GREAT LAKES
DREDGED MATERIAL TESTING AND EVALUATION MANUAL

APPENDIX G
BIOLOGICAL EFFECTS TESTING PROCEDURES

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Acid volatile sulfide - Sulfide forms present in sediments which react with divalent metallic cations and render sediment metals unavailable to the surrounding water and biota.

Acute toxicity test - A test to determine the short-term toxicity of a chemical or material to an organism. The test usually measures lethality.

Bioaccumulation - The net accumulation of chemicals by an aquatic organism via direct partitioning from the water plus ingestion of the chemicals with its food items.

Brood board - A sheet of material (e.g., styrofoam) with an array of openings designed to hold culture containers with brood stock of the test species.

Brood stock - Organisms which are or will be utilized as parents for the test organisms.

Chemical bioavailability - The potential of a chemical to be readily partitioned from water or particles into plant and animal tissue.

Chronic toxicity test - A test to determine the toxic effects of a chemical or material of sufficient duration to extend over the entire life cycle of the test organism.

Control sediment - A sediment essentially free of contaminants and compatible with the biological needs of the test organisms such that it has no discernable influence on the response being measured in the test. Performance of the test organisms in the control sediment is evaluated to determine the health of the organisms and the test acceptability.

Dilution factor - The decimal fraction that a given toxicant exposure level is multiplied by to indicate the reduction in toxicant concentration at the next lower exposure level (e.g., a 0.5 dilution factor results in a 50 percent reduction; a 0.6 dilution factor results in a 40 percent reduction, etc.).

Dilution water - Water of choice for preparing dredged material elutriate and for diluting the elutriate when necessary.

Disposal site sediment - Sediment sample representative of the surficial sediments at the proposed disposal site. Used as point of comparison for interpretation of dredged material bioassay and

bioaccumulation data for regulatory decision making.

Dredged material - Aquatic sediments that have been moved by dredging operations.

EC50 - Effect concentration at which 50 percent of the test organisms elicit the defined response (usually a sublethal response).

Elutriate - Supernatant prepared by mixing sediment or dredged material with dilution water, and used for chemical analysis and toxicity testing.

Embryo - Fertilized egg or ova.

Endpoint - A response in a toxicity test such as lethality, growth or behavioral change.

Hypothesis testing - A statistical approach designed to confirm or deny the null hypothesis that organisms in a given treatment (i.e., dredged site sediment or elutriate) are not affected differently than organisms in the disposal site sediment or elutriate.

Illumination - Amount or energy of light incident upon a unit of surface area, measured in lumens/m^2.

Imago - Adult or sexually mature stage in an insect life cycle.

Infaunal species - Benthic organism which largely dwells within the sediment, as distinguished from one that largely dwells upon the sediment.

Instars - Successive developmental stages of the larval insect between molts.

LC50 - The median lethal concentration, or the concentration of a substance that kills 50 percent of the organisms tested in a laboratory toxicity test of specified duration.

Larvae - For insects, the immature, worm-like life-stages; for fish, the immature life-stages between hatching and becoming covered with scales.

Light intensity - brightness of light at a standard distance from a source, measured in lumens, foot-candles or μE/m^2/S.

LOEC - Lowest Observable Effect Concentration; in an elutriate toxicity test, the lowest toxicant concentration (or lowest

percentage of full-strength elutriate) resulting in a statistically significant difference from the control in one or more toxicity test endpoints.

May - Word meaning "is (are) allowed to".

Must - Word expressing an absolute requirement. It is used in connection with factors that directly relate to the acceptability of a test.

Neonate - Newly born organism in its first day of life (i.e., ≤24 h of age).

"New" solutions - In a toxicity test in which solutions of toxicant are periodically renewed with fresh solutions, the test solutions immediately following solution renewal.

NOEC - No Observable Effect Concentration; in an elutriate toxicity test, the highest toxicant concentration (or highest percentage of full-strength elutriate) resulting in no statistically significant difference from the control in any of the toxicity test endpoints.

"Old" solutions - In a toxicity test in which solutions of toxicant are periodically renewed with fresh solutions, the test solutions immediately prior to solution renewal.

Parthenogenesis - Reproduction directly and solely by a female of the species without participation or contribution by a male of the species.

Point estimation techniques - Statistical approaches designed to provide a point estimate of an effect of the treatments relative to the controls via regression analysis.

Pupa - In insects which undergo complete metamorphosis, the life cycle stage during which the larval structures are rebuilt into adult form.

Pupation - Process of transforming from a larval form to an adult form of insect for those insects which undergo complete metamorphosis.

Quality control criteria - Measures that are taken before and during a toxicity test to ensure that the test results are of high quality and the interpretation of the test results is valid.

Randomized complete block design - An experimental test design in which the individual experimental chambers are randomized within

a block (row) rather than over the entire set of chambers.

Reference organism - Organism from laboratory culture that is saved and preserved for examination to verify the validity of species identification.

Reference toxicant - A chemical used periodically to monitor the sensitivity of a test organism culture to determine their suitability for testing purposes.

Sediment - Material such as sand, silt, clay or organic matter, usually suspended in or settled on the bottom of a water body. The term dredged material refers to material that has been dredged from a water body, while the term sediment refers to material in a water body prior to the dredging process.

Should - Word stating that a specified condition is recommended and ought to be met, if possible. Although a violation of one "should" is rarely a serious matter, violation of several will often render the results questionable.

Solid-phase - Solid sediment or dredged material consisting of both an inorganic mineral component and an organic component.

Static toxicity test - A toxicity test without flowing water; can be without periodic water exchange or with daily exchange of new test water.

Steady-state - An equilibrium condition for the tissue burden of a chemical when there is no net change over time (i.e., chemical influx to the organism equals the efflux from the organism).

Subchronic test - A test in which exposure to a test material is abbreviated relative to a complete life-cycle test, but which occurs over the sensitive life-stages of the test organism. The results approximate those of a full life-cycle chronic test.

Substrate - Material in which benthic organisms live, either natural (e.g., sediment) or artificial (e.g., paper pulp).

1.0. PURPOSE

This appendix provides detailed instructions for the completion of biological effects-based tests for dredged material. These protocols are intended to be used to evaluate the potential for contaminant-related impacts from proposed discharges of dredged material into the U.S. waters of the Great Lakes basin. The protocols should be used and interpreted as described in the Great Lakes Dredged Material Testing and Evaluation Manual (GLTEM). Other applications of these protocols were not intended.

This appendix contains protocols for six test organisms. These tests were designed to evaluate the potential contaminant effects of dredged material discharges on water column toxicity, benthic toxicity, and benthic bioaccumulation. The development of these protocols drew upon the accumulated knowledge and expertise of several research organizations and individuals. Information is heavily utilized from several ASTM Standards and Guides, many USEPA publications, several USACE publications and the collective experience of the authors and contributors of this appendix.

2.0. APPLICABILITY

Water column (elutriate) toxicity tests are presented for three organisms: the cladocerans, *Daphnia magna* and *Ceriodaphnia dubia*, and the fathead minnow, *Pimephales promelas*. Protocols for both short-term (acute) and long-term (chronic) exposures have been presented for each of these test species. However, the GLTEM only recommends that the acute exposures and survival endpoint be used at this time for tier 3 testing. The GLTEM further recommends that tests with any one of these species should be adequate for tier 3 evaluations. The interpretive guidance for the chronic exposures and non-survival endpoints has not been adequately developed for application in tier 3, and the protocols for the chronic exposures are presented for consideration only in the rare cases where tier 4 testing is necessary.

Benthic (solid phase) toxicity tests are presented for two test organisms, including an insect, *Chironomus tentans*, and an amphipod, *Hyalella azteca*. Protocols for the measurement of survival and growth endpoints have been presented for each test species. However, the GLTEM recommends for tier 3 testing that the survival endpoint be used with both species and the growth endpoint with *C. tentans* only. The interpretive guidance for the growth endpoint with *H. azteca* is not adequately developed for application in tier 3 testing, although the protocol for its

measurement is included herein for potential use in tier 4 testing.

A benthic bioaccumulation test is presented for the oligochaete worm, *Lumbriculus variegatus*. The GLTEM recommends this test be used in tier 3, where necessary, to evaluate bioaccumulation potential.

The protocols presented here represent a significant enhancement to the methods previously used to evaluate potential contaminant related effects of proposed dredged material discharges in the Great Lakes. The USEPA and USACE expect a sharp "learning curve" during the first few years of implementation of the GLTEM, and intend on making revisions to this manual and these protocols where appropriate, based on the experiences of their application. The USEPA and USACE invite the comments and opinions of laboratories performing these protocols, particularly any refinements to these protocols which might improve their execution or reduce costs.

3.0. GENERAL LABORATORY REQUIREMENTS

Certain requirements must be met for a laboratory to successfully perform biological evaluations of sediment or sediment elutriates. The laboratory should have (1) the necessary facilities to conduct a carefully controlled test, (2) safeguards in place for protecting the health and safety of employees working with the sediment samples, and (3) the requisite qualifications among personnel involved in the performance and evaluation of the test.

3.1. Laboratory Conditions

The organism culturing or holding facility must be sufficiently separated from the testing facility to eliminate the possibility for contamination from a test to the culture organisms, particularly of volatile chemicals. Both the culturing/holding facility and the testing facility should have the capability for accurate control of temperature and light, in addition to having a supply of clean air.

3.1.1. Laboratory Equipment

Equipment that will come into contact with sediments, overlying water, elutriates, or dilution water must be constructed of a material that will not contribute any toxicants to the culture or test system. Such equipment should also be of materials that will minimize the sorption of test materials from water. Acceptable materials include glass, type 316 stainless steel, nylon, high-density polyethylene, polycarbonate and fluorocarbon plastics (USEPA 1994). These materials should be

cleaned prior to use. The procedure for cleaning glass is given in Section 5.0. The other materials listed, with the exception of stainless steel, should be similarly cleaned. Stainless steel should not be acid-rinsed.

Cast-iron pipe, copper, brass, lead, galvanized metal, natural rubber and neoprene rubber should not come into contact with the overlying water, stock solutions, elutriates or dilution water. Concrete and high-density plastic containers may be used for holding or culturing chambers, and in the water-supply system.

3.1.2. Temperature Control

For elutriate toxicity testing, the control of temperature may be accomplished by placing the test chambers into a temperature-controlled water bath or in a temperature-controlled environmental chamber/room. For solid- phase sediment toxicity and bioaccumulation tests, temperature may be controlled in the same manner as indicated above if the tests are performed statically. If an automated water renewal system is used, additional temperature control may be provided by the renewal water itself.

The test protocols for the six species considered in this Appendix require uniform test water temperatures of from 20^{o} to 25^{o}C. For each of the test species, the overall mean water temperature should be within 1^{o}C of the selected test temperature.

3.1.3. Laboratory Water

Water used in culturing and testing should be of uniform quality. Acceptable water should allow for satisfactory survival, growth or reproduction of the test organisms. For tests with elutriates, a synthetic, reconstituted water or a diluted mineral water may be used. For tests with solid-phase sediments, the overlying water may be well water, test site water, reconstituted water or water from a municipal supply that has been specially treated to remove certain chemicals.

When deionized water is used, the water-deionizing system should provide a sufficient quantity of at least 1 mega-ohm water. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deioinizer with preconditioned water from a mixed-bed water treatment system.

A natural water is considered to be of uniform quality if monthly ranges of the hardness, alkalinity, and specific conductance are less than 10% of their respective averages and if the monthly range of pH is less than 0.4 (USEPA 1994). Natural

waters should be obtained from an uncontaminated well or spring, if possible, or from a surface-water source. If surface water is used, the intake should be positioned to: (1) minimize fluctuations in quality and contamination, (2) maximize the concentration of dissolved oxygen, and (3) ensure low concentrations of sulfide and iron. Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 μm or less.

Municipal-water supplies may be variable and may contain unacceptably high concentrations of materials such as copper, lead, zinc, fluoride, chlorine, or chloramines. Chlorinated water should not be used for culturing or testing because residual chlorine and chlorine-produced oxidants are toxic to many aquatic organisms. Use of tap water is discouraged unless it is dechlorinated and passed through a deionizer and carbon filter (USEPA 1993a).

If reconstituted water is used, water should be prepared by adding specified amounts of reagent-grade chemicals to high-purity distilled or deionized water (ASTM 1993a, USEPA 1993). Acceptable high-purity water can be prepared using deionization, distillation, or reverse-osmosis units (USEPA 1993). In some applications, test water can be prepared by diluting natural water with deionized water (Kemble et al. 1993).

Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured on each batch of reconstituted water. The reconstituted water should be aerated before use to adjust pH and dissolved oxygen to the acceptable ranges. USEPA (1993) recommends using a batch of reconstituted water for no longer than two weeks.

3.1.4. Laboratory Air Supply

A supply of clean air is necessary for both the test organism culturing/holding unit and the testing laboratory. The air used for water aeration should be free of oil and fumes. Oil-free air pumps are recommended, where possible. Air line filters should be used to remove oil, water and bacteria. The culturing/holding unit and the testing facility should be well ventilated and free of fumes, as well.

3.1.5. Laboratory Lighting

Lighting should be provided by wide-spectrum fluorescent bulbs, with an intensity at the surface of the test water from approximately 300 to 1,100 lux. An automatic timer should provide a photoperiod of 16 hours of light and 8 hours of dark each day.

3.1.6. Test Organism Food

Facilities for culturing the test organism food supply are essential for tests using *Daphnia magna*, *Ceriodaphnia dubia*, fathead minnows or *Hyalella azteca*, as test organisms. *D. magna* and *C. dubia* are fed live green algae, *Selenastrum capricornutum*, in addition to a suspension of trout chow for *D. magna* and a suspension of yeast, Cerophyll® and trout chow for *C. dubia*. Fathead minnows are fed newly hatched nauplius larvae of brine shrimp. Specific instructions and required equipment items for culturing *Selenastrum capricornutum* and brine shrimp are provided in the following toxicity test protocols for *D. magna*, *C. dubia* and fathead minnows. During culturing, *H. azteca* are fed green algae (e.g., *Ankistrodesmus* sp.) in addition to a mixture of yeast, Cerophyll® and trout chow (YCT). Specific equipment needs and instructions for culturing such algae are provided in the following *Hyalella azteca* test protocol. *Chironomus tentans* culture and test organisms are fed Tetrafin® goldfish food, and *Lumbriculus variegatus* is fed commercial trout chow during culture.

3.1.7. Refrigerated Storage

The testing laboratory should have a cold storage facility of sufficient size to store sediment samples for the period of time between receipt of samples and test initiation (or successful test completion for a portion of the sample). Depending upon the volume of sediment to be tested and the frequency with which such tests are performed, the cold storage facility could range in size from one or more refrigerators to a specially designed cold storage room. The sediment is to be stored in the dark at 4^{o}C until it is used in a test.

3.1.8. Biological Decontamination

The testing laboratory should have an effluent containment facility into which the test effluents (from tests having automatic renewal of overlying water) or the renewal water (from tests with manual overlying water renewals) enter and are treated to kill any exotic, nuisance species that may have been present in the test or disposal site sediment samples. The effluent containment tanks should be of sufficient volume to retain the test effluents for 24 h or more prior to discharge. This is to allow for sufficient contact time between the nuisance organisms and the chemical or heat treatment to produce complete mortality. Chlorination of the effluent is one chemical treatment option, in which case the chlorine concentrations in the effluent holding tank should be 20 mg/L or greater. In the case of heat treatment, the effluent should be heated to 50^{o} C for 2 h before being discharged to a wastewater treatment facility (Sims et al. 1993). The actual size of the containment facility needed will vary depending upon the testing volume for each laboratory.

3.1.9. Hazardous Material Storage

A testing laboratory should have an area or facility for the safe storage of sediments or effluents that are judged to be highly hazardous. These materials should be safely contained in the storage area until they are properly disposed.

3.1.10. Computational Capability

Each testing laboratory should have the necessary computer hardware and software that will allow for tracking samples, reduction of test data, and report preparation. Specific statistical tests for each type of sediment evaluation test are described in the appendix. Several software packages for data reduction are mentioned in the protocols. Other software packages that contain the recommended statistical tests are also commercially available.

3.2. Health and Safety Precautions

Chemical contaminants in field-collected sediments may include carcinogens and mutagens, as well as infectious microorganisms. The laboratory should have an ongoing commitment to the maintenance of a work environment that will not endanger the health of the staff. Special equipment (e.g., respiratory masks, special clothing) or supply items (e.g., disposable gloves) should be present in the physical facility in which the sediments are handled and the tests are performed. Laboratory personnel should periodically receive training in appropriate safety procedures.

3.2.1. Ventilation

Sediment handling and testing should be performed only with adequate ventilation. Sediment handling may be accomplished under a hood, in a ventilated glove box, or, at a minimum, in a well-ventilated room. The testing of sediments should be performed in a system that is well-ventilated.

3.2.2. Personnel Safety

For personal safety, contact of the dredged material or overlying water with skin and eyes should be avoided. Both may be contaminated to various degrees with chemicals and infectious microorganisms. Laboratory coats, disposable gloves and safety glasses should be worn while working with dredged material. The dredged material should be mixed under a hood or, at a minimum, in a well-ventilated room. Test systems should be enclosed and under negative atmospheric pressure to avoid contamination of laboratory air. Should skin or eye contact with sediment occur, immediately wash the skin with soap and water or flush the eyes with water. If the dredged material should contact a dermal wound, wash the skin and apply a topical antibiotic. For individuals regularly involved with dredged material processing, current immunizations against infectious disease microorganisms,

including hepatitis B, tetanus, typhoid fever and polio, are recommended (USEPA 1993).

3.2.3. Hazardous Waste Disposal

For environmental safety, dredged material that is to be discarded should be disposed of in full compliance with existing state Environmental Protection Agency and Department of Transportation regulations. The method of disposal should comply with a protocol for waste disposal approved by the equivalent of an Environmental Safety Officer at the laboratory performing the test. Efforts should be made (e.g., chemical or heat treatment) to destroy any life-stages of exotic nuisance species that may be present in the sediment or associated water, such as zebra or quagga mussels (*Dreissena* sp.) and Asiatic clams (*Corbicula* sp.).

3.3. Personnel Qualifications

A laboratory that conducts biological evaluations of sediments or elutriates should have experienced personnel for culturing and/or holding test organisms, for performing the toxicity or bioaccumulation tests, for performing the requisite chemical measurements that accompany the tests, for statistically analyzing the test data, for preparing a report of the test, for performing quality assurance/quality control (QA/QC) audits and reviews of the test, and for compliance with local, state and federal laws regarding the disposal of contaminated sediment and water.

One individual may fulfill more than one of the above responsibilities. However, the QA/QC audits and review must be performed by an individual not involved with the tests. It is also necessary to either have on staff or have access to an individual that is knowledgeable regarding the identification of the different test species. This will allow for verification of the test organisms as being of a given species.

4.0. QUALITY ASSURANCE REQUIREMENTS

Quality assurance/quality control (QA/QC) of test results for dredged material toxicity tests is based upon guidance offered in the document entitled "Quality assurance/quality control (QA/QC) guidance for laboratory dredged material bioassays" (Moore et al. 1994). The document offers guidance on the subjects of data quality objectives; biological procedures; sample handling, storage and shipment; data recording, reduction, validation and reporting; internal quality control checks, and corrective action. These issues are briefly addressed in this section as they specifically pertain to the toxicity tests mentioned in this appendix.

4.1. Minimum Requirements for Managing Culture Quality

4.1.1. Test Laboratory Cultured Organisms

A laboratory that cultures organisms for dredged material toxicity testing must have a culture of organisms of a single species, and species identification should be verified by a competent taxonomist. Organisms must be disease-free and not from an unusually tolerant or intolerant genetic strain. The history of the parents of the test organisms for at least one generation should be known. Organisms used for toxicity tests should be from a minimum of three female parents. The culturing laboratory should keep records of rate of reproduction and rate of survival of offspring to demonstrate that the test organisms are within normal limits based upon a particular laboratory's recorded results. It is recommended that organisms from the culture be periodically (i.e., monthly if tests are performed routinely, or with each test if performed infrequently) subjected to a toxicity test with a reference toxicant. Suggested reference toxicants are cadmium, copper, sodium or potassium chloride. Results must be within the limits established by the laboratory as a normal response (e.g., ±2 standard deviations of the mean). A minimum of 5 reference toxicant tests with each test species is recommended to document the condition of the culture animals (USEPA 1994).

4.1.2. Purchased Test Organisms

Organisms purchased from a supplier for toxicity tests with dredged material must be disease-free and from an established culture. The supplier should provide with the organisms a record of their history for at least one generation showing no unusual survival trends. Certain physical and chemical characteristics (i.e., temperature, pH, hardness) of the water used to culture the organisms should be supplied. The organisms should be of known age and their diet described. A record of reference toxicant test results should be provided by the supplier of the test organisms, but a reference toxicant test should also be conducted by the laboratory receiving the purchased organisms. If the supplier has not conducted 5 reference toxicant tests with the test organism, the testing laboratory should perform these five tests from five different groups of organisms before starting a sediment toxicity test (USEPA 1994).

4.2. Minimum Requirements for Water and Feed Quality

4.2.1. Water Quality

Water for culturing test organisms must be of suitable quality for good health of the test organisms. The water can be from the regular water supply for the laboratory, disposal site water or reconstituted water. Physical and chemical characteristics of the water must be within the range suitable

for the good health of the test organism at all times. Water quality for the dredged material toxicity test must be similar to the quality for the culture water, insuring no adverse effects upon the organisms due to water quality differences. Specific requirements for water quality are described for each test species in its respective testing protocol.

4.2.2. Food Quality

Food used during the dredged material toxicity test should be of the same type and supply as used during the culturing of the organisms. Information supplied with the food from the supplier or received through direct contract with the supplier must be reviewed to insure that no unusual ingredients or unusually high levels of contaminants are present. If chemical assays are conducted for a chemical of concern for the toxicity test, then the food should also be assayed for this chemical. Food should be suitably stored to maintain quality. Specific food requirements and preparation are described for each test species in its respective testing protocol.

4.3. Toxicity Test Pre-treatment Criteria

4.3.1. Test Water Conditions

Dissolved oxygen concentrations in exposure chambers should exceed 90 percent of saturation before the test organisms are added, but must not exceed 110 percent. Water temperature should be within 1°C of the desired test temperature.

4.3.2. Daphnia magna Test

Individualized brood-board cultures must be analyzed for adequate survival and reproduction prior to the start of a test. Only neonates from females with production of $\geq$9 young/brood should be used in tests. Neonates from about the fourth brood are recommended for testing. Survival of adults in the stock culture must exceed 80 percent. Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.3. Ceriodaphnia dubia Test

Individualized brood-board cultures must be analyzed for adequate survival and reproduction prior to the start of a test. Only neonates from a brood-board set with a history of $\geq$15 young/female in 7 days or 3 broods should be used. Survival of adults in the stock culture must exceed 80 percent. Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.4. Pimephales promelas Test

Survival of adults in the brood culture must exceed 90 percent. Hatching success of embryos should exceed 80 percent.

Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.5. Chironomus tentans Test

Egg mass hatching must be adequate to produce the number of larvae needed for the test. Late fourth instar larvae from the culture aquaria should be monitored on a monthly schedule for adequate dry weight. Dry weight of fourth instar culture larvae must average $\geq$0.6 mg to be acceptable. Larvae must be 8 to 12 days old (post-hatch) at the start of the test. Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.6. Hyalella azteca Test

Young production by adults in the culture should be 75 to 100 young/50 adults/week. Survival of adults and young must exceed 80 percent. Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.7. Lumbriculus variegatus Test

Animals must come from a healthy culture. Health is indicated by organisms having normal coloration, high level of reproduction (i.e., a doubling of population density every 10 to 14 days in a fresh culture), and normal reflexive action to stimuli. Results of the reference toxicant test (see Section 4.3.8.) must meet the requirements to indicate adequate organism health.

4.3.8. Reference Toxicant Test

It is recommended that an assessment of test organism condition be conducted by performing a toxicity test using a reference toxicant (e.g., $CdCl_2$, $CuCl_2$, KCl, NaCl). The test can be conducted concurrently with dredged material toxicity tests or on a routine basis for which a monthly test is recommended. The results of any test should be within two standard deviations of the mean of all previous reference tests to consider the animals suitable for use in a dredged material toxicity test. A minimum of 5 reference toxicant tests should be performed prior to the testing of a sediment (USEPA 1994).

The reference toxicity tests should be a minimum of 48 h in duration with five replicated control and toxicant concentrations. The test should be a static exposure and should use either the culture water or test water for exposure of the organisms. These reference toxicant tests should be conducted with careful technique, and each additional test performed requires a recalculation of the mean and standard deviation.

4.4. Toxicity Test Post-treatment Criteria

4.4.1. Test Water Conditions

Dissolved oxygen should be between 40 and 100 percent of saturation at all times during the test. Light aeration is sometimes necessary to maintain the minimum percent saturation. Water temperature must be consistent amongst all test chambers. The temperatures should be within 2°C of each other at all times, and the mean water temperature for the test should be within 1°C of the desired test temperature. Total ammonia concentrations in the water should not exceed 1.0 mg/L in any test chamber during the test, and the mean total ammonia concentration should not be greater than 0.1 mg/L.

4.4.2. Daphnia magna Test

The results of an acute test should be considered acceptable only if survival in the control chambers averages ≥90 percent. In a chronic test, survival must average ≥80 percent, and production of young by control organisms must average ≥60 young/surviving female after 21 days. For both acute and chronic tests, hardness and alkalinity of reconstituted water used to initiate a test and for renewal of test solutions must fall within the ranges of 160 to 180 mg/L (as $CaCO_3$) for hardness and 110 to 120 mg/L (as $CaCO_3$) for alkalinity. The pH of reconstituted water must be between 6.8 and 8.5. The time-weighted average measured concentration of dissolved oxygen for each test chamber must be between 50 and 100 percent of saturation for the test (ASTM 1993a).

4.4.3. Ceriodaphnia dubia Test

The results of an acute test should be considered acceptable only if mean survival in the control chambers is ≥90 percent. In a chronic test, survival must average ≥80 percent, and young production by control animals must average ≥15 young/surviving female. At least 60 percent of the surviving females in the control chambers should have produced three broods. The time-weighted average measured concentration of dissolved oxygen must be between 50 and 100 percent of saturation for the test (ASTM 1993a).

4.4.4. Pimephales promelas Test

The results of an acute test should be considered acceptable if survival in the control chambers average ≥90 percent. The results of a chronic test should be considered acceptable if survival in the control chambers averages ≥80 percent and if the dry weight of control organisms averages ≥0.25 mg per larva. Dissolved oxygen must average (time-weighted) >40 percent of saturation for all test chambers and must not fall below 40 percent in any test chamber at any time.

4.4.5. Chironomus tentans Test

The results of a test should be considered acceptable if survival in the control chambers averages ≥70 percent. Dissolved oxygen saturation should average >40 percent in all test chambers. Dry weight of the controls must average 0.6 mg for the test to be considered acceptable.

4.4.6. Hyalella azteca Test

The results of a test should be considered acceptable if survival in the control chambers averages ≥80 percent. Dissolved oxygen saturation must be >40 percent in all chambers at all times and should average (time-weighted) between 50 and 100 percent of saturation for the test.

4.4.7. Lumbriculus variegatus Test

The results of a test should be considered acceptable if a sufficient mass of organisms is available after 28 days of exposure to dredged materials. Test organisms should have been observed to burrow into the sediment at the start of the test. Dissolved oxygen should be ≥40 percent of saturation in all test chambers at all times.

4.5. Biological Test Procedures

4.5.1. Standard Operating Procedures (SOPs)

The six testing procedures described in this Appendix can be adapted for use as SOPs. The testing procedures contain checklists and schedules for each of the important aspects of test organism preparation and for conducting a test.

4.5.2. Good Laboratory Practices

Good laboratory practices should be employed when conducting a test to eliminate bias and opportunity for contamination of a test. Many potential problems can be eliminated by proper acclimation, test conduct, use of proper controls, statistical design and randomization, and reference toxicant testing.

4.5.3. Statistical Design and Randomization

The appropriate statistical design should have a minimum of five replicates for all toxicity tests. Reference toxicant tests should have two to five replicates per chemical concentration. The chemical bioaccumulation test also requires a minimum of five replicates. A power analysis should be run before increasing the number of replicates to ensure cost effectiveness of increased sensitivity. A randomized block design is recommended to remove the bias of positional effects in the test.

4.6. Sample Handling, Storage and Shipment

4.6.1. Chain of Custody

Documentation which includes dates and signatures should accompany all samples from the origin of the sample to its destination at the sample testing facility. Chain of custody documentation should continue in the laboratory as the sample is stored, processed, tested and disposed.

4.6.2. Sample Preparation

Sediment or dredged material samples must be treated in a consistent manner to avoid bias in the toxicity test. For example, all elutriate samples must be centrifuged even if they appear to lack suspended material. Disposal site sediment must be treated identically with test site sediment. All samples must be kept at all times in appropriate containers that are clean.

4.6.3. Sample Storage

Samples must be stored in appropriate containers that are full (i.e., zero or minimal headspace) and tightly covered. They must be kept in the dark at 4ºC and tested within 8 weeks. Samples must be re-homogenized prior to use in a test.

4.7. Data Recording, Reduction, Validation and Reporting

4.7.1. Use of Laboratory Notebooks

Data for a test should be recorded (in indelible ink) on data forms and stored in a bound notebook. Storage of data solely on electronic media is not acceptable. Data forms should be marked with a dash when data were purposely omitted. Erasures are not permissible. If data must be corrected, they should be lined-through and initialed by the person making the correction. All data forms should identify the person reporting the data.

4.7.2. Data Management

Standardization of data reporting and statistical analysis is very important. Standardization helps reduce bias and results in consistent interpretation of test results. Duplicate copies of all data (preferably stored at different locations) are recommended to minimize loss.

4.7.3. Unacceptable Data or Outliers

If data are immediately identified as being erroneus (e.g., instrument not properly calibrated), a new measurement can be made and recorded, replacing the initial measurement. If, however, the data are unexpected, but not obviously erroneus, a second measurement may be taken to provide verification. If unexpected or erroneus data are discovered later, they must be dealt with in a scientifically defensible manner. If the outlier

data can be explained, they can be removed from the data set. Otherwise they are either used in calculations or tested statistically for their eligibility as outliers (see numerous statistical texts for outlier tests).

4.8. Internal Quality Control

A testing laboratory should have a person (e.g., a Quality Control Officer or another person under the direction of the officer) not associated with the toxicity test conduct an audit to determine if all planned procedures and measurements were completed. Results of each audit should be submitted to the testing organization's chief officer and to the study director. Additionally, there should be a verification of the taxonomy of the test organism, and a review of the acceptability of control organism survival (and growth in some cases) in the test. The reference toxicant test results must be within acceptable limits.

4.9. Corrective Action

Deficiencies in the completeness of data records and quality of test results obtained for the sample must be addressed. Some deficiencies are less important than others such as unreported water quality measurements. Retesting of a sample is usually required when there is excessive test organism mortality in control exposures, out-of-range water quality measurements, lack of randomization, lack of required reference, control, or reference toxicant tests, and out-of-range reference toxicant results. The laboratory logbook and sample file/report should document any actions taken, the reasons for such actions and the success of the actions taken.

5.0. SAMPLE HANDLING AND PREPARATION PROCEDURES

Proper handling procedures of the sediment samples from the time of collection to the final disposition of the samples following their use in a test are very important. The samples must be properly labeled and tracked using a chain-of-custody form (Moore et al. 1994).

Samples of dredged or disposal site material should be stored in the dark at 4°C with minimal headspace above the sediment. Glass storage containers should be thoroughly pre-cleaned using the following recommended procedure (ASTM 1993d): (1) non-phosphate detergent wash, (2) triple water rinse, (3) water-miscible organic solvent wash (acetone followed by pesticide grade hexane), (4) water rinse, (5) acid wash (such as 5-10% concentrated hydrochloric acid), and (6) triple rinse with deionized-distilled water. Container cleanliness should be documented according to specific QA/QC guidelines (USEPA 1990). New polyethylene containers, if used, should similarly be

thoroughly cleaned before use. Due to the difficulty in completely cleaning polyethylene containers that have stored contaminated sediments, they should be used only once, and then discarded. Storage containers should be filled completely to minimize headspace. It is prudent to complete the testing of sediments with a minimum of storage time (probably less than 2 weeks), to minimize changes in sediment chemistry (ASTM 1993d). Sediment holding time should not exceed 8 weeks (USEPA and USACE 1994). Various standard chemical extraction methods have storage time limits ranging from less than 7 days to less than 6 months (USEPA 1985, ASTM 1993e). The maximum allowable holding time may change in the future, as more information becomes available.

The dredged, disposal site or control material should be thoroughly mixed to a homogeneous state prior to use in a toxicity test. The sediment may first be screened through a coarse-mesh screen (e.g., 5 mm or no. 5 mesh) to remove large objects, such as rocks and sticks. The removal of any materials should be carefully documented. Mixing can be accomplished by hand or with mechanical mixers, depending on the sample volume. The water should not be drained from the sediment sample, but should be mixed with the sediment as much as possible. Mixing should be sufficient to homogenize the sample without significantly elevating its temperature. Mixing may need to be minimized if there are known or suspected volatile or semi-volatile contaminants of concern. Some volatilization during mixing is unavoidable and adequate ventilation should always be provided. If the sediment has been in storage following an initial mixing, it should be re-homogenized immediately prior to being tested.

For solid-phase toxicity or bioaccumulation tests, the required volume of the homogenized sample is placed into each of the clean replicate test chambers. Overlying water is then added, and the sediment is allowed to settle for 24 h before the test organisms are added.

For elutriate toxicity tests, the elutriate should be prepared on a weekly basis for the exposures, and stored for no longer than 7 days. The 100 percent or stock elutriate is prepared in a 1:4 volume ratio of sediment to test water by mixing vigorously for 30 min with a magnetic stirrer, shaker or tumbler. At 10 min intervals, the contents are manually stirred to ensure complete mixing. After the 30 min mixing period, the mixture is allowed to settle for 1 hr, and the supernatant is decanted or siphoned off from each container. Centrifuge the supernatant at 4,000 X g in a refrigerated centrifuge for 45 min (Ankley et al. 1990) to precipitate suspended solids. This supernatant may be combined with other supernatants from the same sample to provide a sufficient volume for a test. It should be

used directly as the 100 percent test site elutriate water.

The required volumes of elutriate and dilution water for test initiation and each renewal vary with each test and are provided with the specific test protocol. The elutriate should be stored in the dark at 4°C until just prior to use in a test. It should then be equilibrated to the desired test temperature before being used in the test. This can be accomplished by placing the renewal solutions into a water bath or another temperature-controlled environment maintained at the test temperature.

6.0. *Daphnia magna* WATER COLUMN TOXICITY TESTS

Daphnia magna (Figure G-1) is a planktonic freshwater cladoceran of the family Daphniidae. Under appropriate culture conditions at 20°C, it reproduces parthenogenetically, producing its first brood at 7-10 days, and subsequent broods every 2-3 days. It is quite sensitive to some toxicants (Slooff and Canton 1983, Adams et al. 1986, Nebeker et al. 1984, 1986a, Dutka et al. 1989), and has been commonly used in toxicity studies (Knight and Waller 1987). The use of *D. magna* in the evaluation of sediment quality has involved acute and chronic exposures to elutriates, organic solvent extracts of sediment samples, whole sediments and pore waters (Hoke and Prater 1980, Laskowski-Hoke and Prater 1981, Maleug et al. 1984a,b, LeBlanc and Surprenant 1985, Nebeker et al. 1986a,b, Giesy et al. 1988, 1990, Burton et al. 1989, Hoke 1989, Larson 1989, Wiederholm and Dave 1989, Stemmer et al. 1990, Davenport and Spacie 1991). These and other studies with effluents or sediments have utilized test durations ranging from 2 to 21 days; typical test durations have included 2, 4, 7, 10, 14 or 21 d. In general, test lengths of 7 or more d have been used to provide measures of subchronic or chronic toxicity, i.e., reproductive or growth effects (Adams and Heidolph 1985, Knight and Waller 1987, Lewis and Horning 1988, Winner 1988, Gersich and Milazzo 1990). Although several test durations have been used, probably the best defined protocol is for the 21-d exposure (Biesinger et al. 1987, ASTM 1993a).

This document has adapted the standardized 21-d chronic toxicity test with chemicals in water for application to dredged material elutriates. If standardized protocols of shorter duration for estimating chronic toxicity to *D. magna* are developed, it would be appropriate to shorten the test duration of this protocol in the future.

Methods for performing a 48-h acute toxicity test either with full-strength elutriate alone or with full-strength elutriate plus several dilutions of the elutriate are presented

in this document. It is recommended that an acute toxicity test be performed prior to conducting a chronic test.

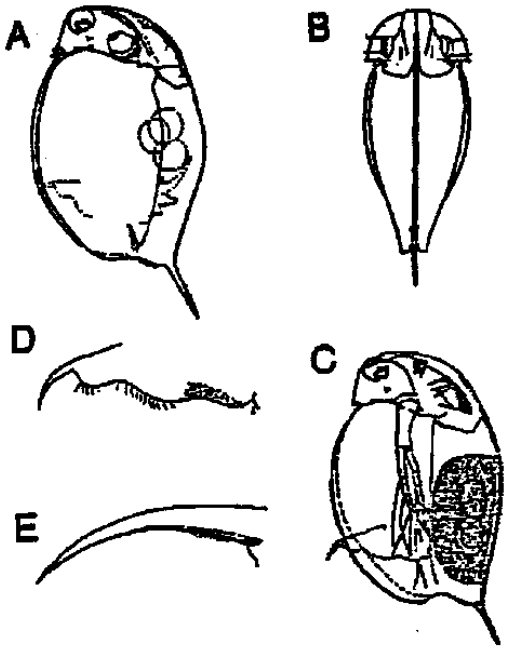

Figure G-1. *Daphnia magna* adult female (X7) and distinguishing body features. A. Lateral aspect, parthenogenetic female; B. Dorsal aspect; C. Ephippial female; D. Postabdomen showing sinuate posterior margin; E. Postabdominal claw (From Brooks 1957).

6.1. CULTURE METHODS

The culturing methods recommended are based on methods described by Biesinger et al. (1987) and ASTM (1993b). Required materials are listed in Attachment A.

6.1.1. Organism Source

Organisms for the initiation of a laboratory culture may be obtained from any source which has a culture of *D. magna* that is periodically verified by a qualified taxonomist. Brood stock organisms are available from various government laboratories and commercial sources.

6.1.2. Acclimation of New Brood Stock

Environmental stress on the daphnids in the starter culture must be minimized to facilitate normal culture growth and brood production. Measure the temperature of the water containing the

stock animals upon their arrival and gradually adjust it to the desired culture temperature. A temperature of 20°C is recommended for both culturing and testing (Biesinger et al. 1987, ASTM 1993b, Lewis and Horning 1991). Water quality in the brood stock container in which the new daphnids were transported should be gradually adjusted over a period of two or more days to meet the conditions of the water in which the organisms will be cultured. Changes in temperature >3°C in any 12-h period should be avoided (ASTM 1993b). To accomplish a gradual change, it is important to know the basic chemical characteristics (i.e., pH, hardness, alkalinity) of the water used by the laboratory from which the brood stock was received, and over a period of two or more days to dilute that water incrementally with the culture water to be used. This is continued until the water meets the requirements for the desired culture water. In preparation for a 21-d test, it is recommended that a minimum of two (and preferably five) generations be raised using the same water, food and temperature as will be used in the test (ASTM 1993b).

6.1.3. Reference Organism

It is recommended by EPA (USEPA 1989) for *Ceriodaphnia dubia* that a new laboratory culture be started with a single animal. The same is recommended here for *D. magna*. It should be killed after producing a supply of young, and definitively identified as *D. magna* using a taxonomic key. This specimen should then be permanently mounted on a slide for future reference. Procedures are available (USEPA 1989) for making slide mounts according to the method of Beckett and Lewis (1982).

Once the starter culture has arrived and neonates are being produced, adults should be separated and one offspring from a large brood (i.e., >15 young) selected as the source of the new laboratory stock culture. The remaining starter culture animals may be maintained as a back-up culture.

6.1.4. Culture Chambers

D. magna may be cultured in 2,000-mL glass beakers, each containing 1,600 mL of culture water and 20 daphnids. The beakers should be covered with glass to minimize evaporation.

