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Testing the Toxicity of Field Collected Sediments

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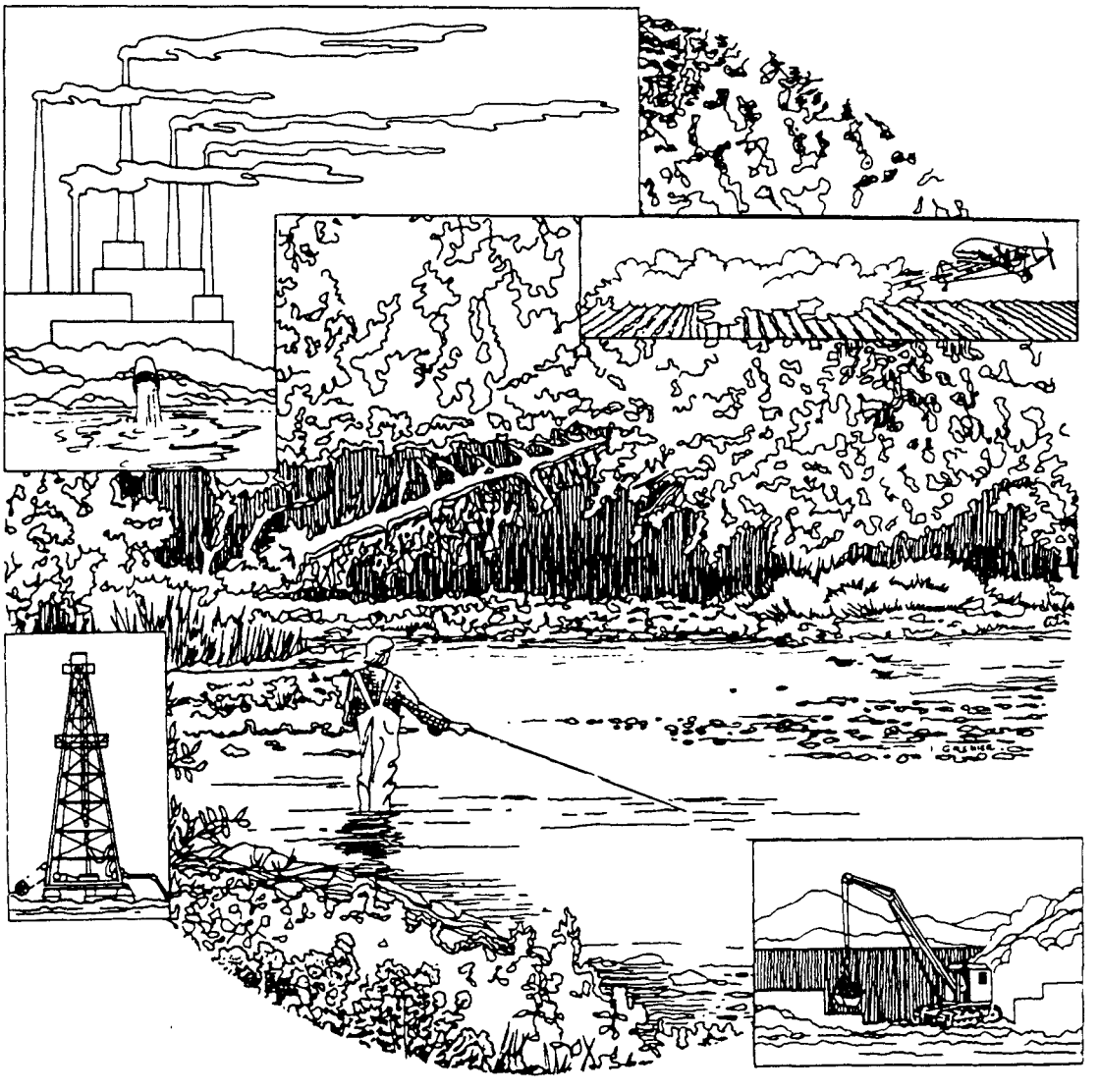

"TESTING THE TOXICITY OF FIELD COLLECTED FRESHWATER SEDIMENTS"

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I. Introduction. **Nelson**

A. Extent of sediment contamination.

II. Sediment Assessments. **Nelson**

A. U.S. EPA 1989 Sediment Methods Compendium.
B. ASTM Sediment Sub-committee activities, E47.03.
C. Assessment and Remediation of Contaminated Sediments (ARCS), Great Lakes National Program Office.

III. EPA Sediment Management Strategy. **Wood**

A. Sediment management strategy.
B. Extent.
C. Research driving regulatory solutions.
D. NPDES Program adapting to prevention of sediment contamination.
E. Needs to address sediment contamination prevention.

IV. Safety Precautions and Considerations. **Coyle**

A. Minimizing exposure.
B. Proactive safety management.
C. Primary, secondary, and tertiary protection
D. Physiological and psychological factors.
E. Perception of hazard.
F. Route of exposure.

V. Sediment Manipulation. **Coyle**

- A. Collection.
- B. Shipping.
- C. Storage.
- D. Preparation.
  1. Mixing.
  2. Aqueous extractions (i.e., pore water, elutriates).
     - a. Methods.
     - b. Practical considerations.
     - c. Factors influencing composition and toxicity of pore water and elutriates.
- E. Water quality.
     - a. Routine measurements.
     - b. Potential problems and solutions.
- F. Sediment disposal considerations and requirements.

VI. Sediment and aqueous extract chemistry. **Nelson**

- A. Metals and other inorganics.
- B. Organics.

VII. Whole sediment characterization. **Nelson**

- A. Total organic carbon.
- B. Particle size distribution (percent sand, silt, clay).
- C. pH.
- D. Total volatile sulfides.
- E. Water content (percent).

VIII. Sediment Toxicity Testing.

- A. Microtox testing of aqueous sediment extractions. **Coyle**
  1. Methods review.
     - a. Future approaches (Direct Contact).

- B. Aqueous extract testing. **Burton and Coyle**
  1. Test organisms (Daphnia magna, Ceriodaphnia dubia, Pimphales promelas).
  2. Methods review.
  3. Test set-up.
  4. Monitoring test.
  5. Ending test.
  6. Water Quality.
  7. Interpreting results.
     - a. Tests reflect acute toxicity of water soluble contaminants.
     - b. Tests results not stand-alone descriptions, but are parts of a larger toxicity appraisal process.

- C. Microbial and In situ Testing. **Burton**

- D. Whole Sediment Testing. **Nelson and Burton**
  1. Initiating tests.
     - a. Experimental design.
  2. Test organisms (Hyalella azteca, Chironomus riparius, Chironomus tentans, Daphnia magna, Ceriodaphnia dubia).
     - a. Culture.
     - b. Handling.
     - c. Test preparations.
       - (1) Diluter calibration.
       - (2) Food preparation.

(3) Temperature in water bath.

3. Test set-up.
   a. Day -1.
      (1) Sediment into test chambers.
      (2) Overlying water.
      (3) Aeration.
   b. Day 0.
      (1) Water quality determinations.
4. Monitoring tests.
   a. Biological.
      (1) Feeding.
      (2) Qualitative observations.
         (a) Test organisms.
         (b) Sediment and overlying water conditions.
   b. Equipment operation.
      (1) Diluter functioning.
      (2) Aeration.
      (3) Screens cleaned.
   c. Water quality determinations.
      (1) Day 7, etc. to end of test.
5. Ending tests.
   a. Water quality.
   b. Sieving sediments.
   c. Retrieving test organisms.
   d. Preserving test organisms.
6. Interpreting results.
   a. Test acceptability.

IX. Strengths and Limitations of Sediment Toxicity Testing. **OPEN FORUM**

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Watersned Protection Division

Draft
Final
Report

Sediment Classification Methods Compendium

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Draft Final Report

SEDIMENT CLASSIFICATION METHODS COMPENDIUM

by

U.S. Environmental Protection Agency

Portions of this document were prepared by Tetra Tech, Inc., under the direction of Michael Kravitz, U.S. EPA Work Assignment Manager

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CONTENTS

FIGURES

TABLES

ACKNOWLEDGMENTS

This compendium was prepared by the U.S. Environmental Protection Agency, Sediment Oversight Technical Committee. Chaired by Dr. Elizabeth Southerland of the Office of Water Regulations and Standards, the committee has representation from a number of Program Offices in Headquarters and the Regions. The methods represented here were written by the following authors (also listed at the beginning of their respective chapters):

- Gerald Ankley, Anthony R. Carlson, Phillip M. Cook, Wayne S. Davis, Catherine Krueger, Janet Lamberson, Henry Lee II, Richard C. Swartz, Nelson Thomas, and Christopher S. Zarba (U.S. EPA)
- Gordon R. Bilyard, Gary M. Braun, and Betsy Day (Tetra Tech, Inc.)
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TABLE 1-1. SEDIMENT QUALITY ASSESSMENT METHODS
(Sediment Classification Methods Compendium, U.S. EPA, June 1989)

