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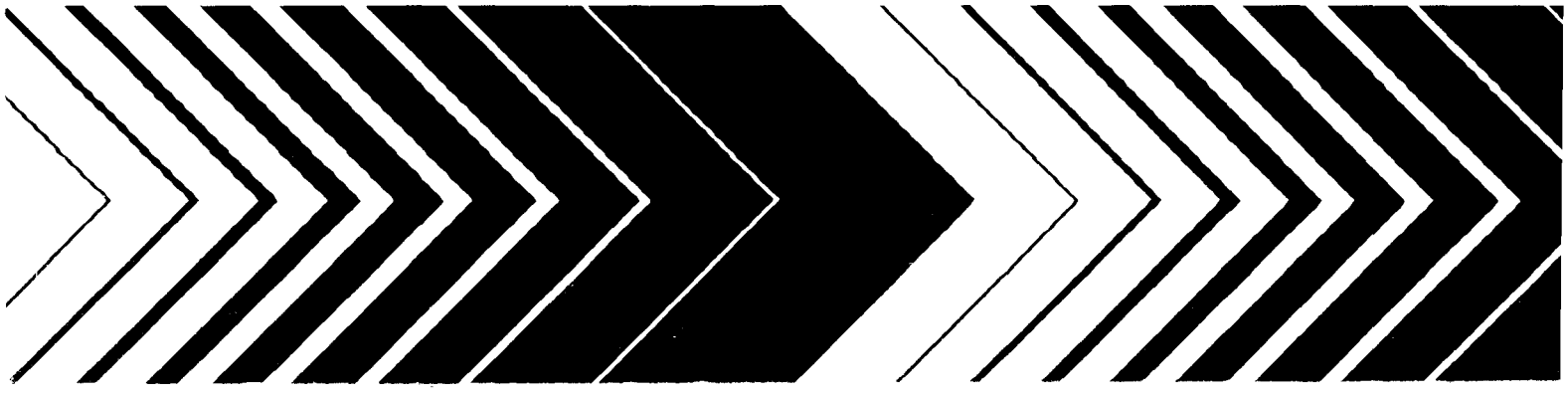
User's Guide:

Procedures for Conducting *Daphnia magna* Toxicity Bioassays



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PROCEDURES FOR CONDUCTING *DAPHNIA MAGNA* TOXICITY BIOASSAYS

Prepared for the Office of Solid Waste

by

Kenneth E. Biesinger
Environmental Research Laboratory
Duluth, Minnesota 55804

and

Llewellyn R. Williams
Environmental Monitoring Systems Laboratory
Las Vegas, Nevada 89114

and

William H. van der Schalie
U.S. Army Medical Bioengineering Research
Fort Detrick, Frederick, Maryland 21701-5010

ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
LAS VEGAS, NEVADA 89114

NOTICE

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FOREWORD

The procedures reported herein for conducting Daphnia magna toxicity bioassays were successfully evaluated in a collaborative study involving eleven government, industry, and academic laboratories with four toxicants. Results of this study were reported at the annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC) in November 1985 and were reported by Williams et al., 1986.

ABSTRACT

A standardized protocol has been developed to provide guidance for conducting acute (death or immobility) and chronic (survival and reproduction) toxicity of solid waste leachates to Daphnia magna. The method with slight modifications is applicable for testing toxicants in general. Acute test results are reported as a 48-hour EC_{50} (concentration at which 50 percent of test organisms are killed or immobilized after 48 hours of exposure) with 95 percent confidence intervals. Chronic test results are reported as 21-day LC_{50}s (concentration at which 50 percent of test organisms were killed during 21-day exposures) with 95 percent confidence limits, the lowest concentration at which there was a significant (95 percent confidence interval) effect on reproduction and the highest concentration at which there was no significant effect.

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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 (21 days for Daphnia) effects such as changes in growth, reproduction, mutations, or death.

LC_{50}: a statistically estimated toxicant concentration at which 50 percent of exposed organisms would be killed at a specific time of observation; for example, 48-hour, 7-day, 14-day, or 21-day LC_{50}s for Daphnia.

EC_{50}: a statistically estimated toxicant concentration at which a specific response (i.e., death or immobilization) would be elicited in 50 percent of exposed organisms at a specific time of observation; for example, 48-hour EC_{50} 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 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 percent confidence intervals for toxicity data.

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

Number of young: the total number of young that were produced during the test period by those females that remained alive at the end of a chronic test.

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

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Dr. B. Adams, Monsanto Industrial Chemicals Co.
Dr. K. Biesinger, EPA/ERL-Duluth
Dr. A. Buikema, Virginia Polytechnical Institute
Mr. S. Ells, EPA/OTS Washington, D.C.
Dr. P. Feder, Battelle Columbus Laboratories
Dr. C. Goulden, Academy of Natural Sciences, Philadelphia
Dr. K. Keating, Rutgers University
Mr. T. Kimmel, EPA/OSW Washington, D.C.
Mr. G. LeBlanc, EG&G Bionomics
Dr. M. Lewis, Proctor and Gamble Co.
Dr. A. Maki, Exxon Corporation
Mr. B. McAllister, Analytical Bio-Chemistry Laboratories
Dr. C. Muska, E.I. Dupont DeNemours and Co.
Dr. A. Nebeker, EPA/ERL-Corvallis
Mr. M. Palmieri, Allied Chemical Corp.
Mr. B. Parkhurst, Western Aquatics, Inc.
Mr. J. Pearson, EPA/EMSL-Las Vegas
Dr. K. Porter, University of Georgia
Dr. W. van der Schalie, USAMBRDL, Ft. Detrick
Dr. C. Weber, EPA/EMSL-Cincinnati
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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 percent during the 14 days prior to testing. Culturing and testing are conducted at a constant temperature of 20 ± 1°C with a 16 hour photoperiod. Daphnids are cultured and tested in hard reconstituted water for dilution (American Public Health Association et al., 1980) and fed trout food and Selenastrum capricornutum.

A 48-hour 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 using a control and 1, 10, and 100 percent of the leachate or effluent being tested (or a wide range of concentrations when testing other forms of toxicants). Five <24-hour-old Daphnia magna in 80 mL of solution are used in each 100 mL beaker at a test concentration. A 48-hour static acute test is also conducted using five <24-hour-old Daphnia per 80 mL of solution in a 100 mL beaker. Five or more concentrations and a control (plus an acetate or alternative solvent control, if needed) are tested in quadruplicate (i.e., there are 20 daphnids per experimental condition). The daphnids are tested unfed. Immobilization or death is recorded at test termination, and a 48-hour EC_{50} concentration is calculated. The beakers in both tests are covered with glass to minimize evaporation.