6.1.5. Culture Water

Reconstituted water with a hardness of 160-180 mg/L as $CaCO_3$ and a pH between 6.8 and 8.5 is recommended as culture water (Biesinger et al. 1987); however, other types of water are acceptable provided adequate survival (i.e., >80 percent survival in 21 days) and reproduction (i.e., >4 young per female per reproductive day) of *D. magna* are documented. Methods for preparation of the reconstituted water are given in Attachment B. The culture water is renewed weekly by transferring adult daphnids to new water. Each batch of renewal water should be

monitored for temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness; and the measurements recorded in a culture maintenance logbook. Renewal water temperature should be within 1°C of the culture water being renewed. Renewal should not occur until the correct temperature is attained. Dissolved oxygen, pH, conductivity, alkalinity and hardness of the renewal batch water should all be within 10 percent of the values for the same parameters for the initial batch of water. If not, adjustments should be made by aeration (in the case of low dissolved oxygen) or preparation of a new batch of renewal water. Reconstituted water more than one month old should not be used.

6.1.6. Temperature and Photoperiod

The temperature for culturing *D. magna* should be maintained at 20±2°C. The recommended photoperiod is 16 h light and 8 h dark with a light intensity of 30 to 100 foot-candles (Biesinger et al. 1987).

6.1.7. Food and Feeding

A diet that has been used successfully by USEPA to culture *D. magna* consists of trout chow and the green alga, *Selenastrum capricornutum*. Preparation methods for the trout chow and algal diets are given in Attachment C.

Each 2,000 mL culture beaker should receive volume additions of dietary ingredients resulting in final concentrations of 5 mg/L dry wt. of trout chow and 10^8 cells/L of *S. capricornutum* three times weekly (each Monday, Wednesday and Friday). The appropriate volumes of trout chow and algal cell suspensions to produce final concentrations of 5 mg/L dry wt. and 10^8 cells/L are determined as described in Attachment C.

The unused trout chow and algal concentrate should be refrigerated after use. Stored refrigerated trout chow may be used for up to one week, and *S. capricornutum* for up to 12 d (Biesinger et al. 1987).

6.1.8. Handling

A fire-polished pipet of at least 5 to 6 mm bore diameter is recommended for transferring adult daphnids (ASTM 1993b). A smaller diameter pipet may be used to transfer young, but should have an inside diameter of about 1.5 times the size of the organisms (Biesinger et al. 1987). Pipets should be stored in 100 percent methanol (which is replaced weekly), and rinsed three times with tap and distilled water prior to use. Care should be exercised to avoid injury to the daphnids during transfer and to ensure that they are gently introduced below the surface of the water in the new chambers.

6.1.9. General Culture Maintenance

Cultures should be maintained at 20±2°C in a controlled constant temperature environment (e.g., water bath, incubator, environmental chamber or room). Temperature of the culture water should be monitored daily, and a log of the temperatures maintained. Adult daphnids are transferred to new culture media weekly, and are fed each Monday, Wednesday and Friday. Young daphnids are either disposed of or used to start new cultures. Young from the second to sixth broods of the adults are used to start new cultures each week. Adults are disposed of at 4 weeks of age (Biesinger et al. 1987).

6.1.10. Pre-Test Culture Maintenance

Two weeks prior to the start of an acute or chronic test, adult brood stock about to have their second to sixth broods are placed into individual 100 mL beakers (as in the test itself) and observed. A healthy condition is indicated by the absence of floaters (i.e., animals on surface), absence of ephippia (i.e., specialized detached brood chambers with fertilized eggs, which develop under stressful conditions), large size of adults (i.e., >4 mm in total length at 21 d), dark coloration, absence of external parasites, and presence of acceptable numbers of young (four or more young per female per reproductive day). Sixty young daphnids produced from healthy adults are then transferred individually into 100 mL beakers containing new media and reared for at least two weeks. Young from these daphnids are used for the actual toxicity tests (Biesinger et al. 1987, ASTM 1993b).

6.1.11. Culture Evaluation

The general and pre-test cultures are observed daily for their condition of health and for water temperature measurements. Production of young should be at an acceptable level (i.e., 4 or more young per female per reproductive day) in both the general and pre-test cultures. A reproductive day for a given female is each day from the time of the first brood, inclusive of the day of the first brood. Daphnids should not be used to start a test if they fail one or more of the following pre-test culture criteria (ASTM 1993b):

(a) Young for a test must be from adults that appear healthy and uninjured.
(b) Young for a test must be selected from a brood later than the third brood.
(c) Young for a test must be from an adult that produced young before day 10.
(d) Young for a test must be from an adult that produced at least nine young in the previous brood.
(e) Young for a test must be from a culture which did not produce ephippia and which did not have substantial mortality in the week immediately prior to the test.

If the health of the general or pre-test culture is questionable, culturing conditions should be scrutinized and adjustments made to restore the health and increase young production. Any adjustments made may be considered to have resulted in an acceptable state of health for the culture when the pre-test culture meets the above criteria.

6.1.12. Culture Records

A separate set of record books should be kept for the culture unit. Sample culture record forms are provided in Attachment D. Records must be kept on the survival of brood organisms in both the general and pre-test cultures. In the pre-test culture, the time to first brood and the number of young produced should be recorded.

6.2. ACUTE TEST

6.2.1. Elutriate Preparation (Acute Test)

The GLTEM currently recommends that acute exposures of 48 h duration be performed for routine tier 3 testing. Chronic tests (discussed in Section 6.3) may be used for tier 4 testing, if needed. The culturing of organisms is the same for both acute and chronic tests. Animals used to start an acute test are of the same age (i.e., $\leq$24 h old) and are handled in the same way as in a chronic test. The same general test conditions of temperature, lighting and dilution water characteristics apply to both acute and chronic tests. The GLTEM recommends that an acute test first be performed with the 100 percent elutriate, and followed by a dilution series only if survival in the 100 percent elutriate is less than 50 percent. This will require 125 mL of 100 percent elutriate for the five replicate exposures. To obtain a sufficient volume of elutriate water for an acute test with a complete dilution series (i.e., about 250 mL), place 70 mL of well-mixed sediment into a clean 500 mL beaker, add 280 mL of dilution water (same as culture water), and follow the elutriate preparation procedure described in Section 5.0. The test methods follow standard procedures for measuring the acute toxicity of effluents and receiving waters (USEPA 1993). Table G-1 provides a summary of the volumes of elutriate and dilution water required in a test with five different elutriate concentrations using a 0.5 dilution factor and a water-only control.

Table G-1. Volumes (mL) of Dredged Material Elutriate and Dilution Water Required for the *Daphnia magna* 48-h Acute Toxicity Test.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required	Total Dilution Water Volume Required
100	25.0	0.0	125	0
50	12.5	12.5	62.5	62.5
25	6.25	18.8	31.2	93.8
12.5	3.12	21.9	15.6	109.5
6.2	1.6	23.4	8.0	117.0
0.0	0.0	25.0	0	125

6.2.2. Acute Test Design

The basic design and conditions for performing a 48 h acute toxicity test are given in Table G-2. The exposure is static without renewal of the test solution. The test is performed with neonates (<24 h old) which have been provided with food during the holding period prior to test initiation. Newly released young should have YCT and *Selenastrum* available for a minimum of 2 h prior to their use in a test. The animals are not fed during the actual test.

Table G-2. Overview of Recommended Dredged Material Elutriate Test Conditions for the *Daphnia magna* 48-h Acute Toxicity Test.

1.	Test type	Static
2.	Temperature (°C)	20±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 $\mu E/m^2/s$, 540-1080 lux or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	30 mL minimum
7.	Test solution volume	25 mL minimum
8.	Age of test organisms	Less than 24 h
9.	No. neonates per test chamber	5

Table G-2 (continued)

10.	No. replicate test chambers per concentration	5 minimum
11.	No. neonates per test concentration	25 minimum
12.	Feeding regime	No food provided during test. Neonates are fed once during the holding period.
13.	Aeration	None or light aeration prior to test initiation if dissolved oxygen ≤90 percent.
14.	Dilution water	Hard reconstituted water of 160-180 mg/L as $CaCO_3$ hardness and a pH range of 6.8-8.5.
15.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations with a performance control (water-only).
16.	Dilution factor	≥0.5
17.	Water quality monitoring	Daily measurements of water temperature, dissolved oxygen and pH. Single measurements of hardness, alkalinity and specific conductance.
18.	Test duration	48 hr
19.	Endpoints	Survival and complete immobilization.
20.	Test acceptability	90% or greater survival in the water-only control solutions.
21.	Sample storage	Store sediment and elutriate in dark at 4ºC. Elutriate should be prepared for testing within 8 weeks of sample collection and used to initiate the test within 24 h of preparation. Elutriate should be stored for no longer than 7 d.
22.	Sediment volume required	100 mL minimum from each site sampled.

6.2.3. Organism Introduction

Fifty neonates less than 24 h old are required for an acute toxicity evaluation of 100 percent elutriate; 25 in the dredged material elutriate and 25 in the control water. A total of 150 neonates is required to start a test with five serial dilutions of the 100 percent elutriate and a control. Neonates should be randomly selected and distributed to the test chambers in a two-stage transfer process. 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 (Bentley et al. 1986).

6.2.4. Test Organism Monitoring

Immobilization and lethality are the endpoints in an acute test. Test organisms are observed at 24 and 48 h for complete immobilization. Complete immobilization is frequently used as an endpoint for toxicity tests with this species, resulting in an EC50 estimate. This endpoint includes those animals that are dead. Affected animals that are completely immobilized are observed to lie motionless on the bottoms of the test chambers, and do not respond to gentle prodding. Observations may be made with the use of a microscope. If survival data are desired for calculation of an LC50, the immobilized organisms should be examined for heartbeat using a dissecting microscope.

6.2.5. Water Quality Monitoring

Water quality should be carefully measured and documented for each test. Daily measurements of temperature, dissolved oxygen concentration and pH should be taken in each chamber. Hardness alkalinity and specific conductance should be measured once for the batch of water used in the test.

6.3. CHRONIC TEST

6.3.1. Elutriate Preparation (Chronic Test)

To obtain a sufficient volume of elutriate water for each week (i.e. 4.65 L) of a chronic test with *D. magna*, separately place three 500 mL subsamples of well-mixed dredged material into 3 clean 4 L containers. The dredged material may first be screened through a coarse-meshed sieve (e.g., 5 mm or No. 5 mesh) to remove large objects, such as rocks and sticks. At room temperature, add 2,000 mL of culture water (i.e., hard reconstituted water) into each container to produce a 1:4 volume ratio. Prepare the elutriate as indicated in Section 5.0. The supernatant should be centrifuged at 4,000 x g for 45 min, as in Ankley et al. (1990). Decant or siphon off the supernatant from each container and combine the supernatants for use directly as the 100 percent test site elutriate water. The required volumes of elutriate and dilution water for test initiation and each renewal of test solutions are indicated in Table G-3 for a 0.5 dilution factor. The elutriate should be stored in the dark at 4^{o}C until just prior to acclimation to test temperature and use in the test. It should not be stored for longer than 7 days.

Table G-3. Volumes (mL) of Dredged Material Elutriate and Dilution Water Required Per Renewal for the *Daphnia magna* 21-d Chronic Toxicity Test.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required	Total Dilution Water Volume Required
100	80.0	0.0	800	0
50	40.0	40.0	400	400
25	20.0	60.0	200	600
12.5	10.0	70.0	100	700
6.25	5.0	75.0	50	750
0.0	0.0	80.0	0	800

6.3.2. Chronic Test Design

The basic design and conditions for performing a chronic elutriate toxicity test are given in Table G-4. The test consists of a series of 5 dilutions of dredged material elutriate and its performance control (culture water with no elutriate). Each of the experimental units in a definitive chronic test is replicated 10 times for a total of 60 exposure chambers per test site. A dilution factor of 0.5 or greater is used for determining the dilutions to be made from the full-strength (100-percent) test sediment elutriate.

Table G-4. Overview of Recommended Dredged Material Elutriate Test Conditions for the *Daphnia magna* 21-d Chronic Toxicity Test.

1.	Test type	Static with renewal
2.	Temperature (°C)	20±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 µE/m²/s,540-1080 lux or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	100 mL
7.	Test solution volume	80 mL
8.	Renewal of test solutions	Days 2, 4, 7, 9, 11, 14, 16, and 18
9.	Age of test organisms	Less than 24 h

Table G-4 (continued)

10.	No. neonates per test chamber	1
11.	No. replicate test chambers per concentration	10
12.	No. neonates per test concentration	10
13.	Feeding regime	Feed three times weekly when solutions are renewed to result in final concentrations in each test chamber of 5 mg/L of trout chow suspension and 10^8 cells/L of *Selenastrum capricornutum*.
14.	Aeration	None or light aeration prior to test initiation if dissolved oxygen $\leq$90 percent.
15.	Dilution water	Hard reconstituted water of 160-180 mg/L as $CaCO_3$ hardness and a pH range of 6.8-8.5.
16.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations with a performance control (water-only).
17.	Dilution factor	$\geq$0.5
18.	Water quality monitoring	Daily measurements of water temperature, dissolved oxygen and pH. Weekly measurements of hardness, alkalinity and specific conductance.
19.	Test duration	21 d
20.	Endpoints	Survival and reproduction.
21.	Test acceptability	80% or greater survival and an average of 60 or more young/surviving female in the water-only control solutions.
23.	Sediment volume required	4.5 L from each test site.

A randomized complete block design is used (see Figure G-2 for example). Neonates are added in a specific manner (see Section 6.3.6.), which allows for the performance of each female parent to be tracked.

A summary of daily activities prior to and during a test are presented in Attachment E. This schedule assumes that all materials are in hand, and that healthy cultures of *D. magna* and *S. capricornutum* are being maintained.

6.3.3. Test Chambers

In a chronic test, sixty 100 mL glass beakers are required for each test site including its performance control. Ten

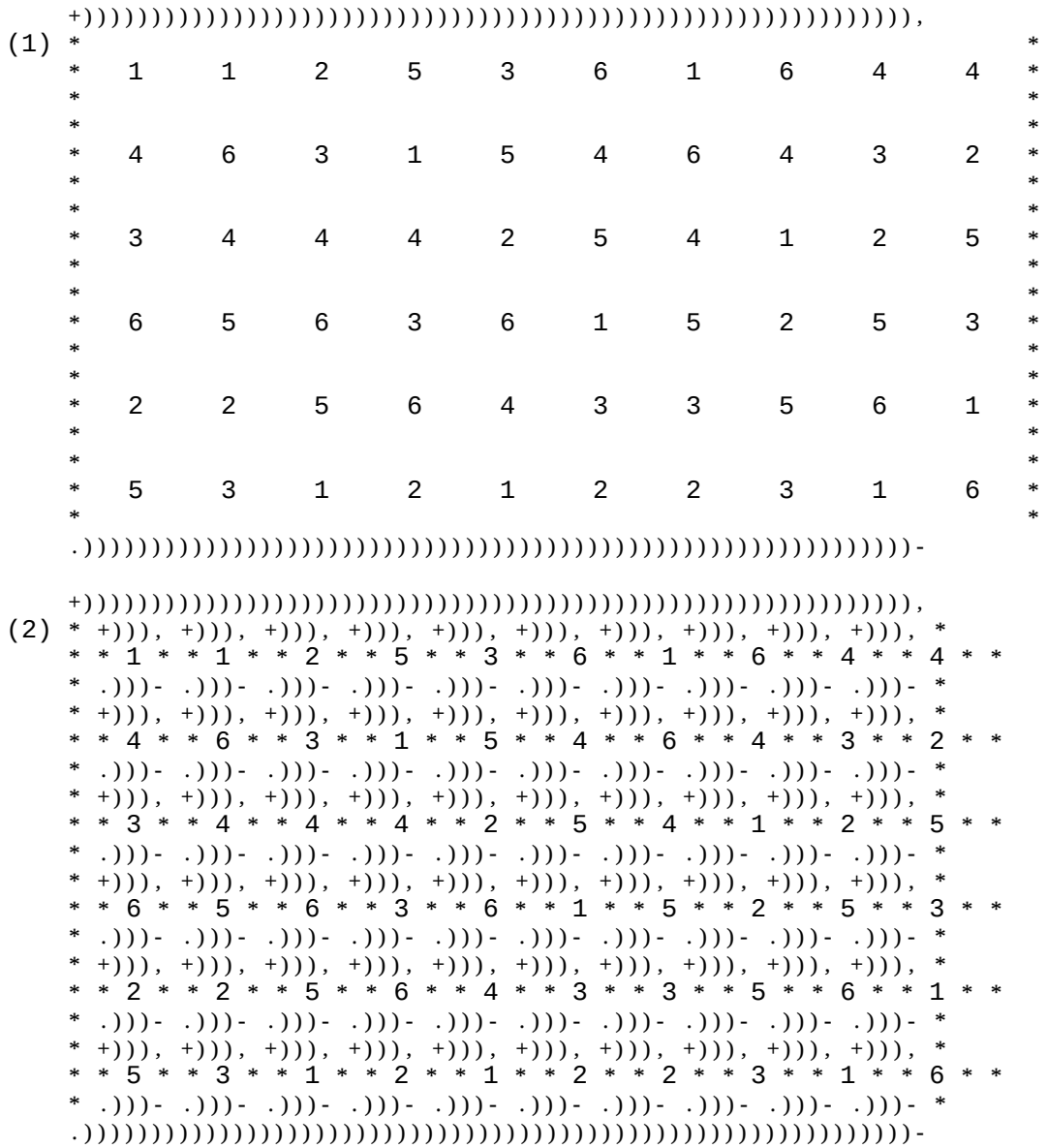

Figure G-2. Examples of a randomizing template (1) and a block-randomized arrangement of beakers for a toxicity test (2). The randomizing template is prepared from a random numbers table for each row. Each of the 10 rows contains one replicate of each treatment.

beakers are used for each of the 5 elutriate exposures and 10 for the performance control. All test beakers should be covered with a sheet of glass to reduce evaporation of water.

6.3.4. Water Renewal

All test chambers must receive a renewal of elutriate or control diluent three times weekly, or on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. The renewal elutriate for the first week of the test should be made at the same time as the elutriate for test initiation, and should be stored in the dark at 4° C. Similar volumes will need to be prepared at the start of the second and third weeks. The diluent for preparation of the elutriate test solution is culture water (control diluent). Adult organisms are gently transferred by pipet to freshly prepared test solutions at each renewal. The renewal solutions should be allowed sufficient time to equilibrate to the desired test temperature prior to transfer of the animals.

6.3.5. Temperature and Photoperiod

Tests should be performed in a temperature-controlled unit at 20 ± 1°C. The daily photoperiod should be 16 L: 8 D.

6.3.6. Organism Introduction

A total of 60 neonates less than 24 h old is required to start the test. Neonates are taken from the pre-test culture adults that have 9 or more young in their fourth or subsequent broods, and are less than 21 d old. Ten brood cups, each with 9 or more young, are selected from the pre-test culture for the test. Into a block-randomized arrangement of test beakers (Figure G-2), from one beaker pipet one neonate into each of the 6 beakers representing one complete subset (i.e. performance control, and five elutriate dilutions)of the 10 replicates. It is recommended that several randomized templates be prepared in advance that can be alternated for use. Discard the additional neonates from that beaker. Repeat this process 9 times for the test site dredged material sample, using neonates from a new pre-test culture beaker for each of the 6 bioassay chambers in a replicate.

6.3.7. Food and Feeding

Three times weekly, all test chambers receive an allotment of food resulting in a final concentration 5 mg/L dry wt. of trout chow suspension and 10^8 cells/L of *S. capricornutum* (see Attachment C for food preparation).

6.3.8. Test Organism Monitoring

Test organisms are observed daily for survival and young production. Organisms also are observed for their behavior. Any abnormal behavior (e.g., rapid or slow swimming, spinning) in adults and offspring should be recorded. Observations are generally made without the aid of a microscope. A light box may be used for illuminating the young animals during examination. To aid in counting live young on renewal days, a few drops of 1N HCl added to the chamber after the adult has been transferred to

new media will kill the young and result in their settling to the bottom.

6.3.9. Water Quality Monitoring

Water temperature should be monitored daily in at least five exposure chambers (four corners and center of chamber array). At the time the water temperature is measured, the temperature reading for a continuously recording monitor in the constant temperature environment should be observed. Dissolved oxygen and pH should be measured daily in a control chamber (C) and in one of the low (L), medium (M) and high (H) exposure chambers (Attachment D, Form D2). At the time of renewal (days 2, 4, 7, 9, 11, 14, 16, and 18), these parameters should be measured in both "old" and "new" solutions. Hardness, alkalinity and specific conductance should be measured in the initial control (or culture) water and the low, medium and high elutriate concentrations at the start of the test and on days 7, 14 and 21.

Preferred ranges for water quality characteristics are presented in Table G-5. If temperature and dissolved oxygen of renewal water are outside of the preferred ranges, the water should be handled accordingly to adjust these characteristics so they fall within the preferred ranges. If pH, hardness and alkalinity of renewal dilution water are not within the preferred ranges, a new batch of renewal dilution water should be prepared. All water quality characteristics for the new batch must fall within the preferred ranges before the renewal dilution water is used.

Table G-5. Preferred Means and Ranges for Water Quality Characteristics in the *Daphnia magna* 21-d Toxicity Test.

Characteristic	Preferred Mean	Preferred Range
Temperature (°C)	20	19-21
Dissolved Oxygen (% of Saturation)	>75	50-100
pH	7.8	6.8-8.5[a]
Hardness (mg/L as $CaCO_3$)	170	160-180
Alkalinity (mg/L as $CaCO_3$)	115	110-120

[a] pH adjustments using NaOH or HCl are necessary if the test solution pH is less than 6.8 or greater than 8.5 (Biesinger 1987).

Individual measurements of water quality parameters that fall outside of the preferred ranges do not necessarily stop or invalidate the test. The test will be invalidated only if the

departures from preferred water quality values are sufficiently great or of sufficient frequency to prevent conformance with the quality assurance criteria indicated in Section 4.4.2. of this Appendix.

6.3.10. Test Termination

The test is terminated on day 21. Adult survival and young production are recorded as on previous days, and summed for the duration of the test (see Attachment D for sample data forms).

Four to six broods totaling 60 to >100 young are commonly obtained in a 21-d test at 20 ± 1°C. The first brood may be expected on d 7 to 10 with subsequent broods produced every 2-3 d. If a brood is being released at the time of transfer, and partial broods are released in each of the old and new media, consider it a single brood (USEPA 1993).

6.4. *Data Reporting and Statistical Analysis*

See Section 12.

7.0. *Ceriodaphnia dubia* **WATER COLUMN TOXICITY TESTS**

Ceriodaphnia dubia, a freshwater cladoceran in the family Daphniidae, is widely distributed in North America and Europe (Berner 1986). It is considerably smaller in size than some of the other commonly tested daphnids, such as *Daphnia magna,* and has a shorter life-cycle. Under optimal conditions, 3-4 broods may be produced parthenogenetically in one week (Berner 1986). Its utility as a test species was described by Mount and Norberg (1984), and it is routinely used in effluent biomonitoring programs (USEPA 1993). *C. dubia* has been used in acute toxicity tests to evaluate elutriates (Ankley et al. 1991b), and chronic tests with this species have been used in recent years to conduct evaluations of comparative chemical toxicity (Cowgill et al. 1985, Winner 1988, Cowgill and Milazzo 1991, Oris et al. 1991), of stream water quality (Burton et al. 1987, Nimmo et al. 1989), of the toxicity of sediment pore waters (Adams et al. 1986, Ankley et al. 1990), and of elutriates (Hoke et al. 1990). This report describes the methods used to culture *C. dubia* and to perform acute and chronic toxicity tests of dredged material elutriates in static test systems.

7.1. CULTURE METHODS

The culturing methods described below are largely taken from methods described by EPA (USEPA 1993) and ASTM (ASTM 1993c), and from Standard Operating Procedures at the USEPA National Effluent Toxicity Assessment Center, Environmental Research Laboratory-Duluth, Duluth, MN. Required materials are listed in

Attachment F.

7.1.1. Organism Source

Organisms for the initiation of a laboratory culture may be obtained from a source which has a verified culture of *Ceriodaphnia dubia*. Verification should be made by a recognized taxonomist. Starter cultures are available from either the USEPA Aquatic Biology Branch, Quality Assurance Research Division, EMSL-Cincinnati Newtown Facility, 3411 Church Street, Newtown, OH 45244 (513-533-8114); or the USEPA National Effluent Toxicity Assessment Center, Environmental Research Laboratory-Duluth, 6201 Congdon Boulevard, Duluth, MN 55804 (218-720-5529).

7.1.2. Acclimation of New Brood Stock

Environmental stress on the organisms in the starter culture must be minimized to facilitate normal culture growth and brood production. The temperature of the water containing the stock animals should be measured upon their arrival and gradually adjusted to the desired culture temperature of 25±1°C. Changes in temperature >3°C per any 12-hr period should be avoided (ASTM 1993c). Instantaneous pH changes >0.5 units have been found to cause mortalities (Mount and Norberg 1984). Mount and Norberg (1984) tentatively recommended that instantaneous pH changes should not exceed 0.2 units. If the new culture medium is different from the medium used in the laboratory from which the starter culture was obtained, animals from the starter culture should be transferred to the new culture medium gradually over a period two or more days to avoid mass mortality (ASTM 1993c). To accomplish a gradual change, it is important to know the basic chemical characteristics (i.e. pH, hardness, alkalinity) of the water used by the laboratory from which the culture was received, and to incrementally dilute that water with the culture water to be employed over two or more days. This is continued until the water essentially consists of 100 percent desired culture water. In preparation for a three-brood toxicity test, it is recommended (ASTM 1993c) that two (and preferably five) generations be raised using the same water, food and temperature as will be used in the test.

7.1.3. Reference Organism

It is recommended (USEPA 1993) that a new laboratory culture be started with a single animal, which is sacrificed after producing young. It should be permanently retained on a microscope slide for future reference. Procedures for making slide mounts according to the method of Beckett and Lewis (1982) are available in USEPA (1993). Once the stock culture has arrived from the supplier and neonates are being produced, adults should be separated and one offspring from a large brood should be selected as the source of the new laboratory culture. This organism should be from a third (or later) brood of at least eight neonates, as in the selection of neonates for conducting

toxicity tests. This will indicate that the culture has originated from a genetic line capable of a high level of reproduction. The remaining starter culture animals may be maintained as a back-up mass culture.

7.1.4. Culture Chambers

Chambers for mass culturing may be large crystallizing dishes, battery jars or aquaria containing 1 to 2 L of culture water for 40 to 80 adults, respectively. Chambers for individualized culturing are new 30 mL (1 oz) polystyrene souffle cups or 30 mL glass beakers containing 15 ml of culture water. Double-strength safety glass or 6 mm plastic panels may be used as cover material for brood board or mass cultures.

7.1.5. Culture Water

Moderately hard synthetic water made with deionized or Millipore Milli-Q^{R} water and reagent grade chemicals or 20 percent diluted mineral water (DMW) are recommended as culture water. Methods for their preparation are given in Attachment G.

Culture water is renewed a minimum of three times weekly (typically Monday, Wednesday and Friday) in both mass and individual cultures. Each batch of renewal water should be monitored for temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness, and the measurements recorded in a culture maintenance logbook. Water should not be used if it is more than one month old.

7.1.6. Temperature and Photoperiod

The temperature for culturing *C. dubia* should be maintained at 25±1^{o}C. A photoperiod of 16 h light and 8 h dark is recommended.

7.1.7. Food and Feeding

A diet that has been used successfully by EPA consists of a combination of yeast, Cerophyll^{R} and trout chow (i.e., the YCT diet,) supplemented with the unicellular green alga, *Selenastrum capricornutum* (USEPA 1973). Methods of preparing the YCT diet and of culturing and concentrating *Selenastrum* are given in Attachment H.

Mass cultures receive 7 mL YCT and 7 mL of algal concentrate per L of culture medium daily. Individual culture vessels receive 0.10 mL YCT and 0.10 mL of algal concentrate per 15 mL of culture medium daily. The YCT mixture must be measured for solids content. The dry weight of solids in a YCT batch should be 1.7-1.9 g/L, resulting in culture or test solution dry solid weights of 12-13 mg/L. Dry weight of solids in each batch of YCT diet is determined by oven-drying duplicate 5 mL samples of well-mixed YCT suspension in oven-dried pre-weighed weigh pans. The

mean dry weight per 5 mL is converted to a lL volume, and the resultant concentration of suspended solids is checked to see that it falls within the range of 1.7-1.9 g/L. The algal concentrate cell density must be measured, and contain between 3.0×10^7 and 3.7×10^7 cells/mL.

The unused YCT and algal concentrate should be refrigerated after use. The YCT should not be refrozen, and any unused portion of YCT should be discarded after two weeks. Algal concentrates should be discarded after one month.

7.1.8. Handling

A fire-polished pipet[1] of 2 mm bore diameter is recommended for transferring animals. Care should be exercised to avoid injury to the daphnids during transfer and to ensure that the neonates are introduced to the chambers below the surface of the water. At the time of culture medium renewal, organisms are transferred to the new medium, and food is added either immediately before or after the transfer.

7.1.9. Culture Maintenance-Mass Cultures

Animals (approximately 40 neonates <48 h old) are placed into the culture water on d 0 (typically a Friday). Culture water is renewed on d 3, 5 and 7. At the time of each water renewal, adult survival is recorded, and the offspring and old medium are discarded. On d 7 a new culture is started. The first culture is renewed on d 10 and 12 of the second week, and on d 14 the adults are discarded. A new culture is started with neonates from 7-d old adults. Mass cultures of overlapping ages are recommended to ensure the availability of animals should one or more cultures be lost due to a reduction in the quality of food or water.

7.1.10. Culture Maintenance-Brood Board Cultures

Neonates that are to be used in toxicity tests must be obtained from females individually cultured in brood boards. On d 0, one neonate *C. dubia* ($\leq$24 h old) is pipetted into each of 60 culture chambers contained in a brood board (Figure G-3). When a new brood board is started, the board should be labeled with initiation date, water type, initial animal age, and generation of adults that the young were collected from. Neonates selected for use in testing must be from the third (or later) brood of a female, and the brood should contain at least eight neonates. The medium is renewed and organisms transferred to fresh medium on d 3, 5 and 7. On d 6, chambers with no young present are

[1] Pipettes are stored in 100 percent methanol (which is replaced weekly) and are rinsed three times with tap and distilled water prior to use.

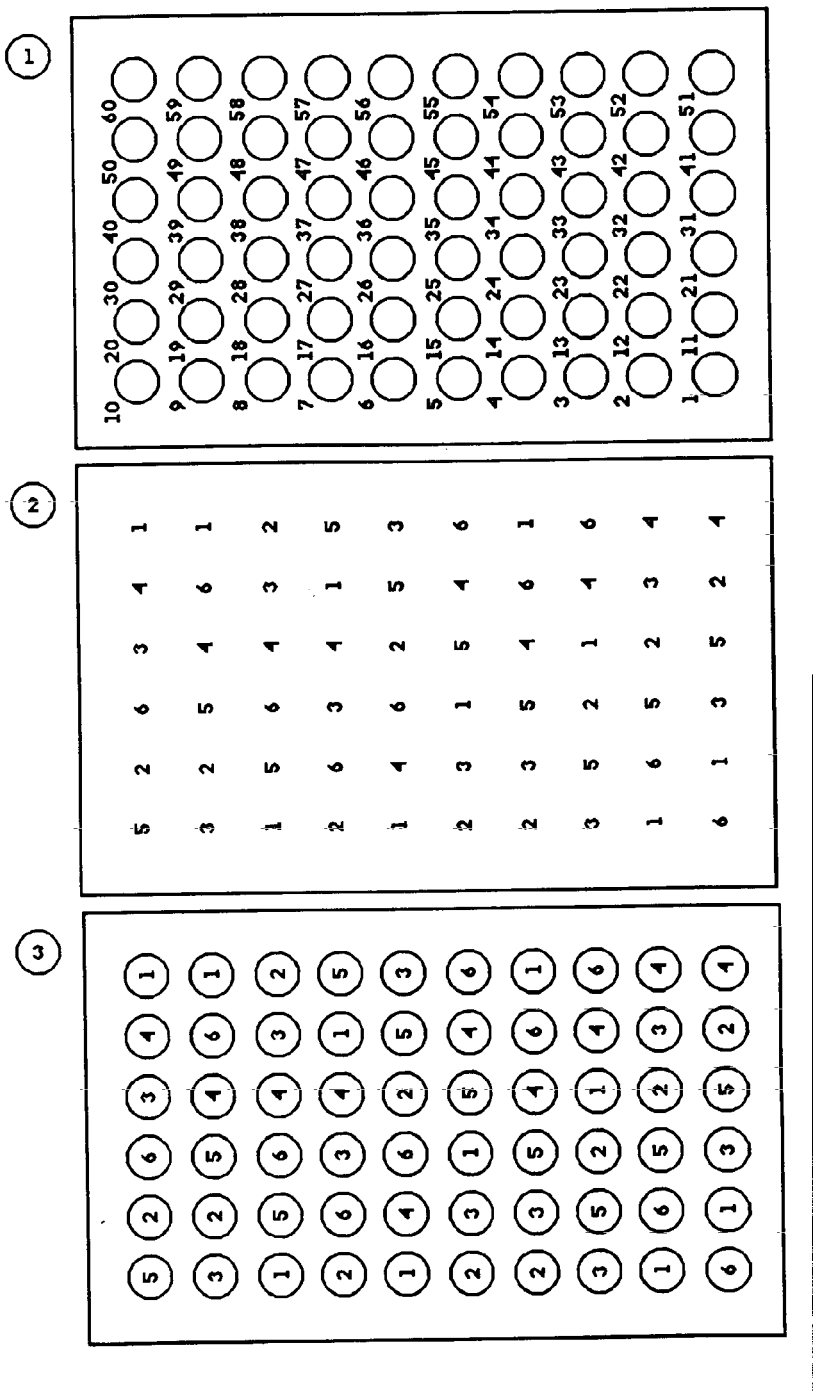

Figure G-3. Examples of a culture brood board, randomizing template, and block-randomized test board: (1) brood board with positions for six columns of ten replicate test chambers, (2) randomizing template prepared from a random numbers table for each row, and (3) randomized test board developed from (1) and (2) for the purpose of assigning treatment positions within each row on the board.

clearly marked with a black marking pen. On d 7, a new culture is started on a second brood board from broods of 10-20 neonates in the marked containers. If young are lacking in the marked cups, the second brood of young from unmarked cups may be used. If necessary, due to insufficient neonate production, new brood boards may be started with neonates from adults which produce 6-10 young in their third or fourth brood. These adults may serve as a source of neonates until they are 14 days old, at which time the adults are discarded. During the second week, the medium is renewed, adults transferred, and offspring discarded unless needed on days 10 and 12.

Two brood boards of individual cultures, each 7 days apart, should provide for a supply of organisms to initiate cultures and tests. Cultures which are well maintained and in good health will produce at least 15 young per adult in three broods (~6-8 days). A culture of this size (i.e. 60 individuals per brood board) should produce more than the minimum number of neonates needed (120) for two acute tests with five elutriate dilutions (150 neonates) or two chronic tests (120 neonates) weekly. Fifty neonates will be required for an acute toxicity test with the 100 percent elutriate only and a performacne control. Each chronic test will require 60 neonates for five elutriate concentrations of the test sediment and a performance control.

7.1.11. Culture Evaluation

Cultures are observed daily at the time of renewal and/or feeding. Test temperature is measured and recorded daily in the middle and four corner cups. Individual cultures should produce a minimum of 15 neonates each week. Production is determined monthly by randomly selecting 10 females from a brood board, and counting the young produced in the first three broods (within 7 days). If brood size and total young production in the cultures is less than 15 neonates for three broods in 60 percent of these 10 animals or overall survival is <80 percent (USEPA NETAC 1990; USEPA 1993), culturing conditions should be scrutinized and adjustments made to increase production.

Any males that may be present either in the mass cultures or the individual cultures should be removed on d 3. They are distinguished from females at this age by their smaller size, lighter color, and different body shape, having a more elliptical shape than the females (Mount and Norberg 1984). Distinguishing features of adult males include a broad cervical notch, large eye, long and cylindrical antennule with a terminal male seta 1.5 times the length of the antennule which terminates in a curved hook, and a clasper on the second thoracic appendage which is long and thin, curving to a small terminal hook (Berner 1986). Illustrations (Figure G-4) of female and male organisms are from Berner (1986).

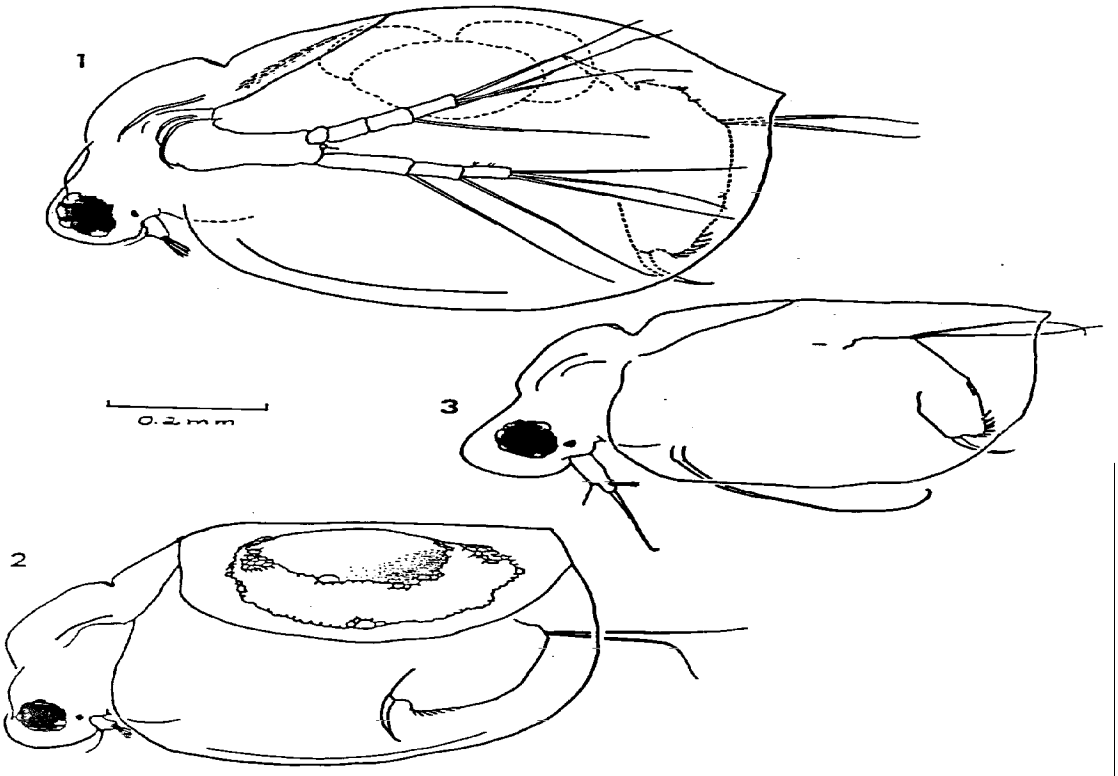

Figure G-4. *Ceriodaphnia dubia*: 1. parthenogenetic female; 2.sexual (ephippial) female; 3. male.

7.1.12. Culture Records

A separate set of record books should be maintained for the culture unit. Sample culture record forms are provided in Attachment I (Form I1). Records must be kept on the survival of brood organisms, in both the mass and individual cultures and of production of young in individual cultures. Adult mortality in excess of 20 percent or production of fewer than 15 young per three broods in 60 percent of the adults per week in a brood board are indicative of problems such as poor water or food quality. Organisms from this board should not be used in elutriate toxicity tests.

At the time of each culture medium renewal, the following information is entered into the culture record book: date, water type, culture water preparation date, preparation dates of YCT diet and *Selenastrum, Ceriodaphnia* age, animal appearance, and percent survival.

Records are maintained on each batch of culture water. Information on preparation date, pH, alkalinity, hardness, conductivity, and the last Millipore^{R} filter change is recorded in the culture log book. A record is also maintained on daily measurements of temperature in the culture unit.

7.2. ACUTE TEST

7.2.1. Elutriate Preparation (Acute Test)

The GLTEM currently recommends that acute exposures of 48 h duration be performed for routine tier 3 testing. Chronic tests (discussed in Section 7.3) may be used for tier 4 testing, if needed. The culturing of organisms is the same for both acute and chronic tests. Animals used to start an acute test are of the same age (i.e., $\leq$24 h old) and are handled the same way as in a chronic test. The same general test conditions of temperature, lighting and dilution water characteristics apply to both acute and chronic tests.