Method (Chapter)	Num	Descr	Comb	Concept
Bulk Sediment Toxicity (2.0)		▪		Test organisms are exposed to sediments which may contain unknown quantities of potentially toxic chemicals. At the end of a specified time period, the response of the test organisms is examined in relation to a specified biological endpoint.
Spiked Sediment Toxicity (3.0)	▪			Dose-response relationships are established by exposing test organisms to sediments that have been spiked with known amounts of chemicals or mixtures of chemicals.
Interstitial Water Toxicity (4.0)	▪			Toxicity of interstitial water is quantified and identification evaluation procedures are applied to identify and quantify chemical components responsible for sediment toxicity. The procedures are implemented in three phases to characterize interstitial water toxicity, identify the suspected toxicant, and confirm toxicant identification.
Equilibrium Partitioning (5.0)	▪			A sediment quality value for a given contaminant is determined by calculating the sediment concentration of the contaminant that would correspond to an interstitial water concentration equivalent to the U.S. EPA water quality criterion for the contaminant.
Tissue Residue (6.0)	▪			Safe sediment concentrations of specific chemicals are established by determining the sediment chemical concentration that will result in acceptable tissue residues. Methods to derive unacceptable tissue residues are based on chronic water quality criteria and bioconcentration factors, chronic dose-response experiments or field correlation, and human health risk levels from the consumption of freshwater fish or seafood.
Freshwater Benthic Community Structure (8.0)		▪		Environmental degradation is measured by evaluating alterations in freshwater benthic community structure.
Marine Benthic Community Structure (9.0)		▪		Environmental degradation is measured by evaluating alterations in marine benthic community structure.
Sediment Quality Triad (9.0)	▪	▪	▪	Sediment chemical contamination, sediment toxicity, and benthic infauna community structure are measured on the same sediment. Correspondence between sediment chemistry, toxicity, and biological effects is used to determine sediment concentrations that discriminate conditions of minimal, uncertain, and major biological effects.
Apparent Effects Threshold (10.0)	▪		▪	An AET is the sediment concentration of a contaminant above which statistically significant biological effects (e.g., amphipod mortality in bioassays, depressions in the abundance of benthic infauna) would always be expected. AET values are empirically derived from paired field data for sediment chemistry and a range of biological effects indicators.
International Joint Commission (11.0)[1]				Contaminated sediments are assessed in two stages: 1) an initial assessment that is based on macro-zoobenthic community structure and concentrations of contaminants in sediments and biological tissues, and 2) a detailed assessment that is based on a phased sampling of the physical, chemical, and biological aspects of the sediment, including laboratory toxicity bioassays.

1 The IJC approach is an example of a sequential approach, or "strategy" combining a number of methods for the purpose of assessing contaminated sediments in the Great Lakes.

American Society for Testing and Materials
E-47 Biological Effects and Environmental Fate (Main Committee)
E-47.03 Sediment Toxicity Subcommittee
Christopher G. Ingersoll, Chair
USFWS, NFCRC
Columbia, MO, 314/875-5399

ASTM Sediment Subcommittee Activities

Document #1: E 1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates (Task Group Chair: Marcia Nelson, NFCRC, Columbia, MO, 314/875-5399).

Proposed additional species-specific annexes.

(1) Daphnia and Ceriodaphnia (Allen Burton, Wright State University, Dayton, OH, 513/873-2201).

(2) Diporeia spp. (formerly Pontoporeia hoyi; Peter Landrum, NOAA, Ann Arbor, MI, 313/668-2276).

(3) Ostracods (Arthur Stewart, Oak Ridge National Laboratory, Oak Ridge, TN, 615-574-7835).

(4) Hexagenia spp. (Donna Bedard, Ontario Ministry of the Environment, Rexdale, Ontario, 416/235-5970 and Mary Henry, USFWS, U. of Minn, Minneapolis, MN).

(5) Tubificid oligochaetes (Trefor Reynoldson, Environment Canada, Burlington, Ontario, 416/336-4783).

(6) Naidid oligochaetes (Dave Smith, Bio-Aquatics Testing, Carrollton, TX, 214/247-5928).

(7) Lumbricus sp. (Gary Phipps, ERL-Duluth, MN, 218/720-5550).

(8) Mollusks (Don Wade and Anne Keller, TVA, Muscle Shoals, AL, 205/386-2068).

Document #2: E 1367 Guide for Conducting 10-d Static Sediment Toxicity Tests with Estuarine and Marine Amphipods (Task Group Chair: Janet Lamberson, USEPA, Newport, OR, 503/867-4043).

Document #3: E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediment for Toxicological Testing (Task Group Chair: A. Burton, WSU).

Document #4: Guide For Designing Sediment Toxicity and Bioaccumulation Tests (Task Group Chair: John Scott, SAIC, Narragansett, RI, 401/782-3017).

Document #5: Sediment Resuspension Testing Methods (Allen Burton, WSU).

Document #6: Guide for Conducting Sediment Toxicity Tests with Polychaetes (Task Group Chair: Don Reish, California State University-Long Beach, Long Beach, CA, 213/431-7064).

Document #7: Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Fish (Draft #2, 04/17/90, Task Group Chair: Mike Mac, USFWS, Ann Arbor, MI, 313/994-3331).

Document #8: Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates. (Task Group Chair: Henry Lee, USEPA, Newport, OR, 503/867-4042).

Document #9: Use of Oysters and Echinoderm Embryos and Larvae in Sediment Toxicity Testing (Task Group Chair: Paul Dinnel, University of Washington, Seattle, WA, 206/543-7345).

Document #10: Toxicity Identification and Evaluation (TIE) for Sediment Water Extracts (Task Group Chair: vacant).

SEDIMENT MANAGEMENT: A REGULATORY PERSPECTIVE

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I. Introduction.
- A. Topics to be covered.
  1. EPA agency-wide sediment management strategy.
  2. Why a strategy now?
     - a. Extent of sediment contamination problem.
     - b. What we have learned through research that is driving regulatory solutions.
  3. How EPA envisions NPDES Program adapting to address prevention of sediment contamination caused by point sources.
  4. What do we need (research, procedures, policy) in order for the NPDES Program to address sediment contamination prevention?

II. EPA sediment management strategy.
- A. The strategy will state EPA's policy on sediments in light of latest science and understanding of the extent of the problem. It is very early in the strategy development process. EPA is committed to involving the public in the process.
- B. The strategy will likely have 4 basic components.
  1. Assessment and risk identification.
     - a. Statement of the sediment contamination problem, why we think its a national problem, how we know it is a problem in some locations.
     - b. What EPA intends to do to better define the extent of the national problem.
  2. Prevention.
     - a. Statement of policy on point and non-point source prevention, pesticide regulation, and toxic substances control.
  3. Remediation.
     - a. Roles and responsibilities.
     - b. Consistent identification of sites for remediation.
     - c. Consistent cleanup goals.
  4. Dredged material management.
     - a. Balancing economic and environmental factors.
     - b. Applicability of RCRA.

III. Why a sediment management strategy now?
- A. What data is telling us about risk and ecological impact.
  1. 1989 National Academy of Sciences Report on contaminated marine sediments.
  2. Site-specific studies showing human health risk from consumption of fish and shellfish.
     - a. Quincy Bay, MA: cancer risk from consuming lobster tomalley.
     - b. Lake Michigan: developmental problems in children whose mothers consumed large amounts of fish.
     - c. Los Angeles-Long Beach Harbor: 10^{-3}-$^{-4}$ cancer risk from consuming white croaker.
     - d. Puget Sound: As much as 2×10^{-4} cancer risk for moderate seafood consumers and 4×10^{-3} risk for high-quantity consumers.

3. Site-specific studies showing harm to aquatic life, waterfowl, and up the food chain.
   a. Elizabeth River, VA: Severe fin and gill erosion, tumors, and mortality.
   b. Black River, OH: fish tumors.
   c. Great Lakes: reproductive problems in Forster's tern, reproductive failures and mortality in mink.
   d. Commencement Bay, WA: mortality in amphipods and oyster larvae.

B. Improved ability to identify sediment toxicity and classify sediments based on their impact on aquatic life and human health.
   1. Criteria documents.
      a. Scheduled for public review and comment in 1991. (6 non-polar organics).
   2. Advances in whole sediment toxicity tests.
   3. Advances in sediment TIE research and method development making TIE methodologies increasingly useful for identifying causative agents and sources.

C. Congress is interested. Seven separate pieces of legislation introduced in 89 and 90 that address sediments.
   1. National inventory of sites.
   2. Sediment criteria and standards.
   3. Accelerated point and non-point source controls.

IV. NPDES Program

A. EPA fully intends to use sediment criteria, sediment toxicity analysis, and sediment TIE as the basis for point source controls to protect sediment quality.
   1. EPA believes the science of sediment classification and source identification is solid and getting better and that implementing point source controls will therefore not require any great leap of faith.

B. What is on the horizon. Point source sediment quality controls are probably inevitable.
   1. Source identification using refined sediment TIE procedures.
   2. Chemical-specific permit limits based on sediment quality criteria.
   3. Whole effluent limits based in some way on ambient sediment toxicity (measured or projected).
   4. Chemical-specific permit limits based in the presence of bioconcentratable compounds on effluent, ambient sediment and/or ambient tissue (measured or projected).

V. How NPDES gets from here to there.

A. Assessment needs.
   1. We know a good deal about the extent of sediment contamination, but we need more and better information, particularly on source identification.
   2. EPA is wrestling with the assessment question. How extensive should an assessment be?
      a. Data base of existing information on sites?
      b. Fill in gaps in existing data on sites?
      c. Full blown comprehensive assessment (new data) on sites and sources.

B. Need to continue sediment criteria development.
   1. First set of 6 non-polar organics.
   2. Metals.
   3. More organics, inorganics.

C. Need to continue refinement of TIE methodologies.
   1. Research so far has been mostly on identifying causative agents in highly complex sediments. Upcoming research will focus also on less complex samples with defensible source identification as an objective. EPA is currently selecting candidate sediment samples for this purpose.

D. Continued refinement of promising sediment toxicity protocols that are user friendly and suitable for wide use by regulatory authorities.