The 21-day chronic test is conducted using ten 100-mL beakers, each of which contains one <24-hour-old daphnid in 80 mL of solution, at each concentration tested. The acute EC_{50} values are used as the basis for selecting the test concentrations to use in the chronic test. The solution is changed, and endpoints are recorded three times weekly (Monday, Wednesday, and Friday). 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 5 mg/L of trout food which must be obtained from the Environmental Research Laboratory in Duluth, Minnesota, plus 10^8 cells/L (10^5 cells/mL) of Selenastrum capricornutum three times weekly (Monday, Wednesday, and Friday). Endpoints determined for each test include survival and the number of young produced by each female that is alive at the end of the test period. An optional endpoint is the length of those adult daphnids that are 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.

The trout food for these tests must be obtained from the Environmental Research Laboratory-Duluth (ERL-D) where it has been tested (see Appendix G-2).

GENERAL CULTURE PROCEDURE FOR BROOD STOCKS

Daphnia magna may be cultured in 2,000-mL glass containers, each having 20 daphnids per 1,600 mL of hard (160-180 mg/L $CaCO_3$) reconstituted water (American Public Health Association et al., 1980; also see Appendix A). The culture must be maintained at 20 $\pm$ 2°C in a constant temperature bath (or in an incubator or room regulated to narrow temperature tolerances) and exposed to a 16-hour photoperiod. The *Daphnia* must be transferred to fresh water weekly (minimum) and must be fed 5 mg/L of trout food (Appendix B) plus 10^8 cells/L (10^5 cells/mL) of *Selanastrum capricornutum* (Appendices C and D) each Monday, Wednesday, and Friday (this number of cells is equivalent to about 1.8 mg dry weight of *Selanastrum*). At the time of transfer, only adults are transferred; the young are disposed of or used to initiate additional cultures. The young from the second to sixth broods from these adults are used to start new cultures each week. When the adults are 4 weeks old, they are disposed of. If new cultures are initiated every 7 days there will be a continuous source of animals ready for acclimation. Maintaining cultures by this method minimizes overcrowding, male production, ephippia formation, and population "crashes." It also helps to control bacteria and fungi.

For transferring *Daphnia* use pipettes that have an inside diameter at least 1.5 times the size of the animals. Care must be taken not to bump or bruise the daphnids while transferring them to new media; they must be introduced below the surface of the new medium 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. Their health is indicated by: survival; absence of

floaters; absence of ephippia; large size of adults; dark coloration; absence of external parasites; and presence of large numbers of young (three or more young per female per reproductive day). 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 (i.e., when the medium is changed).

Methods

Offspring (<24 hours old) of the adults set aside for acclimation must be placed in culture chambers and must be 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 (not sealed) to minimize evaporation. Daphnids must be transferred into clean containers every Monday, Wednesday, and Friday, when the medium is changed, by using a fire-polished pipette; all transferring must be done under the water surface. Note the survival of the test animals 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 medium is changed. The young used for starting an experiment must come from the second to the sixth broods.

Containers

One-hundred-milliliter glass containers (beakers are usually used) that contain 80 mL of the dilution water (medium).

Replication

A sufficient number of replicates to assure that enough healthy young daphnids are available to begin a test.

Aeration

Must not be used.

Cleaning

All glassware must be scrubbed with a 1-percent solution of Liquinox or another non-phosphate detergent, rinsed with tap water until sudsing has ceased, and then rinsed three more times with tap water. The glassware is then rinsed three times with distilled water, once with 10 percent HNO_3, once with distilled water, once with acetone, and six more 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. Do not exceed a range of light intensity from 30 to 100 foot candles. Close regulation in the range of 50 to 70 foot candles is recommended to stabilize growth rates of the live alga Selenastrum capricornutum used as feed for the daphnids.

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 should be measured with a maximum-minimum thermometer.

Water Quality Measurements

Hardness, alkalinity, and pH measurements must be made on each batch of water used.

pH

The pH must be between 6.8 and 8.5.

ACUTE TESTS

SPECIFIC PROCEDURES

All data will be recorded by using the form provided in Appendix F (1 and 2).

Organisms

Young daphids 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 (<24-hour-old) Daphnia in the chambers and subject them to test conditions for 48 hours. Daphnia must be transferred with a fire-polished pipette (with an inside diameter about 1.5 times the size of the daphnids) 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 (effluents or toxicants) must be stored at 4°C in the dark but must be allowed to come gradually to 20 ± 1°C before adding daphnids. Dilutions are made in volumetric flasks and are then poured into the test beakers.

Dilution Water

Dilution water must be the same as the acclimation and culture water.

Controls

Controls must be set up and treated the same as test solutions, with regard to experimental conditions, except that no leachate (toxicant, etc.) is added. No more than 10 percent 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 percent mortality may occur in 48 hours among acetate-control daphnids for the test to be valid. (If other 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 percent, 50 percent, etc.) or greater (0.75, e.g., 100 percent, 75 percent, 56 percent, etc.) must be used for 48-hour tests. The highest concentration to test may be determined by a 48-hour screening test using order of magnitude leachate (effluent) dilutions (i.e., 100 percent, 10 percent, and 1 percent), 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 the appropriate concentrations to use in the 48-hour acute test. For example, if all animals die at 100 percent of the leachate and if no animals die at 10 percent, then the following concentrations should be tested for 48-hour acute tests: 100 percent, 50 percent, 25 percent, 12.5 percent, and 6.25 percent. (See Standard Methods [APHA, 1980] for suitable toxicant concentrations.)

Randomization

Daphnids are transferred randomly from the acclimation stock to beakers containing the appropriate experimental conditions. The beakers are, in turn, placed at random locations in a water bath or a controlled temperature incubator or room.

Replication

Four containers, each containing five daphnids (for 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 must be rinsed with tap water. Because most leachates are unknown mixtures, a 10 percent nitric acid rinse which is followed by both a distilled water and an acetone rinse and then is followed in turn by at least three distilled water rinses is required. Test containers and flasks should have an additional rinse with the dilution water to be used for testing just before a test is started. (If testing only inorganic toxicants, acetone is not needed; if testing only organic toxicants, nitric acid is not needed.)

Light and Photoperiod

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

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 continuously.