To obtain a sufficient volume of elutriate for an acute test (i.e., ~120 mL), place 40 mL of well-mixed sediment into a clean 500 mL beaker and add 160 mL of dilution water (same as culture water), and follow the elutriate preparation procedure described in section 5.0. The GLTEM recommends that the acute test first be performed with the 100 percent elutriate, and followed by a dilution series only if survival in the 100 percent elutriate is less than 50 percent. Table G-6 provides a summary of the volumes of elutriate and dilution water required in an acute test with five different elutriate concentrations and a water-only control using a 0.5 dilution level.

Table G-6. Volumes (mL) of Dredged Material Elutriate and Dilution Water Required per Renewal for the *Ceriodaphnia dubia* 7-d Acute Toxicity Test.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required	Total Dilution Water Volume Required
100	15.0	0.0	75.0	0
50	7.5	7.5	37.5	37.5
25	3.8	11.2	19.0	56.0
12.5	1.9	13.1	9.5	65.5
6.25	0.95	14.1	4.8	70.5
0.0	0.0	15.0	0	75.0

7.2.2. Acute Test Design

The test methods follow standard procedures for measuring the acute toxicity of effluents and receiving waters (USEPA, 1991). The basic design and conditions for performing a 48 h acute toxicity test are given in Table G-7. The test is performed with neonates (<24 h old) which have been provided with food for a minimum of 2 h during the holding period prior to test initiation. The animals are not fed during the test.

Table G-7. Overview of Recommended Dredged Material Elutriate Test Conditions for the 48-h *Ceriodaphnia dubia* Acute Toxicity Test.

1.	Test type	Static or static-renewal
2.	Temperature (°C)	25±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 µE/m^2/s, 540-1080 lux or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	30 mL
7.	Test solution volume	15 mL
8.	Renewal of test solutions	None (if dissolved oxygen is adequate) or at 24 h

Table G-7 (continued)

9.	Age of test organisms	Less than 24 h
10.	No. neonates per test chamber	5
11.	No. replicate test chambers per concentration	5 minimum
12.	No. neonates per test concentration	25 minimum
13.	Feeding regime	Do not feed during test. Feed 0.1 mL each of YCT and *Selenastrum capricornutum* suspension per holding chamber prior to test initiation so that young have food available for a minimum of 2 h prior to test initiation.
14.	Aeration	None during exposure; elutriate may be aerated before renewal if dissolved oxygen is low (i.e., ≤50% of saturation).
15.	Dilution water	Moderately hard synthetic water prepared using either (1) Millipore Milli-Q^{R} (or equivalent) deionized water and reagent grade chemicals or (2) 20% DMW.
16.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations with a performance control (water only).
17.	Dilution factor	≥0.5
18.	Water quality monitoring	Daily measurements of water temperature, dissolved oxygen and pH. Single measurements of hardness, alkalinity and specific conductance.
19.	Test duration	48 h
20.	Endpoints	Survival and complete immobilization
21.	Test acceptability	90% or greater survival in the water-only control solutions.
22.	Sample requirements	Storage of dredged material is at 4°C in the dark. Elutriate water should be prepared for tests within 8 weeks of collection.
23.	Dredged material volume required	40 mL from each test site

7.2.3. Organism Introduction

Fifty neonates less than 24 h old are required to start an acute test with 100 percent elutriate and a performance control. A total of 150 neonates is required to start an acute test with 5 elutriate concentrations and a performance control. Neonates should be randomly selected and distributed to the test chambers as described in Section 6.2.3. for *Daphnia magna*. Neonates should be from broods of at least 8 young. Organisms should be transferred from the culture chambers to the test chambers with a widemouth pipette with an opening of approximately 4 mm diameter. The tip of the pipette should be kept under the surface of the water to prevent air from being trapped under the carapace (USEPA 1993).

7.2.4. Test Organism Monitoring

Immobilization and lethality are the endpoints in an acute test. Test organisms are observed at 24 and 48 h for complete immobilization. Complete immobilization is frequently used as an endpoint for toxicity tests with this species, resulting in an EC50 estimate. This endpoint includes those animals that are dead. Affected animals that are completely immobilized are observed to lie motionless on the bottoms of the test chambers, and do not respond to gentle prodding. A microscope may be desirable for determining this endpoint. If survival data and the calculation of an LC50 are desired, the immobilized organisms should be examined for heartbeat using a dissection microscope.

7.2.5. Water Quality Monitoring

Water quality should be carefully measured and documented for each test. Daily measurements of temperature, dissolved oxygen concentration and pH should be taken in each chamber. Hardness, alkalinity and specific conductance should be measured once for the batch of water used in the test.

7.3. CHRONIC TEST

7.3.1. Chronic Test Design

The basic design and conditions for performing a chronic toxicity test are given in Table G-8. The test consists of a series of 5 dilutions of the test site dredged material elutriate and its performance control (culture water with no elutriate). Each of these experimental units is replicated 10 times, for a total of 60 exposure chambers. A dilution factor of 0.5 or greater is used for determining the dilutions to be made from the full-strength (100 percent) test sediment elutriate. Table G-9 is an example of a 0.5 dilution factor. A randomized complete block design is used (see Figure G-3 for example of randomization of test board). Neonates are added in a specific manner (see Section 7.3.5.) which allows for the performance of each female parent to be tracked.

Table G-8. Overview of Recommended Dredged Material Elutriate Test Conditions for the *Ceriodaphnia dubia* 7-d Chronic Toxicity Test.

1.	Test type	Static renewal
2.	Temperature (°C)	25±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 µE/m^2/s, 540-1080 lux or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	30 mL
7.	Test solution volume	15 mL
8.	Renewal of test solutions	Days 3 and 5
9.	Age of test organisms	Less than 24 h; and all released within an 8-h period
10.	No. neonates per test chamber	1
11.	No. replicate test chambers per concentration	10
12.	No. neonates per test concentration	10
13.	Feeding regime	Feed 0.1 mL each of YCT and *Selenastrum capricornutum* suspension per test chamber daily
14.	Aeration	None during exposure; elutriate may be aerated before renewal if dissolved oxygen is low (i.e., ≤50% of saturation)
15.	Dilution water	Moderately hard synthetic water prepared using either Millipore Milli-Q^R (or equivalent) deionized water and reagent grade chemicals or 20% DMW
16.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations with a performance control (water only)
17.	Dilution factor	≥0.5
18.	Water quality monitoring	Daily measurements of water temperature, dissolved oxygen and pH. Hardness, alkalinity and specific conductance are measured at the beginning and end of test.

Table G-8 (continued)

19.	Test duration	Until 60% of control females have three broods (may require more or less than 7 days)
20.	Endpoints	Survival and reproduction
21.	Test acceptability	80% or greater survival and an average of 15 or more young/surviving female in the control solutions. At least 60% of surviving females in controls should have produced their third brood.
22.	Sample requirements	Storage of dredged material is at 4°C. Elutriate water should be prepared for tests within 8 weeks of collection.
23.	Dredged material volume required	300 mL from each test site

Table G-9. Volumes (mL) of Dredged Material Elutriate and Dilution Water Required per Renewal for the *Ceriodaphnia dubia* 7-d Chronic Toxicity Test.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required	Total Dilution Water Volume Required
100	15.0	0.0	150	0
50	7.5	7.5	75	75
25	3.8	11.2	38	112
12.5	1.9	13.1	19	131
6.25	0.95	14.1	9	141
0.0	0.0	15.0	0	150

A summary of daily activities prior to and during a test is presented in Attachment J. This schedule assumes that all materials are in hand, and that a healthy culture of organisms is being maintained.

7.3.2. Test Chambers

For a chronic test, sixty new polystyrene 1 oz souffle cups (30 mL) or 30 mL glass beakers are required for each test site and its performance control. Test Chambers may be reused for renewals in a test, provided they are used at the same exposure levels. Polystyrene cups should be discarded after the test.

7.3.3. Water Renewal

All test chambers must receive a renewal of elutriate or

control diluent on d 3 and 5. The renewal elutriates for the test sediment should be the same as the initial elutriate, having been stored in the dark at 4°C. The diluent for preparation of elutriate test solution is culture water (control diluent). The renewal solutions should be warmed to the test temperature of 25±1°C prior to transfer of test organisms.

7.3.4. Temperature and Photoperiod

Tests should be performed in a temperature-controlled unit at 25±1°C. The daily photoperiod should be 16 L: 8 D.

7.3.5. Organism Introduction

A total of 60 neonates less than 24 h old, and all within 8 h of the same age, is required to start the test. Neonates are taken from the individual culture brood boards from adults that have 8 or more young in their third or subsequent broods.

Ten brood cups, each with 8 or more young, are selected from a brood board for the test. On a block-randomized brood board (prepare several randomized templates in advance that can be alternated for use), pipet from one cup one neonate into each of the 6 cups representing one complete subset (i.e. performance control, and five elutriate dilutions) of the 10 replicates. Discard the additional neonates from that brood cup. Repeat this process 9 times for the test site dredged material sample, using neonates from a new brood cup for each of the 6 bioassay chambers in a given row.

7.3.6. Food and Feeding

All test chambers receive a daily allotment of 100 uL of YCT diet and 100 μL of *Selenastrum* per 15 mL of medium (see Attachment I for food preparation). At the time of water renewal, (d 3 and 5), food should be added immediately prior to or after organism transfer.

7.3.7. Test Organism Monitoring

Test organisms are observed daily for survival and young production. Organisms also are observed for their behavior. Any abnormal behavior (e.g. rapid or slow swimming, spinning) in adults and offspring should be recorded. Observations are made using a dissecting microscope with substage lighting. A light box may be used for illuminating the animals during examination. To aid in counting live young on d 3 and 5, 2 drops of 1N HCl added to the chamber after the adult has been transferred to new media will kill the young and result in their settling to the bottom.

7.3.8. Water Quality Monitoring

Water temperature should be monitored daily in at least five locations on the test board (four corners and center). Dissolved

oxygen and pH should be measured daily in a control chamber (C) and in one of the low (L) medium (M) and high (H) exposure chambers (Attachment I, Form I2). At the time of renewal (d 3 and 5), these parameters should be measured in both "old" and "new" solutions. Hardness, alkalinity and specific conductance should be measured in the initial control (or culture) water and the low, medium and high elutriate concentrations at the start of the test and at termination on d 7 or 8. Preferred ranges for water quality parameters are presented in Table G-10.

7.3.9. Test Termination

The test is terminated when 60 percent of the control females have had three broods or at the end of d 8, whichever occurs first. Adult survival and young production are recorded as on previous days, and summed for the duration of the test (see Attachment I for sample data forms). Three broods totaling 20-35 young are commonly obtained in a 7-d test at 25 ± 1°C. The first brood may be expected on d 3 or 4, and typically consists of two to five young. The second and third broods are released from 36 to 48 h after the first brood, and typically consist of 8 to 20 young. If a brood is being released at the time of transfer, and partial broods are released in each of the old and new media, consider it a single brood (USEPA 1993).

Table G-10. Preferred Means and Ranges for Water Quality Parameters in the *Ceriodaphnia dubia* 7-d Chronic Toxicity Test.

Parameter	Preferred Mean	Preferred Range
Temperature (°C)	25	24-26
Dissolved Oxygen (% of saturation)	>75	50-100
pH	7.6[a], 8.1[b]	7.4-7.8[a], 7.9-8.3[b]
Hardness (mg/L as $CaC0_3$)	90	80-100
Alkalinity (mg/L as $CaC0_3$)	65	60-70

[a] Mean and range for moderately hard water prepared with reagent grade chemicals.

[b] Mean and range for moderately hard water prepared by addition of mineral water.

7.4. Data Reporting and Statistical Analysis
See Section 12.

8.0. *Pimephales promelas* **WATER COLUMN TOXICITY TESTS**

The fathead minnow, *Pimephales promelas* Rafinesque (Figure G-5), is a freshwater fish included in the family Cyprinidae. This species was chosen as a test organism for several reasons: (1) ease of culturing and testing, (2) widespread occurrence, (3) rapid growth, (4) ecological importance, and (5) sensitivity to a variety of environmental pollutants. Fathead minnows are omnivorous feeders which eat a variety of plant and animal life (Devine 1968, Held and Peterka 1974). They are tolerant of a wide range of physical and natural chemical conditions. Adults can attain a total length of 90-101 mm and mature to reproduction in 90 to 120 days under ideal conditions. Spawning may be impacted at extremes of pH but has been successful at pH values ranging from 5.9 (Mount 1973) to 9.5 (McCarraher and Thomas 1968). The species is tolerant of water temperatures ranging from 2 to 33°C (Bardach et al. 1966) and spawns successfully in the temperature range of 15.6 to 29.8°C (Brungs 1971). Fathead minnows tolerate high total alkalinity concentrations of up to 1,800 mg/L as $CaCO_3$ (McCarraher and Thomas 1968) and turbidity as high as 15,000 mg/L total solids (Rawson and Moore 1944).

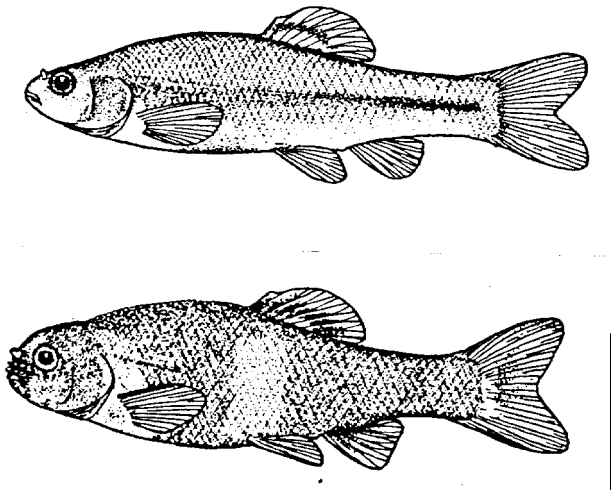

Figure G-5. Mature female and male fathead minnow, *Pimephales promelas*, approximately life-sized (From Eddy and Underhill 1974).

A large database of acute and chronic toxicity information is available for the fathead minnow (e.g., Brooke et al. 1984,

Geiger et al. 1985, 1986, 1988, 1990, Mayer and Ellersieck 1986). These studies indicate that the fathead minnow is sensitive to low levels of most industrial organic chemicals, pesticides, and metals. Fathead minnows are also extremely sensitive to ammonia (Ankley et al. 1990) and hydrogen sulfide (Smith et al. 1976), both common toxicants in sediments.

This report describes the methods used to culture fathead minnows and to perform acute and subchronic toxicity tests of larvae in static test systems with regular renewal of the elutriate. The endpoint in a 4-d acute test is survival. Endpoints in the 7-d fathead minnow toxicity test include both survival and growth.

The use of growth as a routinely monitored endpoint in chronic toxicity tests with fish was first suggested by Sprague (1971) because it serves as an integrator of all sublethal effects. Growth is the result of a suite of physiological and behavioral interactions which may be adversely affected by chemical contaminants in sediments, the water column or food (McKim 1977). If fish experience a decreased growth rate, the larval-juvenile period (the period of highest mortality for fish) will be extended. The probability of mortality due to predation also will be increased, because the period of greatest susceptibility to predation is extended (Werner and Hall 1974; Werner and Gilliam 1984; Post and Prankevicius 1987). Thus, minor reductions in growth can result in significant reductions of survival and recruitment of larval fish to adult populations, and subsequent population level impacts (May 1971; Laurence 1974; Werner and Blaxter 1980: Leiby 1984; Adams and DeAngelis 1987). With specific regard to growth as an endpoint in the 7-d fathead minnow embryo-larval test, a number of studies have demonstrated a significant correlation between growth reduction and different synoptic measures of impact upon aquatic community structure (Mount et al. 1984; Mount et al. 1985; Norberg-King and Mount 1986; Birge et al. 1989; Dickson et al. 1990).

8.1. CULTURE METHODS

The culturing methods described in this report are based on the methods recommended by USEPA (Denny 1987, USEPA 1993), and ASTM (1993a, 1993f). Required materials are listed in Attachment K. Fathead minnow embryos and larvae for use in testing are available from a variety of commercial sources.

8.1.1. Organism Source

Organisms for the initiation of a laboratory culture should be obtained from a source which has a verified culture of *P. promelas*. Fish that are adapted to laboratory conditions and free of disease must be used as the brood stock. Embryos make the best initial stock of fish because they are easiest to

transport and most likely to be free of disease. Stock from wild populations should be avoided unless cultured through at least one generation to ensure they are disease-free and of adequate vigor (Denny 1987). Starter cultures are available from the USEPA Aquatic Biology Branch, Quality Assurance Research Division, EMSL-Cincinnati Newtown Facility, 3411 Church Street, Newtown, OH 45244 (Telephone: 513-533-8114); the USEPA Environmental Research Laboratory-Duluth, 6201 Congdon Boulevard, Duluth, MN 55804 (Telephone: 218-720-5500); or any of several commercial suppliers with species verified stocks.

8.1.2. Acclimation

Environmental stress on the organisms in the starter culture must be minimized to facilitate normal growth and embryo production. The temperature of the water containing the brood stock animals should be measured upon their arrival and gradually adjusted to the desired culture temperature of 25±1°C. Changes in water temperature >3°C in any 12-h period should be avoided and, in general, water temperature should not change more than 3°C in a 72-h period (ASTM 1993f). The dissolved oxygen concentration should be maintained between 60 and 100% of saturation. Gentle aeration with oil-free compressed room air is desirable. Supersaturation by dissolved gases should be avoided to prevent gas bubble disease. If the culture medium differs in hardness, alkalinity or pH from that in which the organisms were received, animals from the starter culture should be transferred to the new culture medium gradually over a period of 1 to 2 days to avoid stressing the organisms (ASTM 1993f).

Reproductively mature (≥120-d old when cultured at 25°C) individuals must be segregated into breeding groups to successfully produce embryos. When sexes can be distinguished (Denny 1987), a male is placed with one or two females and a spawning substrate. This sex ratio accelerates the onset of spawning activity. If misidentification of sexes occurs, replace with properly sexed individuals. Group spawning with 3-4 males and 10-15 females also works well.

8.1.3. Reference Organism

It is recommended that several organisms in the brood stock be examined by a biologist competent in fish taxonomy to ascertain that the brood stock are *P. promelas*. Several taxonomic references are available to distinguish members of the fish family Cyprinidae (e.g. Eddy and Underhill 1974, Scott and Crossman 1973). Verification should be documented in writing and should include the name of the individual responsible for the taxonomy, the taxonomic key used, the date of identification and the source of the individual organisms used in the identification.

8.1.4. Culture Chambers

Chambers for mass culturing of fathead embryos may be constructed of glass, fiberglass, or stainless steel, although glass is preferred. An example of a single culturing system would be a 57 L (15 gallon) glass aquarium (31 cm x 61 cm x 32 cm, WxLxH) with a standpipe drain adjusted to provide 10 to 20 cm of water depth (Denny 1987). Each aquarium can be divided into quarters to accommodate four breeding males. The divisions are made with stainless steel mesh (5 mm opening), fastened in place with silicone glue. The entire unit must be acid washed with 1N HCl or HNO_3 to remove any manufacture residues. One water inlet, drain and airstone can serve each aquarium.

White plastic dish-washing pans commonly available in stores (e.g. 53 cm x 40 cm x 12 cm, LxWxH) make good hatching trays for embryo incubation because white pans facilitate viewing the newly hatched larvae. The water temperature within this pan should be maintained at 25±1ºC. Newly hatched larvae are small (<5 mm total length) and can easily escape to a drain if flowing water is used. Therefore, static water conditions are best with water temperature controlled by a water bath, in-tank electric heaters, or constant room temperatures. A generous supply of low pressure (≈3 psi) air (compressed oil-free room air) and an airstone are needed to mix the water in the pan and aerate the water that is in contact with the developing embryos.

8.1.5. Spawning Substrates

Fathead minnows deposit their eggs on the underside of submerged or floating objects. For cultures, suitable spawning substrates have been provided with PVC (polyvinylchloride) pipe, 7.6 to 10.2 cm diameter, cut into 7 to 10 cm lengths. The pipes are then halved lengthwise, the underside roughened with a wire brush (Gale and Bunyak 1982) and placed into each breeding tank with the arch down. This creates a room-like spawning area under which fathead minnows will deposit the eggs. Other spawning substrate materials have been used successfully, such as, stainless steel shaped like the PVC pipe with sand coated to the underside with silicone glue (Benoit 1982).

8.1.6. Culture Water

An adequate supply of water such as from a spring, well or controlled surface water of consistent high quality is necessary to culture fathead minnows. Water quality parameters such as hardness, alkalinity, conductivity and pH should fall within the following ranges: hardness, 25-300 mg/L as $CaCO_3$; alkalinity, 25-300 mg/L as $CaCO_3$; conductivity, 50-500 umhos/cm; and pH, 6.5 to 9.0. Dechlorinated water can be used when dechlorinated with sodium bisulfite (sodium sulfite can be used but is less desirable) which also removes chloramines (ASTM 1993a, 1993f) or by dechlorination with aeration in an open chamber of sufficient

retention time (>24 h) to remove chlorine and chloramines. Chemical monitoring of the water for residual chlorine or chloramine concentration must be conducted to ensure concentrations of these chemicals do not exceed 3 μg/L. Municipal drinking water also often contains copper, lead, zinc and fluoride which can be removed, when excessive, by using appropriate ion exchange resins (ASTM 1993a, 1993f). The national water quality criteria for the chronic values for freshwater organisms exposed to copper, lead and zinc are 12, 3.2 and 110 μg/L, respectively, at water hardness of 100 mg/L as $CaCO_3$ (USEPA 1987). Different chronic values must be calculated if the culturing water differs from 100 mg/L as $CaCO_3$. No criterion is available for fluoride.

8.1.7. Temperature and Photoperiod

Water temperature for culturing fathead minnows should be maintained at 25±1°C. This temperature is suitable for reproduction, incubation and growth. Temperatures below 22°C and above 26°C reduce reproduction of fathead minnows (Brungs 1971).

A photoperiod of 16 h light and 8 h dark during each 24-h period is recommended. Wide spectrum fluorescent lights with intensity of 10-20 µE/m^2/s (540-1080 lux, 50-100 ft-c) at the water surface are preferred.

8.1.8. Food and Feeding

Adult fathead minnows and fish over 30-days old are fed adult brine shrimp (*Artemia* sp.). These shrimp are purchased frozen from suppliers and allowed to slightly thaw prior to feeding. It is not necessary to rinse the shrimp with culture water to remove brine before feeding. Fish are fed *ad libitum* twice daily (i.e. approximately 1 to 2 mL per feeding per breeding pair). A rule of thumb is that most of the food will be consumed in about 10 min (Denny 1987) if the amount is appropriate.

Larval fathead minnows are fed freshly hatched (<24-h old) brine shrimp nauplii a minimum of twice daily. Before feeding the nauplii, they should be concentrated on a fine Nitex® screen rinsed with culture water, and resuspended with a minimum of culture water before feeding to the fish. Feeding should begin the same day as larvae hatch. The size of the nauplii is important; they must be small enough to be ingested by the larvae. Feeding of fresh nauplii is continued until fish reach ~25-30 days old when they can be fed adult brine shrimp.

Culturing of brine shrimp nauplii is done in a 25°C brine (NaCl) incubator. Hatchery designs and necessary apparatus are simple (Denny 1987). ASTM (1993g) has published guidelines for using brine shrimp nauplii in aquatic toxicology.

Other commercial diets are acceptable. However, they must exhibit growth of larval fathead minnows comparable to the recommended diet.

8.1.9. Chamber Cleaning

Chambers containing adult breeding fish should be scraped and siphoned a maximum of once weekly. Clean only a few chambers each day so that the whole brood culture is not disturbed by the cleaning. Some algal growth in the chambers is desirable as the fish eat it to supplement their diet. Chambers containing larvae and fish less than 30-d old should have the bottom siphoned carefully each day to remove uneaten brine shrimp nauplii and feces. Dead brine shrimp promote growth of a fungal mat which may entrap and kill larval fish.

8.1.10. Handling

Care must be taken to avoid disturbance of the adults and young fish due to unnecessary movement, noise, and extraneous lighting. Fish handling should be kept at a minimum. An adult fish should be moved carefully using only nets made of soft knotless material, and must be released quickly into water to avoid stress. Fish too small (<50 mg) to net should be handled with glass pipets or tubing with fire-polished ends. Organisms that are injured or dropped during handling or that touch dry surfaces should be discarded (ASTM 1993a, 1993f).

8.1.11. Water Quality Monitoring

Water used to culture fathead minnows should be monitored for temperature (25±1°C), dissolved oxygen (5.0-8.3 mg/L), hardness (25-300 mg/L as $CaCO_3$), alkalinity (25-300 mg/L as $CaCO_3$), conductivity (50-1,000 µmhos/cm), pH (6.5-9.0 pH units), ammonia (<0.1 mg/L total ammonia), and any other characteristics [e.g. chlorine and chloramines (residual chlorine ion specific electrode method, Rigdon et al. 1978), sulfides (iodometric method, APHA 1985)] useful to indicate consistent quality. Temperature should be measured daily and dissolved oxygen twice weekly in the breeding and culturing tanks. Hardness and alkalinity should be measured weekly at the water supply source to the tanks.

8.1.12. Embryo Incubation

Visually inspect the spawning substrates daily for deposited embryos by removing them from the water with your hands or check for embryos without removing the substrates from the water by feeling with your fingers. When handling substrates, clean hands or wear latex gloves. When embryos are present, transfer the substrate to the incubation pan and replace the substrate with a clean one. Estimate the number of embryos on each substrate so that, if hatching is successful, the number of fry available on a certain date can be determined. Report this information on the

embryo production record form (Attachment L, Form L-1).

Incubate embryos on substrates in the incubation pan by standing the substrates on edge and placing an airstone near each substrate. For successful incubation, abundant air-induced circulation of the water is needed to maintain oxygen to the embryos. Check each substrate daily for white unfertilized and fungus-infected embryos. When defective embryos are located they must be removed with a forceps to prevent the spread of fungus to healthy embryos. Embryos will form dark eye-spots at about 48 h in 25ºC water and will begin hatching by 96 h. Hatched embryos may remain in the incubation pan until hatching of all viable embryos is complete by 120 h. Larvae are then counted and transferred to rearing tanks or used for tests. Larvae used for tests should be <24-h old when reared at the testing facility or must be <48-h old when larvae are received from remote sites. Larvae should be within 12 h of each other in age for use in the tests.

8.1.13. Culture Evaluation

Brood stock evaluation is based upon survival and reproductive rate of the fish. Rarely do mature fathead minnows die in the culture tanks. Any more than an occasional random death of one of the brood stock should be cause for concern. The first symptom of problems in the culture tanks is a reduction in the reproductive rate. Typical spawning rates can be as high as a spawning every 2 or 3 d with one male and two females present. Once a spawning rate has been established any decrease in rate can be attributed to a change in water or food quality, brood stock health, or "spawned out" fish. Spawning pairs usually continue to reproduce for several months.

Embryos are evaluated daily for suitability as test organisms by observing the embryos for changes in development or fungal attack. Generally, when one-half or more of the embryos on a spawning substrate show either lack of development or fungal infection, the entire group of embryos from that substrate should be discarded (Denny 1987). After hatching and until used for testing, each batch of larvae should be observed for rate of survival. When approximately 20% or more of the hatched larvae have died during this time interval, that batch of larvae should be considered unsuitable.

8.1.14. Culture Records

A separate set of records should be maintained for the culture unit. The records should show dates of spawnings by each breeding pair and the estimated number of embryos per substrate. In addition, there should be daily records (Attachment L) of water temperature, feedings, chamber cleaning, aeration, water flow, spawning substrate inspections, mortalities and hatching

success of embryos.

8.2. ELUTRIATE ACUTE TOXICITY TEST METHODS

8.2.1. Acute Test Design

The basic design and conditions for performing a 4-d dredged material elutriate toxicity test are given in Table G-11. Additionally, a daily activity schedule (Attachment M) is provided to facilitate planning and starting a toxicity test. The GLTEM recommends that the acute test first be performed with the 100 percent elutriate, and followed by a dilution series only if survival in the 100 percent elutriate is less than 50 percent. Each exposure is replicated five times. Table G-12 provides a summary of the volumes of dredged material and water required for a complete dilution series, using a 0.6 dilution factor. If more than 50% of the test organisms die at the lowest dilution treatment (10%) then another test must be conducted starting at about a 10% elutriate solution concentration and reducing the elutriate concentration by a factor that will allow the organisms to survive sufficiently to calculate an LC50.

A completely random design or a randomized complete block design can be used to arrange the test chambers. The randomized complete block design is used if the possibility exists for an effect due to test chamber placement by such things as slight differences of water temperature or lighting. The randomized complete block design requires three groupings (one for each replicate) with randomization within each grouping (Figure G-6). Larvae are introduced to the test chambers in a specific manner (see Section 8.2.6.).

Table G-11. Overview of Recommended Dredged Material Elutriate Test Conditions for the Fathead Minnow 4-d Acute Toxicity Test.

1.	Test type	Static with one renewal at 48 h
2.	Water temperature	25±1°C
3.	Illumination quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 $\mu E/M^2/s$, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	250 mL minimum
7.	Test solution volume	200 mL minimum
8.	Renewal of test solutions	Daily
9.	Age of test organisms	24 to 48 h

Table G-11 (continued)

10.	No. of larvae per test chamber	10
11.	No. replicate test chambers per concentration	5 minimum
12.	No. of larvae per test concentration	50 minimum
13.	Feeding regimen	Feed 0.1 mL brine shrimp nauplii suspension per test chamber three times daily; or 0.15 mL twice daily
14.	Aeration	None unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min.
15.	Dilution water	Culture water, test site water, or reconstituted water
16.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations, and a performance control (diluent or culture water only)
17.	Dilution factor	≥0.5
18.	Test duration	4 days
19.	Endpoint	Mortality (LC50 or NOEC)
20.	Test acceptability	Dissolved oxygen ≥40% of saturation, mean temperature of 25±1°C; 90% or greater survival in the controls and satisfactory results from reference toxicant tests
21.	Sample requirements	Storage of dredged material is at 4°C; elutriate water should be prepared and tests initiated within 8 weeks of collection
22.	Dredged material required	A minimum of 2400 mL for each test site

8.2.2. Test Chambers

Glass or non-toxic disposable plastic test chambers are required for each concentration and control. Test chambers must be clean and of 250 mL capacity or greater. To avoid potential contamination from the air and to reduce evaporation of the test solutions, the test chambers should be covered with sheets of safety plate glass or plastic (6mm, 1/4 inch thickness; USEPA 1989). If plastic sheets are used they should not be of fresh construction and should not emit chemical odors. Fresh plastic sheets should be immersed in culture water for a minimum of 24 h to reduce chemical odors.

Table G-12. Volumes (mL) of Dredged Material Elutriate and Dilution Water for Each Renewal of a Single Test Elutriate in the Fathead Minnow 4-d Acute Toxicity Test Using a 0.6 Dilution Factor.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required/5 Reps	Total Dilution Water Volume Required/5 Reps
100.0	200.0	0.0	1000.0	0.0
60.0	120.0	80.0	6000.0	400.0
36.0	72.0	128.0	360.0	640.0
21.6	43.2	156.8	216.0	784.0
13.0	25.9	174.1	129.5	870.5
0.0	0.0	200.0	0.0	1000.0

8.2.3. Cleaning of Glassware

All glassware used in the tests should be thoroughly cleaned before contact with test solutions or test organisms. Glassware should be washed with detergent, rinsed three times each with tap water and deionized water, and then placed in a clean 10 percent HC1 or HNO_3 bath for approximately 4 h. This should be followed by several rinses with deionized water, an acetone rinse, and a final rinse with deionized water. Disposable plastic test chambers should need no cleaning prior to use in a toxicity test but should be rinsed with deionized water. If an oil-sheen is seen in the rinse water, discard the test chambers. Plastic test chambers must not be reused.

8.2.4. Elutriate Renewal

All test chambers must receive fresh elutriate and/or diluent water a minimum of once at 48 h. The renewal solutions must be prepared on the day of renewal with the elutriate stock solution prepared at the beginning of the test. The diluent water is either culture or site water. The renewal solutions must be prepared in advance to allow temperature adjustment to the test temperature of 25°C. As the solutions warm from the 4°C storage temperature, supersaturation of the dissolved gasses may occur. If the dissolved oxygen concentration exceeds 100% of saturation (≈8.3 mg/L), gentle aeration may be necessary to reduce the supersaturation of gases. If the dissolved oxygen in test solutions drops below 4.0 mg/L during the test, gentle aeration should be added. The aeration should be added through a disposable glass pipet tip that has been inserted to near the bottom of the test chamber and adjusted to deliver at a rate of

about 100 bubbles/min.

8.2.5. Temperature and Photoperiod

Tests should be performed in a temperature-controlled room or in a temperature-controlled water bath. The test temperature recommended for this method is 25±1°C and the photoperiod recommendation is 16 h light and 8 h dark in each 24-h period. The light quality and intensity should be at ambient laboratory levels (USEPA 1989), which is approximately 540-1080 lux, or 50-100 foot candles (ft-c) at the water surface in the test chambers.

8.2.6. Organism Introduction

A total of 300 larval fathead minnows 24-48 h old are required to start the test. Larvae are captured from a common pool of larvae with an appropriate pipet (fire-polished opening, 5 mm diameter) for young larvae and a beaker in conjunction with a small dip net for larger larvae, and placed into the exposure chambers containing test solutions equilibrated to 25°C. The test chambers are randomized before introducing the larvae. Larvae are introduced in the order the chambers have been randomized either by row or within a block (Fig. G-6). Two or three larvae are consecutively added beneath the surface of the test solutions in each test chamber until a total of ten organisms are in each. Care should be taken to count them and to add the least possible volume of culture water. The pipet may become contaminated with elutriate constituents; however, this source of potential contamination can be eliminated by rinsing the pipet in a beaker of diluent water after each larval transfer.

8.2.7. Food and Feeding

The fish in each test chamber are fed 0.1 mL (approximately 700-1,000 nauplii) of a concentrated suspension of brine shrimp nauplii. The brine shrimp must be recently hatched (less than 24-h old). One method to achieve this feeding rate is to allow the brine culture of nauplii to settle a few minutes in a 2 L separatory funnel without aeration. As soon as the nauplii are mostly near the bottom of the container, take 125 mL of them by pipette and place them in a beaker with a fine-meshed bottom and rinse one to three times with diluent water to reduce the sodium chloride concentration. Immediately back-rinse the beaker with 10 to 15 mL of diluent water into another beaker. This solution contains the appropriate density of nauplii for feeding. Feedings should be three times daily at 4-h intervals. If twice-daily feedings are used, 0.15 mL of nauplii, rather than 0.10 mL, are fed to each test chamber at 6-h intervals.

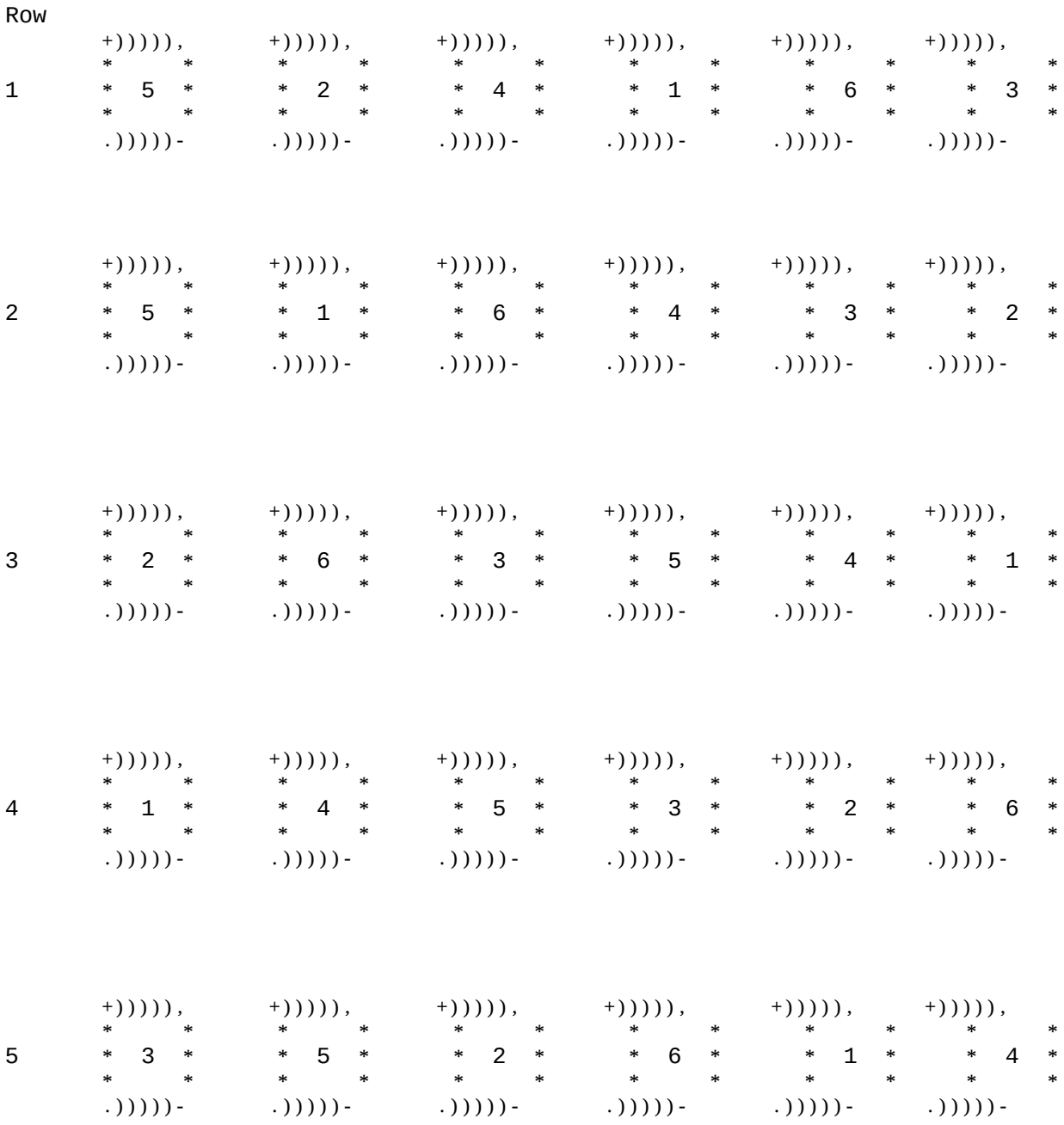

Figure G-6. Example of an exposure chamber arrangement for a randomized complete block design. Each row contains one replicate of all treatments.

The feeding schedule will be dependent on when the test solutions are renewed. If the test is initiated after 1200 PM, the larvae may be fed once or twice the first day. On following days, the larvae normally would be fed at the beginning of the work day, or a minimum of 2 h before test solution renewal,

during mid-day, and at the end of the work day after test solution renewal. However, if test solutions are renewed at the beginning of the work day, the first feeding should be after test solution renewal and the remaining feedings would be at appropriate intervals (USEPA 1989).

8.2.8. Cleaning Test Chambers

At the time of renewal of test solutions, uneaten and dead brine shrimp nauplii and other debris are removed from the bottom of the test chamber with a small siphon hose (1/8" i.d. made of Tygon® or Teflon®). Three possible techniques for cleaning are: (1) a hose is held with a loop in one hand to close the hose with a crimp if a fathead minnow larva is endangered by the siphon, (2) use a spring-loaded hose clamp to control flow, or; (3) an alternative to the siphon is a glass tube or 50 mL pipet fitted with a rubber bulb to suction the bottom. Because the larvae may be small during the test, cleaning may be facilitated by placing the test chamber on a light box during cleaning. Siphoning or discharging the suction bulb into a white plastic container is recommended to insure that no larvae escape the test chambers unnoticed. Any larvae found in the white container should be returned to the study and a record made of it in the log. The test chambers are siphoned to within 5-7 mm of the bottom leaving approximately 15-20% of the previous test solution volume in place (USEPA 1993).

8.2.9. Test Solution Renewal

Test solutions can be renewed each day but must be renewed at 48 h from the stock elutriate solution. The stock solution must be prepared the day before the test begins due to the several hours of time required to prepare the necessary elutriate volume. The test solutions are made in the same manner as at test initiation. Make certain that the temperature of the new solutions is 25°C and that supersaturation of gasses has not occurred. Immediately after siphoning the test chambers, the test solutions are renewed by adding the new solution down the inside of the test chamber being careful not to disturb the larvae with excessive turbulence.