E. Simplified models of sediment fate and transport that are user friendly and suitable for wide use by regulatory authorities.

F. Validation

1. Audience can appreciate the need for validation of predictive methodologies to show that whatever the methodology, it is reasonably accurate at projecting and defining real aquatic life and human health risk.
2. EPA is committed to basing point source sediment quality controls in good solid science. Want to target regulatory efforts at real problems.

G. Need input from scientific community, regulators, and industry. There will be key opportunities for this.
1. Public comment on agency-wide sediment management strategy (early 1991).
2. Public comment on proposed sediment criteria for 6 non-polar organics (1991).
3. Continued exchanges like today.

VI. Summary.
A. There is strong momentum toward point source sediment contamination controls.
B. In an atypical fashion, the research is driving policy and the regulatory program. This is a good thing that is likely to yield informed, fair regulatory decisions.
C. We are at a point where we know we are on the right track technically.
D. EPA focus will continue to be on refining methodologies in order to make point source sediment contamination controls real.

VII. Questions.

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SEDIMENT STORAGE, HANDLING AND TESTING PROCEDURES, FISH AND INVERTEBRATE TOXICOLOGY

I. General:

This SOP describes the procedures to minimize exposure of personnel and the facility while conducting laboratory tests with sediments or sediment extracts. Sediment is often a storage reservoir for many contaminants introduced into surface waters. These contaminants may include polychlorinated biphenyls, polynuclear aromatic compounds and inorganic contaminants including heavy metals. Contaminants present in sediment may include carcinogens, mutagens, or potentially toxic compounds. Bioassessment tests (toxicity and bioaccumulation) are used to estimate potential biological impact that may result from exposure to these contaminants associated with sediment. Since field sediments may contain potentially toxic materials they should be treated with caution to minimize occupational exposure to workers.

II. Safety:

A. Site Section: Prior to collection of sediment for laboratory tests, information on known or suspected contaminants associated with the sediment at the site must be identified. Historical data (e.g., types of industry, known contaminant inputs, STORET) or additional chemical analyses will be needed before sediments are collected for laboratory tests.

B. Personal protection: This section deals with the procedures that will be implemented by all personnel working with contaminated sediment. It should be noted that research conducted with sediment varies considerably depending on the scope and objective of the research. Therefore, the guidelines set forth in this SOP may not be applicable to all situations dealing with potentially contaminated sediments (1,2,3,4).

1. Medical Surveillance. Health monitoring will be provided for personnel working with sediments. The health monitoring establishes a baseline to which all subsequent medical finding can be compared.

2. Personal precautions. Workers must always be aware of possible points of contamination as described by the supervisor. Hands should always be kept away from the eyes and mouth. After completion of a manipulation involving sediment or the removal of possibly contaminated laboratory clothing (gloves, lab coat, etc.), the hands, forearms, and other areas of suspected contact should be washed with hand soap and water at a sink located within the laboratory work area. Do not use organic solvents to clean the skin. These solvents may increase penetration of the contaminant into the skin.

3. Laboratory clothing. When working with sediments it is of the utmost importance to avoid skin contact. A fully fastened knee length lab coat must be worn in the laboratory work area at all times. Disposable Tyvec^{R} lab clothing must be worn for sediment manipulation and when water quality is determined. Cloth lab clothing may be worn during non-hazardous activities, such as feeding test organisms, entering data, or checking diluters. Any laboratory clothing containing holes or tears will not be used. The lab coat must be removed and stored in the proper bag prior to leaving the laboratory work area. All lab clothing may only be handled while wearing gloves. The procedure for putting on gloves and a lab coat is: (a) put on one pair of clean gloves, (b) put on the lab coat, and (c) put on a second pair of gloves. The procedure for removing the gloves and lab coat is: (a) remove the outer pair of gloves making sure not to contact the skin with the surface of the outer glove, (b) remove the lab coat, (c) remove the second pair of gloves, and (d)

wash hands at the sink. Clothing should be examined daily for possible contamination.

4. Hand protection. Hands will be the most frequent point of potential contact with contaminants. Gloves must be worn to avoid skin contamination. Disposable gloves must be discarded after each use in appropriate containers designated for this use. Double gloves will be used with the outer glove being striped off after any potential exposure. Torn or punctured gloves must be discarded and replaced immediately. It must be remembered that rubber, latex or vinyl gloves do not provide full protection. Contaminants may diffuse into the gloves. When sediment is handled gloves should be changed frequently (3). Cuffs must be tight fitting or taped to the sleeve to prevent inward migration of contaminants.

5. Eye protection. Safety glasses must be worn at all times. In addition, face shields will be made available in the laboratory work area.

6. Further precautions. Protective disposable footwear is recommended during sediment manipulation. Long hair should be tied back and loose clothing should be covered by the lab coat. Eating, drinking, smoking, chewing gum, smokeless tobacco and shorts are prohibited in the laboratory work area where sediments are being used or stored. Food must not be stored in the laboratory work area. Oral pipetting will never be performed. In addition, respirators, a glove box, or a vented hood will be used when sediment is manipulated. Respirators will be labeled with the workers name, date of filter replacement and stored in individual lockers when not in use. These lockers are located in the change area outside the laboratory work area. Reusable protective gear will be placed in a cabinet located outside the laboratory work area (see Section C below).

C. Facility engineered protection: The following guidelines are for the laboratory work area where sediments will be tested.

1. Area identification and access control.

   a. The laboratory work area where sediments are used or stored will be properly identified. A sign stating "Authorized personnel only" will be visible. Access to the designated laboratory work area will be limited. Access doors to the building will be kept closed while sediment is manipulated.

   c. Animals and plants not related to the experiment shall not be permitted in the laboratory.

2. Eyewash stations and hand washing facilities are available in the laboratory work area.

3. Containment devices. Work with sediment will be performed in an appropriate containment device. Procedures involving sediment will not be conducted on an open bench due to the potential hazard of generating contaminated dusts, aerosols, or fumes. Hoods, glove boxes, and enclosed vented water baths for testing are used to minimize the worker exposure to contaminants associated with sediment. All containment devices will be constructed out of smooth, unbreakable material, such as Teflon®, stainless steel, polyethylene, fiberglass, or plexiglass. Exhaust air from hoods, glove boxes, or water baths which contain sediments does not have to be filtered (1). The discharge must be out of the building, as far from the air intake supply as possible (1).

4. Equipment. Use of instruments such as pH, dissolved oxygen or conductivity meters will be used in a glove box or hood. This equipment will be enclosed in plastic to reduce the potential for contamination. Instruments will be serviced or

calibrated in the work area. All calibration and maintenance log books should be kept with the equipment. All equipment that has come in contact with potentially contaminated sediment must be kept either under negative pressure (e.g., a hood) or sealed in an air tight container (e.g., a Tupperware^{R} container) before it is cleaned.

5. Work surfaces. All work surfaces potentially exposed to sediments must be covered with Teflon^{R} sheets, plastic trays, dry absorbent plastic-backed paper, foil, or other impervious or disposable material. If a surface becomes contaminated or if a spill occurs, the work surface should be decontaminated or disposed of immediately.

6. Housekeeping. The laboratory work area shall be kept clean and orderly. Clean-up shall follow every operation or, at a minimum, at the end of each day. Containers for disposal of contaminated materials will be placed in the work area.

7. Spill control. A sediment spill will be treated as a "Chemical Spill: Organic solvent." The sediment spill will be contained with the appropriate absorbent material. If a spill occurs the worker should (a) pour absorbent material on the spill quickly, using enough material to adsorb all fluid and cover the mass with excess dry absorbent to control vapors; (b) sound the air horn to signal for help if necessary; (c) close doors to all labs in the building; (d) increase ventilation by turning on exhaust hoods in the laboratory work area; (e) if problems are encountered in containing the spill, consideration should be given to evacuating the building, route personnel away from the problem area; (f) clean up adsorbents and dispose of them properly. (g) allow personnel to return to the laboratory work area.

III. Storage of sediment:

A. Solid-phase sediment and sediment extracts will be stored at 4°C in air-tight containers in the dark. All samples must be accompanied with proper identification and sample tracking information. Sediment extracts can be temporarily stored at 4°C in refrigerators located in the laboratory work areas.

IV. Homogenization and preparation of elutriate samples:

A. Sediment will always be transferred using double containment. Transfer of sediment from the storage container is a procedure which involves a potential hazard for personal contamination. During this procedure, the number of investigators in the laboratory work area should be minimized. Other workers in the building must be notified of the handling of the sediment.

B. Mixing and sampling of solid-phase sediment or sediment extracts will be done in the original storage container under a hood. If the containers holding sediment are removed from the hood, an intermediate non-breakable container must be used. The worker must use a respirator with organic vapor-acid gas filters and appropriate clothing as described in Section II when solid-phase sediment or sediment extracts are not under a hood or in a glove box.

V. Placing sediment (or sediment extracts) into test chambers:

A. Sediment will always be transferred using double containment. Sediment transfer into test chambers is a procedure which involves a potential hazard for personal contamination. During this procedure, the number of investigators in the laboratory work area should be minimized. Other workers in the building must be notified of the handling of the sediment.

B. Solid-phase sediment will be distributed into the test chambers using a spoon within the glove box or hood located in the laboratory work area. Mixing and sampling of solid-phase sediment ing will be done in the original storage container. An aliquot of the solid-phase sediment is added to each test chamber using a spoon. The solid-phase sediment aliquot in the test chamber is settled by smoothing with a spoon. Overlying water is place over the sediment for the test chamber is removed from the hood. Sediment extracts will always be handled under a hood. When the test chambers are removed from the glove box hood, or water bath, an intermediate non-breakable container must be used. The worker must use a respirator with organic vapor-acid gas filters and appropriate clothing as described in Section II when test chambers containing solid-phase sediment or sediment extracts are not under the vented water bath, hood, or in a glove box.