Water Quality Measurements

Hardness and alkalinity must be measured in the high and low concentrations and in the control at 0-hour. Dissolved oxygen and pH measurements must be made at 0 and 48 hours in the high, middle, and low concentrations, and the control. Control concentrations for hardness, alkalinity, and pH of the hard reconstituted water should be as follows: 170 $\pm$ 10 mg/L $CaCO_3$, 115 $\pm$ mg/L $CaCO_3$; and 7.6-8.5, respectively (American Public Health Association et al., 1980); dissolved oxygen must be from 90 to 100 percent saturation at the time the test is started.

pH

The pH of the test water must be from 7.6 to 8.5. If the pH of the leachate (toxicant, etc.) is initially between 6.8 and 8.5, no adjustments are required. If not, the pH of each concentration tested must be adjusted by using sodium hydroxide to raise the pH to $\geq$6.8 or by using hydrochloric acid to lower the pH to $\leq$8.5. The pH must be measured and, if needed, adjusted just prior to beginning the acute test.

Leachate Measurements

Test solutions of leachates or effluents 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 dilution; if not, either conductivity or total organic carbon may be used. Concentrations of known individual toxicants must be measured directly.

Test Apparatus

Test equipment should consist primarily of high grade borosilicate glass or stainless steel. Fluorocarbon and high-density polyethylene equipment is acceptable if the toxicant tested is an inorganic chemical or mixture. 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-hour-old daphnids will be available for testing.

2. Prepare test solutions by adjusting the temperature to 20 $\pm$ 1°C and by 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; therefore, the time must be recorded.

6. Randomly add <24-hour-old daphnids into each beaker until each beaker contains five daphnids. This should be accomplished in less than one hour.

7. Randomly place control and test concentrations into rows; then randomly place the beakers within each row and place a glass cover on each.

8. At the end of 24 and 48 hours, count and record the number of immobilized or dead Daphnia per beaker.

9. Measure dissolved oxygen, pH, hardness, and alkalinity of the control and of 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 either directly or indirectly at the beginning and at the end of the test.

11. Calculate the 48-hour EC_{50} and its 95 percent confidence limits unless 100 percent of the leachate or effluent is nontoxic. If when testing individual toxicants the highest concentration is not toxic, repeat the test unless the solubility in water is exceeded.

STATISTICAL EVALUATIONS

An acceptable EC_{50} test will have at least two test concentrations where the number of immobile (dead) animals bracket 50 percent unless there is less than 50 percent response in the 100 percent solution or at the solubility limit of the toxicant in water. If the lowest test concentration results in excess of 50 percent response, the test must be repeated.

The analysis of the data must include:

1. A preliminary scatterplot of the response rates (number of dead or immobile/48 hour) observed in each test or control beaker, versus group number and percent of logarithm of concentration, to look for patterns of response and outlying beakers.

2. EC_{50} estimates based on the responses in the treatment groups, unless they cannot be calculated for the reasons stated previously. EC_{50} estimates should be accompanied by estimates of their standard errors

and of their 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 EC_{50}.

3. The results of outlier tests, as described below, and the preliminary scatterplot should be used to detect outlying beakers (clearly atypical) within a treatment or control group.

4. If the results from one or more beakers are determined to be outliers, then EC_{50} estimates, standard errors, and confidence intervals will be calculated both by including and by 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 will be discarded as appropriate, and the analyses will proceed. If the outlying values are not the obvious result of 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 that value, and both sets of estimates will be reported. 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 EC_{50} 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. Use of any other method of estimation must be justified by citing generally accepted references in which the estimation method is described and recommended for similar testing situations.

CHRONIC STATIC-RENEWAL TESTS

Specific Procedures

All data wil be recorded by using the form in Appendix F (3 and 4).

Organisms--

Test animals must come from a healthy culture and must be raised under controlled acclimation 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 less than 24 hours prior to starting a test.

Food and Feeding--

Trout 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 (<24-hour-old) daphnids 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 (plate or watchglass) to minimize evaporation and to keep out debris. Daphnids must be transferred into clean containers every Monday, Wednesday, and Friday when the medium is changed by using a fire-polished pipette (with the inside diameter about 1.5 times the size of the animal being transferred); all transferring of organisms must be done under the surface of the water. Survival of the test organisms must be noted each time the medium is changed. Reproduction must be noted by counting the number of 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 (or effluents to be tested) must be stored at 4°C in the dark but must be allowed to come to 20 $\pm$ 1°C before adding daphnids. Dilution of the leachate or toxicant solution and the mixing of food 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 adding food and toxicant (test) solution to clean beakers and then transferring adult daphnids to the resulting test cells. Daphnids must be added within one hour after the test solutions have been prepared.

Dilution Water--

Dilution water must be the same as that used for culturing.

Controls--

Controls must be set up and treated the same as test containers with regard to experimental conditions except that no leachate (toxicant, etc.) is added. Control animals must produce a minimum average of 40 young in 21 days for the experiment to be valid. At least 80 percent of the adults must survive in the control water for the 3-week test period for the test to be valid.

Acetate Controls--

Acetate controls must be run in addition to water controls whenever acetate is used in generating solid waste leachate for testing. The acetate concentration in the control and all concentrations tested must be the same as that in the highest leachate concentration. At least 80 percent of the adults must survive for 21 days in the acetate control solution for the test to be valid. If other solvents are used, the same procedure (solvent control testing) is applicable.

Concentrations--

The test design should include at least five concentrations of test material (toxicant) made up in a geometric progression with a dilution factor of 0.5 (e.g., 100 percent, 50 percent, 25 percent, etc.) or greater (100 percent, 75 percent, 56.25 percent, etc.). Initial concentrations selected

for testing should bracket (i.e., above and below) previous results or should be based on results from acute tests (let the highest test concentration equal the 48-hour EC_{50}).

Randomization--

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

Replication--

Ten containers, each containing one daphnid (a total of ten animals), are required for each experimental condition.

Aeration--

Must not be used.

Cleaning--

All glassware must be cleaned as follows: scrub with a 1 percent solution of Liquinox or other suitable 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, once with 10 percent HNO_3, once again with distilled water, once with acetone, and finally six more times with distilled water.

Light and Photoperiod--

Fluorescent light bulbs must provide a color rendering index ≥ 90 with a closely controlled 16-hour photoperiod. A light intensity of 30 to 100 foot candles must be used. The narrower range of 50 to 70 foot candles may be desirable because green algae acclimated to low light conditions can photosythesize at a rapid enough rate toward the upper end of the allowable range that pH levels increase to or beyond the recommended pH range. If that occurs, raise or mask the light source to reduce effective illumination until the pH range stabilizes within the recommended limits.

