
5	0.50	0.57	114
5	1.50	1.59	106
12	0.50	0.47	94
12	0.50	0.49	98
21	0.50	0.45	90
Laboratory #3			
0	0.50	0.53	106
7	0.50	0.52	104
14	0.50	0.45	90
21	--[a]	--	--

TABLE 3. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #4			
0	0.50	0.55	110
0	1.50	1.15	110
6	0.50	0.48	96
6	1.50	1.59	106
13	0.50	0.49	98
21	--[b]	--	--
Laboratory #5			
0	0.50	0.40	80
0	1.50	1.46	97
7	1.50	1.44	96
14	0.50	0.42	84
14	1.50	1.43	95
21	0.50	0.55	110
21	1.50	1.65	110
Laboratory #6			
0	0.50	0.47	94
0	0.50	0.47	94
7	0.50	0.46	92
7	0.50	0.55	110
14	0.50	0.45	90
14	0.50	0.45	90
21	0.50	0.45	90

TABLE 3 . (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #7			
0	--[a]	--	--
7	1.00	1.10	110
14	1.00	0.69	69
21	0.50	0.64	128
Laboratory #8			
0	0.50	0.53	106
8	0.50	0.47	94
15	0.50	0.45	90
15	0.50	0.46	92
20	0.50	0.45	90
20	0.50	0.45	90
Laboratory #9			
0	0.50	0.45	90
7	0.50	0.40	80
7	1.50	1.45	97
14	0.50	0.39	78
14	1.50	1.42	95
21	0.50	0.50	100
21	0.50	0.52	104

TABLE 3. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #10			
0	0.50	0.55	110
0	1.50	1.65	110
7	0.50	0.58	116
7	1.50	1.65	110
14	0.50	0.49	98
21	0.50	0.49	98
Laboratory #11			
0	0.50	0.56	112
7	0.50	0.51	102
7	1.50	1.59	106
14	1.50	1.57	105
21	--[a]	--	--

[a]No samples analyzed

[b]No samples provided

Mean and standard deviation - 97±11

(n=62)

TABLE 4. Results of $CuCl_2$/GAA (unknown #124) fortified quality assurance blind samples analyzed concurrently with each laboratory's samples. Results are presented as mg/ℓ of the tracer fluorescein.

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #1			
0	0.50	0.41	82
0	1.50	1.43	95
7	0.50	0.41	82
14	1.50	1.44	96
21	0.50	0.41	82
21	1.50	1.46	97
Laboratory #2			
0	0.50	0.49	98
0	1.50	1.56	104
8	1.50	1.56	104
14	0.50	0.49	98
14	1.50	1.56	104
21	0.50	0.49	98
Laboratory #3			
0	0.50	0.50	100
0	1.50	1.58	105
6	0.50	0.50	100
6	1.50	1.60	107
13	0.50	0.50	100
21	1.50	1.59	106

TABLE 4. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #4			
0	0.50	0.51	102
0	1.50	1.47	98
7	0.50	0.51	102
7	1.50	1.46	97
18	0.50	0.47	94
21	0.50	0.51	102
21	1.50	1.46	97
Laboratory #5			
0	0.50	0.49	98
0	1.50	1.58	105
7	0.50	0.46	92
7	1.50	1.57	105
14	0.50	0.51	102
14	1.50	1.58	105
21	0.50	0.52	104
21	1.50	1.62	108
Laboratory #6			
0	0.50	0.47	94
0	1.50	1.60	107
7	0.50	0.48	96
7	1.50	1.60	107
14	1.50	1.60	107
21	0.50	0.49	98
21	1.50	1.54	103

TABLE 4. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #7			
0	1.00	1.20	120
7	1.00	1.10	110
14	0.50	0.38	76
14	1.50	1.43	95
21	0.50	0.41	82
21	1.50	1.42	95
Laboratory #8			
0	0.50	0.47	94
7	0.50	0.49	98
7	1.50	1.57	105
14	1.50	1.57	105
14	0.50	0.47	94
21	1.50	1.58	105
Laboratory #9			
0	0.50	0.48	96
0	1.50	1.59	106
7	0.50	0.47	94
7	1.50	1.59	106
14	0.50	0.49	98
14	1.50	1.60	107
21	1.50	1.58	105

TABLE 4. (Cont.)

Day	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
Laboratory #10			
0	1.50	1.59	106
0	0.50	0.50	100
7	0.50	0.49	98
7	1.50	1.56	104
14	0.50	0.51	102
14	1.50	1.57	105
21	0.50	0.51	102
21	1.50	1.60	107
Laboratory #11			
0	0.50	0.48	96
0	1.50	1.58	105
7	0.50	0.47	94
7	1.50	1.58	105
14	0.50	0.47	94
14	1.50	1.59	106
21	--[a]	--	--

[a]No samples provided

Mean and standard deviation - 100±7
(n=74)

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