VI. Conducting sediment tests:

A. Hoods or incubators will be used to manipulate and solid-phase sediment and sediment extracts.

B. Water baths are covered with a vented plexiglass hood. These hoods will only be opened when: (1) transferring test chambers in and out of the water bath, (2) placing animals into the test chambers to start a test, (3) feeding the animals, or (4) during water sampling.

VII. Terminating sediment tests:

A. Removal of sediment containing test chambers from plexiglass vented hoods is a procedure which involves a potential hazard for personal and surface contamination. The number of investigators in the laboratory work area should be minimized. If the test chambers are removed from the glove box hood, or water bath, an intermediate non-breakable container must be used.

B. The worker will use a respirator and appropriate clothing as described in Section II during transfer of sediment test chambers to the glove box or hood. Sediments may need to be sieved to enumerate and observe animals.

C. All test chambers and equipment coming in contact with the sediment will be rinsed of excess sediment in the glove box or hood.

VIII. Clean-up of equipment after sediment tests:

A. Glassware and equipment coming in contact with sediment will be cleaned as soon as possible. Cleaning glassware poses an increased exposure hazard, all glassware must be cleaned under the vented sinks or hoods located in the laboratory work area.

References:

1. Dornhoffer, M.K. 1986. Handling chemical carcinogens: A safety guide for the Laboratory Researcher. Chemical Sciences Laboratories, Lenexa, KS. 62 p.

2. Federal Register, Vol. 43(247):60109-60129.

3. Castegnaro, M. and E.B. Sansone. 1986. Chemical Carcinogens. Some Guidelines for handling and disposal in the laboratory. Springer-Verlag, New York. 97 p.

4. Prudent Practices for Disposal of Chemicals from Laboratories. 1983. Committee on Hazardous Substances in the Laboratory. Commission on Physical Sciences, Mathematics, and Resources. National Research Council. National Academy Press, Washington D.C. 282 p.

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STANDARD GUIDE FOR COLLECTION, STORAGE, CHARACTERIZATION, AND MANIPULATION OF SEDIMENTS FOR TOXICOLOGICAL TESTING

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1. Scope

1.1 This guidance document describes procedures for obtaining, storing, characterizing, and manipulating saltwater and freshwater sediments, for use in laboratory sediment toxicity evaluations. It is not meant to provide guidance for all aspects of sediment assessments, such as chemical analyses or monitoring geophysical characterization, or extractable phase/fractionation analyses. Some of this information might, however, have applications for some of these activities. for guidance on toxicity test design and exposure method considerations, see Guide for Designing Biological Tests with Sediments (Draft #2) or specific sediment toxicity test methods. (see Section 2.1). Methodological considerations which affect toxicity studies will be reviewed and the apparent consensus approach for test methods discussed. Currently, the state-of the-art is in its infancy, and the development of standard methods is not feasible; however, it is crucial that there be an understanding of the significant effect which these methods have on sediment quality evaluations. It is anticipated that recommended methods and this guide will be routinely updated to reflect progress in our understanding of sediments and how to best study them.

1.2 There are several regulatory guidance documents (1-16) concerned with sediment collection and characterization procedures, which might be important for individuals performing Federal or State agency-related work. Discussion of some of the principles and current thoughts on these approaches can be found in Dickson et al., 1987 (17).

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1.3 This guide is arranged as follows:

1.4 Field collected sediments might contain potentially toxic materials and thus should be treated with caution to minimize occupational exposure to workers. Worker safety must also be considered when working with spiked sediments containing various organic or inorganic contaminants, or both; and those that are radio-labeled. Careful consideration should be given to those chemicals which might biodegrade, volatilize, oxidize, or photolyze during the test period.

1.5 This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use. Specific hazard statements are given in Section 8.

2. Referenced Documents

2.1 ASTM Standards:

D 1129	Definitions of Terms Relating to Water
D 4387	Guide for Selecting Grab Sampling Devices for Collecting Benthic Macroinvertebrates
D 4822	Guide for Selection of Methods of Particle Size Analysis of Fluvial Sediments (Manual Methods).
D 4823	Guide for Core Sampling Submerged, Unconsolidated Sediments
E 380	Practice for Using the International System of Units (SI) (the Modernized Metric System)
E 729	Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians
E 943	Definitions of Terms Relating to Biological Effects and Environmental Fate
E 1023	Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E 1367	Guide for Conducting Solid Phase 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods
E ???	Guide for Conducting Solid Phase Sediment Toxicity Tests with Freshwater Invertebrates
E 1295	Guide for Conducting Three Brood Renewal Toxicity Tests with Ceriodaphnia dubia

3. Terminology

3.1 The words "must", "should", "may", "can", and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with the factors that directly relate to the acceptability of the test. "Should" is used to state that the specified condition is recommended and ought to be met in most tests. Although a violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable", "is often desirable", and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to", "can" is used to mean "is (are) able to", and "might" is used to mean "could possibly". Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can".

3.2 Definitions. For definitions of terms used in this guide, refer to Guide E 729, Definitions E 943, and Definitions D 1129, and Guide D 4387; for an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 This guide provides a review of widely used methods to collect, store, characterize, and manipulate sediments for toxicity testing. Where the science permits, recommendations are

provided on which procedures are appropriate, while identifying their limitations.

5. Significance and Use

5.1 Sediment toxicity evaluations are a critical component of environmental quality and ecosystem impact assessments, used to meet a variety of research and regulatory objectives.

5.2 The manner in which the sediments are collected, stored, characterized, and manipulated can greatly influence the results of any sediment quality or process evaluation. Addressing these variables in a systematic and uniform manner will aid interpretations of sediment toxicity or bioaccumulation results and may allow comparisons between studies.

6. Interferences

6.1 Maintaining the integrity of a sediment environment during its removal, transport, and testing in the laboratory is extremely difficult. The sediment environment is composed of a myriad of microenvironments, redox gradients, and other interacting physicochemical and biological processes. Many of these characteristics influence sediment toxicity and bioavailability to benthic and planktonic organisms, microbial degradation, and chemical sorption. Any disruption of this environment complicates interpretations of treatment effects, causative factors, and in situ comparisons. For additional information see Section 9.

7. Apparatus

7.1 A variety of sampling, characterization, and manipulation

methods exist using different equipment. These are reviewed in Sections 9 through 14.

7.2 Cleaning: Test chambers and equipment used to prepare and store dilution water and stock solutions should be cleaned before use. New and used sample containers should be washed following these steps: (1) non-phosphate detergent wash, (2) triple water rinse, (3) water-miscible organic solvent wash, (acetone followed by pesticide grade hexane (2,8)), (4) water rinse, (5) acid wash (such as 5% concentrated hydrochloric acid), and (6) triple rinse with deionized-distilled water. Altering this cleaning procedure might result in problems. Many organic solvents might leave a film that is insoluble in water (Step 3). A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid (Steps 3 through 5), but it might attack silicone adhesive. (See 9.10 for cleaning during sample collection.)

8. Safety Hazards

8.1 Many substances can adversely affect humans if adequate precautions are not taken. Information on toxicity to humans (18) and recommended handling procedures of toxicants (19) should be studied before tests are begun with any contaminant or sediment. Health and safety precautions should be considered before beginning a test.

8.2 Field collected sediments might contain a mixture of hazardous contaminants and/or disease causing organisms such that proper handling to avoid human exposure is important. Therefore, skin contact with all test materials and solutions should be

minimized by such means as wearing appropriate protective gloves especially when washing equipment or putting hands in dilution water over sediments, or into sediments. Proper handling procedures might include: 1) sieving and distributing sediments under a ventilated hood or an enclosed glove box, 2) enclosing and ventilating the toxicity test water bath, and 3) using respirators, aprons, safety glasses, and gloves when handling potentially hazardous sediments. Special procedures might be necessary with radiolabeled test materials (20) and with materials that are, or are suspected of being, carcinogenic (19).

8.3 Disposal of sediments, dilution water over sediments, and test organisms containing hazardous compounds might pose special problems. For tests involving spiking sediments with known toxicants, removal or degradation of the toxicant(s) before disposal is sometimes desirable. Disposal of all hazardous wastes should adhere to the requirements and regulations of the Resource Conservation and Recovery Act and any relevant State or local regulations.

9. Sampling and Transport

9.1 Sediments have been collected for a variety of chemical, physical, toxicological and biological investigations. These collections have been made with both a series of grab sampling devices and core samplers (See Table 2, Guide D 4823). The advantages and disadvantages of the various collection methods have been previously reported (3,4) and are summarized in Table 1. All sampling methods disturb the sediment integrity to a degree. For purposes of sediment toxicity evaluations it is

important to obtain sediments with as little disruption as possible, to allow for realistic laboratory evaluations of in situ conditions. Choosing the most appropriate sediment sampler for a study will depend on the sediments characteristics, the efficiency required, and the study objectives. Several references are available which discuss the various collection devices (3,4,21,22,23). The efficiency of these samplers for benthic collections have been compared and in general the grab samplers are less efficient collectors than the corers but are easier to handle, work in heavier seas, often require fewer personnel and are more easily obtained (21,23-31).