Temperature--

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

Water Quality Measurements--

Hardness, alkalinity, pH, and dissolved oxygen measurements must be made for the high and low concentrations and the control when solutions are prepared. Dissolved oxygen and pH measurements must be made in the high, middle, and low concentrations after transfer of daphnids on 2-3-day-old solutions. 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 (i.e., set up additional

controls once or twice during the experiment specifically for checking dissolved oxygen).

pH--

The pH of the leachate (or toxicant, etc.) to be used in testing must be adjusted (so that the resulting test solution is within the recommended range) 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 test solutions must be measured and adjusted prior to the beginning and just before each renewal for chronic tests. If the pH is initially between 6.8 and 8.5, no adjustments are required.

Leachate (Effluent or Toxicant Solution)--

Test solution concentrations should be measured either directly or indirectly. If leachates or effluents 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. Individual toxicant concentrations must be measured directly.

Test Apparatus--

Test equipment should consist primarily of high grade borosilicate glass or stainless steel. Fluorocarbon and high density polyethylene equipment are acceptable if the chemical or mixture being tested is inorganic in nature. Equipment constructed with rubber or plasticized materials that could contact tests solution 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-hour-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.

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

7. Randomly place control and test concentration beakers into rows, and place beakers in random positions within each row; then cover with glass, and record the time.

8. Every Monday, Wednesday, and Friday:

    a. Count number of dead or immobilized adults.

    b. Mix fresh test solutions containing food for each experimental condition.

    c. Pour test solutions into clean beakers and transfer daphnids.

    d. Count number of young per surviving female.

    e. Discard dead adults and all young.

9. Measure dissolved oxygen, pH, hardness, and alkalinity when the experiment is set up and measure dissolved oxygen and pH on 2- or 3-day-old samples. Perform sufficient (a minimum of six times during an experiment) measurements on subsequent set-ups to characterize these parameters.

10. Record and evaluate adult mortality and young per female for animals living 21 days by using the appropriate statistical procedures. As an option, measure the length of Daphnia surviving 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 body lengths may be presented at the discretion of the investigator. Analyses of reproduction and growth (length) responses will be carried out only on those daphnids that survive to the end of the test.

For analysis of the mortality results, a distinction will be made between toxicant-related and accidental mortality. The causes, if known, of all accident-related deaths will be documented. Accident-related deaths per treatment level must not be >20 percent of the daphnids tested. Final (21-day) mortality results will be adjusted for accident-related mortality by disregarding those daphnids that died (i.e., those daphnids are excluded from both the numerator and the 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-observed effect concentration (NOEC) and of a statistically significant effect concentration. The NOEC 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 effect concentration is the next highest concentration.

Estimates of the LC_{50} for toxicant-related mortality, along with associated standard errors and confidence intervals, will be reported.

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, p, will be transformed by the arcsine variance-stabilizing transformation to [arcsin /r/n+1 + arcsin /(r+1)/(n+1)] for small sample sizes. The transformed proportions will be tested for equality by a one-way analysis of variance. 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 by the Bonferroni t procedure (Miller, 1966) to determine which treatment groups have significantly different mortality rates (at the 95 percent confidence level) from the control group.

The LC_{50} and LC_{10} 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 is appropriate for estimating the LC_{50}.

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 the total number of offspring per female for animals living 21 days. 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 will be examined for suspected outliers. If these records are found to contain clerical or experimental errors leading to erroneous values, the errroneous values will be corrected or discarded and the analysis will proceed. If the outlying values are not the obvious result of 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 susequent analyses will be carried out both with and without the response, and both sets of estimates will be reported. 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, use a log transformation. 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 by the Bonferroni t procedure (Miller, 1966) or by the Kruskal-Wallis rank-sum-based procedure (Hollander and Wolfe, 1973) to determine which treatment groups have significantly different response rates (at the 95 percent level) from the control group.

CONFIDENCE INTERVALS AND AFTER-THE-FACT POWER CALCULATIONS

The determination of NOECs and statistically significant concentrations does not impart information about the sensitivity of the inferences, i.e., an insensitive test might not reveal statistically significant differences in group average responses even when the differences are clearly of biological significance.

After-the-fact power calculations can be carried out to determine how large a treatment response must be before it can be statistically differentiated from the control response with high probability. Power calculations for length and productivity responses will be based on the noncentral t distribution when adjustment is made for multiple comparisons by Bonferroni's method. Power calculations for mortality responses will be based on 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 which account for multiple comparisons and for possibly heterogeneous variances will be based on the t-distribution. Confidence intervals for mortality responses will be based on the Poisson approach (Feder, 1981; Nelson, 1970) and will account for multiple comparisons.

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 so that an EC_{50} can be determined. If calculating an optional LC_{50}, the daphnids that are immobile must be carefully transferred with a glass pipette into a petri dish or watch glass. Using a 30X dissecting microscope, observe each daphnid individually for heartbeat. Absence of a heartbeat will denote a dead daphnid and will provide data for the determination of an LC_{50}.

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 by counting them. An automatic counter is not recommended as this will count food particles, etc., which are similar in size to the daphnids.

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.

A 21-day LC_{50} and the number of young per female for animals surviving 21 days must be reported. The lowest concentration causing an effect on reproduction at the 95 percent confidence level must be reported and will constitute the toxic concentration. The next lower concentration will constitute the no-significant-effect (NOEC) concentration at the 95 percent 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 effluent or toxicant, including its source and any physical and chemical properties known.
- A description of the extraction procedure used if testing a leachate.
- The chemical characteristics of the dilution water.
- The scientific name and the source of the test organism.
- A description of the test procedure.
- The methods used for measuring hardness, alkalinity, dissolved oxygen, pH, and temperature, and the results of these measurements.
- Direct or indirect measurements of leachates, effluents, or toxicants.
- Methods used for all chemical analyses.

For acute test results:

- A description of the endpoint used and of the results of the statistical analyses conducted.
- The percent of organisms that survived in each experimental solution.
- An EC_{50} value and the 95 percent confidence limits.
- The methods used for statistical analyses of the data.

For chronic test results:

- The number of mortalities and effects observed in controls.

- A nonsignificant and a significant effect concentration at the 95 percent confidence level for the number of young produced by each female that survived 21 days.
- A 21-day LC_{50} with 95 percent 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
   - $NaHCO_3$
   - $CaSO_4.2H_2O$
   - $MgSO_4$
   - 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 percent solution of nitric acid. Slowly pour out acid solution into cold running water. Rinse carboy thoroughly with deionized distilled water at least five 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 $CaSO_4.2H_2O$

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

2.28 g $MgSO4$

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, and mix 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. To assure complete mixing of chemicals and saturation with dissolved oxygen, stir with the lid removed (but covered with a foam plug or glass wool) for 24 hours using a magnetic stirrer.

5. Measure hardness, alkalinity, dissolved oxygen, and pH. The hardness must be from 160-180 mg/l $CaCO_3$; the alkalinity from 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.