8.2.10. Test Organism Monitoring

Test organisms are observed daily for survival and abnormal behavior. Mortalities and any abnormal behavior (e.g. rapid or slow swimming, swimming at the surface, spinning) should be recorded. Organisms can be observed without magnification. Dead organisms must be removed immediately upon discovery or a minimum of once daily.

8.2.11. Water Quality Monitoring

Water temperature should be measured daily in at least five locations in the test chamber array (four corners and center)

with an accurate thermometer precise to 0.1ºC. Dissolved oxygen and pH should be measured daily with precision to 0.1 mg/L and 0.1 pH unit, respectively, in a control chamber and in one each of the low, medium and high concentration exposure chambers. Measure ammonia once before starting the test. The same measurements should be made in the fresh renewal solutions as well as the old solutions. Hardness and alkalinity determinations should be precise to 1-2 mg/L as $CaCO_3$ and specific conductance measured with precision to 5 µmhos/cm in the control (diluent) water and the low, medium and high elutriate concentrations at the start of the test and at test termination on d 4.

8.2.12. Test Termination

The test is terminated on d 4 at the same hour of the day that the test was begun. Survival of the larvae is recorded as on previous days and summed for the duration of the test.

8.3. ELUTRIATE CHRONIC TOXICITY TEST METHODS

8.3.1. Test Design

The basic design and conditions for performing a 7-d dredged material elutriate toxicity test are given in Table G-13. Additionally, a daily activity schedule (Attachment M) has been prepared to facilitate planning and starting a toxicity test. The test consists of a series of 5 dilutions of the test site dredged material elutriate and its performance control (culture water). Each of these experimental units (treatments) is replicated a minimum of 5 times, for a total of 25 exposure chambers per dredged material elutriate, plus controls. Thus, a chronic test of one dredged material elutriate with five dilutions requires 30 exposure chambers. A dilution factor of 0.5 or greater is used to determine the dilutions to be made from the full-strength (100 percent) test elutriate (Table G-14). An alternative design that could be used if there are several test sites would be to initially test only the 100% elutriate for each site (with an appropriate performance control). Then, test with a series of dilutions could be performed only for those dredged materials exhibiting chronic toxicity in the 100% elutriate solution.

Table G-13. Overview of Recommended Dredged Material Elutriate Test Conditions for the Fathead Minnow 7-d Chronic Survival And Growth Toxicity Test.

1.	Test type	Static with daily renewal
2.	Water Temperature	25±1°C
3.	Illumination quality	fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 μE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber size	250 mL
7.	Test solution volume	100 mL
8.	Renewal of test solutions	Daily
9.	Age of test organisms	Less than 24 h preferred when in-house culture available; must be less than 48 h when larvae shipped from remote sites
10.	No. larvae per test chamber	10
11.	No. replicate test chambers per concentration	5 minimum
12.	No. larvae per test concentration	50 minimum
13.	Feeding regimen	Feed 0.1 mL brine shrimp nauplii suspension per test chamber three times daily; or 0.15 mL twice daily.
14.	Aeration	None, unless DO drops below 4.0; rate should not exceed 100 bubbles/min.
15.	Dilution water	Culture water, test site water, or reconstituted water.
16.	Elutriate concentrations	Minimum of 5 test site elutriate concentrations, and a performance control (diluent or culture water only)
17.	Dilution factor	≥0.5
18.	Test duration	7 d
19.	Endpoints	Survival and growth (dry weight)
20.	Test acceptability	80% or greater survival in the control solutions; mean weight per control fish ≥0.25 mg; satisfactory results from the reference toxicant test
21.	Sample requirements	Storage of dredged material is at 4°C; elutriate water should be prepared and tests initiated within 6 weeks of collection
22.	Dredged material required	A minimum of 1,800 mL for each test site

Table G-14. Volumes (mL) of Dredged Material Elutriate and Dilution Water for Each Renewal of a Single Test Elutriate in the Fathead Minnow 7-d Chronic Survival and Growth Toxicity Test Using a 0.5 Dilution Factor.

Percent Elutriate	Elutriate Volume Per Replicate	Dilution Water Volume Per Replicate	Total Elutriate Volume Required/5 Reps	Total Dilution Water Volume Required/5 Reps
100.0	100.0	0.0	500.0	0.0
50.0	50.0	50.0	250.0	250.0
25.0	25.0	75.0	125.0	375.0
12.5	12.5	87.5	62.5	437.5
6.25	6.25	93.75	31.2	468.8
0.0	0.0	100.0	0.0	500.0

A completely random design or a randomized complete block design can be used to arrange the test chambers. The randomized complete block design is used if the possibility exists for an effect due to test chamber placement by such things as slight differences of water temperature or lighting. The randomized block design requires five groupings (one for each replicate) with randomization within each grouping (Fig. G-6). Larvae are introduced to the test chambers in a specific manner (see Section 8.2.6.) to insure random selection of test organisms.

8.3.2. Test Chambers

Test chambers must be clean and of 250 mL capacity or greater. To avoid potential contamination from the air and to reduce evaporation of the test solutions, the test chambers should be covered with sheets of safety plate glass or plastic (6mm, 1/4 inch thickness; USEPA 1993). If plastic sheets are used they should not be of fresh construction and emitting chemical odors.

8.3.3. Cleaning of Glassware

See Section 8.2.3. "Cleaning of Glassware".

8.3.4. Elutriate Renewal

All test chambers must receive fresh elutriate and/or diluent water each day except d 7. The renewal solutions must be prepared daily with the elutriate stock solution prepared at the beginning of the test. The diluent water is either culture or

site water. The renewal solutions must be prepared in advance to allow temperature adjustment to the test temperature of 25°C. As the solutions warm from the 4°C storage temperature, supersaturation of the dissolved gasses may occur. If the dissolved oxygen concentration exceeds 100% of saturation (≈8.3 mg/L), gentle aeration may be necessary to reduce the supersaturation of gases. If the dissolved oxygen in test solutions drops below 4.0 mg/L during the test, gentle aeration should be added. The aeration should be added through a disposable glass pipet tip that has been inserted to near the bottom of the test chamber and adjusted to deliver at a rate of about 100 bubbles/min.

8.3.5. Temperature and Photoperiod

See Section 8.2.5. "Temperature and Photoperiod".

8.3.6. Organism Introduction

A total of 300 larval fathead minnows less than 24-h old (maximum of 48-h old when shipped from remote sources) are required to start the test. Larvae are captured from a common pool of larvae with an appropriate pipet (fire-polished opening, 5 mm diameter) and placed into the exposure chambers containing test solutions equilibrated to 25°C. The test chambers are randomized before introducing the larvae. Larvae are introduced in the order the chambers have been randomized either by row or within a block (Fig. G-6). Two or three larvae are consecutively added beneath the surface of the test solutions in each test chamber until a total of ten organisms are in each. Care should be taken to count them and to add the least possible volume of culture water. The pipet may become contaminated with elutriate constituents; however, this source of potential contamination can be eliminated by rinsing the pipet in a beaker of diluent water after each larval transfer. A randomly selected group of ten fish must be weighed in the same manner as the fish at test termination (Section 8.3.12) to measure control fish growth during the study.

8.3.7. Food and Feeding

See Section 8.2.7. "Food and Feeding".

8.3.8. Cleaning Test Chambers

See Section 8.2.8. "Cleaning Test Chambers".

8.3.9. Test Solution Renewal

Test solutions must be renewed each day except on d 7 from the stock elutriate solution. (The stock solution must be prepared the day before the test begins due to the several hours of time required to prepare the necessary elutriate volume). The test solutions are made in the same manner as at test initiation. Make certain that the temperature of the new solutions is 25°C

and that supersaturation of gasses has not occurred. Immediately after siphoning the test chambers, the test solutions are renewed by adding the new solution down the inside of the test chamber being careful not to disturb the larvae with excessive turbulence.

8.3.10. Test Organism Monitoring

See Section 8.2.10. "Test Organism Monitoring".

8.3.11. Water Quality Monitoring

Water temperature should be measured daily in at least five locations in the test chamber array (four corners and center) with an accurate thermometer precise to 0.1°C. Dissolved oxygen and pH should be measured daily with precision to 0.1 mg/L and 0.1 pH unit, respectively, in the old and new solutions in a control chamber and in one each of the low, medium and high concentration exposure chambers. Ammonia concentrations should be measured at least once in the high elutriate concentration. Hardness and alkalinity determination should be precise to 1-2 mg/L as $CaCO_3$ and specific conductance measured with precision to 5 µmhos/cm in the control (diluent) water and the low, medium and high elutriate concentrations at the start of the test and at test termination on d 7.

8.3.12. Test Termination

The test is terminated on d 7 at the same hour of the day that the test was begun. Survival of the larvae is recorded as on previous days and summed for the duration of the test. Surviving larvae are quickly killed (by overdosing with an anesthetic or freezing), and transferred to aluminum weighing boats which have been oven-dried. The fish larvae are dried in an oven at 100°C for at least 4 h (until steady weight is achieved), cooled in a dessicator, and weighed. Larvae are weighed as groups with all surviving larvae from one test chamber in one weighing boat. The weighing boats are marked with the exposure chamber code for proper identification. Weighing must be done with a balance capable of weighing to 0.01 mg (0.00001 gm). The dried weights and number of organisms weighed are reported on prepared data forms (Attachment L).

8.4. Data Reporting and Statistical Analysis

See Section 12.

9.0 *Chironomus tentans* **SOLID-PHASE TOXICITY TEST**

Chironomus tentans Fabricius (Diptera:Chironomidae) is a widely distributed (holarctic) non-biting midge (Townsend et al. 1981). It and several other species of chironomids are commonly referred to as "bloodworms" due to their red coloration during

their aquatic larval stages. *Chironomus tentans* is commonly found in eutrophic ponds and lakes (Flannagan 1971, Driver 1977), where it serves as an important dietary component for various species of fish and waterfowl (Sadler 1935, Siegfried 1973, Driver et al. 1974, McLarney et al. 1974).

Larvae of *C. tentans* prefer soft sediments, and normally inhabit the uppermost portion of the sediment. They inhabit sediments having particle sizes ranging between 0.15 mm to 2.0 mm, and were found to be adapted in British Columbia lakes to the following ranges of environmental factors (Topping 1971): temperature, 0-23.3°C; dissolved oxygen, 0.22-8.23 mg/L; pH, 8.0-9.2; conductivity, 481-4,136 μmhos; organic carbon, 1.92-15.45 percent. They were absent from lakes if the hydrogen sulfide concentration in overlying water exceeded 0.3 mg/L. Abundance of larvae was positively correlated with conductivity, pH, amount of food, percentages of particles in the 0.59-1.98 mm size range, and concentrations of sodium, potassium, magnesium, chloride, sulfate and dissolved oxygen. Other publications (e.g., Curry 1962, Oliver 1971) have extended the ranges for temperature (0-35°C) and pH (7-10) for waters which are inhabited by *C. tentans.*

The *C. tentans* life cycle is mainly aquatic. Adult females that have mated oviposit a single transparent, gelatinous egg mass directly into the water. An average egg mass contains approximately 2,300 eggs (Sadler 1935), which hatch within 2 or 3 days at 19-22°C (ASTM 1993e). Four larval instars are recognized, each lasting for about one week at a temperature of 20°C (ASTM 1993e). Larvae begin to construct minute tubes or cases on the second or third day after hatching. The cases, which are lengthened and enlarged as the larvae grow, are composed of very small particles bound together with threads from the mouths of the larvae (Sadler 1935). The larvae draw food particles, commonly algae, inside the tubes, and also feed in the immediate vicinity of either end of the open-ended tubes with their caudal extremities anchored within the tube. The larval stages are followed by a black-colored pupal stage (3 days) and emergence to a terrestrial adult (imago) stage. Larval and pupal life stages are presented in Figure G-7. The adult stage lasts for several days (3-5), during which the adults mate during flight and the females oviposit their egg masses (2-3 days post-emergence) (Sadler 1935). The complete life cycle requires about 30 days at 25°C (Adams et al. 1985).

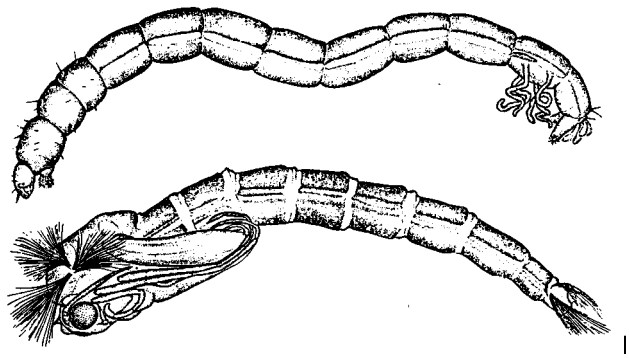

Figure G-7. *Chironomus tentans* 4th instar larvae (top) and pupa (bottom), X4. (From Johannsen and Thomsen 1937; drawing by Dr. Velma Knox).

Sexual dimorphism is readily apparent in adults. Adult males are distinguished from females by the presence of large, plumose antennae, a thinner abdomen and visible genitalia (ASTM 1993e). The male has paired genital claspers on the posterior tip of the abdomen (Townsend et al. 1981). The adult female weighs approximately twice as much as the male, with about 30 percent of the female weight contributed by the eggs.

Various laboratory and field investigations of water and sediment quality in freshwater systems have used *C. tentans*. For example, a field study of *C. tentans* distribution in a heavy metal-contaminated lake showed decreased populations in the most heavily contaminated sediments (Wentsel et al. 1977a). Laboratory evaluations of *C. tentans* survival and growth in sediments contaminated with heavy metals or organic compounds have resulted in decreased survival and/or reduced growth of larvae (e.g., Wentsel et al. 1977b, Adams et al. 1985, 1986, Giesy et al. 1988, 1990, Nebeker 1984b, 1988, Hoke et al. 1990, West et al. 1993). Decreased numbers of emergent adults have also been observed following exposure of larvae to contaminated sediments (Wentsel et al. 1978).

Chironomus tentans has a number of attributes which make it a good choice for the development of standardized laboratory test methods, including: (a) ease of culture, (b) ease of handling, (c) ecological relevance, (d) extreme sensitivity to certain classes of contaminants (e.g., pesticides), and (e) the availability of some basic culture/test conditions (e.g., ASTM 1993e). This report describes methods used to culture *C. tentans* and to perform a 10-day solid-phase sediment toxicity test with the midge. The culture methods described have been used successfully in various laboratories; however, slight

modifications to the culture methods are allowable provided that they result in a stable supply of organisms for testing.

9.1. CULTURE METHODS

The culturing methods described below are based largely on methods in use at ERL-Duluth (USEPA) and described in documents which include the standard operating procedure for culturing *Chironomus tentans* (Denny and Mead 1991, Denny et al. 1992). Required materials are listed in Attachment N.

9.1.1. Organism Source

Organisms for the initiation of a laboratory culture may be collected from the field or obtained from a laboratory (Attachment O) with a verified culture. In either case, organisms collected or received should be carefully examined by a qualified taxonomist using a key to verify the species identity. One or more organisms should be cleared to allow for improved viewing of the important characteristics of the head capsule, and then mounted to serve as reference material. Methods for clearing and mounting aquatic invertebrates are provided in Pennak (1989).

9.1.2. Acclimation of New Brood Stock

Environmental stress on the starter culture should be minimized to facilitate the development of a healthy culture. The temperature of the water containing the egg masses or larvae should be measured upon arrival and gradually adjusted to the desired culture temperature of 23° C. The water temperature change should not exceed 2°C per 24 h (ASTM 1993e). The water in which the brood stock arrived should also be gradually changed to 100% culture water from the new laboratory over at least a 4 h period.

Since culture performance fluctuates with temperature, a constant temperature of 23°C is recommended for uniformity in maturation and emergence of culture midges (Denny et al. 1992). Although toxicity tests have been performed over a temperature range of 19-23°C (e.g., Wentsel 1977b, 1978, Cairns et al. 1984, Nebeker et al. 1984a,b, 1988, Gauss et al. 1985, Adams et al. 1985, Tucker and Adams 1986, Giesy et al. 1988), we recommend testing at 23°C to maintain continuity with the culture temperature.

9.1.3. Culture Chambers

Glass aquaria (e.g., standard 19.0 L capacity, 36 cm x 21 cm x 26 cm high) are recommended for use as culture chambers. A water volume of approximately 7.5 L may be maintained in an aquarium of these dimensions by drilling an overflow hole in one end 11 cm from the bottom. The top should be covered with a mesh material to trap emergent adults. Queen-sized panty hose with

the elasticized waist positioned around the chamber top and the legs cut off and clipped shut have been used at ERL-Duluth (Denny et al. 1992). Fiberglass window screen glued to a glass strip (approximately 2-3 cm wide) rectangle placed on top of each aquarium has been used at the University of Wisconsin-Superior.

9.1.4. Substrate

Both shredded paper toweling and silica sand have been successfully used as artificial substrates. A sand substrate may facilitate ease of larval transfer at test initiation over that from paper toweling. Either substrate may be used if a healthy culture can be maintained.

The paper towel substrate is prepared according to a procedure adapted from Batac-Catalan and White (1982). Plain white kitchen paper towels are first either shredded in a paper shredder or cut into strips with scissors. A mass of the shredded or cut toweling is then loosely packed into a 2 L beaker, submersed in acetone, covered, and, in a fume hood, allowed to soak overnight to solubilize any trace organic contaminants. The acetone is discarded into a waste solvent bottle and the toweling rinsed three times with distilled water. Distilled water is again added and brought to a boil with occasional stirring to drive off the acetone vapors. The boiling and stirring process is performed three times, followed by rinses with cold distilled water. A mass of the toweling sufficient to fill a 150 mL beaker is placed into a heavy duty blender (e.g., commercial style blender) containing 1 L of distilled water, and blended for 30 seconds or until the strips are well broken apart and in the form of a pulp. The pulp is then placed into a 710 micron sieve and rinsed well with distilled water to remove the shortest fibers.

The initial mass of dry shredded paper toweling loosely packed into a 2 L beaker as described above will provide sufficient pulp substrate for about ten 19 L chambers. The toweling from the 150 mL beaker produces a mass of towel substrate that is approximately sufficient for one 19 L chamber. Several masses may be prepared separately at the same time and either stored in deionized water in a suitable container (e.g., 500 mL plastic bottle), or kept frozen for later use.

A sand substrate that has been used successfully at different laboratories consists of silica sand of 0.25 to 0.50 mm grain size (94 percent of total). The sand is rinsed with hot culture water, autoclaved, and oven-dried prior to use. One L of sand is a sufficient volume of substrate for each 19 L chamber.

9.1.5. Culture Water

Chironomus tentans can be successfully cultured in a variety

of types of overlying water. Regardless of the water type used by a given testing laboratory, it is suggested that the culture water be of the same basic qualities as the overlying water to be used in toxicity tests. Water of a quality sufficient to culture other test species such as the fathead minnow, *Pimephales promelas*, or the cladoceran, *Daphnia magna*, generally will be adequate for culturing *C.tentans*.

Chironomus tentans can be cultured under either static or renewal conditions. If a laboratory has a flow-through supply of water of high quality, a culture system which uses an automatic daily renewal of overlying water is recommended to avoid the possibility of fouling of the culture system by excess food and waste, and the resultant death of culture organisms due to oxygen depletion. Water renewal may be either intermittent or continuous. An automatic intermittent renewal for a daily three hour period is used at ERL-Duluth (Denny et al. 1992). A continuous renewal over a 24 h period is used by the University of Wisconsin-Superior.

If a static system is used, the overlying water may be derived from different sources. Untreated well water or dechlorinated water from a municipal supply may be used, or culture water of desired characteristics may be prepared (see Attachment P). In static systems, the overlying water volume should be changed every 4-7 d by siphoning down to a level just above the substrate, and slowly adding freshly prepared water (Batac-Catalan and White 1982). When using a static culture system, extra care must be taken to ensure that proper water quality is maintained. For example, supplemental aeration will likely be required to maintain adequate concentrations of dissolved oxygen. The air supply should be determined to be free of impurities, such as oil, by inclusion of a filter in the air line, if necessary.

9.1.6. Temperature and Photoperiod

The temperature for culturing *C. tentans* should be maintained at 23°C (Denny et al. 1992). A photoperiod of 16 h light and 8 h dark is recommended, with an intensity of 540 to 1080 lux or 50-100 ft-C. A photoperiod of 24 h of light also can result in normal development, but either no light or short periods of light (e.g., 8 L:16 D) prevent completion of the *Chironomus* life cycle (Englemann and Shapiro 1965, Townsend et al. 1981).

9.1.7. Food and Feeding

Various food items have been used for culturing *C. tentans* by different laboratories (ASTM 1993e), although some type of flaked fish food is used by most laboratories. Denny et al. (1992) adopted the use of Tetrafin® goldfish food, and methods

for preparing this food are described here. Tetrafin® flake food contains a minimum of 32% crude protein, 3% fat, and a maximum crude fiber content of 2%. The maximum moisture content is 6.5%, and the L-ascorbyl-2-phosphate content is ≥200 mg per 454 g.

In culture systems utilizing daily water renewal or continuous flow conditions, food should be prepared to provide a final concentration of approximately 0.04 mg dry solids/mL of culture water in each aquarium (Denny et al. 1992). Prepare a stock suspension of the solids in culture water such that a total volume of 5.0 mL of food suspension is added daily to each aquarium. For example, if a culture aquarium volume is 7.5 L, 300 mg of food would be added once each day. This would be accomplished by adding 5 mL of a 60 g/L stock suspension. The stock suspension should be initially shaken and then stirred immediately prior to withdrawal of an aliquot for each culture tank to ensure homogeneity of the food. Once prepared, the Tetrafin® food may be used for approximately two weeks if it is refrigerated between use. Specific details for food preparation are provided in Attachment Q.

9.1.8. Initiating a Culture

Organisms for initiating a culture are commonly received as egg masses from a commercial supplier or another laboratory. Two or three egg masses generally provide a sufficient number of organisms to start a new culture. In some cases, the embryos may have hatched during shipment. If the embryos have not hatched, acclimate the egg masses to the culture water and the desired culture temperature of 23°C in a glass beaker or crystallizing dish containing about 100 mL of culture water. The temperature change should not exceed 2°C per d. Allow the embryos to start hatching before adding a small amount (e.g., ≈4 mg dry solids) of suspended food particles to the water. Do not add food until the embryos are hatching to reduce the risk of oxygen depletion. When the hatch is judged to be complete or near completion, transfer the first instar larvae and remaining eggs into a larger culture aquarium. Larvae that have formed cases can be transferred with a gentle stream of water from a squeeze bottle. If larvae are already evident in the shipment of new brood stock, adjust the temperature of the shipped water containing larvae to the desired culture temperature at a rate not exceeding 2°C per 24 h. Add food at the rate of ≈4 mg per 100 mL of water to the shipped water. After proper acclimation, place food (≈4 mg) into a container of culture water (100 mL), and transfer the larvae and remaining eggs into the beaker or crystallizing dish immediately. Allow the larvae to feed in the confined beaker or finger bowl for a day prior to transferring them into a larger culture aquarium containing substrate, overlying water and food.

9.1.9. Culture Maintenance

Beakers or crystallizing dishes containing two to three egg masses should be examined under a dissecting microscope to determine hatching success. When most of the larvae have hatched, transfer the hatched larvae plus the remainder of the egg masses into an aquarium that contains substrate, overlying culture water and an initial increment of food (e.g., in the 19 L aquaria set-up, 5 mL of concentrated food suspension per 7.5 L of culture water to yield a final concentration of 0.04 mg/mL of solids in the aquarium).

The temperature of the aquarium water should be maintained at 23°±1°C. Dissolved oxygen levels should be monitored, and maintained at concentrations of 3.4 mg/L or above (i.e., ≥40 percent of saturation). If dissolved oxygen levels approach or drop below 3.4 mg/L, place an airstone into the chamber, and gently aerate. If the overlying water is automatically renewed, the flow should be regularly monitored to provide a measure of the daily rate of total volume turnover.

If culturing is being performed in a static system, fresh water should be added every four to seven days by siphoning the old water to just above the substrate (Batac-Catalan and White 1982), and slowly adding in the new water. A screened siphon tube should be used to avoid the removal of larvae and substrate (Denny et al. 1992). Care should be taken to avoid disturbance of the substrate and larvae during siphoning and replacement of the water. On days when water is renewed, add food after the water has been changed. Laboratories utilizing static cultures should develop lower feeding rates specific to their systems.

At a culture temperature of 23°C, larvae should have readily attained the second instar by 8 d post-hatch (Denny et al. 1992). The second and third instars are the desired ages for initiation of a 10-d toxicity test. Adult emergence will begin approximately 24 d post-oviposit at this temperature.

Once adults begin to emerge, they should be gently siphoned into a dry aspirator flask on a daily basis. An aspirator can be readily made from a 250 or 500 mL Erlenmeyer flask, a two-hole stopper, some short sections of 0.25 inch glass tubing, and Tygon or rubber tubing for collecting and providing suction (Figure G-8).

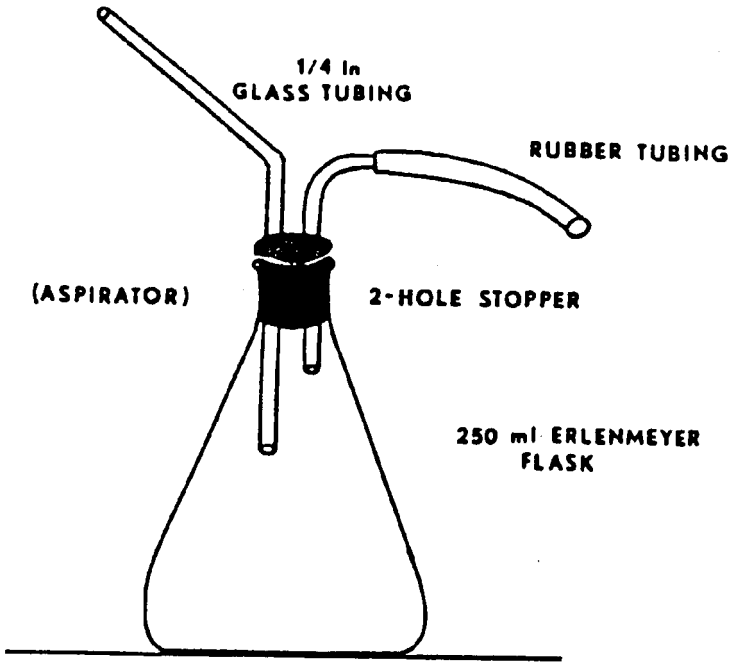

Figure G-8. Aspirating flask for collection of adults (from Batac-Catalan and White, 1982).

Aspirate with short, sharp inhalations to avoid injuring the adults. Check the sex ratio of collected adults to ensure that a sufficient number of males are available for mating and fertilization. Males are readily distinguished from females by their large plumose antennae (ASTM 1993e). One male may fertilize more than one female. However, a ratio of 50% or more males will ensure good fertilization.

A mating and oviposition chamber may be prepared in several different ways (Figure G-9). The flask in which the adults were collected may be used by simply adding a volume of water (e.g., 50-75 mL) to the flask and tipping the flask (Fig. G-9A, Batac-Catalan and White 1982). Denny et al. (1992) used a 500 mL collecting flask (Fig. G-9B), which included a length of Nitex® screen positioned vertically and extending into the water when water was subsequently added. The Nitex® screen is used by the females to position themselves just above the water during oviposition. The two-hole stopper and tubing of the aspirator should be replaced by screened material, a cotton plug, or perforated aluminum foil to allow for adequate air exchange in the oviposition chamber.

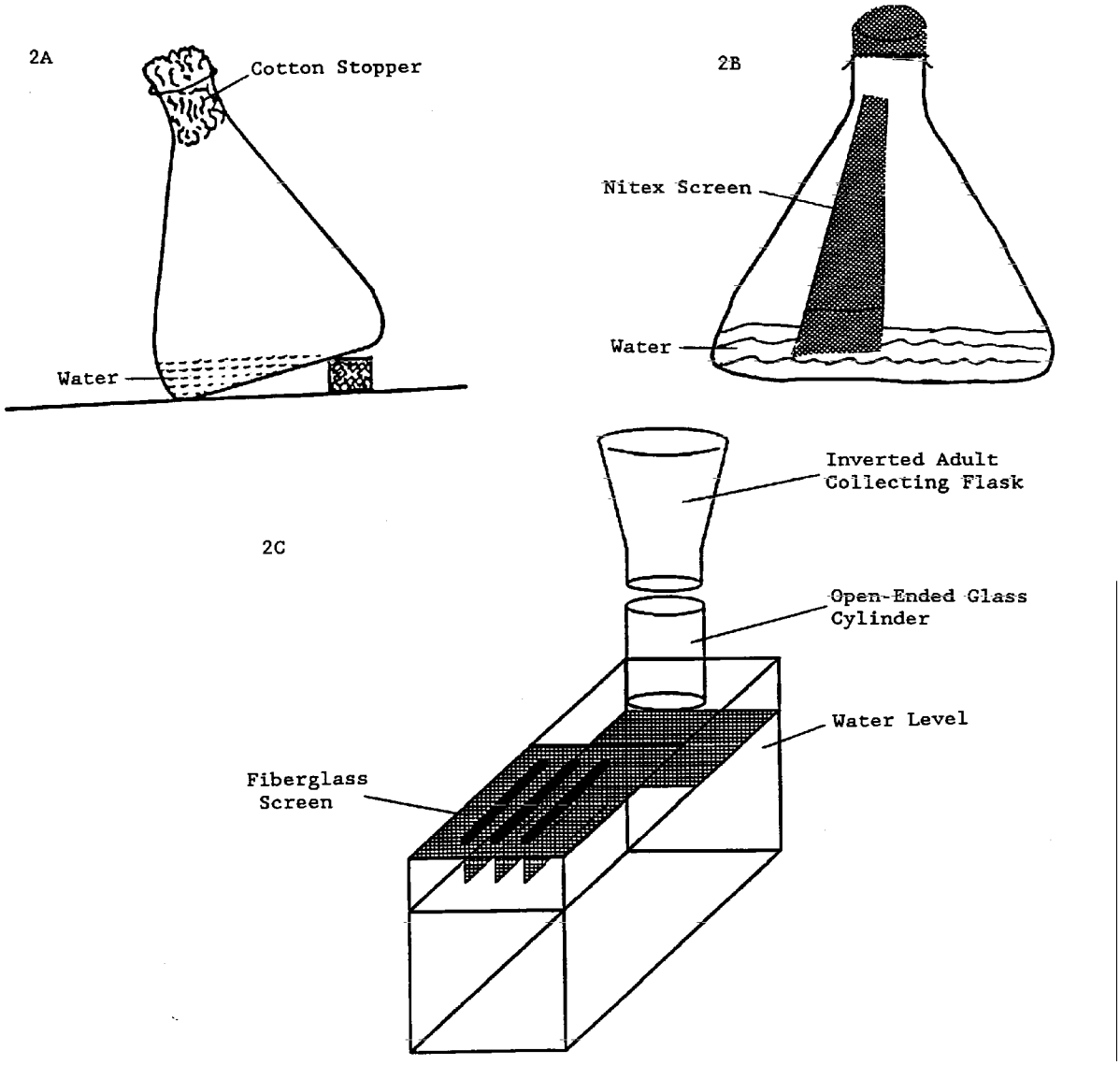

Figure G-9. Several styles of mating and oviposition chambers: 2A from Batac-Catalan and White, 1982; 2B from Denny et al., 1992; 2C from R. Venkataramani and S. McGovern, University of Wisconsin-Superior, unpublished.

A chamber designed by the University of Wisconsin-Superior (Fig. G-9C) provides for attachment of an inverted 250 mL Erlenmeyer adult collecting flask, sufficient room for mating, and a surface area for females to position themselves for egg deposition in the water. The overall dimensions are

approximately 20 cm x 10 cm x 14 cm (L x W x H). The rectangular chamber consists of two sections to allow for removal of the upper section and collection of the egg masses. The inverted adult collection flask is taped to the mouth of a 120 mL glass bottle with the bottom cut away and glued to a glass portion of the chamber cover. The lower portion of the chamber (~20 cm x 10 cm x 10.5 cm, L x W x H) should contain a volume of water of 8-9 cm depth and sufficient Nitex® or fiberglass window screen positioned such that it either extends into the water or is just above the water to allow for deposition of egg masses upon the water.

Egg masses should be collected daily, and depending upon the culture and testing needs of the laboratory, should regularly (e.g., weekly, twice weekly) be used to initiate a new larval culture tank. Egg masses should be collected with a wide bore pipet and placed into a crystallizing dish where they will begin to hatch within 48 h at 23°C. Upon hatching, they can be assigned to rearing tanks. Egg masses to be used for larval rearing should be documented as to their date of deposition, so that an accurate record will be available of the age of the larvae throughout their life cycle.

9.1.10. Culture Evaluation

Cultures should be observed daily at the time of feeding to ensure that a healthy culture is maintained. Larvae should be actively feeding within their cases in the substrate, as a requirement of health. Water temperature is measured and recorded daily in each aquarium used for larval rearing. Dissolved oxygen concentrations should be monitored and recorded weekly. Observations of the larvae should indicate good growth as the larvae progress through the four instars. Records should be kept on the time to first emergence and the success of emergence for each aquarium.

A culture evaluation chart (Attachment R, Form R1) should be maintained, and updated monthly. If the culture is not showing normal growth (i.e., mean dry weight of at least 0.6 mg for ten 22-day old 4th instar larvae), survival, emergence of adults, hatching success, or performance in reference toxicant tests, the culturing conditions should be scrutinized and adjustments made to restore culture health. Any adjustments made may be considered to have resulted in an acceptable state of health for the culture when the culture produces a regular supply of vigorous larvae that perform acceptably in reference toxicant tests (see Section 4.3.8.).

9.2. TOXICITY TEST METHODS

9.2.1. Solid-Phase Sediment Preparation

See Section 5.0.

All glassware used in the tests must be initially clean and should also be thoroughly cleaned after each test. Glassware should be washed with detergent, and rinsed 3 times each with tap water and distilled or deionized water. Glassware should then be rinsed with clean 10% HCl, followed by several rinses with distilled or deionized water. Cleaning is completed by an acetone rinse followed by several rinses with distilled or deionized water.

9.2.2. Test Design

The basic design and conditions for performing a 10-d toxicity test with solid-phase dredged material are given in Table G-15. In a typical test, one or more dredged material samples will be compared to a disposal site sediment sample. Ideally, disposal site sediment samples will have similar physical properties (e.g., grain size, organic matter) as the dredged material samples. In addition, a clean control sediment which serves as a basis for evaluating biological performance criteria for the test and determining test acceptability should be run simultaneously. The control material can be laboratory specific; however, previous testing should have demonstrated that the test organisms routinely survive and grow in an acceptable manner in the sediment. Exposures consist of a minimum of five replicates of each test sample plus a minimum of five replicates each of the disposal site material and control sediment in an exposure system designed to renew water overlying the sediment. The replicates for each test, disposal site, or control sediment should all be contained within their respective aquaria. Sediments from different sources should not be mixed within an aquarium.

Table G-15. Overview of Recommended Conditions for the 10-d Larval Survival and Growth Toxicity Test with *Chironomus tentans* and Solid-Phase Dredged Material.

1.	Test Type	Solid-phase sediment toxicity test with daily renewal of overlying water.
2.	Temperature	23±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)

Table G-15 (continued)

4.	Light intensity	10-20 uE/m^2/s, 540-1080 lux, or 50-100 ft-C (ambient laboratory levels).
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber	300 mL high-form beaker with two opposing holes (1.5 cm diameter centered 7.7 cm high above the bottom and covered with 60 mesh stainless steel screen).
7.	Sediment volume	100 mL
8.	Overlying water volume	150-175 mL; variable due to water renewal siphoning cycle.
9.	Renewal of overlying water	Two volume additions per day.
10.	Age of test organisms	Second or third instar larvae (all organisms must be at third instar or younger with at least 50 percent of the organisms at third instar).
11.	No. organisms per test chamber	10
12.	No. replicate test chambers per sample	5 minimum
13.	Total number of organisms per sample	50 minimum
14.	Feeding regime	Feed 1.5 mL daily to each beaker of blended Tetrafin® goldfish food containing 6.0 mg of dry solids.
15.	Aeration	Aerate if dissolved oxygen drops below 40% of saturation (i.e., 3.40 mg/L at 23°C).
16.	Overlying water	Similar to culture water or, if desired, site water should be measured twice during the test.
17.	Water quality monitoring	Daily measurements of water temperature and dissolved oxygen. Hardness, alkalinity, specific conductance, pH and total ammonia
18.	Test duration	10 d
19.	Test endpoints	Larval survival and growth (dry weight).
20.	Test acceptability	70% or greater survival in the control sediment. Maintenance of

		dissolved oxygen at ≥40% saturation and mean temperature of 23 ± 1°C. Test initiated with healthy, 8-12 day old (post-hatch) larvae. Satisfactory performance in reference toxicant test.
21.	Sample requirements	Storage of dredged material is at 4°C. Test should be initiated within 2 weeks of sample collection, and must be initiated with 8 weeks of collection.
22.	Dredged material volume required	A minimum of 500 mL from each test and disposal site.

The automated renewal of overlying water within each test chamber has a definite advantage in reducing labor hours as compared to manual renewal of water (Ankley et al. 1993). Several types of automated water renewal systems have been used, and are presented here as delivery system options. Any method of water renwal is acceptable, provided the recommended volumes of overlying water and their renewal rates are maintained, along with the recommended physical and chemical characteristics of the overlying water. Mount/Brungs (1967) diluters have been modified for use in sediment testing, and other automated water delivery systems have been used, as well (e.g., Maki 1977, Ingersoll and Nelson 1990, Benoit et al. 1993, Zumwalt et al. 1994).

Thoroughly homogenized sediment (100 mL) is added to each 300 mL high-form exposure beaker, and the sediment allowed to settle for 24 h in the test system before introduction of test organisms. The overlying water flows over the sediment during this 24 h period at approximately two volume additions per day.

A summary of daily activities prior to and during a test is presented in Attachment S. This schedule assumes that all materials are on hand, and that a healthy culture of animals is being maintained.

9.2.3. Test Chambers

Five 300 mL high-form beakers with side-walls drilled and screened (two opposing holes of 1.5 cm diameter, centered 7.7 cm up from the beaker floor, and covered with 60 mesh stainless steel screen) are required for each dredged material, control or disposal site sediment sample. The screened holes allow for renewal of overlying water, thereby allowing for a renewed supply of dissolved oxygen.

A single test site sediment and a disposal site sediment can be tested simultaneously in a portable mini-flow exposure system

of the size described in Benoit et al. (1993), using five replicates per sample. A maximum of 12 samples (60 total replicates), including reference and control samples, can be tested simultaneously in a modified mini-diluter system.

9.2.4. Water Renewal

Laboratory culture water or water with similar characteristics may serve as overlying water for the exposures. The overlying water should be of high quality whereby it does not contribute contaminants to the exposure system. In certain projects, it may be desired that disposal site water be used; however, this may prove formidable from a logistical standpoint. The exposure system is set to provide approximately two volume additions per day. This renewal rate will likely require supplemental aeration for many sediments. Aeration of the overlying water should be initiated if the dissolved oxygen concentration drops to 40 percent of saturation or below (i.e., 3.40 mg/L at 23°C). Overlying water within the tanks in which the exposure chambers are positioned should be aerated for all samples if the dissolved oxygen concentration drops to 40 percent or less in one or more exposure chambers.

9.2.5. Temperature and Photoperiod

Tests should be performed at 23±1°C. The daily photoperiod should be 16 L:8 D, using ambient laboratory lighting of 50-100 ft-C.

9.2.6. Organism Introduction

A sufficient number of second and third instar larvae (8-12 d post-hatch) are removed from the paper toweling or sand substrate in the culture rearing chamber to provide 10 organisms per replicate. They should be handled gently in freeing them of substrate, and placed directly into randomly chosen test beakers, after which each beaker is returned to its respective test holding tank. Larvae with their cases may first be withdrawn from the culture chamber with a fire-polished wide-bore pipet and transferred to an enamel pan containing culture water. The larvae may be gently forced out of their cases by touching the ends of their cases with a small, soft-bristled artist's paint brush. Larvae may also be transferred in their cases if they are not readily removed. Their presence inside of their cases can be confirmed by placing them into a transparent dish and inspecting them under a dissecting scope with strong backlight conditions.