9.2 The principal disadvantage of dredge samplers varies; common problems are shallow depth of penetration and presence of a shock wave that results in loss of the fine surface sediments. Murray and Murray (32), however, described a dredge usable in heavy seas which quantitatively samples the top 1 cm of sediment and retains fine materials. Other grab samplers that quantitatively sample surface sediments have been described by Grizzle (33). The depth profile of the sample may be lost in the removal of the sample from the sampler. Dredge sampling promotes loss of not only fine sediments, but also water soluble compounds and volatile organic compounds present in the sediment.

9.3 Studies of macroinvertebrate sampling efficiency with various grab samplers have provided useful information for sampling in sediment toxicity and sediment quality evaluations. The Ekman dredge is the most commonly used sampler for benthic investigations (21). The Ekman's efficiency is limited to less compacted, fine-grained sediments, as are the corer samplers.

The most commonly used corer is the Kajak-Brinkhurst corer. In more resistant sediments the Petersen, PONAR, and Smith-McIntyre dredges are used most often (21). Based on studies of benthic macroinvertebrate populations, the sediment corers are the most accurate samplers, followed by the Ekman dredge, in most cases (21). For resistant sediments, the PONAR dredge was the most accurate and the Petersen the least (21). A comparison of sampler precision showed the van Veen sampler to be the least precise; the most precise were the corers and Ekman dredge (21).

9.4 Many of the problems associated with dredge samplers are largely overcome with the corers. The best corers for most sediment studies are hand-held polytetrarfluoroethylene plastic, high density polyethylene, or glass corers (liners), or large box-corers. The corers can maintain the integrity of the sediment surface while collecting a sufficient depth. Furthermore, the box core can be sub-cored or sectioned at specific depth intervals, as required by the study. The box corer, unfortunately, is large and cumbersome; thus, it is difficult to use. Other coring devices which have been successfully used include the percussion corer (34) and vibratory corers (35-37).

9.5 Corer samplers also have several limitations. Most corers do not work well in sandy sediments; dredge samplers or diver-collected material remain the only current alternatives. In general, corers collect less sediment than dredge samplers which may provide inadequate quantities for some studies. Small cores tend to increase bow waves (that is, disturbance of surface sediments) and compaction, thus altering the vertical profile.

However, these corers provide better confidence limits and spatial information when multiple cores are obtained (21,24,38-41). As shown by Rutledge and Fleeger (42) and others, care must be taken in subsampling from core samples, since surface sediments might be disrupted in even hand-held core collection. They recommend subsampling in situ or homogenizing core sections before subsampling.

9.6 Studies of sediment toxicity, interstitial waters, microbiological processes, or chemical fate probably will require core sampling to best maintain the complex integrity of the sediment. When obtaining cores from shallow waters one must ensure that the vessel does not disturb the sediments prior to sampling (30). Most of the studies in the literature employed grab samplers although box corers (43-45), gravity corers (46) and hand collection (47-49) methods are reported with increasing frequency. For additional information of various core types see reference USEPA (4).

9.7 Subsampling, compositing, or homogenization of sediment samples is often necessary and the optimal methods will depend on the study objectives. Important considerations include: loss of sediment integrity and depth profile; changes in chemical speciation via oxidation and reduction or other chemical interactions; chemical equilibrium disruption resulting in volatilization, sorption, or desorption; changes in biological activity; completeness of mixing; and sampling container contamination. In most studies of sediment toxicity, it is advantageous to subsample the inner core area (not contacting the sampler) since this area is most likely to have maintained its

integrity and depth profile and not be contaminated by the sampler. Subsamples from the depositional layer of concern, for example, the top 1 or 2 cm should be collected with a nonreactive sampling tool, such as, a polytetrafluoroethylene lined calibration scoop (50). Samples are frequently of a mixed depth but a 2 cm sample (51) is the most common depth obtained, although depths up to 40 ft have been used in some dredging studies. For some studies it is advantageous or necessary to composite or mix single sediment samples (16,50). Composites usually consist of three to five grab samples. Subsamples are collected with a nonreactive sampling scoop and placed in a nonreactive bowl or pan. The composite sample should be stirred until texture and color appear uniform.

9.8 Due to the large volume of sediment which is often needed for toxicity or bioaccumulation tests and chemical analyses, it might not be possible to use subsampled cores because of sample size limitations. In those situations, the investigator should be aware of the above considerations and their possible affect on test results as they relate to in situ conditions.

9.9 Assessment of in situ sediment toxicity or bioaccumulation is aided by collection and testing of reference and control samples. For purposes of this guide, a reference sediment is defined as a sediment possessing similar characteristics to the test sediment but without anthropogenic contaminants. Sediment characteristics, such as particle size distribution and percent organic carbon, should bracket that of the test sediment. If there is a wide range of test sediment

types, the reference sediment characteristics should be in an intermediate range unless the test species is affected by particle size. The appropriate ASTM guides for marine and freshwater invertebrates should then be consulted to determine the particle size requirements of the test species. It is preferable that reference sediments be collected from the same aquatic system, located close to, and have similar physical, chemical, and biological characteristics to the test sediment. In some situations, the reference sediment might be toxic due to naturally occurring chemical, physical, or biological properties. For this reason, it is important to also test the toxicity of control sediments. The reference sediment test results might be analyzed as either a treatment or as a control variable, depending on the study objectives. For purposes of this guide, a control sediment might consist of natural or artificially prepared sediments of known composition and of consistent quality that have been used in prior sediment toxicity tests or culturing, and for which baseline data exists which shows they do not cause toxicity. Control sediments have been successfully used in toxicity evaluations (52).

9.10 When collecting sediment grab samples, it is important to clean the sampling device, scoop, spatula, and mixing bowls between sample sites. The cleaning procedure can follow that outlined in Section 7 or the following (53): 1) soap and water wash, 2) distilled water rinse, 3) methanol rinse, 4) methylene chloride rinse, and 5) site water rinse. Waste solvents should be collected in labelled hazardous waste containers.

9.11 In most cases the transport conditions for the samples were not specified in the references reviewed. Where conditions were specified, the sediments were usually transported whole, in both plastic, polyethylene (54-56), and glass (48,49,57) containers and transported under refrigeration or on ice (48,49,51,57-62).

9.12 Collection, transport, storage, and test chamber material composition should be chosen based on a consideration of sorption effects, sample composition, and contact time. For example, in sediments where organics are of concern, brown borosilicate glass containers with Polytetrafluoroethylene (PTF) lid liners are optimal, while plastic containers are recommended for metal samples. PTF or high density polyethylene containers are relatively inert and optimal for samples contaminated with multiple chemical types. Additionally, polycarbonate containers have been shown not to sorb metal species (63). Additional information on sample containers, preservation, storage times and volume requirements, in regards to chemical analyses, are available in other guidance documents (3-6,10,16). In many cases these criteria are applicable to toxicity test chamber requirements.

10. Storage

10.1 Containers for storage were generally not specified although it was assumed that the containers were the same as the transport containers, where specified, and were generally polyethylene (see 9.12). Where sediments contain volatile compounds, transport and storage should be in air tight PTF or

glass containers with PTF-lined screw caps. For further information on storage requirements for chemical analyses see Table 2.

10.2 Drying, freezing, and cold storage conditions all affect toxicity (17,64-69). Often the storage time of sediments used in toxicity tests was not specified and where specified ranged from a few days (70) to one year (55). Storage of sediments after arrival at the laboratory was generally by refrigeration at 4 C (54-56,58-62,67,70-73). Significant changes in metal toxicity to cladocerans and microbial activity have been observed in stored sediments (68,74). Recommended limits for storage of metal-spiked sediments have ranged from within 2 days (64) to 5 days (70) to 7 days (75,76). A study of sediments contaminated with nonpolar organics found that interstitial water storage time did not affect toxicity to polychaetes when samples were frozen (77). Cadmium toxicity in sediments has been shown to be related to acid volatile sulfide (AVS) complexation (78). When anoxic sediments were exposed to air, AVS were rapidly volatilized. AVS is apparently the reactive solid phase sulfide pool that binds metal, thus reducing toxicity. If a study objective is to investigate metal toxicity and the sediment environment is anoxic, then exposure to air might reduce or increase toxicity due to oxidation and precipitation of the metal species or loss of acid volatile sulfide complexation. It is generally agreed that sediments to be used for toxicity testing should not be frozen (17,67,69,70,75,79).

10.3 Although risking changes in sediment composition, several studies elected to freeze samples (51,67,80-84). Fast-

freezing of sediment cores has been recommended for chemical analyses; however, this alters sediment structure and profile distortion occurs (42). Freezing has been reported to inhibit oxidation of reduced iron and manganese compounds (81). It has also been recommended for stored sediments which are to be analyzed for organics and nutrients (85).

10.4 Interstitial water chemistry changed significantly after 24 h storage (86,87), even when stored at in situ temperatures (87). Coagulation and precipitation of the humic material was noted when interstitial water was stored at 4 C for more than one week (88). Oxidation of reduced arsenic species in pore water of stored sediments was unaffected for up to 6 weeks when samples where acidified and kept near 0 C, without deoxygenation. When samples were not acidified, deoxygenation was necessary (89).

10.5 In summary, sediments for toxicity tests and chemical analyses are typically refrigerated or placed on ice in polyethylene containers during transport. If, in addition, samples are to be used for chemical analyses, then the appropriate container should be used as described above. The storage conditions should be refrigeration at 4 C and under anoxic conditions if appropriate (10,16,90). It has been shown that sediments can be stored at 4 C for up to 12 months without significant alterations in toxicity (91). Limits to storage time before testing, therefore, appear to be a function of both sediment and contaminant characteristics. While it is prudent to complete the testing of sediments with a minimum of storage time

(probably less than 2 weeks) this may not be possible for any number of reasons.