6. Reconstituted water may be stored and used for one month.

APPENDIX B

DAPHNIA TROUT FOOD PREPARATION

- Add 15 grams of trout food (obtained from the Environmental Research Laboratory-Diluth) 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 1 mL of dry solids. The final concentration must be 5 mg dry solids per mL of food, and 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 prepared Daphnia trout food in refrigerator for up to one week.

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 $\pm$ 10°C to 24 $\pm$ 10°C

2. Lighting continuous "cool-white" fluorescent light from 4000 $\pm$ 10 percent to 5000 $\pm$ 10 percent lumens; photoperiod from 14L:10D to continuous lighting.

3. The cultures must be maintained sterile in a chemostat (flow-through) system or have continuous aeration and must be 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 percent 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 percent HNO_3, rinse with distilled water, 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 0a*

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
$CaCl_2$	
$CaCl110.2H_20$	36.76
$MgSO_4.7H_20$	36.97
$NaHCO_3$	12.60
$KI2HPO_4$	8.71
$NaNO_3$	85.01
$Na_2SiO_3.9H_20$	28.42**

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	$FeCl_3.6H_2O$	1.575††
3	$CuSO_4.5H_2O$	0.01
	$CoCl_2.6H_2O$	0.01
	$ZnSO_4.7H_20$	0.022
	$MnCl_2.4H_20$	0.18
	$Na_2MoO_4.2H_20$	0.006
	H_3BO_3	1.0

*0a 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.
**Filter sterilize this stock solution and add 1 mL to the culture medium after autoclaving by making use of a sterile technique.
†Stock must be less than 3 months old.
††Use 2 mL/L of 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, other than sodium silicate, 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, add one milliliter of each macronutrient stock (except sodium silicate) and one milliliter (2 mL of $FeCl_2$) of each micronutrient stock to ~900 mL of glass-distilled water. Add nutrients in the order given in 1 and 2 above, stir between each addition, and then make up to a liter with glass-distilled water. Place one liter of medium in a 2-L Erlenmeyer flask 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 O_2 (15 psi) and 1210°C for 15 minutes. Allow to come to room temperature. Add 1 mL $Na_2SiO_3.9H_2O$ stock by using a sterile technique.

5. For agar slants and petri plates, prepare medium as above but, in addition, dissolve 1 percent (w/v) agar (DIFCO Bacto-Agar or equivalent) prior to autoclaving. Place agar solution into test tube for slants; tilt after removal from autoclave but before the agar has jelled. For obtaining uncontaminated algal cultures, pour autoclaved solution into sterile petri plates by using a 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 new agar slants by transfer from uncontaminated 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 uncontaminated 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 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).

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 should be continuously aerated and stirred with a magnetic stir-bar or should be 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 can be removed for use and can be replaced with fresh media 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 hemacytometer counting cell. Enough sets (having 16 squares each) are scanned 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 by using the following formula:

$$\frac{\text{(No. of cells counted) x (4 x 1006)}}{\text{No. of squares counted}} = \text{No. of cells/mL}$$

The number of milliliters needed to get 1008 cells is determined by dividing 1008 cells by the number of cells per mL in the culture. The volume (mL) thus determined is then measured out, placed in centrifuge tubes, and centrifuged at 2,200 RPM (700 x g) for 15 minutes. The algae media is then carefully poured off, and ten milliliters of reconstituted water is added to resuspend the algae (i.e., 10 mL will then contain approximately 10^8 Selenastrum cells). The resuspended Selenastrum is then added to volumetric flasks containing approximately 950 mL of leachate (test solution), 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; it is now ready for dispensing into the test chambers. Algae suspended in centrifuge cells may be stored in the dark at 40°C for 10 to 12 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 x g for 15 minutes is suggested). Pour off the supernatant and leave behind as little of the algal medium as possible. Resuspend the algae in a small amount of the same solution used for culturing the daphnids about to be fed. Remove a small portion from the algal solution 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:

$$\text{Cells/mL} = \text{(cell count) (10,000)}\ \text{(25/the number of double-lined fields counted)}\ \text{(dilution factor)}$$

Dilute the algal solution with the appropriate daphnid culture medium so that milliliter of the resulting dilution, 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 10 to 12 days after harvest.

METHOD 3

A particle counter may be used for counting algal 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 algal stock or your culture technique. Common problems include errors in media preparation or heavy contamination with some other organisms 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 should be 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*
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
Carboys	5 gallon plastic w/spigot
Fluorescent lights (algae and daphnid maintenance)	"Cool-white" for algae "Grown-Lux" and "Vita-Life" for daphnids
Light table	Model GB 11-17 30 watts "Glow Box"
Light meter	Model No. 200
Dissolved oxygen meter	Model 0260 Oxygen Analyzer
pH meter	0-14 pH units + 1/10 pH
Compound microscope	

*Or equivalent

Appendix E, Equipment (continued)

Dissecting microscope	15 x W.F., Cat. 147
Equipment	Model Specifications
Micrometer	0.01 or 0.001 inches at 4X
Hemacytometer	
Centrifuge	Model Pr-2 1000 x g force
Membrane filter apparatus	
Autoclave or pressure cooker	
Drying oven	Temperature capability 1000°C
Dishwasher	L/A-7537 glassware washer
Balance	Accurate to 0.0001 gram

APPENDIX F

STATIC ACUTE TOXICITY TEST

FORMS

FORM ____

STATIC ACUTE TOXICITY TEST

TEST MATERIAL _____

	Date									
	Time									
	Control Temp									
	Data By									
	REP	CONC	DO	pH	HRD	ALK	DO	pH	HRD	ALK

(continued)

FORM ____

STATIC ACUTE TOXICITY TEST (Continued)

TEST MATERIAL _____

STOCK CONCENTRATION _____ SOLVENT _____

PREPARED BY _____ DATE PREPARED _____

TEST CONCENTRATIONS mg/L ug/L	HIGH ----> LOW								
	CONT.	S. CONT.							
Amount of Stock Added									
Amount of Dilution H_2O Added									

(continued)

FORM ____

STATIC ACUTE TOXICITY TEST

TEST MATERIAL _____ _____

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 ug/L	OBSER.	OBSER.	OBSER.	OBSER.	OBSER.	NO. DEAD	OBSER.	NO. DEAD	OBSER.	NO. DEAD	OBSER.	NO. DEAD		OBSER.	NO. DEAD	OBSER.	NO. DEAD	OBSER.	DEAD	OBSER.	NO. DEAD	
Control																						

(continued)

FORM ____

STATIC ACUTE TOXICITY TEST (Continued)