9.2.7. Food and Feeding

Tetrafin® goldfish food should be prepared in distilled water to yield a concentrated suspension of 4.0 mg dry solids/mL (Ankley et al. 1993). Since stock culture food (56 mg dry solids/mL) is 14 times more concentrated than the desired concentration for feeding in a toxicity test, dilute 71.5 mL of

thoroughly mixed culture food concentrate to 1,000 mL with distilled water to yield the test food concentration of 4 mg dry solids/mL. Each replicate test beaker receives 1.5 mL of well-mixed food suspension daily. The food should be stirred between each replicate feeding. A total volume of 75 mL is required for the duration of the test (10 d) for each type of sediment sample (i.e., dredged site, disposal site and control) containing five replicates.

9.2.8. Test Organism Monitoring

Observe the beakers daily. The chironomids will form cases in the sediment, and most likely will not be visible if they are in good health. The openings of their tubes, however, may be visible. Organisms on the sediment surface that are not inside cases may be indicative of a stressful environment. Record the observations for each beaker.

9.2.9. Water Quality Monitoring

Water should be monitored daily for temperature and dissolved oxygen concentrations. The temperature should be maintained within ± 1°C of the desired temperature (23°C) at all times. Dissolved oxygen concentrations should be maintained at or above 40 percent of saturation. Hardness, alkalinity, specific conductance and pH should be measured at the beginning and end of the test from one of the replicates. Because ammonia may be elevated in some test sediments (e.g. Ankley et al. 1991a), measurement of total ammonia may aid in test interpretation. Total ammonia should be measured near the beginning and end of each test. It may be desirable to have an additional replicate for chemistry measurements only. Water quality parameters should be recorded on a data form (see Attachment R, Form R2).

9.2.10. Test Termination

After 10 d of exposure, sediment from each replicate is sieved through a fine-meshed screen sufficiently small to retain the fourth instar larvae (e.g., U.S. Standard No. 30, having a 0.59 mm mesh size). Larvae are placed into a crystallizing dish or beaker containing culture water and, if necessary, viewed under a dissecting microscope to determine if the larvae are alive. A small volume of carbonated water may be added to the volume of water in a beaker to immobilize the larvae, thereby facilitating their transfer to a weighing pan. Surviving larvae are freed of any remaining substrate and placed into a pre-weighed aluminum weighing pan. The larvae are then oven-dried for at least 4 h at 100°C (until a steady weight is obtained). The sample is allowed to come to room temperature in a desiccator, and weighed to the nearest 0.01 mg. The larvae from a given replicate are weighed together.

9.2.11. Data Reporting and Statistical Analysis

See Section 12.

10.0 *Hyalella azteca* **SOLID-PHASE TOXICITY TEST**

Hyalella azteca (Figure G-10) is a freshwater crustacean (Amphipoda:Talitridae) which is widely distributed in North America and South America (Pennak 1989). This species was chosen as a test organism for several reasons: (1) ease of culturing and testing, (2) widespread and common occurrence, (3) rapid growth and short generation time, (4) ecological importance, (5) close association with sediments and (6) sensitivity to a variety of environmental pollutants. *Hyalella azteca* is an omnivorous feeder. It prefers foods high in protein (de March 1981) and will browse on the film of bacteria and microscopic plants, animals and organic debris (aufwuchs) covering leaves, stems and other substrates (Pennak 1989). Bluegreen and green algae are less preferred as food and are not assimilated as efficiently (de March 1981). In most amphipods, and probably *H. azteca* as well, food is held by the gnathopods and anterior pereiopods and chewed directly (Pennak 1989).

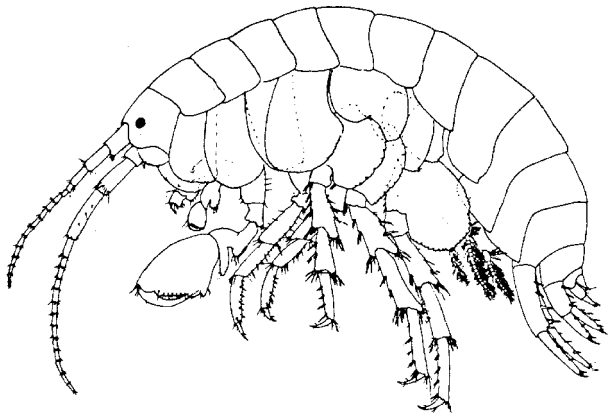

Figure G-10. *Hyalella azteca*, X14 (From Cole and Watkins 1977).

Reproduction by *H. azteca* is obligately sexual. Males pair with females by grasping the females (amplexus) with their enlarged second gnathopods while on the backs of the females. After feeding together for 1 to 7 days the female is ready to molt and the two animals separate for a short time while the female sheds her old exoskeleton. Once the exoskeleton is shed, the two animals reunite and copulation occurs. The male places sperm near the marsupium of the female and her pleopods sweep the

sperm into the marsupium. The animals separate and the female releases eggs from her oviducts into the marsupium where they are fertilized. *H. azteca* averages about 18 eggs per brood (Pennak 1989) with larger animals having the most eggs (Cooper 1965).

The developing embryos and newly hatched young are retained in the marsupium until the next molt. At 24 to 28°C, hatching has been reported to occur from 5 to 10 d after fertilization (Bovee 1950, Cooper 1965, Embody 1911). The time between molts for females is 7 to 8 d at the temperature range of 26 to 28°C (Bovee 1950); therefore, about the time embryos hatch, the female molts and releases the young. *H. azteca* averages 15 broods in 152 d (Pennak 1989). Pairing of the sexes is simultaneous with embryo incubation of the previous brood in the marsupium.

H. azteca has a minimum of nine instars in its life history (Geisler 1944). There are 5 to 8 pre-reproductive instars (Cooper 1965) and an indefinite number of post-reproductive instars. The first five instars form the juvenile stage of development, instar stages 6 and 7 form the adolescent stage when sexes can be differentiated, instar stage 8 is the nuptial stage and all subsequent instars are the adult stages of development (Pennak 1989).

Occurrence of *H. azteca* is most common in warm (20-30°C for much of the summer) mesotrophic or eutrophic lakes which support aquatic plants and periphyton. It is also found in ponds, sloughs, marshes, rivers, ditches, streams and springs, but in lower numbers. They have achieved densities of >10,000m^2 in preferred habitats (de March 1981).

Hyalella azteca avoids bright light, preferring to hide under litter and feed during the day. Activity levels increase at night; however, de March (1977) reported that during a laboratory study conducted with a 16-h light and 8-h dark photoperiod and 20 to 30°C, *H. azteca* reproduced well at 55 μE/m2/s but not at 12 μE/m^2/s light intensity. (Average room light intensity is 10 μE/m^2/s.) She also reported that a photoperiod duration of 16 h or more was conducive to reproductive success. However, some laboratories (e.g., U.S. FWS Columbia, MO; UW-Superior; USEPA, Duluth, MN) have reported successful reproduction at light intensities of 8 to 16 μE/m^2/s.

Temperatures tolerated by *H. azteca* range from 0 to 33°C (Bovee 1949, Embody 1911, Sprague 1963). At temperatures less than 10°C the organisms rest and are immobile (de March 1977, 1978). At temperatures of 10 to 18°C some reproduction occurs and juveniles grow slowly into large adults. Smaller adults result when organisms are grown at temperatures in the range of 18 to 28°C and reproductive output is high. The highest

reproduction occurs in the temperature range of 26 to 28°C (de March 1978) while lethality, due to temperature, occurs at 33 to 37°C (Bovee 1949, Sprague 1963).

Hyalella azteca is found in widely varying water conditions in which dissolved oxygen can range from saturation to very stagnant conditions. Sprague (1963) reported a 24-h LC50 at 20°C of 0.7 mg/L dissolved oxygen, and Pennak and Rosine (1976) reported a similar value. Little is known of *H. azteca's* preference for various ions or ionic concentrations in water. de March (1981) reported that *H. azteca* was not observed when calcium was less than 7 mg/L and that salinity to the concentration of sea water is tolerated if the organisms are acclimated slowly to increasing concentrations of seawater ions.

Hyalella azteca tolerate a wide range of substrate conditions. Ingersoll and Nelson (1990) reported that they tested *H. azteca* in long-term studies using sediments ranging from more than 90% silt- and clay-sized particles to 100% sand-sized particles without detrimental effects on either survival or growth of the organisms. Ankley et al. (1993) found that organisms tested on quartz sand with four water renewals/d without food had poor survival (30%) and that feeding a 0.8 mg/d ration of yeast, cereal leaves and trout chow yielded better survival (90%). Organisms tested in the same system with a sediment containing about 8% organic carbon did not benefit from feeding. Therefore, feeding of tests is necessary to eliminate the confounding effect of sediment organic carbon content.

A number of studies have used *H. azteca* to assess toxicity of sediments (e.g., Nebeker et al. 1984a, Borgmann and Munawar 1989, Ingersoll and Nelson 1990, Ankley et al. 1991a,b) with favorable results. The amphipods are often among the most sensitive species tested.

This report describes methods used to culture *H. azteca,* to perform a 10-d exposure of this organism to solid-phase sediments with either an intermittent- or continuous-flow overlying water renewal system; and methods for data analysis. The endpoint in the toxicity test is survival, although growth can also be monitored as an endpoint in this test (Ingersoll and Nelson 1990).

10.1. CULTURE METHODS

The culturing methods described in this report are based on the methods developed by USEPA (Denny and Collyard 1991, Denny et al. 1993). Other culture methods have been successfully used for *Hyalella azteca* (e.g., ASTM 1993e); any of these are acceptable provided that a stable stock of healthy, reproducing test animals results. Required materials are listed in Attachment T.

10.1.1. Organism Source

Organisms for the initiation of a laboratory culture should be obtained from a source which has a verified culture of *H. azteca*. Organisms that are adapted to laboratory conditions and free of disease must be used as the brood stock. Juveniles or adults are equally suited for the initial stock and are easy to transport. Stock from wild populations should be avoided unless cultured through at least one generation to ensure their identity is verified, they are disease-free and of adequate vigor (Denny et al. 1993). Starter cultures are available from several government and commercial suppliers (Attachment U).

10.1.2. Acclimation

Environmental stress on the organisms in the starter culture must be minimized to facilitate normal growth and embryo production. The temperature of the water containing the brood stock animals should be measured upon their arrival and gradually adjusted to the desired culture temperature of 23±1°C. Changes in water temperature >2°C in any 24-h period should be avoided and, in general, water temperature should not change more than 3°C in a 72-h period (ASTM 1993a). The dissolved oxygen concentration should be maintained between 60 and 100% of saturation. Gentle aeration (1 bubble/sec/L of water from an air line terminating with a disposable glass pipet with an approximate 1mm diameter opening) with oil-free compressed room air is desirable. Supersaturation by dissolved gases should be avoided to prevent entrapment of organisms at the water surface. If the culture medium differs in hardness, alkalinity or pH from that in which the organisms were received, animals from the starter culture should be transferred to the new culture medium gradually over a period of 1 to 2 d to avoid stress (ASTM 1993a).

Reproductively mature (≥30-d old when cultured at 23°C) individuals must be segregated into breeding groups to successfully produce embryos of known age.

10.1.3. Reference Organism

It is recommended that several organisms in the brood stock, especially when obtained from wild populations, be examined by a competent invertebrate taxonomist to ensure that the brood stock is a pure culture of *Hyalella azteca*. Several taxonomic references are available to distinguish members of the crustacean Order Amphipoda (e.g., Bousfield 1958, Pennak 1989, Covich and Thorp 1991). Verification should be documented in writing, including the name of the individual responsible for the taxonomy, the taxonomic key used, the date of identification and the source of the individuals used in the identification.

10.1.4. Culture Chambers

Chambers for mass culturing of *H. azteca* may be constructed

of glass, plastic, fiberglass, or stainless steel, although glass or plastic is preferred. An example of a single culturing system would be a 2-L glass battery jar or polycarbonate beaker, although larger aquaria work well, too. The unit must be acid washed with 1N HCl or HNO_3 and rinsed with deionized water to remove any manufacturing residues. One air line with a disposable glass pipet attached serves each chamber.

White translucent plastic dishwashing pans commonly available in stores (e.g., 53 cm x 40 cm x 12 cm, L x W x H) make good sorting containers for separation of adults from juveniles. The water temperature within this pan should be maintained at 23±1°C. Juveniles and adults can easily be seen in the pans if placed in bright light or on a light table. A supply of low pressure (≈3 psi) air (compressed, oil-free room air) is needed to mix the water in the culture chambers to keep the culture water from becoming supersaturated with dissolved oxygen due to the abundance of green algae in the cultures.

10.1.5. Culturing Substrates

Hyalella azteca hide beneath any available materials during the light portion of the photoperiod. For cultures, suitable culturing substrates have been provided with presoaked maple (*Acer* ap.), poplar (*Populus* sp.), alder (*Alnus* sp.) or birch (*Betula* sp.) leaves (Ingersoll and Nelson 1990; Nebeker et al. 1984a). Other more standardized choices are plastic mesh and presoaked cotton gauze. Plastic mesh of 10 to 15 meshes/cm and either 100% cotton cheesecloth or surgical gauze works well (Borgmann and Munawar 1989, Denny et al. 1993). Plastic mesh large enough to stand obliquely in the culture chamber or a single 10 x 15 cm piece of cotton gauze added to each culture chamber works well with about 50 adults present in the chamber.

10.1.6. Culture Water

An adequate supply of water, such as spring, well, reconstituted (ASTM 1993a; Attachment B) or controlled surface water, is necessary to culture *H. azteca*. Water quality parameters of hardness, alkalinity, conductivity and pH should fall within the following ranges: hardness, 60-300 mg/L as $CaCO_3$; alkalinity, 50-300 mg/L as $CaCO_3$; conductivity, 50-500 μmhos/cm; and pH, 6.5 to 9.0. Dechlorinated water can be used when dechlorinated with sodium bisulfite (sodium sulfite can be used but is less desirable), which also removes chloramines (ASTM 1993a), or by dechlorination with aeration in an open chamber of sufficient retention time (>1h) to completely remove the chlorine and chloramines. Chemical monitoring of the water for residual chlorine or chloramine concentration must be conducted to ensure that concentrations of these chemicals do not exceed 3 μg/L. Municipal drinking water may contain copper, lead, zinc and fluoride which can be removed, when excessive, by using

appropriate ion-exchange resins (ASTM 1993a). The national water quality criteria to prevent chronic effects on freshwater organisms exposed to copper, lead and zinc are 12, 3.2 and 110 μg/L, respectively, at a water hardness of 100 mg/L as $CaCO_3$ (USEPA 1987). Different chronic values must be calculated if the hardness of the culture water differs from 100 mg/L as $CaCO_3$. No criterion is available for fluoride.

10.1.7. Temperature and Photoperiod

Water temperature for culturing *H. azteca* should be maintained at 23±1°C. This temperature is suitable for reproduction, incubation and growth. Temperatures below 15°C and above 28°C reduce reproduction of *H. azteca* (de March 1977, 1978).

A photoperiod of 16 h light and 8 h dark during each 24-h period is recommended. Wide spectrum fluorescent lights with minimum luminescence of 10 to 20 $\mu E/m^2/s$ (540-1080 lux; 50-100 ft-c) at the water surface are preferred.

10.1.8. Food and Feeding

Adult and juvenile *H. azteca* are fed three times each week (MWF) 10 to 15 mL/L of yeast-Cerophyll®-trout chow (YCT) mixture, and a 60-mL inoculum of a green alga (a single cell alga such as *Ankistrodesmus* sp. works well but filamentous green algae will also work) at the time of culture renewal (Attachment V). This results in a large green algal population in the culture chamber in 48 to 72 h.

Other commercial diets (e.g., Tetrafin®, TetraMin®, rabbit chow) also are acceptable. However, they should result in a similar rate of growth in adults and a similar rate of young production as the recommended diet.

10.1.9. Chamber Cleaning

Chambers containing breeding *H. azteca* should be washed weekly when the culture chambers are renewed. Satisfactory cleaning is accomplished by washing the culture chambers with soap and rinsing with either distilled, deionized or culture water.

10.1.10. Handling

Care must be taken to avoid disturbance of the juvenile and adult *H. azteca* by unnecessary movement, noise, or extraneous lighting. Organism handling should be kept at a minimum. Juvenile and adult *H. azteca* can be carefully transferred using a glass or clear plastic pipet which has a polished end with a 6 mm diameter opening. Organisms must be quickly released below the water surface to avoid stress.

10.1.11. Water Quality Monitoring

Water used to culture *H. azteca* should be monitored for temperature (standardized alcohol or electronic thermometer), dissolved oxygen (titrimetric or ion selective electrode), hardness (titrimetric total hardness), alkalinity (titrimetric total alkalinity), conductivity (conductivity meter), pH (electrode method) and any other characteristics [e.g. chlorine and chloramines (residual chlorine ion specific electrode method, Rigdon et al. 1978), sulfides (iodometric method, APHA 1985)] useful to indicate consistent quality. Temperature should be measured daily and dissolved oxygen twice weekly in the culturing tanks. Hardness and alkalinity should be measured weekly at the water supply source to the chambers. Because ammonia may be elevated in some test systems, measurement of total ammonia to ensure a concentration of <0.1 mg/L may aid in culturing success. Total ammonia should be measured near the end of each culture solution renewal.

10.1.12. Juvenile Production

Visually inspect the contents of all culture chambers every seventh day for juvenile production by pouring the contents of each culture chamber into a translucent white plastic pan (use of a light box to see the organisms is recommended). After the adults are removed, the remaining organisms will be the juveniles ranging in age from <1 to 7 d. When juveniles are present, transfer them with a 6 mm i.d. pipet to a 1-L beaker for holding for one week in preparation for a toxicity test, or place them into a mass culture chamber for use as brood adults later. Count the number of adults and juveniles in each culture chamber and record the counts on the culture record sheet (Attachment W). These records are useful to determine if cultures are maintaining a vigorous reproductive rate indicative of culture health.

The brood-board method is an alternative method of culturing *H. azteca* to produce juveniles of known ages. Mated adults are placed in a small beaker or plastic cup (one pair per chamber) and fed an amount proportionate to the larger mass culture chambers. A substrate may be added to each chamber but is not needed. The chambers may be inspected daily for production of offspring (3 to 4 young per female per week for peak reproduction rate); therefore, ages of juveniles can be more precisely determined than those produced in mass culture chambers.

10.1.13. Culture Evaluation

Brood stock evaluation is based upon survival and reproductive rate of the adults. Counts of surviving adults, breeding pairs and young production should be made at the time of culture renewals and the information should be recorded (e.g., Attachment W, Form W1). Some adult *H. azteca* can be expected to die in the culture tanks between weekly renewals, but any

unusually high death rate for the week in one of the brood stock chambers when compared to previous weekly mortalities should be a cause for concern. The first symptom of problems in the culture chambers is a reduction in the reproductive rate. Typical reproductive rates in culture chambers containing 50 adults range from 75 to as high as 100 juveniles per week. Once a reproductive rate has been established, any decrease in this rate can be attributed to a change in water or food quality, or brood stock health. Adult females usually continue to reproduce for several months; however, their fertility will gradually decrease as senescence approaches (≈100 d).

The <1- to 7-d old amphipods are held in separate culture chambers with presoaked cotton gauze for an additional 7 d, at which time the 7- to 14-d old *H. azteca* are used in sediment toxicity tests. During the seven days, the juveniles are fed 10 to 15 mL of YCT daily. After separation from the adults, each batch of juveniles should be observed daily for survival until used for testing. If >20% of the juveniles die during this time interval, that batch of juveniles should be considered unsuitable for testing.

10.1.14. Culture Records

A separate set of records should be maintained for the culture unit. The records should show dates of renewal of culture chambers and the estimated number of surviving adults and production of juveniles per culture chamber. In addition, there should be daily records (Attachment W) of water temperature and feedings.

10.2. TOXICITY TEST METHODS

10.2.1. Solid-Phase Sediment Preparation

See Section 5.0.

10.2.2. Test Design

The basic design and conditions for performing a 10-d toxicity test with solid-phase dredged material are given in Table G-16. In a typical test, one or more dredged material samples will be evaluated by comparison with a disposal site sediment sample. In addition, a control sediment which serves as a basis for evaluating biological performance criteria for the test should be run simultaneously. The control material can be laboratory specific; however, previous testing should have demonstrated that test organism survival is routinely >80 percent, and that they grow and reproduce in the sediment. Exposures consist of a minimum of five replicates of each test sample and disposal site material in an exposure system designed to renew water overlying the sediment. The replicates for each test disposal site or control sediment should all be contained

within their respective aquaria. Sediments from different sources should not be mixed within an aquarium.

Table G-16. Overview of Recommended Test Conditions for the 10-d Solid-Phase Dredged Material *Hyalella azteca* Survival Toxicity Test.

1.	Test Type	Solid-phase sediment toxicity test with renewal of overlying water (2 volume additions/day).
2.	Temperature	23±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber	300-mL high-form beaker
7.	Test sediment volume	100 mL
8.	Overlying water volume	150-175 mL; variable due to water renewal siphoning cycle
9.	Renewal of overlying water	2 volume additions/day done continuously or intermittently, such as one volume addition every 12 h
10.	Age of test organisms	7- to 14-days old
11.	No. of organisms per test chamber	10
12.	No. replicate test chambers per treatment	5 minimum
13.	No. organisms per treatment site	50 minimum
14.	Feeding regimen	YCT[a] food, fed 1.5 mL daily to each test chamber.
15.	Aeration	Add aeration to each test chamber if dissolved oxygen in overlying water falls below recommended minimum in any test chamber.
16.	Overlying water	Culture water, test site water, well water, surface water or reconstituted water.

Table G-16. (continued)

17.	Test chamber cleaning	If test chamber screens become clogged during the test, gently brush outside of screen only.
18.	Dissolved oxygen minimum	40% of saturation; if this saturation level cannot be maintained, add aeration to each large test chamber.
19.	Test duration	10 d
20.	Endpoint	Survival and growth.
21.	Test acceptability	80% or greater survival in the control sediments; dissolved oxygen $\geq$40% saturation; mean test temperature 23±1°C; and satisfactory results from a reference toxicant test.
22.	Sample requirements	Storage of dredged material at 4°C; sediment should be sieved and homogenized and tests initiated as soon as possible but must be within 8 weeks of collection.
23.	Sediment volume required	A minimum of 500 mL from each test and disposal site.

[a] YCT is a food mixture comprised of yeast, cereal leaves and trout chow (see Attachment V).

The automated renewal of overlying water within each test chamber has a definite advantage in reducing labor hours as compared to manual renewal of water (Ankley et al. 1993). Several types of automated water renewal systems are available (e.g., Mount/Brungs 1967, Maki 1977, Ingersoll and Nelson 1990, Benoit et al. 1993, Zumwalt et al. 1994), and are presented here as options. Any method of water renewal is acceptable, provided the recommended volumes of water and their renewal rates are maintained, along with the recommended physical and chemical characteristics of overlying water.

Homogenized [sieved through a coarse (5 mm) screen then blended for a few minutes in the storage container with a wooden or plastic rod] sediment (100 mL) is added to each 300-mL high-form (taller than standard 300-mL beakers) exposure beaker, and the sediment allowed to settle for 24 h in the test system before introduction of test organisms. The overlying water flows over the sediment during this 24-h period at approximately two volume additions/d.

A summary of daily activities prior to and during a test is presented in Attachment X. This schedule assumes that all materials are on hand, and that a healthy culture of animals is being maintained.

10.2.3. Test Chambers

Five 300-mL high-form (taller than standard 300-mL beaker) beakers with side-walls drilled and screened (two opposing holes of 1.5 cm diameter, centered 7.7 cm up from the beaker floor, and covered with 60 mesh stainless steel screen) are required for each sediment sample tested (Benoit et al. 1993). The screened openings facilitate the exchange of water over the sediments when the water renewal system operates. The five beakers containing replicate samples of a test sediment must be in the same larger test chambers containing the overlying water with no mixing of sediments from other sites.

A single test site sediment (5 total replicates) and a disposal site sediment can be tested simultaneously in a portable mini-flow exposure system of the size described in Benoit et al. (1993) using five replicates per sample. Other types of renewal systems can be used (see Section 10.2.2.). At the time of construction, the dimensions of the portable mini-flow exposure system can be enlarged to the proportions necessary to accommodate tests for a larger number of sediment samples. A maximum of twelve samples (60 total replicates) can be tested simultaneously in a modified mini-diluter system (Benoit et al. 1993).

10.2.4. Water Renewal

Laboratory culture water or water with similar characteristics may serve as overlying water for the exposures. In certain projects, it may be desirable to use disposal site water; however, this may prove formidable from a logistical and test organism acclimation standpoint. The exposure system should be set to provide approximately two volume additions/d. This renewal rate may not result in satisfactory dissolved oxygen levels for most sediments. If the recommended minimum dissolved oxygen concentration cannot be maintained in all test beakers, then each chamber containing the five replicate test beakers, including control and disposal site sediment containing chambers, must be aerated for the remainder of the test.

10.2.5. Temperature and Photoperiod

Tests should be performed at 23±1°C. The daily photoperiod should be 16 L:8 D with a light intensity of 50-100 ft-C provided by wide spectrum fluorescent lamps.

10.2.6. Organism Introduction

A sufficient number of 7- to 14-d old juveniles are removed

from the juvenile culturing beakers to provide 10 organisms per replicate. They should be handled gently (use a glass or plastic pipet with 6 mm diameter opening that has been fire polished) and placed beneath the water surface directly into randomly chosen test beakers, after which each beaker is returned to its respective test holding tank. Any organisms trapped (floaters) in the water surface tension must be submerged with a drop of water or a blunt-ended probe. If some test organisms persist in floating, they can be removed and replaced during the first 24 h of the test.

10.2.7. Food and Feeding

Previously prepared YCT (Attachment V), which has been kept refrigerated (it should not be more than 14-d old), is fed daily at the rate of 1.5 mL to each test chamber. Food should be added at the end or after an overlying water renewal cycle to prevent food from leaving the test systems. YCT is prepared (Attachment Y) at a concentration of 1800 mg/L dry solids; therefore, feeding 1.5 mL results in 2.7 mg dry solids/feeding/test beaker. The food should be stirred or shaken before each feeding. A total volume of 75 mL is required for the duration of the test (10 d) for each sample of five replicates.

10.2.8. Test Organism Monitoring

Observe the contents of the beakers daily. The amphipods will burrow in the sediment or graze upon the sediments and may not be visible if they are in good health. A stressful environment may be indicated when all or most organisms in a beaker are observed to be persistently darting about in the overlying water and are apparently not feeding on the sediments. Dead or severely affected organisms, when they occur, may be seen lying motionless on the surface of the sediment or floating at the water surface. Record the observations for each beaker.

10.2.9. Water Quality Monitoring

Water should be monitored daily for temperature, and dissolved oxygen concentrations must be measured on even numbered days plus d 1. The temperature should be maintained within ±1°C of 23°C. Dissolved oxygen concentrations must be maintained at or above 40 percent of saturation. Hardness, alkalinity, specific conductance and pH should be measured near the beginning (d 1) and near the end (d 9) of the test from one of the replicates (Attachment W). Because ammonia may be elevated in some test sediments (e.g., Ankley et al. 1990), measurement of total ammonia may aid in test interpretation. Total ammonia should be measured near the beginning (d 1) and end (d 9) of each test. Determination of the worst case conditions for dissolved oxygen and ammonia is made by measuring concentrations just above the test sediment prior to the next overlying water renewal cycle. Water quality parameters should be recorded on a data

form (see Attachment W, Form W2).

10.2.10. Test Termination

After ten d of exposure, easily captured organisms can be removed with a pipette to save time before sediment from each replicate is sieved through a fine-meshed screen sufficiently small to retain the juvenile amphipods (e.g., U.S. Standard No. 30, having a 0.59 mm mesh size). Other methods of test organism removal from the test beakers such as swirling the overlying water with the pipet to lift organisms from the sediment may save time. Amphipods are placed into a clear viewing pan or beaker containing culture water and, if necessary, viewed under a dissecting microscope to determine if any movement occurs to indicate viability. Once survival determinations have been made, a determination for growth differences may be made. The live organisms should first be quickly killed by over dosing with an anesthetic or by freezing. There they are placed in a pre-dried and weighed metal pan, and placed in an oven at 100°C for about 4 h or at 60°C for about 12 h. After cooling the pans in a desiccator, the organisms are weighed to 0.01 mg.

10.2.11. Data Reporting and Statistical Analysis

See Section 12.

11.0. *Lumbriculus variegatus* CHEMICAL ACCUMULATION

Aquatic sediments are well known to act as sinks or reservoirs for nonionic, hydrophobic organic chemicals (Larsson 1985, 1986, Bierman 1990) and heavy metals (Malueg et al. 1984, Fallon and Horvath 1985, Poulton et al. 1988, Ankley et al. 1991a, West et al. 1993). They are also sources of toxicants, releasing chemicals into aquatic ecosystems through processes such as diffusion, resuspension and bioaccumulation through benthic and pelagic food chains (Rice and White 1987, Chapman 1988, Schuytema et al. 1988). The extent to which sediment-associated chemicals may be available to benthic organisms is of serious concern (DiToro et al. 1991).

Several studies have shown that hydrophobic organic compounds are bioaccumulated from sediment by freshwater infaunal organisms including larval insects, such as *Chironomus tentans* (Adams et al. 1985, Adams 1987) and *Hexagenia limbata* (Gobas et al. 1989); oligochaete worms, such as *Tubifex tubifex* and *Limnodrilus hoffmeisteri* (Oliver 1984, 1987, Connell et al. 1988); and by marine organisms, such as polychaete worms, *Nephtys incisa,* and molluscs, *Mercenaria mercenaria* and *Yoldia limatula* (Lake et al. 1990). Since these and related organisms are components of food webs containing higher consumers from all of

the vertebrate classes, the possibility exists for chemical bioaccumulation and/or biomagnification at higher trophic levels. It is important, therefore, to examine the uptake of chemicals by the benthos from contaminated sediments, as well as the toxicity of contaminated sediments to benthos.

Various species of organisms have been suggested for use in studies of chemical bioaccumulation from aquatic sediments. Several criteria should be considered before a species is adopted for routine use. These criteria include: ready availability of healthy organisms throughout the year, known chemical exposure history, adequate tissue masses for trace chemical analysis, ease of handling and tolerance of a wide range of sediment physico-chemical conditions (e.g., particle size), amenability to long-term exposures, and ability to accurately reflect concentrations of contaminants in field organisms (i.e., exposure is realistic). With these criteria in mind, the advantages and disadvantages of several potential freshwater taxa are discussed briefly below.

Freshwater clams provide an adequate tissue mass, are quite easily handled, and can be used in long-term exposures. However, few freshwater species are appropriate for testing, and the exposure is uncertain due to valve closure. Chironomids can be readily cultured, are quite easily handled, and reflect appropriate routes of exposure. However, large numbers of individuals are required to provide an adequate tissue mass for low-level residue analysis, and their rapid life-cycle makes it difficult to perform long-term exposures with highly hydrophobic compounds which equilibrate very slowly between sediment, pore water and animal tissue. Larval mayflies (i.e., *Hexagenia limbata*) reflect appropriate routes of exposure, have adequate tissue mass for residue analysis and can be used in long-term tests. However, they cannot be continuously cultured in the laboratory and consequently are not always available. Furthermore, the exposure history and health of field-collected individuals may be uncertain. Amphipods (e.g., *Hyalella azteca*) can be cultured in the laboratory, are easily handled, and reflect appropriate routes of exposure. However, their collective tissue mass may be insufficient for convenient trace residue analysis, and they are relatively sensitive to chemical parameters in the sediment. Although fishes (e.g., fathead minnows) provide an adequate tissue mass, are readily available and easily handled, and can be used in long-term exposures, they do not have the same routes of exposure to sediment-associated contaminants as benthic invertebrates.

As a group, oligochaetes represent infaunal benthic organisms that meet many of the test criteria described above. Certain oligochaete species are easily handled and cultured, provide reasonable biomass for residue analyses, and are tolerant

of varying sediment physical/chemical characteristics. Oligochaetes are exposed to contaminants via all appropriate routes of exposure, including pore water and ingestion of sediment particles. Various oligochaete species have been used in toxicity and bioaccumulation evaluations (Chapman et al. 1982a,b, Wiederholm et al. 1987, Keilty et al. 1988a,b), and field populations have been used as indicators of pollution of aquatic sediments (Brinkhurst 1980, Spencer 1980, Lauritsen 1985, Robbins et al. 1989).

Lumbriculus variegatus (Figure G-11) is a freshwater oligochaete that has been successfully cultured in the laboratory, and used in both chemical toxicity and bioaccumulation studies. Toxicity studies have been performed in water-only exposures of toxicants (Bailey and Liu 1980, Hornig 1980, Ewell et al. 1986, Nebeker et al. 1989, Ankley et al. 1991a,b), in effluent tests (Hornig 1980), and in solid-phase sediment toxicity evaluations (Nebeker et al. 1989, Ankley et al. 1991a,b, 1992b,c, Call et al. 1991, Carlson et al. 1991, Phipps et al. 1993, West et al. 1993). Several studies have reported the use of *L. variegatus* to examine chemical bioaccumulation from the sediment (Schuytema et al. 1988, Nebeker et al. 1989, Ankley et al. 1991a, 1992a, Call et al. 1991, Carlson et al. 1991).

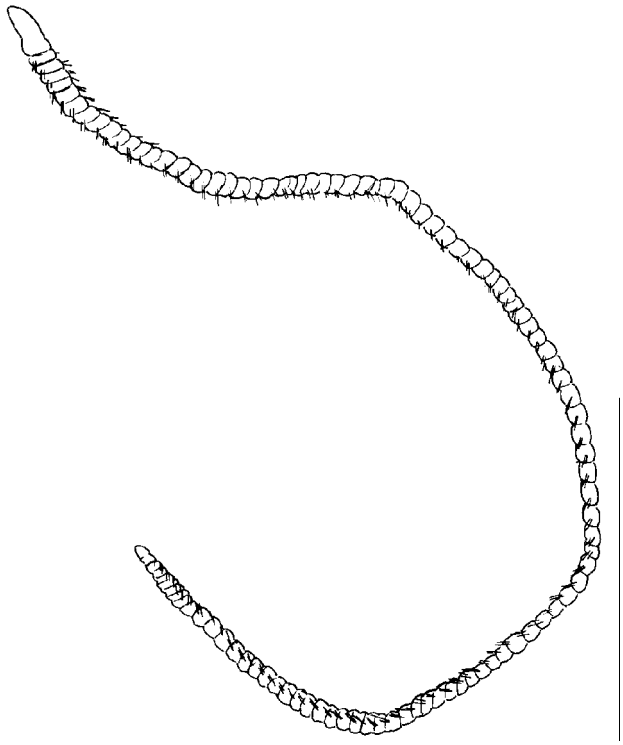

Figure G-11. *Lumbriculus variegatus* adult, X 10.

Lumbriculus variegatus inhabits a variety of sediment types throughout the United States and Europe (Chekanovskaya 1962, Cook 1969, Spencer 1980, Brinkhurst 1986). It typically inhabits the upper aerobic zone of sediments from reservoirs, rivers, lakes, ponds and marshes, in which it will tunnel through the sediment while actively feeding. When not tunneling, it will bury its anterior portion in the sediment and undulate its posterior portion in the overlying water for respiratory exchange.

Lumbriculus variegatus adults attain body lengths as great as 40 to 90 mm and a diameter from 1.0 to 1.5 mm (Phipps et al. 1993). They may vary in wet weight from about 5-12 mg (Call et al. 1991, Phipps et al. 1993). The lipid content of the animals is about 1.0 percent on a wet weight basis (Ankley et al. 1992b). They most commonly reproduce asexually by architomy or budding, although they can reproduce sexually (Chekanovskaya 1962). Sexual reproduction appears to occur infrequently, at least in culture, as newly hatched worms have never been observed in cultures at the University of Wisconsin-Superior or the Environmental Research Laboratory (ERL)-Duluth (Phipps et al. 1993). Rather, the cultures appear to consist of adults of various sizes. Under culturing conditions at ERL-Duluth, the population doubles every 10-14 d at 20°C (Phipps et al. 1993).

The use of *L. variegatus* in laboratory bioaccumulation studies has been field-validated with natural populations of oligochaetes. After a 30-d laboratory exposure of *L. variegatus* to sediments from the lower Fox River and Green Bay, Wisconsin, total PCB concentrations in laboratory-exposed *L. variegatus* compared well with concentrations measured in field-collected oligochaetes from the same sites (Ankley et al. 1992a). PCB homologue patterns also were similar between laboratory-exposed and field-collected oligochaetes, with a tendency for the more highly chlorinated PCBs to show slightly greater bioaccumulation in the field-collected organisms. In contrast, a comparison of total PCBs in laboratory-exposed fish (*Pimephales promelas*) and field-collected fish (*Ictalurus melas)* revealed poor agreement in bioaccumulation relative to sediments.

This report provides methods used in studies of chemical bioaccumulation from aquatic sediments using the oligochaete, *L. variegatus*. It describes methods for maintaining a continuous culture, collection and preparation of sediment, preparation of the exposure system, performance of the exposure, and treatment of the data.

11.1. CULTURE METHODS

The culturing methods described below are based on methods in use at ERL-Duluth (USEPA) and described in "Standard Operating

Procedure for the Culture of *Lumbriculus variegatus*" (Juenemann and Denny 1992) and a methods paper by Phipps et al. (1993). Required materials are listed in Attachment Y.

11.1.1. Organism Source

Organisms for the initiation of a laboratory culture may be obtained from a laboratory (Attachment Z) with a verified culture. Collection from the field should be avoided to eliminate the possibility of initiating a culture with a different species. Organisms received should be carefully examined by a qualified taxonomist using a key (e.g., Brinkhurst and Cook 1966, Brinkhurst 1986, Pennak 1989) to verify the species. One or more organisms should be cleared and mounted to serve as reference material. Methods for clearing and mounting aquatic oligochaetes are provided in Stimson et al. (1982) and Pennak (1989). Organisms should all be of a single species, *Lumbriculus variegatus*, and be disease-free. They should possess very low contaminant body burdens.

11.1.2. Acclimation of New Brood Stock

Environmental stress on the starter culture should be minimized to facilitate the rapid development of a healthy culture. Although *L. variegatus* is generally tolerant to changes in temperature, dissolved oxygen and pH (Phipps et al. 1993), it is prudent to habituate newly acquired organisms gradually to their new culture water. Measure the temperature of the water containing the stock animals upon their arrival and gradually adjust it to the desired culture temperature. A temperature of 20-23°C has been used for culturing (Juenemann and Denny 1992, Phipps et al. 1993). A temperature of 23°C is recommended for both culturing and testing, although many toxicity tests have been performed over a temperature range of 17-22°C. A gradual adjustment of the new brood stock water characteristics to those of the desired culture and test water may be accomplished by incremental dilution of the brood stock water with culture water over a period of two or more days. This is continued until the water meets the requirements for the desired culture water. Culture water should be maintained at the same temperature as the test water.

11.1.3. Culture Chambers

Standard 57-L glass aquaria are recommended for use as culture chambers. The water level should be maintained at a depth of about 25 cm, thereby providing a water volume of approximately 45 L.

11.1.4. Water Renewal

Due to the potential for a rapid increase in biomass, a relatively high culture water renewal rate (i.e., 20 volume exchanges per day) is recommended in a flow-through system. If a

lower renewal rate is used and oxygen concentrations are diminished, the animals will aggregate in clusters, necessitating aeration of the aquaria. The culture water can be from a variety of sources including untreated well water, dechlorinated tap water, natural surface (e.g., lake) water, or various reconstituted waters. Methods for preparing synthetic, reconstituted culture and dilution water are available (e.g., ASTM 1993h, USEPA 1989, 1993). When provided with adequate food, *Lumbriculus variegatus* appears quite tolerant of a wide variety of water quality characteristics (e.g., hardness, alkalinity, pH).

A static culture system can be used successfully if it is well aerated and carefully maintained. A regular schedule of water replacement is recommended. Static systems should be monitored frequently for dissolved oxygen concentrations.

11.1.5. Temperature and Photoperiod

The recommended temperature for culturing *L. variegatus* is 23±1°C. The recommended photoperiod is 16 h light and 8 h dark with a light intensity of approximately 50-100 ft-C (10-20 $\mu E/m^2/s$) at the water surface.

11.1.6. Substrate

Several substrates have been found to work well for culturing *L. variegatus*, including maple and/or poplar leaves, sand, clean sediments of high organic carbon content, and brown paper toweling (Bailey and Liu 1980, Phipps et al. 1993, Juenemann and Denny 1992). Toweling is recommended because it is readily available, uniform in composition, and allows for easy removal of animals.