11. Collection of Interstitial Water

11.1 Isolation of sediment interstitial water can be accomplished by several methods: centrifugation, squeezing, suction, and equilibrium dialysis. In general, methods for recovery of relatively large volumes of interstitial water from sediments are limited to either centrifugation (57,88,92,93) or squeezing (94-97). Other methods, such as suction (98), gas pressurization (50), in situ samplers (99), and equilibration by using dialysis membrane or a fritted glass sampler (100-103), do not produce large quantities of interstitial water. In the case of the dialysis, sufficient time must be allowed to ensure that the sample has come to equilibrium with the interstitial water. The suction and dialysis equilibrium methods are most useful for laboratory studies. Some pore water constituents, for example, dissolved organic carbon or dimethylsulfide, might significantly affected by the collection method (99). Other constituents, such as, salinity, dissolved inorganic carbon, ammonia, sulfide, and sulfate, might not be affected by collection methods providing oxidation is prevented (99). If sediments are anoxic, all steps involved in sample processing might need to be conducted in inert atmospheres to prevent oxidation of reduced species (99,104,105).

11.2 If interstitial water is collected by centrifugation and filtration, then effects on the interstitial chemistry need to be considered after centrifugation. Centrifugation followed by 2μm filtration yielded similar metal concentrations to dialysis methods

(106). However, filtration with glass fiber or plastic filters is not appropriate in some cases and has been shown to remove nonpolar organics (107). Centrifugation at 7600 x g with glass contact only was shown to be superior to filtration methods (107). Other studies have produced contrary results, recommending filtration with polycarbonate filters (98,108). Filtration is normally conducted to remove particles with a 0.45 μm pore size, however 0.20 μm or smaller pore size membranes have been recommended (81). Removal of all bacteria and colloidal materials might require filter pore sizes of less than 0.2 μm. Immediate collection of interstitial water is recommended since chemical changes might occur even when sediments are stored for short periods at in situ temperatures (87) (see 10.4).

12. Characterization

12.1 The characteristics that have been most often measured in sediments are moisture content, organic carbon or volatile matter content, and particle size. When attempting to characterize a sediment, quality assurance should always be addressed (3,4,16). Sediments, by their nature, are very heterogenous; they exhibit significant temporal and spatial heterogeneity in the laboratory and in situ. Replicate samples should be analyzed to determine the variance in sediment characteristics and analytical methods. Sediment characterization will depend on the study objectives and the contaminants of concern, however, a minimum set of characteristics should be included which are known to influence toxicity and will aid data interpretation: in situ temperature, particle size distribution, moisture or interstitial water content,

ash free weight, organic carbon (determined by titration or combustion), pH, Eh, acid volatile sulfides, ammonia, and cation exchange capacity. Many of the methods of characterization have been based on analytical techniques for soils and waters and the literature should be consulted for further information (15,23,109,110).

12.2 The moisture content of sediments is measured by drying the sediments at 50 to 105°C to a constant weight (23).

12.3 Volatile matter content is often measured instead of, and in some cases in addition to, organic carbon content as a measure of the total amount of organic matter in a sample. This measurement is made by ashing the sediments at high temperature and reporting the percent ash free dry weight (7,111,112). Although the exact method for ashing the sample is often not specified, the normally accepted temperature is 550 ± 50 C (16,23).

12.4 Carbon fractions which may be of importance in determining toxicant fate and bioavailability include: total organic carbon (16,113-115), dissolved organic carbon (88), dissolved inorganic carbon, sediment carbonates, and reactive particulate carbon (116,117). Reactive particulate carbon is that portion which equilibrates with the aqueous phase. The organic carbon content of sediments has been measured by wet oxidation which is also useful for the determination of the organic carbon content of water (118). Organic carbon analyses have also been conducted by titration (119), modification of the titration method (120), or combustion after removal of carbonate by the addition of HCl and subsequent drying (73).

12.5 Particle sizing of sediments can be measured by numerous methods (15,121, see Guide D 4822) dependent on the particle properties of the sample (122). Particle size distribution is often determined by wet sieving (2,15,16,23,123). Particle size classes might also be determined by the hydrometer method (124,125), the pipet method (15,126), settling techniques (127), X-ray absorption (123,126) and laser light scattering (128). The pipet method may be superior to the hydrometer method (129). To obtain definite particle sizes for the fine material, a Coulter (particle size) counter method might be employed (130,131). This method gives the fraction of particles with an apparent spherical diameter. Another potential method for determining the particle size distribution of a very fine fraction is through the use of electron microscopy (132). The collection technique for the very fine materials can result in aggregation to larger colloidal structures (132-135). Comparisons of particle sizing methods have shown that some produce similar results and others do not. These differences might be attributed to differences in the particle property being measured, that is, the Malvern Laser Sizer and Electrozone Particle Counter are sizing techniques, and the hydrophotometer and SediGraph determine sedimentation diameter based on particle settling (122,136-138). It is preferable to use a method which incorporates particle settling as a measure, as opposed to strictly sediment sizing.

12.6 Various methods have been recommended to determine bioavailable fractions of metals in sediments (78,139-141). One extraction procedure, cation exchange capacity, provides information relevant to metal bioavailability studies (109). Amorphic oxides of iron and manganese, and reactive particulate carbon have been

implicated as the primary influences on metal sorption potential in sediments (81,140,142-144). Measurement of acid volatile sulfide (AVS) and divalent metal concentrations associated with AVS extraction provides insight into metals availability in anaerobic sediments (78). Easily extractable fractions are usually removed with cation displacing solutions, for example, neutral ammonium acetate, chloride, sodium acetate, or nitrate salts (145). Extraction of saltwater or calcareous sediments, however, is often complicated by complexation effects or dissolution of other sediment components (141,146). Other extractants and associated advantages and disadvantages have been recently discussed (141,144,147,148). Some extractants which have been successfully used in evaluations of trace metals in nondetrital fractions of sediments are EDTA or HCl (141,149,150). Metal partitioning in sediments might be determined by using sequential extraction procedures which fractionate the sediments into several components such as interstitial water, ion exchangeable, easily reducible organic and residual sediment components (93,148,151,152). Unfortunately at this time no one method is clearly superior to the others (147). This might be due, in part, to site specific characteristics which influence bioavailability, for example, desorption and equilibration processes.

12.7 pH is important for many chemicals and can be measured directly (23) or in a 1 to 1 mixture of sediment/soil to water (153).

12.8 Eh measures are particularly important for metal speciation and for determining the extent of sediment oxidation. Redox gradients in sediments often change rapidly over a small depth

and are easily disturbed. Care must be taken in probe insertion to allow equilibration to occur when measuring Eh. These measurements are potentiometric and measured with a platinum electrode relative to a standard hydrogen electrode (23).

12.9 Biochemical oxygen demand and chemical oxygen demand might provide useful information in some cases (23). Sediment oxygen demand might also be a useful descriptor; however, a wide variety of methods exist (90,154-157).

12.10 Analysis of toxicants in sediments is generally performed by standard methods such as those of the EPA (2,23). Soxhlet extraction is generally best for organics but depends on extraction parameters (158,159). Concentrations are generally reported on a dry weight basis.

13. Manipulation

13.1 Manipulation of sediments is often required to yield consistent material for toxicity testing and laboratory experiments. The manipulations reviewed in this section are: spiking (dosing) regimes for laboratory and control sediments; mixing; sieving for attainment of maximal particle sizes; dilutions for concentration-effect determinations; elutriates; capping; air drying; and sterilization. For discussion of subsampling, compositing, or homogenization effects see 9.7.

13.2 Spiking -- The spiking method to be used is contingent on the study objectives. For example, when attempting to mimic in situ conditions, sediment cores should be spiked by adding aqueous or suspended sediment solution of toxicants to the overlying water column; or when investigating dredging effects or conditions of

sediment perturbation where toxicant sorption processes are accelerated, mixing toxicants into sediment slurries may be advantageous. When investigating the source of sediment toxicity or interactive effects of sediment toxicants, it is useful to spike both reference and control sediments with the toxicant of concern present in the test sediment. Mixing time should be limited to a few hours and temperatures kept to a minimum, due to the rapid alterations which occur in the sediment's physicochemical and microbiological characteristics, which thereby alter bioavailability and toxicity. Recalcitrant organics and some metals, for example, cadmium and copper, might be mixed for extended periods without adverse effects (see 9 through 12 for additional discussion).

13.3 Organic compounds are generally added via a carrier solvent such as acetone or methanol to ensure that they are soluble and that they remain in solution during mixing. While organic compounds are generally added in an organic carrier, metals are generally in aqueous solutions. Compounds are also added to water overlying sediments and the compound allowed to sorb with no mixing (71,160-167). Occasionally the carrier has been added directly to sediment (52,82-84,112,137,168-171) and the carrier evaporated before addition of water. This approach does not seem to result in compounds being sorbed to sediment at the same sites as dosing under aqueous conditions (172). Word et al. (107) compared several sediment-labelling techniques using methylene chloride, ethanol, and glycine as carriers. They found glycine was superior when mixed with sediment for 7 days. In most cases, the compound is either coated on the walls of the flask and an aqueous slurry (sediment and water in various proportions) added, or the carrier containing

mixture is added directly to the slurry. When the sediment to water ratio is adjusted for optimal mixing, sediments that are too dense to mix by slurrying in water have been successfully mixed using a rolling mill (72). Other mixing techniques may be used for spiking specific sediments but care should be taken to ensure complete mixing and analyses of spiked compounds run to ensure that labelling is uniform in the mixed material. The use of a polar, water soluble carrier such as methanol has little effect on the partitioning of nonpolar compounds to dissolved organic matter at concentrations up to 15% carrier by volume (173). Another study, however, shows that changes in partitioning of a factor of approximately two, might well occur with 10 % methanol as a cosolvent for anthracene sorption (174). Thus, caution should be taken to minimize the amount of carrier used. The time between the spiking of the compounds and the use of the test sediment has been variable (46,47,70,72,73,80,111, 168,175) and does seem to effect the biological availability of compounds (37,67,175).