TEST MATERIAL _____

_____

PRINCIPAL INVESTIGATOR(S) _____

Time Added Test Material/ Daphnids	No. of Daphnids Per Vessel	No. of Replicates Per Treatment Level	Type Test Vessel	
Test Chamber Volume	Total Solution Volume		Solution Volume Per Replicate Test Vessel	Age of Daphnid at Test Initiation (Hours)

Water Quality of Dilution Water

Data Transcribed		Total
Notebook _____	Source _____	Alkalinity _____
Page No.	Batch No. _____	Total Hardness _____
Location _____	pH _____	Conductivity _____

Comments _____

_____

_____

NO 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

_____

_____

_____

FORM ___

CHRONIC TOXICITY TEST

Chronic Toxicity Test — Age of D. magna _____

Test Material _____ Sponsor _____ D. magna Source _____ Brood No. Removed From _____

Treatment Level _____ Principal Investigator(s) _____ Photoperiod/Light Intensity _____

Test Day	Data By	Number alive/Number offspring										Soln. Renew Using Dilu. Wtr. Batch	pH New/Old Soln.	DO (mg/L) New/Old Soln.	Temp (°C) New/Old Soln.
		A	B	C	D	E	F	G	H	I	J				

(continued)

FORM ___

CHRONIC TOXICITY TEST (Continued)

TEST DAY	DATA BY	OBSERVATIONS TEST MATERIAL TREATMENT LEVEL

Total Hardness					Total Alkalinity				
Test Day	Sample Size	N of Titrant	mL Titrant Used	Total Hardness (mg/L $CaCO_3$)	Test Day	Sample Size	N of Titrant	mL Titrant Used	Total Alkalinity (mg/L $CaCO_3$)

SUMMARY

X No. Surviving _____

X No. Young/Surviving Adult _____

X Total Length of Daphnids at Day 21 _____

DO Range New/Old Solns.	pH Range New/Old Solns	Hard. Range New/Old Solns.	Alk. Range New/Old Solns.

Temperature Range

APPENDIX G

QUALITY CONTROL AND QUALITY ASSURANCE

USE OF STANDARDIZED METHODS

Close adherence to standardized procedures for toxicity testing such as those provided in this protocol is a first and critical step in affecting reproducible bioassay data quality. Many quality control features have been incorporated into the protocol; some of these may be transparent to the user. These features are integrated into each of the specific procedures for preparation of materials, food and test organisms, and into the conduct of the testing program.

USE OF GOOD LABORATORY PRACTICE STANDARDS

Laboratories performing toxicology testing should have in place Good Laboratory Practice (GLP) Standards that apply to all phases of the operation, i.e., organization, personnel, facilities, equipment, reagents and chemicals, operating procedures, test and control substances, study plan, records, and reporting. Appropriate Good Laboratory Practice Standards are described in 40 CFR Part 792, Volume 48, Tuesday, November 29, 1983, pp. 53937-53944.

AUDIT GUIDELINES

To assist laboratory managers, quality assurance officers, or researchers in evaluating their own operations, a set of guidelines in the form of a detailed questionnaire has been provided (see Appendix H).

REFERENCE TOXICANT

Another key to establishing and maintaining a system to control the bioassay operation is the routine use of a reference toxicant(s) to determine the current responsiveness of the daphnid test system. Routine use of carefully prepared dilutions of reference toxicant stock solution in 48-hour acute tests provides an excellent mechanism for determining whether test organism response is within the expected range (historical control chart limits) for that laboratory.

Sodium pentachlorophenate has been shown in a number of EPA-sponsored studies to provide highly reproducible toxicity to Daphnia magna. Well characterized solutions of sodium pentachlorophenate will be available on a continuing basis from the Environmental Monitoring and Support Laboratory (EMSL), Cincinnati, Ohio. These solutions will be suitable for dilution to test concentrations and are stable for prolonged periods if maintained in accordance with instructions provided with the stock solutions. For

information on obtaining reference toxicant solutions, call Cornelius Weber at (513) 569-7337, EMSL-CIN, or Llewellyn Williams at (702) 798-2138, EMSL-LV.

"STANDARD" TROUT FOOD

Trout food suitable for use with this protocol is stocked by the ERL-Duluth and is available upon request. A batch is maintained, and portions will be provided from that source so long as the potency of the nutrients is retained, and no deleterious changes (e.g., excessive bacterial or fungal contamination) are noted in the material. The potency and contamination checks will be based upon periodic analyses of the material and on continued performance checks to determine that negative and positive (reference-toxicant-tested) controls are within laboratory historical control limits. In advance of the time that the replacement of the master stock of trout chow at ERL-Duluth is required, additional batches will be procured and tested to assure that they conform with the basic specifications provided by the Fish and Wildlife Service and that they meet performance specifications then current at the ERL-Duluth. The batch selected will be blended and will be properly maintained for future availability and use.

COOPERATIVE ACTIVITIES

Laboratories are urged to cooperate with one another in mutual evaluation of testing performance through such mechanisms as split-sample exchanges to compare system performance on identical materials or independent processing of one another's data sets to assure uniformity in test data interpretation. Regular programs established in recognition of the potential mutual benefits are likely to improve the comparability of data among testing laboratories and to improve the overall quality of the resulting data.

EPA TECHNICAL SUPPORT

Under various EPA programs, occasional test samples (natural, spiked, or synthetic matrices) become available from the EMSLs for distribution to laboratories willing to participate in round-robin performance evaluation studies. Involvement in such a program can be very beneficial to the laboratories involved; each participant has an opportunity to evaluate the performance of his laboratory against that of others using the same procedures.

Based on historical precedent, it is expected that the laboratories of the Office of Research and Development under the aegis of the EPA will continue to provide technical assistance to those testing and contract laboratories attempting to improve their bioassay testing performance by using EPA methods. Call the authors for further information.

APPENDIX H

STANDARD OPERATING PROCEDURE (SOP) FOR AUDITING DAPHNIA ACUTE EC_{50} TEST

The following is an audit guideline in the form of a questionnaire prepared by an expert panel knowledgeable in aquatic toxicity testing and quality assurance. This questionnaire may be used by an organizational quality assurance officer to supplement GLP or QA systems audits of a testing operation or may be used effectively by individual investigators to check critical elements of their own performance. It is intended to go beyond the scope of routine GLP audits. Although the SOP is highly specific for the Daphnia Acute EC_{50} Test, many of the questionnaire elements apply as well to Daphnia static renewal tests used to estimate chronic toxicity.