Prepare the substrate by first unfolding ordinary brown paper towels and either cutting them into strips (about 2.5 cm wide) or passing them through a paper shredder. The strips are next placed into a conditioning tank. For conditioning, place a volume of dry towel strips (4,000 mL) into an aquarium equipped with two water lines, each having a flow capacity of 100 mL/min. One line is placed below and one above the towel mass. A glass weight, consisting of several 2.5 cm x 25.4 cm glass strips standing on edge and glued on both ends to glass strips approximately 50 cm in length, is placed on the mass to prevent floating. This method creates a uniform water flow throughout the mass of intertwined toweling strips, and minimizes fouling of the strips. The strips of paper are soaked in this manner for at least one week. Following substrate conditioning, the towel mass is removed and evenly distributed over the entire bottom of a culture chamber. A glass weight as described above is placed over the toweling to keep it in place.

Approximately 500-1,000 oligochaetes are transferred to the new culture chamber. The substrate is renewed with pre-conditioned towels when thin or bare areas appear (see Section 11.1.9., "General Culture Maintenance"). A fresh substrate will generally last for 2-3 months.

11.1.7. Food and Feeding

Aquatic oligochaetes ingest their substrate and are believed to obtain their nourishment from the organic matter in the substrate as it passes through their digestive tract (Pennak 1989). Food is provided to the cultures by distributing 10 mL (~5.5 g) of trout starter on the water surface three times weekly. The particles will temporarily disperse on the surface film, break through the surface tension, and settle out over the substrate.

11.1.8. Handling

Oligochaetes inhabiting substrate can be transferred from culture aquaria to a white or light-colored shallow pan with a fine-meshed brine shrimp dipnet (e.g., 7.6 cm, Penn Plax, Inc., Garden City, NY). Those organisms not associated with the towel substrate can be easily captured and moved with a glass pipette (20 cm long, 5 mm I.D. opening, fire polished on both ends) fitted with a pipette bulb (Phipps et al. 1993). When the annelids aggregate into a cluster, a gentle stream of culture water from the pipet or a squeeze bottle will serve to spread them out for capture either as individuals or small groups of individuals. Organisms should not be handled with forceps, as they may be injured and/or fragmented. Injured organisms should be removed from the culture and not used for testing.

11.1.9. General Culture Maintenance

The culture should be examined daily to assess general condition of health, and to ensure that disruptions in aeration or water flow have not occurred. The temperature of the culture water should be measured daily in each chamber either manually or by a continuous temperature monitor with a chart recorder. *L. variegatus* cultures have the potential to develop low concentrations of dissolved oxygen and/or high concentrations of ammonia; therefore, routine monitoring of dissolved oxygen and total ammonia concentrations is advised. The dissolved oxygen concentration should be maintained at $\geq$40 percent of saturation, while the total ammonia concentration should not exceed 0.1 mg/L.

New pre-conditioned paper toweling should be added when the substrate appears thin or when bare spots are observed in the substrate. Depending upon the required culture size, extra organisms may be used to increase the number in additional tanks. Place a mass of desired size (e.g., 5-15 g) into the new chamber. A doubling in population density occurs about every 10-14 d at

20° C.

Snails (*Helisoma* sp.) added to the culturing chambers at ERL-Duluth assist in keeping the chamber walls clean, thereby reducing or eliminating the necessity of cleaning the chambers frequently. If snails are used, their number will have to be thinned regularly, as their populations proliferate under these culturing conditions. *Helisoma* sp. are available from ERL-Duluth upon request (J. Denny, 218-720-5717).

11.1.10. Culture Evaluation

Reproduction should be at a normal level and organisms should be judged to be in good condition before they are used in a bioaccumulation study. The culture population should be doubling about every 10-14 d. Individual animals should appear to be of normal adult size and coloration, and should be highly responsive to a gentle touch with a probe. A culture evaluation chart (Attachment AA, Form AA1) should be maintained, and updated monthly. If the organisms do not meet the criteria above, the culturing conditions should be scrutinized and adjustments made to restore culture health and increase reproduction. Any adjustments made may be considered to have resulted in an acceptable state of health for the culture when the culture meets the above criteria of reproduction, appearance, and responsiveness.

11.2 ACUTE TOXICITY SCREENING TEST

Prior to or concurrent with the full 28-d bioaccumulation study, a 10-d toxicity screening test should be performed with each sediment. It is important to screen the sediment for toxicity, evidenced either by mortalities or behavioral effects (i.e., avoidance of sediment by not burrowing), to determine if the full 28-d test should be performed.

This screening test can be performed in 300 mL high-form beakers containing screened holes in the walls for exchange of overlying water, as are used in conducting sediment toxicity tests with *Chironomus tentans* or *Hyalella azteca*. Test details are provided in Phipps et al. (1993). Briefly, the test should be performed with 100 mL of sediment placed into each beaker, and the beakers then placed within aquaria which provide overlying water to the beakers. The test system in which the beakers are placed should receive two volume renewals daily of overlying water. Aeration should be available and monitored to ensure that dissolved oxygen levels are maintained at 40 percent of saturation or greater. Ten organisms per replicate beaker should be added after the sediment has been allowed to settle for 24 h. The animals should not be fed during the 10-d test period. After 10 d, sediment samples from the toxicity screening test should be sieved, and the animals counted to determine survival and

reproduction. They should be observed for any abnormal behavior, and then oven-dried and weighed to obtain a measure of growth. Results from each dredged material site are compared to results from control and disposal site sediments. Survival of controls must be ≥70 percent for the test to be considered acceptable. These observations should allow for a determination of the appropriateness of either starting or continuing with a 28-d bioaccumulation study.

11.3. BIOACCUMULATION TEST METHODS

11.3.1. Solid-Phase Sediment Preparation

See Section 5.0.

11.3.2. Test Design

The basic design and conditions for performing a bioaccumulation test with dredged material are given in Table G-17. The exposure consists of five or more replicates of each sample in an exposure system designed to renew overlying water at 1 h intervals at a rate to provide a total renewal of about two volume exchanges each day. Several types of automated water renewal systems have been developed (e.g., Mount/Brungs 1967, Maki 1977, Ingersoll and Nelson 1990, Benoit et al. 1993, Zumwalt et al. 1994), and are presented here as delivery system options. Variations of these systems are acceptable, provided the recommended volumes of overlying water and their renewal rates are maintained, along with the recommended physical and chemical characteristics of the overlying water. It should be noted that rectangular glass tanks are used for exposure chambers rather than beakers, as in sediment toxicity tests. Thoroughly homogenized sediment (1,600 mL) is added into each exposure chamber, and the sediment is allowed to settle for 24 h before introduction of test animals. The overlying water flows over the sediment during this 24 h period. The sediment:water volume ratio should be from 1:1.7 to 1:2.1 over the course of the overlying water renewal cycle.

11.3.3. Test Chambers

Five or more, if desired, replicate 5.5 L [15.8 x 29.3 x 11.7 cm, W x L x H (OD)] rectangular glass chambers are recommended for each sediment sample tested. A maximum of two sediment samples (10 total replicates), or one disposal site sediment and one test sediment, can be tested simultaneously in a standard mini-flow exposure system of the size described in Benoit et al. (1993), using five replicates per sample.

11.3.4. Water Renewal

Laboratory culture water may serve as overlying water for the exposures. The exposure system is set to provide about two volume exchanges per day.

Table G-17. Overview of Recommended Test Conditions for 28-d Bioaccumulation Tests with *Lumbriculus variegatus*.

1.	Test type	Chemical bioaccumulation from sediments with renewal of overlying water.
2.	Temperature	23±1°C
3.	Light quality	Fluorescent bulbs (wide spectrum)
4.	Light intensity	10-20 $\mu E/m^2/s$, 540-1080 lux or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod	16 h light, 8 h dark
6.	Test chamber	5.5 L glass tank (15.8 x 29.3 x 11.7 cm, W x L x H)
7.	Sediment volume	1,600 mL
8.	Overlying water depth	6 to 7.5 cm with top of standpipe siphon at 11 cm
9.	No. of volume renewals of overlying water	2 per day
10.	Age of test organisms	Adults
11.	No. of replicates per sample	Minimum of 5
12.	Initial mass of organisms per replicate	1.0 to 5.0 g, depending upon analytes of concern
13.	Initial No. of organisms per replicate	80-1,000
14.	Feeding regime	No feeding
15.	Aeration	Aerate if dissolved oxygen drops below 40% of saturation (i.e., 3.40 mg/L)
16.	Overlying water	Culture water, (e.g., untreated well water, dechlorinated tap water, reconstituted water)
17.	Test duration	28 d for all chemicals
18.	Measurements (possible-not all would be run on every test)	Animal tissue weight (wet and dry), chemical concentration in sediment and animal tissue, total organic carbon or acid-volatile sulfide content of sediment, organism lipid content.

Table G-17. (continued)

19.	Water Quality Monitoring	Daily measurements of water temperature and dissolved oxygen. Hardness, alkalinity, specific conductance, pH and total ammonia should be measured twice during the test.
20.	Sample storage	Store sediment at 4°C. Test should be initiated within 2 weeks of sample collection, and must be initiated within 8 weeks of collection.
21.	Sediment volume required	8.2 L from each test site (1.6 L for each of five replicates, remainder for analytical chemistry).
22.	Test acceptability criteria	Test initiated with animals from healthy culture; animals burrowed into sediment; 10-day toxicity test survival was not significantly different from controls; dissolved oxygen concentration should exceed 40% of saturation at all times; mean temperature was 23 ± 1°C and did not deviate ≥3°C at any time; total ammonia concentrations averaged 0.1 mg/L; satisfactory results in a reference toxicant test.

This renewal rate will likely require supplemental aeration for many sediments. Aeration of the overlying water should be initiated if the dissolved oxygen concentration drops to 40 percent of saturation or below (i.e., 3.40 mg/L at 23°C).

11.3.5.Temperature and Photoperiod

Tests should be performed at 23±1°C. The daily photoperiod should be 16L:8D, using ambient laboratory lighting of 50-100 ft-C.

11.3.6. Organism Introduction

A biomass of approximately 1.0 to 5.0 g of adult oligochaetes is weighed and added to each chamber on d 0. The initial mass will depend upon the analyte(s) of concern, and their respective lower limits of detection during chemical analysis. Tissue weights at the end of the exposure period required to achieve various analytical detection limits are presented in Table G-18. Assuming that no net weight change occurred during exposure, the initial weight required would be the same as the required final weight. However, either negative or positive weight changes are likely to occur, and the initial weight should be adjusted accordingly. Dependent upon their

size, this will be from 40 to 1,000 animals. An actual count of the organisms is optional but not necessary.

Table G-18. Grams of *Lumbriculus variegatus* Tissue (Wet Weight) Required for Various Analytes at Selected Lower Limits of Detection.

Analyte		Grams of Tissue 1.0	2.0	5.0
		Lower Limit of Detection (μg/g)		
PCBs				
PCB (total)[1]	level of chlorination	0.600	0.300	0.120
PCB (congeners)[2]	1-3	0.025	0.0125	0.005
	4-6	0.050	0.025	0.010
	7-8	0.075	0.375	0.015
	9-10	0.125	0.0625	0.025
Organochlorine Pesticides				
p,p'-DDE[1]		0.050	0.025	0.010
p,p'-DDD[1]		0.050	0.025	0.010
p,p'-DDT[1]		0.050	0.025	0.010
o,p'-DDE[1]		0.050	0.025	0.010
o,p'-DDD[1]		0.050	0.025	0.010
p,p'-DDT[1]		0.050	0.025	0.010
Alpha-Chlordane[1]		0.050	0.025	0.010
Gamma-Chlordane[1]		0.050	0.025	0.010
Dieldrin[1]		0.050	0.025	0.010
Endrin[1]		0.050	0.025	0.010
Heptachlorepoxide[1]		0.050	0.025	0.010
Oxychlordane[1]		0.050	0.025	0.010
Mirex[1]		0.050	0.025	0.010
Trans-Nonachlor[1]		0.050	0.025	0.010
Toxaphene[1]		0.600	0.300	0.120
PAHs				
PAHs (GC Method)[3]		0.012	0.006	0.002

Table G-18. (continued)

Inorganics			
Cadmium[4]	0.005	0.0025	0.001
Copper[4]	0.005	0.0025	0.001
Lead[4]	0.005	0.0025	0.001
Zinc[4]	0.005	0.0025	0.001
	Lower Limit of Detection (ng/g)		
Dioxins			
TCDD[5]	0.020	0.010	0.004
PAHs			
PAHs (HPLC-FD Method)[6]			
Benzo(a)pyrene[6]	0.01	0.005	0.002
Pyrene[6]	0.03	0.015	0.006
Benzo(k)fluoranthene[6]	0.03	0.015	0.006
Dibenzo(a,h)anthracene[6]	0.03	0.015	0.006
Anthracene[6]	0.10	0.050	0.020
Benz(a)anthracene[6]	0.10	0.050	0.020
Benzo(e)pyrene[6]	0.10	0.050	0.020
Benzo(b)fluoranthene[6]	0.10	0.050	0.020
Benzo(g,h,i)perylene[6]	0.10	0.050	0.020
3-Methylcholanthrene[6]	0.10	0.050	0.020

[1] Schmitt et al. (1990); [2] USEPA (1990b); [3] Vassilaros et al. (1982); [4] Schmitt and Finger (1987); [5] USEPA (1990); [6] Obana et al. (1981).

The annelids and substrate are removed from a culture tank with a 7.6 cm fine-meshed dipnet and placed directly into a glass bowl or stainless steel pan (approximately 20 cm or more in diameter) containing culture water. With a pipette (20 cm long, 5 mm I.D. opening, fire polished on both ends) fitted with a pipette bulb, gently pulse the animals with water to loosen the substrate. Allow the animals to reform in a cluster in the bottom of the container and decant or siphon off most of the water and substrate. Repeat this process until the animals are free of substrate. Refill the pan with stream of water from a squeeze bottle. Pick them up with a dissecting needle or dental pick, blot the mass on paper toweling, and place into a tared weighing pan. Experience will help approximate the size of the mass of annelids to equal the desired 1.0 to 5.0 g per replicate.

The weighing must be done rapidly, as the animals should not be allowed to desiccate or warm up in the weighing pan. When the desired weight is attained, water should be added to the weighing pan. Each aliquot of annelids should take 2-3 min. to dilution water and gently separate masses of annelids of desired size with a portion and weigh, and each aliquot should be randomly added to its respective exposure chamber immediately after weighing. The annelids should be observed to determine if they immediately burrow into the sediment.

Organism loading should result in an organic carbon ratio between animal tissue (dry weight) and sediment (dry weight) that preferably lies between 1:50 and 1:100. The ratio should not be less than 1:10.

11.3.7. Food and Feeding

No food is provided during the bioaccumulation exposure. The addition of food would alter the organic carbon content of the sediment, which could influence bioavailability of chemicals in the sediment (Phipps et al. 1993).

11.3.8. Test Organism Monitoring

The health of the animals should be observed at least twice daily. Animals which are not actively feeding and tunneling will be observed to be oriented with the anterior portion of their bodies in the sediment and the posterior portion in the overlying water. If no animals are evident with this orientation, it is possible that the sediment is toxic, and an insufficient biomass will be available for chemical analysis at the end of the exposure period. In such a case, it may be desirable to terminate the exposure early. Results from the 10-d acute toxicity screening test, if performed concurrently with the 28-d bioaccumulation study, will provide evidence for a decision regarding termination. See Section 11.2. on the 10-d acute toxicity screening test for specific criteria regarding test acceptability.

11.3.9. Water Quality Monitoring

Water should be monitored daily for temperature and dissolved oxygen concentrations. The temperature should be maintained within ± 1°C of 23°C at all times. Dissolved oxygen concentrations should be maintained at or above 40 percent of saturation. Hardness, alkalinity, specific conductance and pH should be measured at the beginning and end of the test from one of the replicates of each sediment. Because ammonia may be elevated in some test sediments (Ankley et al. 1990, Call et al. 1991), measurement of total ammonia should be performed regularly. Total ammonia concentrations should be monitored twice weekly in the overlying water from one of the replicates for each sample. Total ammonia concentrations should not exceed

0.1 mg/L. Water quality parameters should be recorded on a data form (see Attachment AA, Form AA4 as an example).

11.3.10. Test Duration

The duration of the test should be sufficient to allow time for the chemicals to equilibrate between sediment, pore water and oligochaete tissue. At present, a minimum exposure period of 28 d is recommended for all chemicals. In general, the larger the K_{ow} (octanol-water partition coefficient) of a nonionic organic chemical, the longer it will take to come into equilibrium or steady-state in animal tissue. For the purposes of environmental risk assessment, it is essential that decisions be made based on steady-state concentrations from the laboratory exposure. There are two ways to help ensure that steady-state concentrations are reached in laboratory tests with *L. variegatus*. The first is to run tests for longer than 28 d if it is suspected that chemicals of concern will not come to equilibrium tissue concentrations in this time period. This may be expected to be the case when the log K_{ow} of the analyte of concern is $\geq$5.5 to 6.0 (Ankley et al., unpublished manuscript). Alternatively, if it is impractical to run the bioaccumulation test for more than 28 d, but the target nonionic chemicals are in a log K_{ow} range where there may be concern about equilibrium (i.e., $\geq$5.5 - 6.0), it is possible to conduct multiple samplings during the 28 d test (e.g., d 1, 3, 4, 7, 14, and 28) and use a kinetic analysis to project or estimate steady-state tissue concentrations. This kinetic approach is described in detail elsewhere (USEPA and USACE 1993). Note that if this approach is used, it will be necessary to set up correspondingly greater numbers of replicate test chambers for the multiple sampling.

11.3.11. Test Termination

The sediment from each replicate is sieved through a fine-meshed screen sufficiently small to retain the oligochaetes (e.g., U.S. Standard No. 35 or 40, having 500 or 425 μm mesh sizes, respectively). The organisms may then be transferred for removal of associated substrate to a light-colored shallow pan by a gentle stream of water. Upon cleaning, they are transferred to a 1 L beaker containing test water with no sediment present for elimination of sediment from the alimentary canal. The beaker should be well aerated with an airstone to maintain a satisfactory level of dissolved oxygen. The animals should be held in the water for a 24-h period to allow for alimentary tract clearance of most of the sediment. Brooke et al. (unpublished manuscript) observed that *Lumbriculus variegatus* cleared most of its gut contents within 12 h in water without sediment. Following clearance of the alimentary canal, the annelid mass is collected, blotted to remove excess water, and weighed to determine wet weight (biomass). The animal tissue mass should not be dried prior to preparation for chemical analysis. If a

dry weight is desired, subsample 0.25 to 0.50 g of annelids, place the subsample into a pre-weighed pan, and obtain a total wet weight. Then oven-dry the annelids for at least 4 h at 100°C (until a steady weight is obtained). Allow to come to room temperature in a desiccator, and weigh to the nearest 0.01 mg.

The remaining mass of animals is then weighed and placed into a suitable clean container (e.g., 10 mL glass vial), sealed, and frozen for later analysis. The containers should be free of contaminants achieved by a thorough cleaning by approved methods (USEPA 1990a) prior to use. Vials should be placed inside of freezer containers to minimize "freezer burn" and dehydration during storage.

11.4. General Analyses

Certain chemical analyses may aid in the interpretation of test results. Measurement of tissue total lipid content in the test organism and total organic carbon (TOC) content of the sediment may help explain the partitioning of some organic chemicals between sediment and biota. Measurement of acid volatile sulfide (AVS) content of the sediment may help explain the bioavailability of divalent metals in the sediment. Their methods of analysis are referenced below.

11.4.1. Annelid Total Lipid Analysis

Take a subsample (≈1 g) of the total oligochaete mass of each thawed replicate for total lipid analysis. Various methods of lipid analysis can yield considerably different results. Consequently it has been suggested (Randall et al. 1991) that the analytical method used for lipid analysis should be calibrated against the chloroform/methanol extraction method described by Folch et al. (1957) and Bligh and Dyer (1959).

11.4.2. Sediment Total Organic Carbon (TOC) Analysis

Sediment TOC may be determined for sediments when bioaccumulation of nonionic organic chemicals is of concern. A subsample from each replicate is analyzed at the end of the exposure. The analysis should be a measure of TOC and not a measure of "loss on ignition" or "total volatile solids". The method used should be specific to the measurement of TOC, such as that described by Cowan and Riley (1987).

11.4.3. Sediment Acid Volatile Sulfide Analysis

AVS and simultaneously extracted metals (SEM) may be determined in wet sediment samples when metals are being assessed for bioaccumulation. SEM measurements apply for cadmium, copper, lead, nickel and zinc. A USEPA methods manual is available for the analysis of AVS and SEM (Allen et al. 1991).

11.4.4. Data Reporting and Statistical Analysis

At the conclusion of the exposure and gut clearance period, information is recorded on chemical residues in the annelids and the sediment. If the optional analyses were performed, data would also be recorded on such measurements as total lipids in the annelids, or the TOC or AVS content of the sediment. A sample data form is presented in Attachment AA (Form AA2). See Section 12.0 for statistical analysis.

12.0. DATA REPORTING AND STATISTICAL ANALYSIS

12.1. Data Reporting

Data are reported for the various tests using the proper reporting forms. Examples of suitable forms are shown in the various Attachments (A through AA). Great care should be taken on each day to ensure that observations from the randomized array of exposure chambers are accurately transferred to the data forms. Once the data have been organized and suitably summarized, analysis can be accomplished using an appropriate method. Toxicity assessment protocols for five species are offered in this Appendix (H). Those for *D. magna*, *C. dubia*, and *P. promelas* assess dredged material elutriates. Acute toxicity tests use organism survival as an endpoint. Chronic toxicity tests with *D. magna* and *C. dubia* use both organism survival and reproduction as endpoints, while the 10-d exposure with *P. promelas* examines survival and growth. Assessments of solid-phase dredged materials are made using *C. tentans, H. azteca* and *L. variegatus* as test organisms. The *C. tentans* test uses survival and growth as endpoints. The latter endpoint is an option in the toxicity test with *H. azteca*. *L. variegatus* is used to assess tissue contamination due to chemical bioaccumulation from solid-phase dredged materials by comparing tissue concentrations of specific chemicals in organisms exposed to dredged material to concentrations in organisms exposed to disposal site sediment, or to an action level.

12.2. Statistical Analysis

12.2.1. Toxicity Test Data Analysis

Methods described in this section are based upon and in agreement with statistical methods described in the Inland Testing Manual (USEPA/USACE 1998) for analysis of Tier 3 test results. Statistical analysis of Tier 4 tests may differ from these methods, as Tier 4 tests are case-specific. Program statements and sample data set analyses are provided for survival and bioaccumulation data. It is highly recommended that the reader refer to this manual. The statistical treatment of all test data follow either a parametric or nonparametric approach (Figures G-12, G-13 and G-14). If the data are found to be

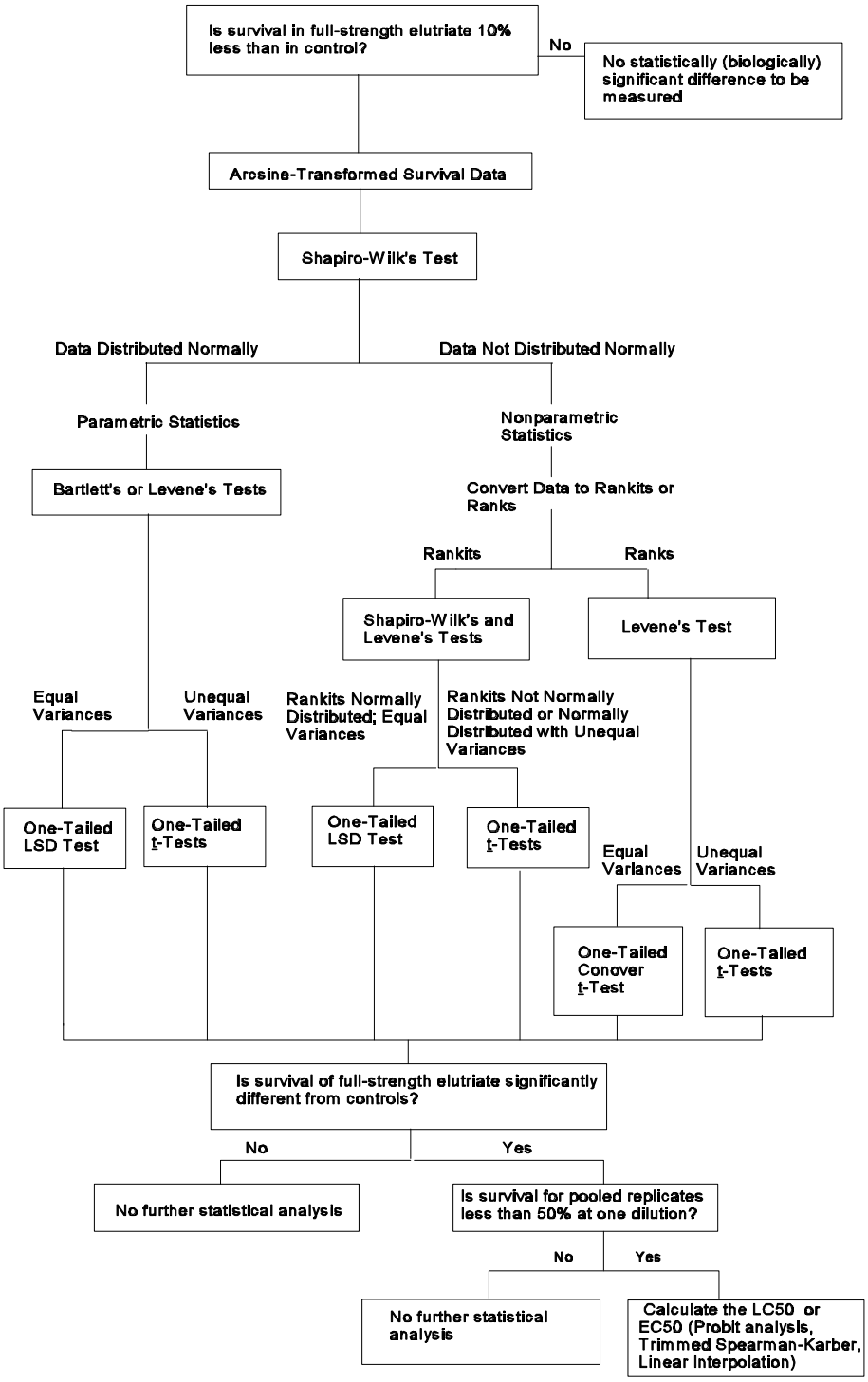

Figure G-12. Statistical treatment of survival data from toxicity tests with dredged material elutriates and *Ceriodaphnia dubia*, *Daphnia magna* and *Pimephales promelas* (adapted from USEPA/U.S. ACE 1994).

normally distributed, a parametric approach is applied. If the data are not normally distributed, a nonparametric approach is used.

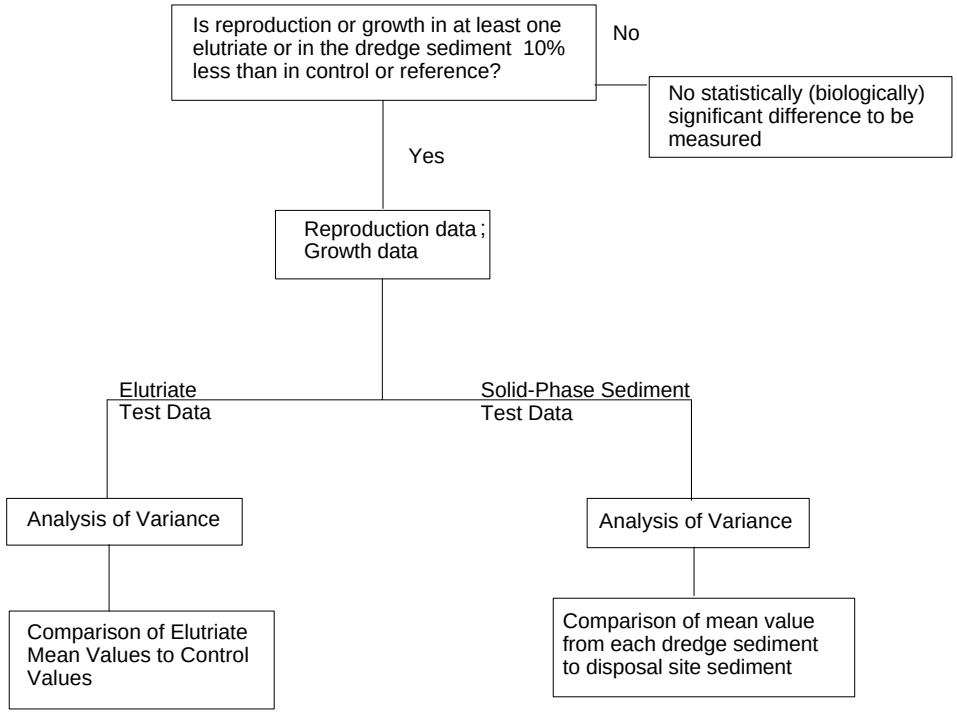

Figure G-13. Statistical treatment of reproduction or growth data from toxicity tests with dredged material elutriates and *Ceriodaphnia dubia, Daphnia magna* or *Pimephales promelas* or with solid-phase sediment and *Chironomus tentans* or *Hyalella azteca*.

Hypothesis testing is used initially to compare endpoints from either a full-strength elutriate to the control or from a dredged sediment to a disposal site sediment. The null hypothesis for toxicity tests is that there is no significant decrease between the specific endpoints (i.e., survival, reproduction or growth) of the test organism exposed to dredged sediments or elutriates when compared to either organisms exposed to disposal site sediment in the case of solid-phase tests or to dilution water (controls) in the case of elutriate tests. If survival in the full-strength elutriate is not reduced $\geq$10 percent relative to survival in the controls, no further statistical analysis is required. If survival is reduced $\geq$10 percent, the survival data are arcsine-transformed, tested for assumptions of normality of distribution and homogeneity of variances, and the survival means at each elutriate concentration compared to the mean survival of controls by a *t*-test. If *t*-test

results indicate that survival in the full-strength elutriate is not significantly different from survival in the controls, no further statistical analysis is required. If *t*-test results indicate that survival in the full-strength elutriate is significantly different from survival in the controls, then the data may be subjected to a point estimation of an effect level such as an LC50 or EC50. The point estimate can be used in a mixing model described in Appendix C of the Inland Testing Manual (USEPA/USACE 1998).

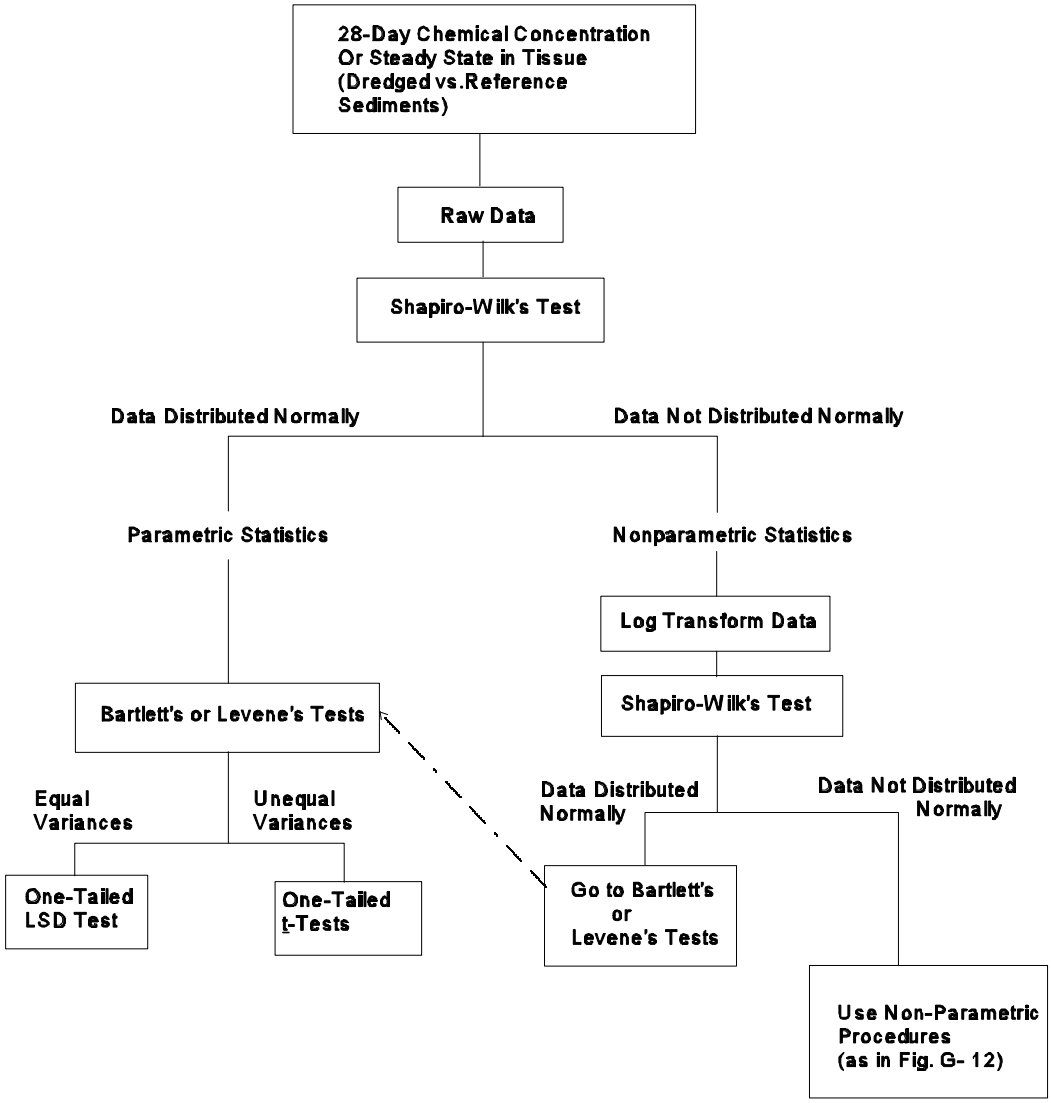

Figure G-14. Comparison of chemical residues in *Lumbriculus variegatus* tissues exposed to dredged site and disposal site sediments (based upon USEPA/USACE 1998).

The methods described for survival data are based upon methods described in the Inland Testing Manual (USEPA/USACE 1998). The methods provided in the Inland Testing Manual, complete with program statements and example data, use software products of the SAS Institute, Inc. (SAS 1985). These are IBM-compatible PC programs. Other acceptable hardware and software products are commercially available and may be used to perform the necessary analyses. While the specific statistical tests included in different software packages may vary in methods for determining data normality or equality of variances, it is important that these tests of assumptions are included in the software package used.

12.2.1.1. Two-Sample t-Test

The two-sample *t*-test (Snedecor and Cochran 1989) may be used in cases where an individual sediment or elutriate is being compared with a reference or control. Survival data should first be arcsine-transformed to reduce the heterogeneity of variance. A table for conversion of binomial percentage data is available in statistical tests (e.g., Steel and Torrie 1980, Snedecor and Cochran 1989). Data should then be tested for normality of distribution using a test such as the Shapiro-Wilk's Test. The normality test is run on the residuals (observations minus treatment mean) rather than on the raw data. Methods for determining the normality statistic, W, are found in USEPA (1993). Data that are normally distributed should then be tested for equality of variances to determine the proper equation for calculating the t-statistic. A calculated F-ratio [larger variance (S_1^2) over smaller variance (S_2^2)] is compared to a table value of F-ratios for the appropriate degrees of freedom for the two samples (see, e.g., Steel and Torrie 1980, Snedecor and Cochran 1989) to determine if the variances are significantly different. If variances are not significantly different, the equation is:

$$t = (\bar{x}_1 - \bar{x}_2) / \sqrt{S^2_{pooled}(1/n_1 + 1/n_2)}$$

where S^2_{pooled}, the pooled variance, is calculated as:

$$S^2_{pooled} = [S_1^2(n_1 - 1) + S_2^2(n_2 - 1)]/(n_1 + n_2 - 2),$$

and S_1^2 and S_2^2 are the sample variances of the two groups.

The calculated *t*-statistic is compared with the student *t* distribution (this is a one-tailed *t*-test and the table of *t* values must be used appropriately) in a statistics text (e.g., Steel and Torrie 1980, Snedecor and Cochran 1989) to determine if the null hypothesis should be rejected. If variances are unequal, the *t*-statistic is calculated by the equation:

$$t = (\bar{x}_1 - \bar{x}_2)/\sqrt{S_1^2/n_1 + S_2^2/n_2},$$

and the degrees of freedom calculated as follows (Satterthwaite 1946):

$$df = \frac{(S_1^2/n_1 + S_2^2/n_2)^2}{(S_1^2/n_1)^2/(n_1 - 1) + (S_2^2/n_2)^2/(n_2 - 1)}$$

Fractional degrees of freedom should be rounded down to the nearest integer (USEPA/USACE 1998). Suggested significance levels (") for normality and variance tests vary dependent upon number of replicates and evenness of statistical design and are given in Table G-19.

Table G-19. Suggested " Levels to Use for Tests of Assumptions.

Test	Number of Observations[a]	" When Design Is	
		Balanced	Unbalanced[b]
Normality	N = 3 to 9	0.10	0.25
	N = 10 to 19	0.05	0.10
	N = 20 or more	0.01	0.05
Equality of Variances	n = 2 to 9	0.10	0.25
	n = 10 or more	0.05	0.10

[a] N = total number of observations (replicates) in all treatments combined
n = number of observations (replicates) in an individual treatment.

[b] $n_{max} \geq 2n_{min}$.

(From USEPA/USACE 1998)

When testing the assumption that the dredged sediment is not significantly different from the disposal site sediment (null hypothesis), an error rate (") must be specified. Biological tests generally set "=0.05 which means that, on the average, the null hypothesis will be rejected in 5% of the tests when it is true. The recommended " is 0.05; however, there is nothing magical about the " level of 0.05 and the evaluator of a toxicity test may desire to use a larger ", such as 0.10. The larger " results in a more environmentally protective sediment evaluation

by rejecting a null hypothesis when it is true a higher percentage of the time (i.e., sediments may be considered contaminated when they are not).

12.2.1.2. Multiple Sample t-Test

A flow-diagram of statistical tests is presented (Figure G-12) with examples of specific statistical tests to test for assumptions, to compare means and to calculate point estimates. These will be discussed briefly. As in the case of the two-sample test, the data are first arcsine-transformed to reduce heterogeneity of variance. A table for conversion of binomial percentage data to arcsine data is available in statistical texts (e.g., see Steel and Torrie 1980, Snedecor and Cochran 1989). The arcsine-transformed survival data are tested for normality of distribution by a test such as Shapiro-Wilk's Test. Methods for determining the normality test statistic, W are found in USEPA (1993). Data that are normally distributed should then be tested for equality of variances to determine the proper equation for calculating the *t*-statistic. If the variances are equal, survival at each elutriate concentration can be compared to control survival by a one-tailed LSD test. If the variances are unequal, this survival comparison is accomplished by a one-tailed *t*-test (USEPA/USACE 1998).

Nonparametric statistics are used to compare data that are not distributed normally based upon Shapiro-Wilk's Test. The survival data are first converted to rankits or ranks. If converted to rankits, Shapiro-Wilk's and Levene`s Tests are performed to determine the assumptions of normality and equal variances. If the rankits are distributed normally and have equal variances, a one-tailed LSD test is recommended for comparing the mean survival at each elutriate concentration to mean survival of controls. If the rankits are not normally distributed, or if they are normally distributed but with unequal variances, a one-tailed *t*-test is used (Fig. G-12). If the data are converted to ranks, Levine's Test is applied to determine the equality of variances. If the variances are equal, a one-tailed Conover *t*-test is used. If the variances are unequal, a one-tailed *t*-test is used to compare mean survival in the various elutriate concentrations to mean survival of controls.

Following analysis by one of the *t*-tests listed in Fig. G-12, the statistical results are examined to determine if organism survival at the full-strength elutriate was significantly different ($p \leq 0.05$) than survival in the controls. If not, no further statistical analysis is required. If the difference is significant, further statistical analysis (i.e., a point estimation) may be performed for subsequent use in a mixing model. If survival for the pooled replicates of a given elutriate concentration is less then 50 percent, an LC50 may be

calculated. Probit analysis is recommended to provide a point estimate of the elutriate concentration that decreases survival to some level. Software with the capacity to calculate various LC values from LC1 to LC99 with 95 percent confidence limits is available through EPA. A compiled version of a program written in IBM PC Basic for IBM compatible PCs may be obtained by sending a double-sided (DS), high density (HD) diskette with a written request to: Environmental Monitoring Systems Laboratory-Cincinnati, Office of Modeling, Monitoring Systems and Quality Assurance, Office of Research and Development, U.S. Environmental Protection Agency, 3411 Church Street, Cincinnati, OH 45268. Other methods for determining a point estimate, such as the Trimmed Spearman-Karber method, or the Logistic Method, may also be used. Programs for these methods are available from EPA. If none of these three methods is available or the data do not meet the requirements of these methods, then the Linear Interpolation Method may be used. The Trimmed Spearman-Karber Method and the Linear Interpolation Method, with the 95 percent confidence intervals provided in addition to a point estimate, are both available on the same diskette as indicated above for Probit Analysis. The program for the Linear Interpolation Method accepts data that do not follow a pattern of monotonically decreasing survival with increasing elutriate concentration.