13.4 Highly volatile compounds have been spiked into sediments in a similar manner to the less volatile materials using cosolvents and mixing in an aqueous slurry by shaking. These experiments were tested immediately in covered flow through systems (108).

13.5 If a solvent other than water is used, both a sediment solvent control and a sediment negative control or reference sediment; or both, must be included in the test. The solvent control must contain the highest concentration of solvent present and must use solvent from the same batch used to make the stock solution (see Practice E 729).

13.6 Because the organic carbon content of the sediments might be one of the most important characteristics affecting the biological availability of contaminants, modifications of the carbon content have been made in many studies. Methods used include dilution with clean sand (55,56,62,108); although humics (170) and other organics such as sheep manure (52) have also been added. Such dilutions also change the particle composition and the size distribution of the particles; thus, results from such experiments should be interpreted with care. The organic carbon content has also been altered by the use of combustion (14,52). Combustion may alter the type of carbon as well as oxidize some of the inorganic components thus altering greatly the characteristics of the sediment.

13.7 A variety of methods have been used to spike sediments with metals. The two principal categories of methods are 1) metal addition directly to the sediment which is mixed and then water added (64,68,176-178), or 2) addition of the metal to the overlying waters (80,166,179,180). Thorough mixing of spiked sediments has been accomplished using the rolling mill tecnique, Eberbach and gyr rotary shakers.

13.8 Equilibration and mixing conditions vary widely in spiking studies. The duration of contact between the toxicant and sediment particles can affect both the partitioning and bioavailability of the toxicant. This effect apparently occurs because of an initial rapid labile sorption followed by movement of the toxicant into resistant sorption sites or in the particle (181-183). Because of the kinetically controlled changes in the partitioning that results in changes in bioavailability (175,184,

185), the contact time can be important when spiking sediments. Bounds on the sorption time can be estimated from the partition coefficient for the sediment following the calculations in Karickhoff and Morris (182). In addition, it is important to recognize that the quantity of toxicant spiked might exceed the complexation capacity of the test sediment system and not allow reactions to attain equilibrium. These phenomenon will complicate test result interpretation (68,147).

13.9 Mixing and sieving are two other manipulations of sediments that are often performed before toxicity testing (46,52,58-60,67,70,72,111,112,163,168,170,175,186). Sediment samples have been sieved for a variety of reasons including the removal of large debris and stones thereby increasing the samples homogeneity and method replicability; the increased ease of counting organisms; the increased sediment handling and subsampling; the ability to study influence of particle size on toxicity, bioavailability, or contaminant partitioning. Sieving of material to a specific size fraction might alter the concentration of contaminant in the sediment by removing large, low sorptive materials.

13.10 Toxicants and organic carbon concentrations tend to be higher with fine grained sediments (that is, clay and silt) due to increased surface area (in relation to the weight of the sample) and sorptive capacity. Measuring size fractions of less than 63 μm has been recommended in contaminant studies, particularly for metals (172,187). In studies of sediment metal concentrations, normalizing to the less than 63 μm size fraction was superior for describing metal binding in sediments, as compared to sediment concentrations

normalized to dry weight, by organic carbon content, or corrected by a centrifugation procedure (172). Small size fractions are characteristic of depositional areas in aquatic systems; however, sieving of sediments from non-depositional sites to obtain a fine fraction might significantly alter the sediment characteristics. The usual sieve size for toxicity testing is greater than 500 μm. If sieving is performed it should be done for all samples to be tested including control and reference sediments.

13.11 Mixing of various layers of sediments might result in either dilution or enhancement of concentrations. The sediment quality will be influenced by the depth of sampling, depth of biological activity, contaminant solubility and partitioning characteristics, and depth of the contaminant concentration peak which is dependent on historical contamination and sedimentation rates for the study site. see Section 10 for additional relevant discussion.

13.12 Another manipulation of sediments for toxicity testing is sediment dilution. In order to obtain concentration-effect information in solid phase sediment toxicity evaluations, differing concentrations of the test sediment should be used. Currently, there is little information available on the most appropriate method for diluting test sediments to obtain a graded contaminant concentration or concerning the methodological effects of such a dilution. A "clean" noncontaminated sediment should be used as the "diluent" which optimally consists of physicochemical characteristics similar to the test sediment, such as organic matter/carbon, particle size, but does not contain elevated (above background) levels of the toxicants of concern. Refer to the

preceding sections for relevant information.

13.13 Many studies of sediment toxicity have been conducted on the elutriate or water-extractable phase (188). This method was developed to assess the effects of dredging operations on water quality. Sediments are shaken in site or reconstituted water (1 to 4 volume to volume ratio) for 30 min. The water phase is then separated from the sediment by centrifugation, followed by filtration of the supernatant through a 0.45 μm filter when conducting some tests, such as algal growth assays. The filtration step may be removed depending on the study objectives (see Section 11 for interferences).

13.14 Sediment pollution remediation alternatives might include capping the contaminated sediments with "clean" sediments. Laboratory design of such experiments should vary the depth of both the contaminated sediments and the capping sediment layers to evaluate contaminant transport via physicochemical and biological (bioturbation) processes.

13.15 Sometimes sediments have been air dried before use (56,168,189,190) but these sediments have generally been used for laboratory studies after some additional manipulation, such as spiking sediments with various levels of contaminants for concentration-effect data (111,190). Air drying would result in losses of volatile compounds and might result in changes in the sediment characteristics, particularly particle size (see Section 10). The presence of air and air drying have all been shown to change metal availability and complexation (141).

13.16 Sterilization of sediments to inhibit biological activity has been performed in some studies. Autoclaving is used in

most cases (191). Other sterilization techniques have included: antibiotic addition, addition of chemical inhibitors such as HgCl or sodium azide, or gamma irradiation. The technique chosen should be contingent on study objectives. Antibiotics, such as streptomycin and ampicillin, have been successfully used in sediment studies (192,193). Some antibiotics, however, are labile and light sensitive, or readily bind to organic matter. Mercuric chloride appears to be superior to sodium azide as a bacteriocide. Autoclaving is the least desirable method as it causes the greatest alteration to the sediments physical and chemical characteristics. In studies requiring sterility, it is crucial that a sterility control be incorporated.

14. Quality Assurance

14.1 Quality assurance guidelines (3,4,10,16) should be followed. Quality assurance considerations for sediment modeling, QA-QC plans, statistical analyses (for example, sample number and location) and sample handling have been addressed in-depth (10).

14.2 Sediment heterogeneity significantly influences studies of sediment quality, contaminant distribution, and both benthic invertebrate and microbial community effects. Spatial heterogeneity might result from numerous biological, chemical, and physical factors and should be considered both horizontally (such as, the sediment surface) and vertically (that is, depth). Accumulation areas with similar particle size distributions might yield significantly different toxicity patterns when subsampled (79,194); therefore, an adequate number of replicates should be processed to determine site variance. When determining site variance one should

consider within sample (that is, subsample) variance, analytical variance (for example, chemical or toxicological), and the sampling instruments' accuracy and precision. After these considerations a sampling design can be constructed which addresses resource limitations and study objectives.

14.3 As stated in previous sections, the methodological approach used, such as, number of samples, will be dependent on the study objectives and sample characteristics. For information on sediment heterogeneity, splitting, compositing, controls, or determining sample numbers, sampler accuracy and precision, and resource requirements, there are a number of references available (4,10,21,85,172,195,196).

15. Report

15.1 Documentation: The record of sediment collection, storage, handling, and manipulation should include the following information either directly or by reference to existing documents. Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

15.1.1 Name of test and investigator(s), name and location of laboratory, and dates of starting and ending of sampling and sediment manipulation;

15.1.2 Source of control, reference or test sediment, method for handling, storage and disposal of sediment;

15.1.3 Source of water, its chemical characteristics, and a description of any pretreatment;

15.1.4 Methods used for, and results (with confidence limits) of, physical and chemical analyses of sediment; and

15.1.5 Anything unusual about the study, any deviation from these procedures, manipulations, and any other relevant information.

TABLE 1 (continued). Summary of Bottom Sampling Equipment

Device	Use	Advantages	Disadvantages
PONAR Grab Sampler	Deep lakes, rivers, and estuaries. Useful on sand, silt, or clay.	Most universal grab sampler. Adequate on most substrates. Large sample obtained intact, permitting subsampling.	Shock wave from descent may disturb "fines". Possible incomplete closure of jaws results in sample loss. Possible contamination from metal frame construction. Sample must be further prepared for analysis.
BMH-53 Piston Corer	Waters of 4-6 feet deep when used with extension rod. Soft to semi-consolidated deposits.	Piston provides for greater sample retention.	Cores must be extruded on site to other containers - Metal barrels introduce risk of metal contamination.
Van Veen	Deep lakes, rivers, and estuaries. Useful on sand, silt, or clay.	Adequate on most substrates. Large sample obtained intact, permitting subsampling.	Shock wave from descent may disturb "fines". Possible incomplete closure of jaws results in sample loss. Possible contamination from metal frame construction. Sample must be further prepared for analysis.
BMH-60	Sampling moving waters from a fixed platform	Streamlined configuration allows sampling where other devices could not achieve proper orientation.	Possible contamination from metal construction. Subsampling difficult. Not effective for sampling fine sediments.
Petersen Grab Sampler	Deep lakes, rivers, and estuaries. Useful on most subatrates.	Large sample; can penetrate most subatrates.	Heavy, may require winch. No cover lid to permit subsampling. All other disadvantages of Ekman and Ponar.
Shipek Grab Sampler	Used primarily in marine waters and large inland lakes and reservoirs.	Sample bucket may be opened to permit subsampling. Retains fine grained sediments effectively.	Possible contamination from metal construction. Heavy, may require winch.