AUDIT SOP FOR DAPHNIA ACUTE EC_{50} TEST

Section I. Basic Study Information

When possible, the following information should be obtained in advance of the audit being scheduled:

A. Auditor Information:

1. Names(s)/Affiliation: _____
_____
_____

2. Date of Audit: _____ __, 19__

B. Testing Lab Information:

1. Laboratory Name: _____
2. Laboratory Address: _____
_____
_____
_____

3. Laboratory Phone No.: (___) ___ - _____

4. Laboratory Contact: Dr./Mr./Ms. _____

5. Principal Investigators: _____
_____
_____
_____
_____

6. Compound Code or Chemical Tested: _____
_____

7. Physical Description of Compound or Chemical Tested: _____
_____

8. Type of Study: _____

C. Sponsor Information:

1. Sponsor Name: _____

2. Sponsor Address: _____
_____
_____

3. Sponsor Contact: _____

4. Sponsor Phone No.: (___) ___ - _____

II. Water

*1. Was the same water used for culturing as for dilution ___ Yes ___ No

Comments: _____

*2. What was the source of dilution water used?

__ Reconstituted. If reconstituted, was the Marking and Dawson (ASTM) standard used?

___ Yes ___ No. If No, cite reference of other standard used under comments.

Comments: _____

Were reagent-grade chemicals used to prepare the reconstituted water? ___ Yes ___ No

Comments: _____

Was glass-distilled or carbon-filtered deionized water with a conductivity less than microohm/cm used to prepare the reconstituted dilution water?
___ Yes ___ No

Comments: _____

__ Well water

Comments: _____

__ Surface water

Comments: _____

Dechlorinated tap water (specify method of dechlorination under comments).

Comments: _____

If dechlorinated tap water was used, specify the water chemical parameters tested and concentrations measured.

Comments: _____

Parameter	Concentration
__ Particulate matter	_____
__ Total organic carbon	_____
__ Chemical oxygen demand	_____
__ Un-ionized ammonia	_____
__ Residual chlorine	_____

*Asterisked questions are essential and must be addressed during the audit.

II. Water - Continued

__ Total organophosphorous pesticides _____ _____

__ Total organochlorine pesticides _____ _____

__ Polychlorinated biphenyls (PCBs) _____ _____

__ Organic chlorine _____ _____

__ Augmented water Comments: _____

__ Other (identify under comments) Comments: _____

*3. What was the hardness of the dilution water (ppm)? Comments: _____

__ <50 50 to 10

__ >100

III. Daphnia Culturing

*1. What was the average age of the females when their first broods were released? _____ days Comments: _____

*2. What was the reproductive rate of the daphnids for the seven-day period prior to testing? _____ young/adult/day (after release of first brood) Comments: _____

*3. What percentage of the culture stock died during the 48-hour period prior to testing? _____% Comments: _____

*4. Were ephippia produced in the culture? ___ Yes ___ No Comments: _____

*5. What was the initial source of the Daphnia stock? (identify under comments) Comments: _____

*Asterisked questions are essential and must be addressed during the audit.

III. Daphnia Culturing - Continued

*6. How was species identification confirmed?
__ Taxonomic key (cite reference under comments)
__ Specialist confirmation (identify under comments)
__ Other (specify under comments)

Comments: _____

*7. When was the identification of test species last confirmed?
Date: __/__/__

Comments: _____

*8. What food was used to feed culture stock?
__ Algae
__ Synthetic (specify source and batch number under comments)
__ Combination (algae and synthetic
__ Other (specify under comments)

Identify under comments, where applicable, the food organism and source for each food type checked.

Comments: _____

IV. Test Setup: General Procedures

*1. Were glass containers used?
___ Yes ___ No. If No, explain and identify container construction material under comments.

Comments: _____

2. How was equipment that came into contact with the test solutions, test substance or Daphnia cleaned?

Comments: _____

Equipment	Pre-Cleaned	Phosphate-Free Detergent	Acetone Rinse	Acid Rinse	Distilled Water Rinse	Dilution Water Rinse
Exposure chambers	__	__	__	__	__	__
Pipettes/glass tubing	__	__	__	__	__	__
General glassware	__	__	__	__	__	__
Food containers	__	__	__	__	__	__

*Asterisked questions are essential and must be addressed during the audit.

III. Daphnia Culturing - Continued

Test probes	___	___	___	___	___	___
Other equipment (specify under comments)	___	___	___	___	___	___

IV. Test Setup: General Procedures - Continued

Question No. 2. - Continued

Comments: _____

_____

_____

_____

What was the mean of the test temperatures over the entire testing period? _____ C

Comments: _____

_____

_____

4. Were the following acclimation and test conditions the same as the culture conditions?

Comments: _____

_____

_____

Condition	Yes	No	
Photoperiod	___	___	_____
Temperature	___	___	_____
Light Intensity	___	___	_____
Light Source	___	___	_____

(Specify light source under comments.)
If No, identify the difference.

_____

_____

5. What was the test photoperiod?

Comments: _____

_____ hr light

_____

_____ hr dark

_____

*6. How was the test chemical added to the dilution water?

Comments: _____

___ Direct addition

_____

___ Stock solution (if stock solution was used, answer question 7)

_____

_____

*Asterisked questions are essential and must be addressed during the audit.

IV. Test Setup: General Procedures - Continued

*7. How was the stock solution prepared?

__ Deionized water

__ Distilled water

__ Dilution water

__ Solvent (specify under comments). If a solvent was used, answer question 8-10.

Comments: _____

8. What was the highest concentration of solvent used in the test solution? _____ ppm

Comments: _____

9. Was the concentration of solvent in the solvent controls equal to the highest concentration used in the test? ___ Yes ___ No

Comments: _____

10. Did the concentration of solvent differ among exposure chambers? ___ Yes ___ No

Comments: _____

11. Were no more than two Daphnia added to each exposure chamber at one time and placed into each exposure chamber sequentially? ___ Yes ___ No

Comments: _____

12. What was the loading rate used? _____ mL/Daphnia

Comments: _____

13. Were the exposure chambers placed in the testing area in a random manner? ___ Yes ___ No

Comments: _____

V. Test Setup: Definitive Test

*1. What was the basis for selection of the test concentrations?

__ Range finding test

__ Limit of water solubility

__ Upper regulatory limit (cite document under comments)

Comments: _____

*Asterisked questions are essential and must be addressed during the audit.