A SAS program called WATTOX.SAS performs the forementioned arcsine transformation, tests of assumptions, and *t*-tests for elutriate toxicity tests. Program statements from WATTOX.SAS and sample data sets are provided in the Inland Testing Manual (USEPA/USACE 1998). The only test endpoint that is used in WATTOX.SAS is survival, and the program compares survival in the control (dilution) water to survival in full-strength (100 percent) elutriate.

A SAS program called BENTOX.SAS compares benthic survival data from dredged sediments to survival data from a disposal site sediment. Program statements from BENTOX.SAS and the analytical results from sample data sets are provided in the Inland Testing Manual (USEPA/USACE 1998).

The software packages and statistical methods mentioned above (i.e., WATTOX.SAS, BENTOX.SAS, Probit Method, Trimmed Spearman-Karber Method, Logistic Method, and Linear Interpolation Method) are used to analyze survival data. These programs and test methods do not analyze growth or reproduction data. A general approach to analyzing growth or reproduction data from elutriate or solid-phase sediment toxicity tests is presented in Figure G-13. For elutriates, reproduction or growth data are subjected to an analysis of variance, and the treatment (elutriate) means are then compared to the control mean by an appropriate test, such as Dunnett's Test. The diskette that is

available from EPA (Cincinnati, OH) also contains a program for analyzing data by Dunnett's Procedure. This allows for a determination of whether growth or reproduction at the various elutriate concentrations is statistically different from that of the controls, and whether the null hypothesis can be rejected. Various software packages are available to accomplish this comparison. The data are first examined to determine normality of distribution and homogeneity of variance. If the data are not normally distributed and the variance is not homogeneous, data may be analyzed by the LSD test or t-test on the rankits.

Computer software called TOXSTAT® has been developed to analyze data in support of chronic test methods described in "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (USEPA 1989). Originally developed for application with chronic toxicity test data for the fathead minnow (*p. promelas*) and the cladoceran, *C. dubia*, the program may also be applied to chronic data for *D. magna*. The IBM compatible program is menu-driven, and is sold by: WEST, Inc., 1402 S. Greeley Highway, Cheyenne, WY 82007-3031. Another commercial software package that includes the commonly used tests of assumptions, as well as specific methods for performing either hypothesis tests or point estimates is TOXCALC©. This package is sold by TidePool Scientific Software, P.O. Box 2203, McKinleyville, CA 95521.

Growth data from 10-d tests of solid-phase sediment with either *C. tentans* or *H. azteca* can likewise be analyzed by a number of software packages. There is not a graded series of concentrations for each dredged site sediment as in the case of elutriates. Therefore, the growth organisms exposed to the single dredged sediment is compared to the growth of organisms exposed to a disposal site sediment. The data are subjected to tests of normality of distribution and equality of variances prior to a comparison of growth means.

Commercial software packages that may be used to analyze growth data include SigmaStat™ Version 1.01 (Jandel Scientific, San Rafael, CA) or ToxCalc© (TidePool Scientific Software, McKinleyville, CA). Others may also be available. Only decreases in growth or reproduction at a dredged site relative to a disposal site are of concern relative to subsequent decision-making. Therefore, one-tailed tests are appropriate. Growth data may also be statistically analyzed using a SAS program called BIOACC.SAS, with the exception that one is interested in significantly decreased growth, rather than significantly increased bioaccumulation from test sediment exposures compared to disposal site sediment exposures. Program statements for BIOACC.SAS are available in USEPA/USACE (1994).

12.2.2. Bioaccumulation Test Data Analysis

Bioaccumulation data from the *L. variegatus* bioassay may be analyzed from either a single-time point study or from a time-sequenced study (USEPA/USACE 1998). In either case, analyses are performed on the data to provide for comparisons between each dredged sediment and the disposal site sediment, and for comparisons with an action level, when applicable. One-sided tests are appropriate, because the main concern is whether organisms exposed to dredged site material have accumulated significantly greater quantities of the chemical(s) of interest than organisms exposed to the disposal site sediment.

12.2.2.1. Comparison With a Disposal site Sediment

If only one dredged sediment is compared to a disposal site, then the procedure described in section 12.2.1. for comparing two samples is used. If more than one sediment is compared to a disposal site, then the procedures described in section 12.2.2. are used. However, an arcsine transformation of the raw data is not appropriate with residue data. Rather, the data are first analyzed in the raw form for assumptions of normality and homogeneity of variances. If they pass these tests of assumptions, the raw data are further analyzed as raw data. If the raw data fail these tests of assumptions, they should be log-transformed and reanalyzed for normality of distribution (USEPA/USACE 1998). If the transformed data fail the normality of distribution tests, they should be analyzed by nonparametric techniques. The scheme is presented in Figure G-14.

Two SAS programs that provide for statistical analysis of bioaccumulation data are BIOACC.SAS and BIOACCSS.SAS. Program statements and analysis of sample data sets are provided in USEPA/USACE (1994).

12.2.2.2. Comparison with an Action Level

In this comparison, the objective is to determine whether the mean bioaccumulation of contaminants in animals exposed to a dredged sediment is significantly less than a specified action level or standard (USEPA/USACE 1998). If the mean tissue concentration of one or more contaminants of concern is greater than or equal to the applicable action level, then no statistical testing is required. The conclusion would be that the dredged sediment does not meet the guidelines associated with the action level. If the mean tissue concentrations of a contaminant of concern are less than the applicable action level, then a confidence-interval approach is used to determine if these means are *significantly* less than the action level. One-sided tests are appropriate since there is concern only if bioaccumulation from the dredged sediment is not significantly less than the action level. There are two different approaches to conducting these tests, and both are acceptable.

The first is to calculate a value of *t*, much as in a *t*-test (this approach is often called a one-sample *t*-test):

$$t = \frac{\bar{x} - actionlevel}{\sqrt{s^2/n}}$$

where $\bar{X}$, s^2 and n refer to mean, variance, and number of replicates for contaminant bioaccumulation from the dredged sediment.

If tests of equality of variances in the comparison of dredged sediments with the disposal site indicate that variances are equal for all sediments, the MSE from the ANOVA is used as s^2, and calculated *t* is compared to $t_{0.95}$, with *N - k* degrees of freedom. If the variances are not equal, then s^2 for the individual sediment is used, and calculated *t* compared with $t_{0.95}$, with *n* - 1 degrees of freedom. If the data were log-transformed to normalize the distributions or equalize variances, then all calculations should be carried out on log-transformed values.

Another approach is to calculate the upper one-sided 95% confidence limit (UCL), and compare it to the action level:

$$UCL = \bar{x} + (t_{0.95,v})(\sqrt{s^2/n})$$

As in the first approach, the MSE is used in place of s^2 if variances are not significantly different, and the degrees of freedom (v) are N - k. If variances are significantly different, s^2 for the individual sediment is used, and v for each sediment i = n_1 - 1. There is a 0.95 probability that the true population mean tissue level is below the UCL. If the UCL is below the action level, there is a $\geq$0.95 probability that the population mean tissue level for the dredged sediment is below the action level, and we conclude that the action level is not exceeded. If the UCL is above the action level, it is uncertain whether the mean population tissue level is less than the action level.

Either of the above procedures may be used with the data that have failed the normality test, but the results should be considered approximate. The choice of which approach to use depends on the computer software and the presentation method to be used. In SAS, it is more convenient to calculate the UCL and compare with the action level, as in program BIOACC.SAS. In SYSTAT, it is simpler to conduct a one-sample *t*-test. Both approaches can easily be performed by hand.

12.3. Final Report

A final report for the biological test(s) performed should

be prepared which allows for an evaluation of the toxicity of the sediment to test animals and/or the accumulation of chemicals from the sediment by benthic organisms. This report should be of sufficient thoroughness that it provides readers with the necessary information to determine if the tests performed met the criteria for test acceptability.

Specific types of information in the final report should include the collection, handling and shipment of sediment samples, the date of receipt of sediment samples by the testing laboratory, storage conditions of sediment by the testing laboratory, and the time elapsed between receipt of the sediment and initiation of the specific biological tests performed. It should include a description of test methods used, and any deviations from the protocols described in this Appendix. The report should include raw data for the biological and/or chemical endpoints measured, as well as the accompanying water quality measurements performed during the test.

Statistical reduction methods should be specified, and the data, whether raw or summarized, used to determine statistical differences from controls or from a disposal site sediment should be included. A summary of the test results based upon statistical treatment of the data should be provided.

The final report should include information on quality control procedures implemented during each test. QA/QC audits performed during the tests should be provided in the final report, complete with requisite signatures by the performing laboratory's QA/QC officer and laboratory director.

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Attachment A. Materials for Culturing and Conducting Toxicity Tests with *Daphnia magna*.

Biological Supplies

- *Daphnia magna* starter culture
- *Selenastrum capricornutum* starter culture (see Attachment C for sources)
- Trout chow

Glassware

- Culture beakers (2L)
- Test beakers (100 mL)
- Erlenmeyer flasks (1 and 2 L)
- Volumetric flasks and graduated cylinders (10-1,000 mL, Class A borosilicate glass or non-toxic plastic)
- Volumetric pipets (1-100 mL, Class A)
- Pipets (disposable, plugged, sterile/mL x 1/100 polystyrene)
- Microscope slides
- Counting chamber (Sedgwick-Rafter, Palmer-Maloney or hemacytometer)
- Burettes
- Fire-polished glass tubes (5 and 8 mm inside diameter)
- Plate glass sheets (double strength) for covering culture and test beakers
- Thermometers (National Bureau of Standards Certified, $\pm 0.1^{o}$ C)

Instruments and Equipment

- pH meter ($\pm$ 0.1 pH unit)
- Dissolved oxygen meter ($\pm$ 0.1 mg/L)
- Specific conductivity meter ($\pm$ 5 umhos/cm or equivalent)
- Continuous recording thermometer ($\pm$ 0.1^{o} C)
- Constant temperature environmental chambers for culturing *Daphnia magna* and *Selenastrum capricornutum*
- Light meter
- Deionized water system (MILLIPORE MILLI-Q^{R} or equivalent)
- Analytical balance (capable of accurately weighing to 0.0001 g)
- Reference weights (class S)
- Magnetic stir plates (for preparing elutriate water and algal cultures)
- Teflon®-coated stir bar magnets
- Microscope (compound scope with 10x, 45x and 100x objective lenses, 10x ocular lens, mechanical stage, substage condensor, and light source)
- Microscope (dissecting scope with substage lighting)
- Light box
- Centrifuge (plankton, or with swing-out buckets having a capacity of 15-100 mL)
- Centrifuge tubes (15-100mL, screw cap)

Autoclave
Refrigerator with freezer
Blender
Carboys (5 gal plastic with spigot)
Fluorescent lights ("Cool-white" for algae; "Grow-Lux" and "Vita-Life" for daphnids)
Drying oven

Reagents

Reagent-grade dry chemicals
$MgCl_2 \cdot 6\ H_20$
$MgSO_4$
$CaCl_2 \cdot 2\ H_2O$
$CaSO_4 \cdot 2H_2O$
$H3BO_3$
$MnCl_2 \cdot 4\ H_2O$
$ZnCl_2$
$FeCl_3 \cdot 6\ H_2O$
$CoCl_2 \cdot 6\ H_2O$
$Na_2Mo0_4 \cdot 2\ H_2O$
$Na_2EDTA \cdot 2\ H_2O$
$NaNO_3$
$MgSO_4 \cdot 7\ H2O$
K_2HPO_4
$NaHCO_3$
Na_2SeO_4
KCl
Hardness and alkalinity test reagents

Reagent-grade liquids
Water - MILLIPORE MILLI-Q^R (or equivalent)
pH buffers - 4, 7 and 10
Specific conductivity standards

Miscellaneous
Acid (1N HCl or H_2SO_4, 10% HNO_3)
Pipet bulbs and fillers
Wash bottles
Nitex^R screen (110 mesh)
Tape
Marking pens
0.45 um filters
Foam plugs (non-toxic, 35-45 mm diameter)

Attachment B. Preparation of Water for Culturing and Testing *Daphnia magna*.

Biesinger et al. (1987) recommend using reconstituted hard water to culture *Daphnia magna* and for use in toxicity tests. A volume of 4.8 L is required initially and at each renewal in a chronic test. Biesinger and co-workers recommend preparation of 19 L at a time according to the following 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 $CaSO_4 \cdot 2H_2O$

   2.28 g MgSO4

   0.15 g KC1

   Extra stock mixtures can be weighed out in advance for use 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. Lesser volumes of hard reconstituted water of the same characteristics can be prepared when desired by adding 192.0, 120.1, 120.0 and 8.0 mg each of $NaHCO_3$, $CaSO_4$, $2H_2O$, $MgSO_4$ and KC1, respectively to 1 L of deionized water. This will produce water with characteristics corresponding to the "hard" category of Table B1.

7. This water may be deficient in some trace nutrients, and it may be desirable to add 2 ug/L of selenium (IV) and 1 ug/L of vitamin B_{12} (Keating 1984; Keating and Dagbusan 1984; ASTM 1991).

TABLE B1: PREPARATION OF SYNTHETIC FRESH WATER USING REAGENT GRADE CHEMICALS[a]

	Reagent Added (mg/L)[b]				Final Water Quality		
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KC1	pH[c]	Hardness[d]	Alka-linity[d]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

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

Attachment C. Preparation of Trout Food and *Selenastrum capricornutum* Diets.

Preparation of Trout Food Diet (from National Effluent Toxicity Assessment Center, ERL-Duluth, USEPA)

1. Add 7.5 gm of No. 1 granule trout food to 400 ml of hard reconstituted water and blend for 15 min to liquify.

2. Let stand for 15 min; decant the upper 300 ml and discard the rest.

3. Pour into a graduated cylinder and record the volume.

4. Thoroughly mix the suspension and withdraw one 10 ml aliquot.

5. Dry the aliquot to a constant weight ($\pm$ 0.1 mg) in a pre-weighed pan (e.g. 50^{o} C for 24 hr).

6. At the end of the drying period, remove the sample from the oven, allow to cool in a desiccator, and weigh to the nearest 0.1 mg.

7. Calculate dry solids weight for 1 ml of suspension. The final concentration must be 5 mg dry solids per ml of food, so the volume must be adjusted by adding reconstituted water. The total volume of water (x) to add equals the number of ml in the sample after removal of the aliquot (290 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 removal of the aliquot.

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

$$x = \frac{(290)(6.32)}{5} - 290$$

   x = 76.6 ml of reconstituted water to add to 290 ml to give a concentration of 5 mg/ml of dry food.

Preparation of Selenastrum capricornutum Diet

A concentration of 10^{8} cells/L of *Selenastrum capricornutum* in addition to the trout food has been found to be satisfactory for a sustained culture of *Daphnia magna* (Biesinger et al. 1987). The following *Selenastrum* culturing methods are adapted from Weber et al. (1989) for preparation of a *Ceriodaphnia dubia* diet.

A "starter" culture of *Selenastrum capricornutum* is used to develop "stock" and "food" cultures maintained in a specific culture medium, described below. "Starter" cultures may be obtained in pure form from the following sources (Biesinger et al. 1987):

Selenastrum capricornutum ATC #22662	American Type Culture Collection 12301 Parklawn Drive Rockville, MD 20852
Selenastrum capricornutum UTEX 1648	The Starr Collection Department of Biology University of Texas at Austin Austin, TX 78712

Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table C1.

2. Add 1 mL of each stock solution, in the order listed in Table C1, to approximately 900 mL of MILLI-Q^{R} water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 $\pm$ 0.1, using 0.1N NaOH or HC1, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table C2.

3. Immediately filter the pH-adjusted medium through a 0.45um pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.

4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is place in the culture vessels.

5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

Establishing and Maintaining "Stock" Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one

milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for 6-12 months in a refrigerator (in the dark) at 4°C.

2. The stock cultures are used as a source of algae to initiate "food" cultures for *Daphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Daphnia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained at 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 $\pm$ 8.6 uE/m^2-s, or 400 ft-c).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.

6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Daphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and

maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.

TABLE C1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

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

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.
[b] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.
[c] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #1.
[d] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #1.

TABLE C2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

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

Micronutrient	Concentration (ug/L)	Element	Concentration (ug/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----

2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 $\pm$ 8.6 uE/m^2-s, or 400 ft-c).

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the

cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3^{0}C.

Preparing Algal Concentrate for Use as Daphnia Food

1. An algal concentrate containing 1 x 10^{8} cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three days and siphoning off the supernatant.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemacytometer, fluorometer, or spectrophotometer, and used to determine the dilution (or further concentration) required to achieve a final cell count of 1 x 10^{8}/mL.

3. Assuming a cell density of approximately 1.5 X 10^{6} cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^{9} algal cells. This numberof cells would provide approximately 45 mL of algal cell concentrate (450 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for approximately seven feedings. Food must be administered nine times during the test.

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

Attachment D. Sample Record Forms for Culturing *Daphnia magna* and Performing a Dredged Material Elutriate Chronic Toxicity Test.

Form D1. Sample Record Form of Survival and Young Production Data for *Daphnia magna* in a Pre-Test Culture.

Date Started: _____ Culturist: _____
Culture Water Medium Renewal
Batch No.: _____ Days: _____
Trout Chow Selenastrum
Batch No.: _____ Cell Density: _____

Repl.	Day	10-1	10-2	10-3	10-4	10-5	10-6	Remarks
10	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	9-1	9-2	9-3	9-4	9-5	9-6	Remarks
9	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	8-1	8-2	8-3	8-4	8-5	8-6	Remarks
8	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D1 (Cont.)

Repl.	Day	7-1	7-2	7-3	7-4	7-5	7-6	Remarks
7	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	6-1	6-2	6-3	6-4	6-5	6-6	Remarks
6	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	5-1	5-2	5-3	5-4	5-5	5-6	Remarks
5	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	4-1	4-2	4-3	4-4	4-5	4-6	Remarks
4	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D1 (Cont.)

Repl.	Day	3-1	3-2	3-3	3-4	3-5	3-6	Remarks
3	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	2-1	2-2	2-3	2-4	2-5	2-6	Remarks
2	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

Repl.	Day	1-1	1-2	1-3	1-4	1-5	1-6	Remarks
1	0							
	1							
	2							
	4							
	6							
	8							
	10							
	12							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D2. Sample Data Form for Temperature and Water Chemistry in a Dredged Material Elutriate Chronic Toxicity Test with *Daphnia magna.*

Day	Temp. (°C)	D.O. (mg/L) C	L	M	H	pH C	L	M	H	Hardness (mg/L as $CaCO_3$) L	M	H	C	Alkalinity (mg/L as $CaCo_3$) C	L	M	H	Specific Conductance (μmhos/cm) C	L	M	H
0	____ ____ ____ ____																				
1	____ ____ ____ ____																				
2	____ ____ ____ ____																				
3	____ ____ ____ ____																				
4	____ ____ ____ ____																				
5	____ ____ ____ ____																				
6	____ ____ ____ ____																				
7	____ ____ ____ ____																				
8	____ ____ ____ ____																				

Form D2 (Cont.)

Day	Temp. (°C)	D.O. (mg/L) C	L	M	H	pH C	L	M	H	Hardness (mg/L as $CaCO_3$) L	M	H	C	Alkalinity (mg/L as $CaCO_3$) C	L	M	H	Specific Conductance (μmhos/cm) C	L	M	H
9	____ ____ ____ ____ ____																				
10	____ ____ ____ ____ ____																				
11	____ ____ ____ ____ ____																				
12	____ ____ ____ ____ ____																				
13	____ ____ ____ ____ ____																				
14	____ ____ ____ ____ ____																				
15	____ ____ ____ ____ ____																				
16	____ ____ ____ ____ ____																				
17	____ ____ ____ ____ ____																				

Form D2 (Cont.)

Day	Temp. (°C)	D.O. (mg/L) C	L	M	H	pH C	L	M	H	Hardness (mg/L as $CaCO_3$) C	L	M	H	Alkalinity (mg/L as $CaCo_3$) C	L	M	H	Specific Conductance (μmhos/cm) C	L	M	H
18	____ ____ ____ ____ ____																				
19	____ ____ ____ ____ ____																				
20	____ ____ ____ ____ ____																				
21	____ ____ ____ ____ ____																				

Form D3. Sample Record Form for Survival and Young Production Data From a Block-Randomized *Daphnia magna* Dredged Material Elutriate Chronic Toxicity Test.

Sample I.D.: _____
Sample Collection Date: _____
Test Start-Date/Time: _____
Test Organisms from
Pre-Test Culture No.: _____
Test Organism Age: _____

Selenastrum Cell Density: _____
Dilution Water
Batch No.: _____
Template No.: _____
Test Chamber Vol. _____
Vol. of Test Solution: _____
Investigator: _____

Repl.	Day	10-5	10-2	10-6	10-3	10-4	10-1	Remarks
10	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D3 (Cont.)

Repl.	Day	9-3	9-2	9-5	9-4	9-6	9-1	Remarks
9	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

Repl.	Day	8-1	8-5	8-6	8-4	8-3	8-2	Remarks
8	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D3 (Cont.)

Repl.	Day	7-2	7-6	7-3	7-4	7-1	7-5	Remarks
7	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

Repl.	Day	6-1	6-4	6-6	6-2	6-5	6-3	Remarks
6	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D3 (Cont.)

Repl.	Day	5-2	5-3	5-1	5-5	5-4	5-6	Remarks
5	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

Repl.	Day	4-2	4-3	4-5	4-4	4-6	4-1	Remarks
4	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D3 (Cont.)

Repl.	Day	3-3	3-5	3-2	3-1	3-4	3-6	Remarks
3	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

Repl.	Day	2-1	2-6	2-5	2-2	2-3	2-4	Remarks
2	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D3 (Cont.)

Repl.	Day	1-6	1-1	1-3	1-5	1-2	1-4	Remarks
1	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
	11							
	12							
	13							
	14							
	15							
	16							
	17							
	18							
	19							
	20							
	21							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form D4. Sample Summary Form for Suvival and Young Production in a *Daphnia magna* Dredged Material Elutriate Chronic Toxicity Test. (Summary of data from Form D3)

Sample I.D.: _____ Investigator: _____
Sample Collection Date: _____ Test Start-Date/Time: _____
Test End-Date/Time: _____

Elutriate Concentration	Total No. of Young Per Surviving Adult Replicate										No. of Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control											
6.25%											
12.5%											
25.0%											
50.0%											
100%											

Attachment E. General Activity Schedule for Performing a Dredged Material Elutriate Chronic Toxicity Test with *Daphnia magna*[a].

Day	Activity
-14	Set up a pre-test culture of 60 beakers and add one neonate (≤24 hr old) to each of the beakers in 80 mL of culture medium containing food.
-12	Renew culture medium, add food and transfer individual daphnids.
-10	Renew culture medium, add food and transfer individual daphnids.
- 8	Renew culture medium, add food and transfer individual daphnids.
- 6	Renew culture medium, add food and transfer adult daphnids. Observe for young production, and record those adults which produced their first brood. Discard any young.
- 4	Renew culture medium, add food and transfer adult daphnids. Observe for young production, and record those adults which produced young, along with the brood number (i.e. first or second) and brood size. Discard any adults which have not produced young.
- 2	Renew culture medium, add food and transfer adult daphnids. Observe for young production, and record those adults which produced young, along with the brood number and size. Discard any young.
- 1	Observe the pre-test culture within 24 hr from the start of the test, and mark those beakers containing adults which may produce young that (1) will be <24 hr old, (2) will be producing their third or more brood, and (3) had at least nine young in the previous brood. Prepare the dredged material elutriate water and performance control water.
0	Add trout chow (final concentration of 5 mg/L) and *Selenastrum capricornutum* (final concentration of 10^8 cells/L) to the dredged material elutriate and performance control waters. Select 10 beakers of neonates to be placed into test solution, one beaker per replicate set of treatments. Add one neonate to each test beaker. Monitor all water quality parameters.
1	Observe test beakers for mortalities and monitor water temperature.
2	Renew test solutions and feed daphnids. Monitor water quality parameters in samples of "old" and "new" solutions.
3	Observe test beakers for mortalities and monitor water temperature.
4	Renew test solutions and feed daphnids. Monitor water quality parameters in samples of "old" and "new" solutions.

Day	Activity
5	Observe test beakers for mortalities and monitor water temperature.
6	Observe test beakers for mortalities and monitor water temperature.
7	Record mortalities and brood number and brood size for any daphnids that have young. Renew test solutions and feed adults. Discard the young. Monitor water quality parameters in samples of "old" and "new" solutions.
8	Observe test beakers for mortalities and brood production. Record occurrence of brood. Monitor water temperature.
9	Record mortalities and brood number and brood size for any daphnids that have young. Renew test solutions containing food and transfer adults. Discard the young. Monitor quality parameters in "old" and "new" solutions.
10	(Same as day 8)
11	(Same as day 9)
12	(Same as day 8)
13	(Same as day 8)
14	(Same as day 9)
15	(Same as day 8)
16	(Same as day 9)
17	(Same as day 8)
18	(Same as day 9)
19	(Same as day 8)
20	(Same as day 8)
21	Record mortalities, and brood number and brood size for any daphnids that have young. Monitor all water quality parameters. Discard all daphnids. Terminate test.

a Activity Schedule assumes that cultures of *D. magna* and *S. capricornutum* are already in existence at the laboratory, and that the culture water and diets have been prepared in advance.

Attachment F. Materials for Culturing of and Conducting Toxicity Tests with *Ceriodaphnia dubia*.

Biological Supplies

Ceriodaphnia dubia starter culture
Selenastrum capricornutum starter culture Trout Chow
Yeast
Cereal Leaves (see Attachment H for sources)

Glassware

Mass culturing chambers (1-2 L volume)
30 mL disposable polystyrene salad cups (1 oz) or glass beakers
Erlenmeyer flasks (250 mL - 3 L)
Separatory funnel (2 L)
Beakers (1-2 L)
Volumetric flasks and graduated cylinders (10-1,000 mL, class A borosilicate glass or non-toxic plastic)
Volumetric pipets (1-100 mL, class A)
Pipettor, adjustable volume repeating dispenser
Microscope slide
Counting chamber (Sedgwick-Rafter, Palmer-Maloney or hemocytometer)
Burettes
2-mm ID fire-polished glass tubes
Disposable pipets and droppers
Plate glass (double-strength) for covering brood board
Thermometer (National Bureau of Standards certified)

Instruments and Equipment

pH meter
Dissolved oxygen and specific conductivity meter
Constant temperature environmental chambers for culturing *Ceriodaphnia* and *Selenastrum* and for testing *Ceriodaphnia*.
Deionized water system (MILLIPORE MILLI-Q^{R} or equivalent)
Analytical balance (capable of weighing accurately to 0.0001g)
Reference weights (class S)
Magnetic stir plates (for algal cultures)
Teflon®-coated stir bar magnets
Light meter
Microscope (compound scope with 10X, 45X and 100X objective lenses, 10X ocular lens, mechanical stage, substage condensor and light source)
Microscope (dissecting scope with substage lighting)
Light box
Centrifuge - plankton, or with swing-out buckets having a capacity of 15-100 mL.
Centrifuge tubes - 15-100 mL, screw-cap
Continuous recording thermometer

Autoclave
Refrigerator with freezer
Blender

Reagents

Reagent-grade dry chemicals
- $MgCl_2 \cdot 6\ H_20$
- $MgSO_4$
- $CaCl_2 \cdot 2\ H_2O$
- $CaSO_4 \cdot 2H_2O$
- $H3BO_3$
- $MnCl_2 \cdot 4\ H_2O$
- $ZnCl_2$
- $FeCl_3 \cdot 6\ H_2O$
- $CoCl_2 \cdot 6\ H_2O$
- $Na_2Mo0_4 \cdot 2\ H_2O$
- $Na_2EDTA \cdot 2\ H_2O$
- $NaNO_3$
- $MgSO_4 \cdot 7\ H2O$
- K_2HPO_4
- $NaHCO_3$
- Na_2SeO_4
- KCl
- Hardness and alkalinity test reagents

Reagent-grade liquids
- Water - MILLIPORE MILLI-Q^R (or equivalent)
- pH buffers - 4, 7 and 10
- Specific conductivity standards

Miscellaneous
- Acid (1N HCl or H_2SO_4)
- Pipet bulbs and fillers
- Wash bottles
- Nitex^R screen (110 mesh)
- Brood board material (e.g. styrofoam insulation board, 50 cm x 30 cm x 2.5 cm)
- Tape
- Marking pens
- 0.45 um filters

Attachment G. Preparation of Water for Culturing and Testing *Ceriodaphnia dubia*.

Waters of different chemical characteristics may be used successfully in culturing and testing *Ceriodaphnia dubia*. Prepared water with a hardness (as CaC03) range between 40 mg/L ("soft" water) and 100 mg/L ("moderately hard" water) may be used for culturing. However, moderately hard water is recommended by EPA (Weber et al. 1989). The synthetic medium that is selected as the culture medium and diluent must result in survival and reproduction results that meet the criteria for acceptability.

Tables G1 and G2 below (from Weber et al. 1989) list the ingredients required to prepare either reconstituted synthetic fresh water or diluted mineral water, each at several hardnesses. The pH, hardness, and alkalinity of the prepared water should be measured and fall within the ranges indicated in the tables. The water must be aerated for 24 hr prior to determination of pH. Aeration should be performed with air that is known to be clean and not contain any oil or chemical residues from the compressor. In-line filters should be used if the potential for such contamination exists. It is recommended by the staff of the USEPA National Effluent Toxicity Assessment Center in Duluth, MN that 2 ug/L of selenium (Se^{+6}) be added to the water in the form of sodium selenite (Na_2SeO_3).

TABLE G1: PREPARATION OF SYNTHETIC FRESH WATER USING REAGENT GRADE CHEMICALS[a]

	Reagent Added (mg/L)[b]				Final Water Quality		
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KC1	pH[c]	Hardness[d]	Alka-linity[d]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

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

TABLE G2. PREPARATION OF SYNTHETIC FRESH WATER USING MINERAL WATER[a]

Water Type	Volume of Mineral Water Added (mL/L)[b],	Proportion of Mineral Water (%)	Final Water Quality		
			pH[c]	Hardness	Alka-linity[d]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	60-70
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[e]	---	---	---	---	---

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

Attachment H. Preparation of YCT and *Selenastrum capricornutum* Diets.

Preparation of YCT Diet (from Weber et al. 1989)

The YCT diet is a mixture of yeast, cereal leaves, and trout chow. This produces an organic-rich microbial culture as the food source for *Ceriodaphnia dubia.* Preparation of the diet requires one week. Equal volumes of the yeast, cereal leaves, and trout chow preparations are combined, and divided into smaller aliquots (e.g. 50 mL) to be frozen for later use. The three ingredients are prepared as follows:

1. Trout Chow[a] - Add 5.0g of trout chow pellets to 1 L of deionized water, and thoroughly mix in a blender (~ 15 min). Transfer to a 2-L separatory funnel and, in a fume hood, continuously aerate from the bottom of the funnel for 7 days at room temperature. Replace any water lost due to evaporation. Place the vessel in a refrigerator and allow to settle for 1 hr. Filter through a fine mesh screen and combine with the other ingredients.

2. Yeast - Add 5.0 g of baker's yeast, (e.g. Fleischmann's[R]) to 1 L of deionized water and thoroughly blend with a mixer at a slow speed. Use immediately.

3. Cereal leaves[b] - Add 5.0 g of dried, powdered, cereal leaves to 1 L of deionized water, and mix in a blender at high speed for 5 min. Allow to settle overnight in a refrigerator before using.

---

[a] Trout chow (starter or No. 1 pellets) may be purchased from a commercial supplier of animal and pet foods.

[b] Dried, powdered cereal leaves are available as "CEREAL LEAVES" from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, (800-325-3010); or as CEROPHYLL[R] from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692-9012 (716-359-2502). Dried, powdered alfalfa leaves from health food stores have served as satisfactory substitutes (Weber et al. 1989).

Preparation of Selenastrum Capricornutum Culture and Diet for Ceriodaphnia dubia (from Weber et al. 1989)

A "starter" culture[a] of *Selenastrum capricornutum* is used to develop "stock" and "food" cultures maintained in a specific culture medium, described below.

Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table H1.

2. Add 1 mL of each stock solution, in the order listed in Table D1, to approximately 900 mL of MILLI-Q^{R} water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 $\pm$ 0.1, using 0.1N NaOH or HC1, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table H2.

3. Immediately filter the pH-adjusted medium through a 0.45um pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.

4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.

5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

Establishing and maintaining "Stock" Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4^{0}C.

2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia*

cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained at 25^{0}C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 $\pm$ 8.6 uE/m^2/s, or 400 ft-c).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.

6. Stock cultures should be examined microscopically weekly, at transfer, or microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4^{0}C. It is advisable to prepare new stock cultures form "starter" cultures obtained from established outside sources of organisms every four to six months.

Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for Ceriodaphnia cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.

2. Food cultures may be maintained at 25^{0}C in environmental

chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 $\pm$ 8.6 $uE/m^2/s$, or 400 ft-c).

TABLE H1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

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

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

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

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

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

TABLE H2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

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

Macronutrient	Concentration (ug/L)	Element	Concentration (ug/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by h and. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3^{0}C.

Preparing Algal Concentrate for use as Ceriodaphnia Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three weeks and siphoning off the supernatant.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer. and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.

3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four Ceriodaphnia tests.

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

Attachment I. Sample Record and Data Forms for Culturing *Ceriodaphnia dubia* and Performing Elutriate Chronic Toxicity Tests.

Form I1. Sample Record Form of Survival and Young Production Data for *Ceriodaphnia dubia* in a Culture Brood Board.

Date Started: _____ Culturist: _____
Culture Water Medium Renewal Days: _____
Batch No.: _____ Brood Board No: _____
YCT Batch No.: _____ *Selenastrum* Cell Density: _____

Repl.	Day	10-1	10-2	10-3	10-4	10-5	10-6	Remarks
10	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	9-1	9-2	9-3	9-4	9-5	9-6	Remarks
9	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	8-1	8-2	8-3	8-4	8-5	8-6	Remarks
8	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	7-1	7-2	7-3	7-4	7-5	7-6	Remarks
7	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form I1 (Cont.)

Repl.	Day	6-1	6-2	6-3	6-4	6-5	6-6	Remarks
6	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	5-1	5-2	5-3	5-4	5-5	5-6	Remarks
5	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	4-1	4-2	4-3	4-4	4-5	4-6	Remarks
4	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

Repl.	Day	3-1	3-2	3-3	3-4	3-5	3-6	Remarks
3	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form I1 (Cont.)

Repl.	Day	2-1	2-2	2-3	2-4	2-5	2-6	Remarks
2	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								
Repl.	Day	1-1	1-2	1-3	1-4	1-5	1-6	Remarks
1	0							
	2							
	4							
	7							
	9							
	11							
	14							
Total								

+ = OK 0 = No Young D = Dead M = Male E = Eggs Present

Form I2. Sample Data Form for Temperature and Water Chemistry in a Dredged Material Elutriate Chronic Toxicity Test with *Ceriodaphnia dubia*.

Day	Temp. (°C)	D.O. (mg/L)				pH				Hardness (mg/L as CaCO3)				Alkalinity (mg/L as CaCo3)				Specific Conductance (umhos/cm)			
		C	L	M	H	C	L	M	H	C	L	M	H	C	L	M	H	C	L	M	H
0	____ ____ ____ ____																				
1	____ ____ ____ ____																				
2	____ ____ ____ ____																				
3	____ ____ ____ ____																				
4	____ ____ ____ ____																				
5	____ ____ ____ ____																				
6	____ ____ ____ ____																				
7	____ ____ ____ ____																				
8	____ ____ ____ ____																				

Form I3. Sample Record Form for Survival and Young Production Data from a Block-Randomized *Ceriodaphnia dubia* Dredged Material Elutriate Chronic Toxicity Test.

Sample I.D.: _____
Sample Collection Date: _____
Test Start-Date/Time: _____
Test Organisms from
Brood Board No.: _____
Test Organism Age: _____
YCT Batch No.: _____

Selenastrum Cell Density: _____
Dilution Water
Batch No.: _____
Template No.: _____
Test Chambers
(glass or plastic/vol.): _____
Investigator: _____

Repl.	Day	10-5	10-2	10-6	10-3	10-4	10-1	Remarks
10	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	9-3	9-2	9-5	9-4	9-6	9-1	Remarks
9	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	8-1	8-5	8-6	8-4	8-3	8-2	Remarks
8	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	7-2	7-6	7-3	7-4	7-1	7-5	Remarks
7	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	6-1	6-4	6-6	6-2	6-5	6-3	Remarks
6	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	5-2	5-3	5-1	5-5	5-4	5-6	Remarks
5	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Repl.	Day	4-2	4-3	4-5	4-4	4-6	4-1	Remarks
4	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Form I3 (Cont.)

Repl.	Day	3-3	3-5	3-2	3-1	3-4	3-6	Remarks
3	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								
Repl.	Day	2-1	2-6	2-5	2-2	2-3	2-4	Remarks
2	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								
Repl.	Day	1-6	1-1	1-3	1-5	1-2	1-4	Remarks
1	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
Total								

Form I4. Sample Summary Form for Survival and Young Production in a *Ceriodaphnia dubia* Dredged Material Elutriate Chronic Toxicity Test. (Summary of data from Form I3)

Sample I.D.: _____ Investigator: _____
Sample Collection Date: _____ Test Start-Date/Time: _____
Test End-Date/Time: _____

Elutriate Concentration	Total No. of Young Per Surviving Adult Replicate										No. of Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control											
6.25%											
12.5%											
25.0%											
50.0%											
100%											

Attachment J. General Activity Schedule for Performing a Dredged Material Elutriate Chronic Toxicity Test with *Ceriodaphnia dubia*.[a]

Day	Activity
-7	Set up a brood board of 60 chambers and add one neonate (≤24 hr old) to each of the chambers. Feed brood animals.
-6	Feed brood animals.
-5	Transfer brood animals to new medium. Feed brood animals.
-4	Check chambers for production young. Count, record, and discard young produced. Feed brood animals.
-3	Check chambers for production of young. Transfer adults to new medium. Count, record, and discard young produced. Feed brood animals.
-2	Check chambers for production of young. Count, record and discard young produced. Feed brood animals.
-1	Check chambers for production of young. Mark all chambers with no young production. Transfer adults to new medium. Count, record, and discard young produced. Feed brood animals.
0	In morning, check marked chambers from preceding day for young production. Place a new mark on those with no young. Prepare sediment elutriate and performance control waters, and monitor all water parameters. Place 15 mL in each chamber in randomized test board. Introduce one neonate (≤24 hr old and all within 8 hr of one another in age) to each chamber. Feed test animals.
+1	Monitor water temperature. Observe test animals and record mortalities. Feed test animals.
+2	(Same as Day 1)
+3	Observe test animals and record mortalities and young production. Renew the test media (i.e. sediment elutriate and performance control waters), and transfer animals to new media. Monitor all water parameters of "old" and "new" solutions. Feed test animals.
+4	Observe test animals and record mortalities and young production. Monitor water temperature. Feed test animals.
+5	(Same as day 3)
+6	(Same as day 4)
+7	Observe test animals and record mortalities and young production. If 60 percent or more of the control animals have had their third brood, the test is terminated. If not, the test is continued. Monitor water parameters of temperature, dissolved oxygen, and pH. If test is continued, feed test animals.

+8	Observe test animals and record mortalities and young production. More than 60 percent of the control animals should have had their third brood, and the test is terminated.

---

[a] Activity schedule assumes that mass and individual cultures have already been established, and that the culture water and diets have been prepared in advance.

Attachment K. Materials for Culturing of and Conducting Toxicity Tests with Fathead Minnows.