V. Test Setup: Definitive Test - Continued

__ Other (specify under comments) _____

2. What test concentrations and controls were used? (identify under comments) Comments: _____

3. For each replicate test concentration were separate stock solutions and dilutions prepared? ___ Yes ___ No Comments: _____

4. Were a minimum of 20 Daphnia exposed to each test concentration? ___ Yes ___ No Comments: _____

If No, how many were exposed to each test concentration? _____

5. What was the number of replicate exposure chambers for each test concentration? _____ Comments: _____

6. Did each replicate contain the same number of daphnids? ___ Yes ___ No Comments: _____

7. Did all Daphnia used in the test come from the same stock culture? ___ Yes ___ No Comments: _____

*8. Was the test begun with Daphnia <24 hours old? ___ Yes ___ No Comments: _____

*9. From what brood were the test organisms taken? (specify under comments) Comments: _____

*10. Were immobilized Daphnia left in the exposure chamber for the duration of the test? ___ Yes ___ No Comments: _____

11. Note which of the following water quality parameters were measured in the test solution and at what stage in the test.

Parameter	Test Beginning	Middle of Test	Test End	All Concentrations	Selective Concentrations
Dissolved Oxygen	__	__	__	__	__
Temperature	__	__	__	__	__

*Asterisked questions are essential and must be addressed during the audit.

V. Definitive Test - Continued

pH	___	___	___	___	___
Specific conductance	___	___	___	___	___

Comments: _____

*12. Were any of the following observed during the test?

Parameter	Test Beginning	Middle of Test	Test End	All Concentrations	Selective Concentrations
Surface film	___	___	___	___	___
Precipitate	___	___	___	___	___
Phase separation	___	___	___	___	___
Cloudiness	___	___	___	___	___
Adsorption to exposure chamber walls	___	___	___	___	___
Other (specify under comments)	___	___	___	___	___

Comments: _____

13. What was the lowest dissolved oxygen level measured, expressed as percent saturation? _____% saturation

Comments: _____

VI. Analytical Measurements

1. Were expected test concentrations confirmed? ___ Yes ___ No

Comments: _____

*2. Were standard chemical analytical techniques used to confirm concentrations? ___ Yes ___ No (if Yes, cite reference(s) under comments)

Comments: _____

3. Were expected test concentrations confirmed by methods other than standard chemical analyses? ___ Yes ___ No

Comments: _____

(if Yes, identify method under comments) _____

*Asterisked questions are essential and must be addressed during the audit.

VI. Analytical Measurements - Continued

4. Identify under comments the limit of quantitation of the procedure.

Comments: _____

5. When were the concentrations confirmed and at what test concentrations?

Comments: _____

Time	Concentrations
___ Test beginning	_____
___ Middle of test	_____
___ Test end	_____

6. How many replicate chambers per concentration were analyzed? _____

Comments: _____

*7. What fractions of the test solution were analyzed?

Comments: _____

___ Dissolved

___ Particulate

___ Total

*8. Did the measured concentration of test substance among the replicate exposure chambers in any one test concentration at any given time vary more than 20 percent? ___ Yes ___ No

Comments: _____

9. Specify under comments the highest and lowest percent change in the measured test concentration in each exposure chamber from the beginning to the end of the test.

Comments: _____

10. Specify under comments the highest and lowest mean percent difference between expected and measured test concentrations at time zero.

Comments: _____

VII. Data Analysis

1. At what time during the test were observations of the exposure chambers

Comments: _____

*Asterisked questions are essential and must be addressed during the audit.

VII. Data Analysis - Continued

made for immobilization and abnormal behavior? _____

2. Were greater than 10 percent of either dilution water or solvent controls immobilized? ___ Yes ___ No
Comments: _____

*3. What criteria were used to determine immobilization? (discuss under comments)
Comments: _____

*4. Were Daphnia physically injured during handling included in the data analysis? ___ Yes ___ No
Comments: _____

5. Were partial mortalities on each side of the EC_{50} observed? ___ Yes ___ No
Comments: _____

6. Were 24- and 48-hour EC_{50} response curves and slopes developed for the immobilization data? ___ Yes ___ No
Comments: _____

7. Were concentration-response curves and slopes developed for the immobilization data? ___ Yes ___ No
Comments: _____

8. Was a statistical test of goodness-of-fit performed on the concentration-response curves? ___ Yes ___ No
Comments: _____

9. Identify under comments the statistical method and reference used to calculate 24- and 48-hour EC_{50} values and their respective 95 percent confidence limits.
Comments: _____

VIII. Reporting

The final report contains which of the following information:

___ Name of test(s)
___ Name of sponsor
___ Testing laboratory
___ Name of study director
___ Principle investigator
___ Testing dates

Comments: _____

*Asterisked questions are essential and must be addressed during the audit.

VIII. Reporting - Continued

___ Detailed test chemical description including:

Comments: _____

___ Source
___ Lot number
___ Composition (identify and note concentration of major ingredients and impurities)

___ Physical/chemical properties
___ Carriers or other additives and their concentrations

Comments: _____

___ Dilution water source
___ Chemical characteristics of dilution water (e.g., conductivity, hardness, pH, etc.)
___ Description of any pretreatment

Comments: _____

___ Detailed information on daphnids used as brood stock including:

Comments: _____

___ Scientific name
___ Method of verification
___ Culture method

___ Test chamber description
___ Volume of solution to chambers
___ Description of test start-up (e.g., conditioning, test chemical additions)
___ Test organisms per test chamber
___ Test organism age
___ Replicates per treatment

Comments: _____

___ Concentration of test chemical in each exposure chamber
___ Percent or number of organisms immobilized in each exposure chamber at each observation period
___ Concentration-response curves
___ Goodness-of-fit test results
___ 24- and 48-hour EC_{50} values and 95 percent confidence limits
___ Method of calculating EC_{50} values

Comments: _____

VIII. Reporting - Continued

___ Results of water quality analyses including:

___ Methods
___ Method validations
___ Reagent blanks
___ Spikes

Comments: _____

___ Results of test chemical concentrations including:

___ Methods
___ Method validations
___ Reagent blanks
___ Spikes

Comments: _____

___ Data records of culture, acclimation, and test temperatures.

Comments: _____

___ Data records of culture, acclimation, and test lighting.

Comments: _____

AUDIT SUMMARY

CERTIFICATION OF INFORMATION PROVIDED

I have reviewed the completed audit form and have found the information provided to be true and accurate to the best of my knowledge.

Signature of authorized representative of laboratory being audited	Date
Signature of auditor or audit team leader	Date

GLOSSARY FOR AUDIT SOP

1. Reconstituted Water - Any water where reagent grade chemicals are added to distilled or deionized water.

2. Augmented Water - Any water where reagent-grade chemicals are added to natural waters.

3. Random - Placement of organisms in the exposure chambers and placement of exposure chambers to avoid systematic bias such as differences in light intensity, temperature, swimming ability, etc., of Daphnia.