Biological Supplies

- *Pimephales promelas* starter culture
- Brine shrimp
  - Frozen adult
  - Cysts

Glassware

- Mass culturing chambers (40 or 57 L volume)
- Erlenmeyer flasks (250 mL and 3 L) for exposure chambers and mixing elutriate stocks
- Separatory funnel (2 L)
- Volumetric flasks and graduated cylinders (10-1,000 mL, class A of borosilicate glass or non-toxic plastic)
- Volumetric pipets (1-100 mL, class A)
- Burettes ($\pm$ 0.05 mL)
- 5-mm ID fire-polished glass tubes
- Disposable pipets and droppers
- Plate glass (double-strength) or 1/4 inch plastic sheets for covering exposure chambers
- Beakers (1000 mL)

Instruments and Equipment

- Thermometer ($\pm$ 0.1^{o} C, National Bureau of Standards certified)
- pH meter ($\pm$ 0.1 pH units)
- Dissolved oxygen ($\pm$ 0.1 mg/L) and specific conductivity meter ($\pm$ 5 umhos/cm or equivalent)
- Constant temperature environmental room for culturing fathead minnows and brine shrimp and for testing fathead minnows
- Deionized water system (MILLIPORE MILLI-Q^{R} or equivalent)
- Analytical balance (capable of weighing accurately to 0.00001g)
- Reference weights (class S)
- Magnetic stir plates (for elutriate solution production)
- Light meter ($\pm$ 5 lux)
- Microscope (40 x magnification dissecting scope with substage lighting)
- Light box
- Centrifuge with swing-out buckets having a capacity of 500-1000 mL ($\geq$ 10,000 x g)
- Centrifuge tubes - 100-200 mL, Teflon®-lined screw-cap
- Continuous recording thermometer ($\pm$ 1.0^{o} C)
- Refrigerator with freezer
- Compressed air supply pump
- Plastic dish washing pan (white)

Automatic light control timer
pH/ion meter (± 0.1 mV)
Residual chlorine ion specific electrode
Ice bath

Reagents

(Reagent grade or better)
Hardness and alkalinity test reagents
Na_2CO_3
H_2SO_4 (concentrated)
Bromcresol green sodium salt
Methyl red sodium salt
NH_4C1
NH_4OH
EDTA (magnesium salt)
Eriochrome Black T
Dissolved oxygen meter calibration reagents
$MnSO_4 \cdot 4H_2O$
NaN_3
NaOH
NaI
KI
H_2SO_4
Starch
$Na_2S_2O_3 \cdot 5H_2O$
$KH(I0_3)_2$
Chlorine analysis reagents
Chloramine-T trihydrate
Phenyl arsine oxide (PAO)
Sodium acetate trihydrate
KI

Reagent-grade liquids

Water - MILLIPORE MILLI-Q^R, or equivalent
pH buffers - 4, 7 and 10
Specific conductivity standards

Miscellaneous

Acid (1N HCl, HNO_3 or H_2SO_4)
NaC1
Pipet bulbs and fillers
Wash bottles
Tape (labeling)
Marking pens
Stainless Steel mesh (5 mm openings)
Silicone glue

Attachment L. Sample Record Forms for Culturing Fathead Minnows and Performing Dredged Material Elutriate Toxicity Tests.

Form L1. Record of Fathead Minnow Embryo Production in the Brood Culture and Checklist for Daily Maintenance of Brood Culture and Incubation Chambers.

Date	Chamber 1				Chamber 2				Chamber 3				Chamber 4				Chamber 5				Chamber 6				Chamber 7			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	C	A	B	C	D

Date	Brood Cultures							Incubating Pan					
	Temp. (C)	Feed Fish (2X) √	Water Flow √	Aeration √	Cleaned Chambers (Which)	Check Sub-strates	Mortali-ties (Where)	Temp. (C)	Aeration √	Remove Dead Embryos	No. of Embryos Started	No. of Embryos Hatched	Percent Hatched

Form L2. Data Form for the Fathead Minnow Larval Survival and Growth Test. Routine Chemical and Physical Determinations.[1]

Dredged Material Source: _____ Test Dates: _____
Collection Date: _____ Analyst: _____

	Day								
Control:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

	Day								
Conc:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

	Day								
Conc:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

Form L2. (Cont.)

Dredged Material Source: _____ Test Dates: _____
Collection Date: _____ Analyst: _____

	Day								
Control:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

	Day								
Conc:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

	Day								
Conc:	0	1	2	3	4	5	6	7	Remarks
Temp.									
D.O. Initial									
Final									
pH Initial									
Final									
Alkalinity									
Hardness									
Conductivity									

[1] Adapted from Weber et al. 1989

Form L3. Survival Data for Fathead Minnow Larval Survival and Growth Test.[1]

Dredged Material Source: _____ Test Dates: _____
Collection Date: _____ Analyst: _____

Conc:	Rep. No.	No. Survivors								Remarks
		0	1	2	3	4	5	6	7	
Control										
Conc:										
Conc:										
Conc:										
Conc:										
Conc:										

Comments:

[1]Adapted from Weber et al. 1989.

Form L4. Weight Data for Fathead Minnow Larval Survival and Growth Test.[1]

Test Date(s):_____ Dredged Materia Source:_____
Weighing Date:_____ Collection Date: _____
Drying Temperature (°C):_____ Analyst:_____

Drying Time(h):_____

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

[1]Adapted from Hughes et al. 1987.

Form L5. Summary Data for Fathead Minnow Larval Survival and Growth Test.[1]

Sediment Source: _____ Test Dates: _____
Collection Date: _____ Analyst: _____

Treatment	Control					
No. of live larvae						
Survival (%)						
Mean dry wgt. of larvae (mg) ± SD						
Temperature range (°C)						
Dissolved oxygen range (mg/L)						
Hardness						
Conductivity						

Comments:

[1]Adapted from Hughes et al. 1987.

Attachment M. General Activity Schedule for Performing a Dredged Material Elutriate 7-d Toxicity Test With Fathead Minnow Larvae.[1]

Day	Activity
-14	Call supplier of fathead embryos, if purchasing them from a supplier, to establish test start date.
-4	Pull spawning substrates with enough embryos (~300 per sediment sample) to conduct dredged material elutriate toxicity test.
-3	Check spawning substrates for fungal infected embryos; remove if present.
-2	Check spawning substrates for unfertilized and fungal infected embryos, remove if present. Begin brine shrimp embryo incubation.
-1	Check spawning substrates for unfertilized and fungal infected embryos; remove if present. Prepare elutriate solution from dredged material and refrigerate.
0	Prepare dredged material elutriate dilutions and performance control waters. Place 100 mL in each chamber in randomized arrangement in temperature controlled room or water bath. Place $\leq$24-hr old ($\leq$ 48-hr if shipped from a remote site) larvae into chamber in order until 10 larvae are in each. Feed test animals. Measure water temperature, dissolved oxygen, pH, hardness, alkalinity and conductance in a control chamber and a low, medium and high concentration of elutriate for each dredged material tested.
+1	Observe test animals and record mortalities. Prepare renewal test solutions. Siphon exposure chambers and renew test solutions. Feed test animals. Measure water temperature, dissolved oxygen and pH in a control chamber and a low, medium and high elutriate concentration for each dredged material tested of "old" and "new" solutions.
+2	(Same as day 1)
+3	(Same as day 1)
+4	(Same as day 1)
+5	(Same as day 1)
+6	(Same as day 1)
+7	Do not feed test animals. Observe test animals and record mortalities. Measure water temperature, dissolved oxygen, pH, hardness, alkalinity and conductance in a control chamber and a low, medium and high concentration of elutriate for each dredged material tested. Place all surviving larvae from each test chamber in a preweighed boat and oven dry at 100 C for at least 2 hr. Cool in a dessicator and weigh to 0.00001 gm.

[1] Activity schedule assumes that brood cultures are already producing embryos.

Attachment N. Materials for Culturing of and Conducting Toxicity Tests with *Chironomus tentans*.

Biological Supplies

Chironomus tentans brood stock (egg masses or larvae)
Tetrafin® goldfish food

Glassware

Crystallizing dishes or beakers (200-300 mL volume)
Erlenmeyer flasks (250 and 500 mL)
Larval rearing chambers (e.g., 19 L capacity)
Exposure beakers (300 mL high-form)
Wide bore pipets (5 to 6 mm ID)
¼" glass tubing (for aspirating flask)
Burettes (for hardness and alkalinity determinations)
Graduated cylinders (assorted sizes, 10 mL to 2 L)

Instruments and Equipment

Dissecting microscope
Sieve (e.g., U.S. Standard No. 30 mesh)
Delivery system for overlying water
Photoperiod timers
Temperature controllers
Thermometer
Dissolved oxygen meter
pH meter
Specific ion meter
Ammonia electrode (or ammonia test kit)
Specific conductance meter
Drying oven
Dessicator
Balance (to 0.01 mg)
Blender
Paper shredder, cutter or scissors
Refrigerator
Freezer
Hot plate
Light box

Miscellaneous

White paper toweling (for substrate)
Acetone (for substrate preparation)
Air Supply
Airstones
Screening material (e.g., Nitex, window screen or panty hose)

Stainless steel screen (no. 60 mesh, for test beakers)
Glass hole-cutting bit
Glass glue
Coarse-mesh sieve ($\geq$ 5 mm mesh)
Aluminum weighing pans
Fluorescent light bulbs (for culture and toxicity test)
Tygon® tubing (0.25 inch diameter for aspirating flask)
Nalgene® bottles (500 and 1,000 mL, for food and substrate preparation and storage)
Deionized water
Aspirator top (for collecting adults)
Water squirt bottle
White dishpan

Attachment O. Some Laboratory Sources of *Chironomus tentans* Cultures.

Laboratory	Contact Person	Phone/Fax/email
Environmental Consulting & Testing 1423 North 8th St., Suite 118 Superior, WI 54880	Steven Poirier	T 800-377-3657 F 715-394-7414
Mid-Continent Ecology Division U.S. Environmental Protection Agency 6201 Congdon Blvd. Duluth, MN 55804	Michael Kahl	T 218-529-5179 F 218-529-5003 E epamdj@du4500.dul.epa.gov
Lake Superior Research Institute University of Wisconsin-Superior Superior, WI 54880	Larry Brooke	T 715-394-8318 F 715-394-8454 E lbrooke@staff.uwsuper.edu
Zoology Department Natural Sciences Building Michigan State University East Lansing, MI 48824	John Giesy	T 517-353-2000 F 517-432-1984 E JGIESY@aol.com
Institute for Environmental Quality Wright State University Dayton, OH 45435	G. Allen Burton	T 937-873-2201 F 937-775-4997 E aburton@wright.edu

Attachment P. Methods of Preparing Synthetic Fresh Water.

TABLE P1: PREPARATION OF SYNTHETIC FRESH WATER USING REAGENT GRADE CHEMICALS[a]

	Reagent Added (mg/L)[b]				Final Water Quality		
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KC1	pH[c]	Hardness[d]	Alka-linity[d]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

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

TABLE P2. PREPARATION OF SYNTHETIC FRESH WATER USING MINERAL WATER[a]

			Final Water Quality		
Water Type	Volume of Mineral Water Added (mL/L)[b],	Proportion of Mineral Water (%)	pH[c]	Hardness	Alka-linity[d]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	60-70
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[e]	---	---	---	---	---

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

Attachment Q. Preparation of Food for *Chironomus tentans*.

The following is based upon a procedure presented by Denny and Mead (1991), and is designed for an aquarium containing 7 L of water.

1. One day in advance, place three marked aluminum weighing pans in an oven at 100°C, and heat overnight. Handle pans with forceps only.

2. Remove pans from the oven, allow to cool in a dessicator, and determine the tared weight of each pan.

3. Blend the Tetrafin® flake food for goldfish in distilled water for 30 seconds or until very finely ground. Use 100 mL of water for every 10 g of Tetrafin® food. The food can be frozen, so larger batches (e.g., 1 L) of food are recommended to reduce the effort in preparing food.

4. Filter the slurry through a #202 Nitex screen to remove large particles.

5. Shake well to ensure homogeneity, and pipet 5.0 mL of the slurry into each of the three tared pans. Dry at 100° C for at least 4 hr and reweigh.

6. Subtract the weight of each pan from the total weight (solids plus pan) to obtain the weight of the solids. This should be approximately 70,000-80,000 mg. Divide by a target value of 56,000 (56 g/L) to obtain a dilution factor.

7. Multiply the volume of the food suspension by the dilution factor to obtain the desired final volume. Dilute the food suspension to the final volume with distilled water.

8. Record all weights and calculations in a record book.

9. Pour food into 500 mL Nalgene® bottles. Keep one bottle for current use in a refrigerator. Freeze the remaining bottles for future use.

10. Shake the bottles vigorously prior to feeding and stir well between feeding of each culture aquarium to ensure a uniform distribution of solids.

Attachment R. Culture and Test Data Forms for *Chironomus tentans*.

Form R1. Sample Evaluation Form for the Health and Reproduction of a *Chironomus tentans* Culture.

Culture Aquarium	Date of Egg Mass Deposition	Date 4th Instar Larvae were Weighed	Age of Weighed 4th Instar Larvae	Mean Dry wt. of 4th Instar Larvae (n=10)	Date of Observed First Emergent Adult	Total Number of Egg Masses Produced	General Comments	Initials of Culturist
A								
B								
C								
D								
E								
F								

Form R2. Sample Data Form for Temperature and Overlying Water Chemistry Measurements in a Toxicity Test with *Chironomus tentans*.

Sediment Sample Source _____

Date of Test Initiation_____

Toxicologist Conducting Test _____

Test Day	Test Replicate Sampled	Temperature (° C)	Dissolved Oxygen (mg/L)	pH	Hardness (mg/L)	Alkalinity (mg/L)	Specific Conductance (umhos/cm)	Total Ammonia (mg/L)
0								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Form R3. Data Chart for Performing Reference Toxicant Tests with $CuSO_4$ or KC1 and *Chironomus tentans*.

Brood Stock Source _____ Test Initiation Date _____ Time ____

Age of Test Animals (days post-hatch)_____ Reference Toxicant ($CuSO_4$) or KCl)_____

No. of Animals per Replicate_____ Reference Toxicant Supplier _____

No. of Replicates_____ Reference Toxicant Lot No._____

Dilution Water/Control_____ Reference Toxicant Purity_____

Test Volume_____ Toxicologist_____

Test Type (circle one)[a] SU, SM, RU, RM, FU, FM

	Survival Readings												
	0 h			24 h		48 h		72 h		96 h			
Conc.	pH	D.O.	Temp.	Surv.	pH	Surv.	pH	Surv.	pH	Surv.	pH	D.O.	Temp.

Comments_____

_____

96-hr LC50 =_____
Method of LC50 Estimate_____
Cumulative Mean LC50_____
No. of Tests the Cumulative Mean is Based on_____
Acceptability of Current Test[b] Yes ___ No ___

_____

[a]SU = Static unmeasured, SM = static measured, RU = renewal unmeasured, RM = renewal measured, FU = flow-through unmeasured, FM = flow-through measured.

[b]Based upon two standard deviations around the cumulative mean 96-hr LC50.

Attachment S. General Activity Schedule for Performing a Sediment Toxicity Test with *Chironomus tentans*[a].

Day	Activity
-13	Newly deposited egg masses from the culture unit are assigned for use in the test and placed into hatching dishes.
-12	A larval rearing aquarium is prepared with new substrate.
-11 and -10	Egg masses are examined for hatching success. If successful hatch is occurring, transfer first instar larvae and any remaining unhatched embryos from the crystallizing dishes into the larval rearing aquaria. To begin nutrient enrichment of substrate, add 1.0 mL of concentrated food suspension to the larval rearing aquarium.
-9	Feed 5.0 mL of concentrated food suspension to each larval rearing aquarium. Monitor temperature and dissolved oxygen concentration of overlying water.
-8	(Same as Day -9)
-1	Feed each larval rearing aquarium and monitor temperature and dissolved oxygen, as previously. Add sediment into each of the replicate test beakers, place into exposure system, and activate the automated water renewal system.
0	Remove second instar larvae from the culture chamber substrate. Add 1.0 mL containing 4.0 mg of dry food into each beaker. Transfer 10 larvae into each randomly chosen beaker. Monitor temperature and dissolved oxygen.
1	Add food to each beaker. Monitor water parameters of temperature, dissolved oxygen, pH, hardness, alkalinity, and conductivity.
2	Add food to each beaker. Monitor temperature and dissolved oxygen.
3	Add food to each beaker. Monitor temperature, dissolved oxygen and total ammonia.

4 to 7	(Same as Day 2)
8	Add food to beaker. Monitor temperature, dissolved oxygen and total ammonia.
9	(Same as Day 1)
10	Monitor temperature and dissolved oxygen. Terminate test by collecting the larvae and obtaining dry weight measurements for each replicate.

[a] Activity schedule assumes that a healthy culture has been established, that an exposure system is in place, and that larval rearing substrate and food suspensions have been prepared in advance.

Attachment T. Materials for Culturing of and Conducting Toxicity Tests with *Hyalella azteca*.

Biological Supplies

- *Hyalella azteca* brood stock
- Active dry yeast
- Cerophyl® (dried cereal leaves)
- Trout food pellets
- Algae

Glassware

- Culture chambers (2 L plastic or glass beakers)
- Exposure beakers (300 mL high form)
- Juvenile holding beakers (1 L)
- Wide bore pipets (5 to 6 mm ID)
- Glass disposable pipets
- Burettes (for hardness and alkalinity determinations)
- Graduated cylinders (assorted sizes, 10 mL to 2 L)
- White organism sorting tray

Instruments and Equipment

- Dissecting microscope
- Sieve (e.g., U.S. Standard No. 30 mesh)
- Delivery system for overlying water
- Photoperiod timers
- Photometer
- Temperature controllers
- Thermometer
- Dissolved oxygen meter
- pH meter
- Specific ion meter
- Ammonia electrode (or ammonia test kit)
- Specific conductance meter
- Drying oven
- Dessicator
- Balance (sensitive to 0.01 mg)
- Blender
- Refrigerator
- Freezer
- Light box
- Centrifuge
- Hemacytometer
- Forceps

Miscellaneous

- Ventilation hood for exposure system
- Air supply
- Cotton surgical gauze or cheese cloth
- Stainless steel screen (no. 60 mesh, for test beakers)

Glass hole-cutting bit
Glass glue
Plastic mesh (110 µ mesh opening; Nytex® 110)
Aluminum weighing pans
Fluorescent light bulbs (for culture and toxicity test)
Nalgene® bottles (500 mL, for food preparation and storage)
Deionized water
¼" air line tubing
White plastic dish pan

Chemicals

Detergent (non-phosphate)
Acetone (reagent grade)
Hexane (reagent grade)
Copper sulfate (reagent grade)
Potassium chloride (reagent grade)
Hydrochloric acid (reagent grade)

Attachment U. Some Laboratory Sources of *Hyalella azteca* Cultures.

Laboratory	Contact Person	Phone/Fax/email
Environmental Consulting & Testing 1423 North 8th St., Suite 118 Superior, WI 54880	Steven Poirier	T 800-377-3657 F 715-394-7414
Mid-Continent Ecology Division U.S. Environmental Protection Agency 6201 Congdon Blvd. Duluth, MN 55804	Michael Kahl	T 218-529-5179 F 218-529-5003 E epamdj@du4500.dul.epa.gov
Lake Superior Research Institute University of Wisconsin-Superior Superior, WI 54880	Larry Brooke	T 715-394-8318 F 715-394-8454 E lbrooke@staff.uwsuper.edu
Zoology Department Natural Sciences Building Michigan State University East Lansing, MI 48824	John Giesy	T 517-353-2000 F 517-432-1984 E JGIESY@aol.com
Institute for Environmental Quality Wright State University Dayton, OH 45435	G. Allen Burton	T 937-873-2201 F 937-775-4997 E aburton@wright.edu

Attachment V. Preparation of Food for Feeding *Hyalella azteca* during Culturing and Testing.

The following is a description of the YCT (Yeast-Cerophyl®-Trout food) and algal food preparation. No algal species is specifically mentioned in the algal food preparation. Any green alga will probably work satisfactorily. The procedure is excerpted from: U.S. Environmental Protection Agency. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA 600/4-89/001. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

TABLE V1. YCT PREPARATION

YCT is composed of yeast at a concentration of 5 g/L, Cerophyl® at 10 g/L and fermented trout food at 5 g/L. These three ingredients are combined in equal volumes (1:1:1) to form the final product.

1. Each ingredient is made up as follows:
   A. Yeast
      To be made the same day as the YCT.
      i. Add 2.5 gm active dry yeast to 500 mL dilution water[a].
      ii. Shake vigorously until totally dissolved and use shortly thereafter. Discard excess solution.

   B. Cerophyl®

      To be prepared 24 h before the YCT.
      i. Weigh 5.0 gm Cerophyl®.
      ii. Combine Cerophyl® and 500 mL dilution water[a] in an Erlenmeyer flask.
      iii. Insert a clean bar, cover and stir for 24 hr at medium speed.
      iv. After 24 hr, remove from stir plate, filter through a fine screen (Nitex® 110 mesh).
      vi. Discard the excess and particulates on filter.

   C. Trout Food
      This ingredient must be prepared at least one week in advance, as it must ferment before using. It is best to make a supply ahead of time and freeze it in small batches. Careful planning is needed to avoid being short of this ingredient.
      i. Weigh 5 gm of trout chow pellets (1/8" pellets work well).
      ii. Add 1 L of dilution water[b] to fermentation chamber.
      iii. Place pellets in glass or plastic bottle and aerate, gently rolling the pellets to prevent settling.
      iv. Cover with plastic wrap to decrease evaporation.
      v. Label the container with the date the food should come down (one week from starting date).
      vi. Keep the water level at 1 L by replacing evaporated water each day.
      vii. After one week, shut the air off and filter supernatant through a fine screen (Nitex® 110 mesh). Distribute liquid into smaller containers, label with the current date and freeze.

2. Preparation of YCT

The batch size of YCT may vary, depending upon usage. Batches are made which are used for only two weeks, and new batches should be prepared a day or two in advance to insure a continuous supply.

A. Remove an adequate amount of fermented trout food from the freezer and thaw in a water bath.

B. Shake ingredients well and filter through a fine screen (Nitex® 110 mesh) into a graduated cylinder. Ingredients should be measured out in equal volumes.

C. Combine ingredients in an Erlenmeyer flask and label with the current date.

D. Suspended solid's level must be measured and adjusted to 1800 mg/L before the food is fed.

3. Suspended Solids Monitoring

Solids are monitored on each batch of YCT and adjusted to a constant measure (1800 mg/L) before feeding to keep feeding volumes and food levels consistent.

A. Oven dry labeled weigh pans to a constant weight and weigh.

B. Shake YCT solution well, it is important that the solution be uniform so as to get a good representative sample.

C. Measure 5.0 mL using a 5 mL pipette and dispense into each of two preweighed, oven dried pans.

D. Place pans containing 5 mL YCT in oven and dry completely (at least 4 hr).

E. Weigh pans again and subtract weight of pan alone to get weight of solids in 5 mL YCT.

F. Convert this figure to mg/L and divide by 1800 mg/L to get the dilution factor.

G. Multiply the volume of YCT by the dilution factor to get the final volume and dilute to this final volume. For example:

Pre-weight (g) (oven dried pan alone)	Post-weight (g) (pan & 5 mL YCT dried)	Difference (YCT alone)	Average
1.61665	1.62600	0.009350	
1.62800	1.63750	0.009500	0.009425

Then, 0.009425 g in 5 mL = x mg/L

To find x, multiply 0.009425 x 1000 = 9.425 mg in 5 mL.

Next, divide this quantity by 0.005 liters to get mg/L:

9.425 ÷ 0.005 L = 1885 mg/L

Total Suspended Solids Dilution Factor = 1885 mg/L = 1.05

1800 mg/L

This number (1.05) is multiplied by the volume of YCT prepared to determine final volume obtained after dilution.

H. Repeat the process with 5 mL more of this diluted YCT in pre-weighed pans to confirm suspended solids.

I. If this dilution factor ratio does not work well, it may be best to make a sample dilution of a small aliquot of YCT and check solids levels before diluting the whole batch.

J. Record suspended solids information and mark the solids level of the YCT on the container.

K. Acceptable solids levels are between 1700 and 1900 mg/L (1800 is preferable).

L. Shake well before feeding.

---

[a] 10% Diluted Mineral Water (DMW) is used in cases above. When DMW is not available, a high quality Millipore or distilled water may be substituted.
[b] We use a cut-off 1 gallon Nalgene® jug which is then inverted with a stopper in the neck through which filtered air is supplied.

TABLE V2. ALGAL CULTURE PREPARATION

1. Preparation of Stock Solutions

A. Stock solutions are prepared at a non-specific concentration.

B. Nutrients are added to Millipore water in reagent bottles, mixed until totally dissolved and stored in the refrigerator or cold storage room in the dark. (Stock solutions will remain good for years, barring contamination).

C. Once dissolved, the $NaSiO_3$ stock solution is filter sterilized using a 0.45 µm Millipore filter.

D. Record the date the new stock solutions are prepared. New stock solutions should not be used for 1 month after they are prepared.

E. A 5 mL pipette should be designated and used for each individual stock solution. These pipettes are stored with the stock solutions in a plastic dish pan for each transport.

F. A "pro-pipetter" valved pipette bulb is used for steady and more accurate measurements.

G. The pipette designated for the $NaSiO_3$ stock solution should be

autoclaved or replaced by a new pipette before it is used for a new batch of media. All other stock solution pipettes can be reused indefinitely.

2. Starting Algal Culture

A. Prepare 2 L batches of MBL medium in a 2 L volumetric flask using Woods Hole MBL. Do not add Na_2SiO_3 until after autoclaving.

B. Pour medium into 2 L Erlenmeyer flask, cover with aluminum foil, and autoclave for 15 min.

C. After cooling, add 2 mL of $NaSiO_3.9H_2O$ (1 mL/L of media) using a sterile pipette.

D. Store media at room temperature; prepare more as needed.

E. Transfer media into (previously autoclaved) sterile 2 L separatory funnel.

F. Support funnel with a ringstand using a 10 to 21 cm diameter ring.

G. Inoculate culture media with 2-5 mL of inoculum (see below step N).

H. Place air stem in culture so that the tip is at the stopcock of the flask. This is done to prevent settling of algae.

I. Stopper funnel with foam plug.

J. Cultures are kept at 25 $\pm$ 2° C at a light intensity of approximately 100 ft-c using Grow-lux fluorescent bulbs.

K. Cultures mature in approximately 6-8 days at 25° C under a photoperiod of 16 hr light:8 hr darkness, depending on the amount and concentration of inoculum.

L. When cultures are very green, remove air and transfer culture into a 2 L Erlenmeyer flask.

M. This culture can be stored in the refrigerator if not prepared immediately, or can be centrifuged at this time.

N. New cultures can be started by transferring 2-5 mL of this mature, well mixed culture to sterile media.

O. Approximately four cultures can be started from the same inoculum, after which the next culture should be started from an algal slant to insure purity. Inoculum can be saved from this slant culture to start the next four subsequent cultures.

P. Record data concerning culture and inoculum dates, concentrations and volumes.

3. Starting Algal Cultures From a Slant

Algal slants can be purchased from the Starr Collection at the University of Texas in Austin, Texas or the American Type Culture Collection in Rockville, Maryland.

Slants can be kept and used for several months if stored in a dark refrigerator at 4° C.

All steps, except step G, are followed as in previous procedures. However, between steps D and E do the following:

A. Flame a small wire loop over a bunsen burner and allow to cool.

B. Uncap algal slant and quickly remove a loopful of the algae by pulling the wire loop gently across the surface of the slant so as not to tear up the agar. Try to keep the slant uncapped for as short a time as possible.

C. Flame the mouth of the slant tube to prevent contamination and quickly replace the cap.

D. Immerse the wire loop containing the algae in the MBL medium and swirl until the algae has come off the loop and is in the medium.

4. Preparation of Algae for Feeding

A. Centrifuge mature algal culture in 100 mL tubes at 2000 RPM for 8-10 min.

B. Pour off MBL supernatant. Use diluent[a] in a squirt bottle to resuspend the algal pellet. Dilution water is the same water used for testing and *Ceriodaphnia* culturing.

C. Use only enough dilution water to just break up and suspend the pellet (approximately ¼ the volume of the algal culture which was centrifuged).

D. Transfer resuspended algae into an Erlenmeyer flask.

E. Count and calculate the number of cells per mL using a Coulter counter or hemacytometer[b] and dilute to 35×10^6 cells per mL with diluent.

F. Store algae in refrigerator. Presently, algae is used until it is gone and stock appears to remain viable for several weeks. The algae stock is not viable when it turns yellow in color.

---

[a] 10% Diluted Mineral Water (DMW) is used routinely at ERL-Duluth.

[b] Hemacytometer readings are likely to be higher than Coulter counter readings.

TABLE V3. Algal Media

Prepare stock solutions and use 1 mL of each stock solutions per liter of medium, except add 2 mL of $FeCl_3.6H_2O$ per liter medium.

Macronutrients	Woods Hole MBL[a] gm/L in stock solution
$CaCl_2.2H_2O$[b]	36.76
$MgSO_4.7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$[b]	85.01
$Na_2SiO_3.9H_2O$	28.42 (add after autoclaving)
Micronutrients	
Na_2 EDTA	4.36
$FeCl_3.6H_20$	3.15
$CuSO_4.5H_2O2$[c]	0.01
$CoCl_2.6H_2O$[c]	0.01
$ZnSO_4.7H_2O$[c]	0.022
$MnCl_2.4H_2O$[o]	0.18
$Na_2MoO_4.2H_2O$[b]	0.006
H_3BO_3[c]	1.0

[a] Nichols, H.W. 1973. In: Handbook of Psychological Methods, J.R. Stein, Ed. Cambridge University Press, London. pp. 7-24.
[b] $CaCl_2$ and $NaNO_3$ can be combined as one stock solution.
[c] Micronutrients can be mixed as single stock solution.

Attachment W. Culture and Test Data Forms for *Hyalella azteca*.

Form W1. Sample Evaluation Form for the Health and Reproduction of a *Hyalella azteca* Culture.

Culture Record
Hyalella azteca

Date: _____ Water Bath Temp. _____

Feeding: Amount YCT _____ Amount Algae _____

Comments:

Culture Renewal

Date: _____ Water Bath Temp. _____

Dissolved Oxygen: _____ (any random tank) pH _____

Culture Jar #	# Adults	# Pairs	Approx. # Young	# Young Saved	# Added	Total

Total # Young Saved: _____ Age: _____ days

Form W2. Sample Data Form for Temperature and Overlying Water Chemistry Measurements in a Toxicity Test with *Hyalella azteca*.

Hyalella azteca Toxicity Test
Overlying Water Chemistry Measurements

Project Name _____

Sediment Sample Source _____

Date of Test Initiation_____

Toxicologist Conducting Test _____

Test Day	Test Replicate Sampled	Temperature (°C)	Dissolved Oxygen (mg/L)	pH[a]	Hardness (mg/L)	Alkalinity (mg/L)	Specific Conductance (umhos/cm)	Total Ammonia (mg/L)
0								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

[a]Unshaded areas are for measurements.

Form W3. Data Chart for Performing Reference Toxicant Tests with $CuSO_4$ and *Hyalella azteca*.

REFERENCE TOXICANT TESTING FORM

-*Hyalella azteca* and $CuSO_4$-

Brood Stock Source_____ Test Initiation Date_____ Time

Aquarium No._____ $CuSO_4$ Form_____

No. Animals Per Replicate_____ $CuSO_4$ Purity_____

No. of Replicates_____ $CuSO_4$ Supplier_____

Dilution Water/Control_____ $CuSO_4$ Lot No._____

Test Volume_____ Toxicologist_____

Test Type (circle one)[a]: SU, SM, RU, RM, FU, FM

	Survival Readings											
	0 h			24 h		48 h		72 h		96 h			
Conc.	pH	D.O.	Temp.	Surv.	pH	Surv.	pH	Surv.	pH	Surv.	pH	D.O.	Temp.

Comments_____

96-hr LC50 = _____
Method of LC50 Estimate _____
Cumulative Mean LC50 _____
No. of Tests the Cumulative Mean is Based on _____
Acceptability of Current Test[b] Yes ___ No ___

[a]SU = Static unmeasured, SM = static measured, RU = renewal unmeasured, RM = renewal measured, FU = flow-through unmeasured, FM = flow-through measured.

[b]Based upon two standard deviations around the cumulative mean 96-hr LC50.

Attachment X. General Activity Schedule for Performing a Sediment Toxicity Test with *Hyalella azteca*[a].

Day	Activity
-7	Renew mass cultures. Separate the juvenile amphipods from the mass cultures and place juveniles in 1-L beakers with a piece of presoaked cotton gauze and feed. Begin preparing YCT food for the test if not previously prepared.
-6	Feed the juveniles and observe the cultures for survival, monitor temperature and dissolved oxygen.
-5	Same as Day -6, omit dissolved oxygen monitoring.
-4	Same as Day -6.
-3	Same as Day -6, omit dissolved oxygen monitoring.
-2	Same as Day -6.
-1	Same as Day -6; add sediment into each of the replicate test beakers, place into exposure system, and activate the automated water renewal system.
0	Transfer ten 7- to 14-day old juveniles into each randomly chosen beaker. Feed 1.5 mL of YCT into each test chamber. Monitor overlying water temperature and dissolved oxygen.
1	Add 1.5 mL of YCT food to each test chamber. Monitor overlying water characteristics of temperature, dissolved oxygen, pH, hardness, alkalinity, conductivity and ammonia.
2	Add 1.5 mL of YCT food to each test chamber. Monitor overlying water temperature.
3 to 8	Same as Day 2 except monitor dissolved oxygen on even numbered days.
9	Same as Day 1.
10	Monitor temperature and dissolved oxygen. Terminate test by collecting the juveniles with a sieve and observing for survivors. Oven-dry survivors and weigh for growth determination.

[a] Activity schedule assumes that a healthy culture has been established (monitored at least quarterly with a 96-hr reference toxicant test), that an exposure system is in place, and that YCT food suspensions have been prepared in advance.

Attachment Y. Materials for Culturing of and Conducting Bioaccumulation Studies with *Lumbriculus variegatus*.[1]

Biological Supplies

Lumbriculus variegatus starter culture
Trout starter
Helisoma sp. snails (optional)

Glassware

Aquaria (57 L, e.g.)
Pipette (20 cm long, 5 mm I.D. opening, ends fire-polished)
Glass weights (to hold substrate in place)
Exposure chambers (15.8 cm x 29.3 cm x 11.7 cm, W x L x H), 12
Glass bowl (20 cm diameter)
Glass vials (10 mL)
Beakers (500 mL)

Instruments and Equipment

Sieve, fine-meshed (e.g., U.S. Standard No. 35 or 40 mesh)
Water delivery system
Paper shredder, cutter or scissors
Temperature controller
Thermometer
Continuous recording thermometer
Photoperiod timer
Dissolved oxygen meter
Specific ion meter
pH meter
Ammonia electrode (or ammonia test kit)
Drying oven
Desiccator
Freezer
Tissue homogenizer

Miscellaneous

Brown paper toweling
Small dipnets (e.g. 7.6 cm)
Shallow pan (plastic, light-colored)
Shallow pan (glass or stainless steel)
Dissecting probes
Dental picks
Light bulbs
Air Supply

Airstones
Acetone
Hexane
Chloroform
Methanol
Copper sulfate (reagent grade)

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[1] Does not include the analytical instrumentation, glassware or reagents necessary to analyze for inorganic or organic chemicals that may bioaccumulate.

Attachment Z. Laboratory Sources of *Lumbriculus variegatus* Cultures.

Laboratory	Contact Person	Phone/Fax/email
Environmental Consulting & Testing 1423 North 8th St., Suite 118 Superior, WI 54880	Steven Poirier	T 800-377-3657 F 715-394-7414
Mid-Continent Ecology Division U.S. Environmental Protection Agency 6201 Congdon Blvd. Duluth, MN 55804	Michael Kahl	T 218-529-5179 F 218-529-5003 E epamdj@du4500.dul.epa.gov
Lake Superior Research Institute University of Wisconsin-Superior Superior, WI 54880	Larry Brooke	T 715-394-8318 F 715-394-8454 E lbrooke@staff.uwsuper.edu
Zoology Department Natural Sciences Building Michigan State University East Lansing, MI 48824	John Giesy	T 517-353-2000 F 517-432-1984 E JGIESY@aol.com
Institute for Environmental Quality Wright State University Dayton, OH 45435	G. Allen Burton	T 937-873-2201 F 937-775-4997 E aburton@wright.edu

Attachment AA. Culture and Test Data Forms for *Lumbriculus variegatus*

Form AA1. Evaluation Form for the Health and Reproduction of a *Lumbriculus variegatus* Culture.

Date of Arrival of Brood Stock_____ Culturist_____
Date of Animal Transfer Into Specific Culture Aquaria

Aquarium:	A	B	C	D	E	F
Date :						

Trout Chow Brand and Batch Number_____

Date	Culture Aquarium	Dissolved oxygen (mg/L)	Temp (°C)	Animal Color	Animal Responsiveness	Wet wt. at time of transfer IN	Wet. wt. at time of transfer OUT	Comments
	A							
	B							
	C							
	D							
	E							
	F							
	A							
	B							
	C							
	D							
	E							
	F							

Form AA2. Sample Record Form for Sediment and *Lumbriculus variegatus* Tissue Samples in a Bioaccumulation Study.

Sediment Sample I.D._____ *Lumbriculus* variegatus source_____

Replicate No._____ *L. variegatus* mass added at test start_____

Sediment Collection Date_____ *L. variegatus* mass retrieved at test end _____

Sediment Exposure Start Date_____ Duration of gut clearance period_____

Investigator(s):_____

	Replicate No.			
Annelid Data	1	2	3	4
Wet wt. at end				
Dry wt. at end				
Percent total lipids				
Conc. chemical (wet wt.) (A)				
Conc. chemical (dry wt.) (B)				
Lipid normalized chemical conc., wet wt. basis (C)				
Sediment Data				
Wet wt.				
Dry wt.				
Percent TOC (dry wt. basis)				
AVS (umol/g, dry wt.)				
Conc. of chemical (dry wt.)				
TOC normalized chemical conc., (dry wt.) (D)				
AVS normalized chemical conc., (dry wt.) (E)				
Accumulation factor, AF, for organic compounds (C/D)				
Accumulation factor, AF, for inorganic compounds (B/E)				

Form AA3. Data Chart for Performing Reference Toxicant Tests with $CuSO_4$ and *Lumbriculus variegatus*.

Brood Stock Source _____
Organisms Tested From Culture
Aquarium No. _____
No. of Animals Tested Per Replicate _____
No. of Replicates _____
Method of LC50 Estimate _____

$CuSO_4$ Form _____
$CuSO_4$ Purity _____
$CuSO_4$ Supplier and Lot No. _____
Test Type (circle one)[a]:
SU, SM, RU, RM, FU, FM
Test Initiation Date _____
Toxicologist _____

Number of Mortalities

Exposure Duration (Hr)	Control A	Control B	Exp. 1 A	Exp. 1 B	Exp. 2 A	Exp. 2 B	Exp. 3 A	Exp. 3 B	Exp. 4 A	Exp. 4 B	Exp. 5 A	Exp. 5 B
0												
24												
48												
72												
96												

96 hr. LC50 = _____

Cumulative Mean 96 hr LC50 = _____

Number of Reference Toxicant Tests Used to Determine Cumulative Mean LC50 _____

Acceptability of Current Test[b]
Yes_____ No_____

[a]SU = Static unmeasured, SM = static measured, RU = renewal unmeasured, RM = renewal measured, FU = flow-through unmeasured, FM = flow-through measured.

[b]Based upon two standard deviations around the cumulative mean 96 hr LC50.

Form AA4. Sample Data Form for Temperature and Water Chemistry Measurements in a Bioaccumulation Study with *Lumbriculus variegatus*.

Sediment Sample Source: No. 1 _____ No. 2 _____ No. 3 _____

Date of Test Initiation _____

Toxicologist Conducting Test _____

	Sample Replicate[a]	Temperature (°C)[b]	Dissolved Oxygen[b] (mg/L)	pH[c]	Total Ammonia[c] (mg/L)	Hardness[c] (mg/L as $CaCO_3$)	Alkalinity[c] (mg/L as $CaCO_3$)	Specific Conductance[c] (μmhos/cm)
	1A							
	1B							
	1C							
	1D							
Day _____	2A							
	2B							
(0-28)	2C							
	2D							
	3A							
	3B							
	3C							
	3D							

[a] Sample form is for a study of three sediment samples in quadruplicate. The form may be expanded for larger numbers of sediment samples or increased replication.
[b] To be measured daily.
[c] To be measured twice during the test.