TABLE 1 (continued). Summary of Bottom Sampling Equipment

Device	Use	Advantages	Disadvantages
Orange-Peel Grab Smith-McIntyre Grab	Deep lakes, rivers, and estuaries. Useful on most substrates.	Designed for sampling hard substrates.	Loss of fines. Heavy - may requires winch. Possible metal contamination.
Scoops, Drag Buckets	Various environments depending on depth and substrate.	Inexpensive, easy to handle.	Loss of fines on retrieval through water column.

(modified, 193)

[a] Comments represent subjective evaluations.

TABLE 2. Sampling Containers, Preservation Requirements, and Holding Times for Sediment Samples[a] (EPA, 196,197). See also Rochon and Chevalier (160).

Contaminant	Container[b]	Preservation	Holding Time
Acidity	P,G	Cool, 4C	14 days
Alkalinity	P,G	Cool, 4C	14 days
Ammonia	P,G	Cool, 4C	28 days
Sulfate	P,G	Cool, 4C	28 days
Sulfide	P,G	Cool, 4C	28 days
Sulfite	P,G	Cool, 4C	48 h
Nitrate	P,G	Cool, 4C	48 h
Nitrate-Nitrite	P,G	Cool, 4C	28 days
Nitrite	P,G	Cool, 4C	48 h
Oil and Grease	G	Cool, 4C	28 days
Organic Carbon	P,G	Cool, 4C	28 days
Metals			
Chromium VI	P,G	Cool, 4C	40 h
Mercury	P,G		8 days
Metals except above	P,G		6 months
Organic Compounds			
Extractables (including phthalates, atrosamines organochlorine pesticides, PCB's artroaromatics, isophorone, Polynuclear aromatic hydrocarbons, haloethers, chlorinated hydrocarbons and TCDD)	G, teflon-lined cap	Cool, 4C	7 days (until extraction) 30 days (after extraction)
Extractables (phenols)	G, teflon-lined cap	Cool, 4C	7 days (until extraction) 30 days (after extraction)
Purgables (halocarbons and aromatics)	G, teflon-lined septum	Cool, 4C	14 days
Purgables (acrolein and acrylonitrate)	G, teflon-lined septum	Cool, 4C	3 days
Orthophosphate	P,G	Cool, 4C	48 h
Pesticides	G, teflon-lined cap	Cool, 4C	7 days (until extraction) 30 days (after extraction)
Phenols	P,G	Cool, 4C	28 days
Phosphorus (elemental)	G	Cool, 4C	48 h
Phosphorus, total	P,G	Cool, 4C	28 days
Chlorinated organic compounds	G, teflon-lined cap	Cool, 4C	7 days (until extraction) 30 days (after extraction)

[a] Taken from EPA 600-4-84-075 and EPA 600-4-85-048, see also Ref. 85.
[b] Polyethylene (P) or Glass (G)

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FIELD-COLLECTED SEDIMENT ELUTRIATE PREPARATION

I. General:

This SOP describes the procedures for homogenizing stored sediment samples and preparation of sediment elutriate samples for toxicity testing. Sediment is often a storage reservoir for many contaminants introduced into surface waters. These contaminants may include polychlorinated biphenyls, polynuclear aromatic compounds and inorganic contaminants including heavy metals. Contaminants present in sediment may include carcinogens, mutagens, or potentially toxic compounds. Toxicity tests will be started before chemical analyses can be completed in most cases. Since field sediments may contain potentially toxic materials they should be treated with caution to minimize occupational exposure to workers.

II. Safety.

A. Personal precautions.

1. Workers must always be aware of possible points of contamination as described by the supervisor. Hands should always be kept away from the eyes and mouth. After completion of a manipulation involving sediment or the removal of possibly contaminated laboratory clothing (gloves, lab coat, etc.), the hands, forearms, and other areas of suspected contact should be washed with hand soap and water at a sink located within the laboratory work area. Do not use organic solvents to clean the skin. These solvents may increase penetration of the contaminant into the skin.

B. Containment devices.

1. All work with sediment will be performed in an appropriate containment device. Procedures involving sediment will not be conducted on an open bench due to the potential hazard of generating contaminated dusts, aerosols, or fumes. Hoods, glove boxes, and enclosed vented water baths for testing and rooms equipped with once pass ventilation are used to minimize the worker exposure to contaminants associated with sediment. All containment devices will be constructed out of smooth, unbreakable material, such as Teflon^{R}, stainless steel, polyethylene, fiberglass, or plexiglass.

C. Work surfaces.

1. All work surfaces potentially exposed to sediments must be covered with Teflon^{R} sheets, plastic trays, dry absorbent plastic-backed paper, foil, or other impervious or disposable material. If a surface becomes contaminated or if a spill occurs, the work surface should be decontaminated or disposed of immediately.

III. Storage of sediment

A. Solid-phase sediment and sediment elutriates and extracts.

1. Solid-phase sediment and sediment elutriates and extracts will be stored at 4°C in air-tight containers in the dark.

a. All samples must be accompanied with proper identification and sample tracking information and can be temporarily stored at 4°C in refrigerators located in the laboratory work areas.

IV. Homogenization of sediments

A. Sediment homogenization or manipulation increases the chances for occupational exposure. During sediment homogenization or other manipulations, the number of investigators in the laboratory work area should be minimized. Other workers in the building must be notified of the handling of the sediment.

B. All mixing of solid-phase sediment or preparation of sediment extracts or elutriates will be performed either in a fume hood or while wearing the appropriate clothing and respiratory protective equipment. If the containers holding sediment are removed from the hood, an intermediate non-breakable container must be used.

V. Elutriate preparation

A. Required equipment.

1. Balance capable of weighing at least 1500 ± .01 grams.

2. Polypropylene centrifuge bottles.

3. Modified 60 cc polypropylene disposable syringes.

a. Remove tip from syringe barrel.

b. Drill a 3/8 inch opening at end of barrel.

c. Wash plunger and barrel in soap and water, rinse with well water, rinse with 10% HCl followed by 3 D.I. water rinses.

4. Elutriate mixing apparatus.

a. The elutriate mixing apparatus consists of a 1/10 HP, 14 rpm, shaded pole gear motor (Dayton Model 3M136A) supported horizontally by a metal frame constructed of 1 inch square tubing. The motor drive is attached via a flexible bushing to the end of a stainless steel box measuring 31 x 23 x 18 cm. The top of the box is removable and secured to the box with two wing-nuts. The interior of the box is divided into 6 compartments measuring 10 x 10 x 18 cm. Each compartment accepts one 1000 mL polypropylene centrifuge bottle. The motor rotated the stainless steel box end over end on two lubricated pillow block bearing assemblies.

5. Large volume centrifuge.

a. The centrifuge is an International Equipment Company Model PR-7000, refrigerated, large capacity centrifuge equipped with the Model 966 rotor. Maximum Relative Centrifugal Force with the 966 rotor is 7400 x G at 6900 rpm.

B. Temperature of manipulations.

1. Sediment samples for elutriate preparation will be taken immediately after homogenization. All manipulations will be done at room temperature (~20°C) except for centrifugation which will be performed at ~4°C.

C. Method.

1. Preparing elutriates with 1000 mL centrifuge bottles.

a. Individually weigh 10 - 1000 mL. centrifuge bottles and caps to be used in sample preparation and obtain a mean weight.

b. Round the mean weight obtained up to the nearest gram and record this weight (e.g. if the mean weight of 10 bottles is 90.89 grams, round this value to 91 grams).

c. Place a clean centrifuge bottle (without the cap) on balance. Tare bottle to 0.00 grams.

d. Transfer 200.00 ± .05 grams of sediment using a modified 60 cc polypropylene disposable syringe to the centrifuge bottle.

e. Remove the centrifuge bottle containing the weighed sediment from the and re-zero the balance.

f. Replace the centrifuge bottle (with sediment) and cap on the pan and add dilution water until the combined weight of the bottle, sediment, cap and bottle equals 1000 grams plus the rounded average gram weight of the containers obtained in step V-C-1-b (above). For example if the average rounded weight of the centrifuge bottles was 91.0 grams, water would be added to the centrifuge bottle containing 200 grams of sediment until the combined weight of the bottle, cap, sediment and water was 1091.00 ± .05 grams.

2. Preparing elutriates with 250 mL centrifuge bottles.

a. Individually weigh 10 - 250 mL. centrifuge bottles and caps to be used in sample preparation and obtain a mean weight.

b. Round the mean weight obtained up to the nearest gram. and record this weight (e.g. if the mean weight of 10 bottles is 34.56 grams, round this value to 35 grams).

c. Place a clean centrifuge bottle (without the cap) on balance.

d. Transfer 50.00 ± .05 grams sediment using a modified 60 cc polypropylene disposable syringe to the centrifuge bottle.

e. Remove the centrifuge bottle containing the weighed sediment from the pan and re-zero the balance.

f. Replace the centrifuge bottle (with sediment) and cap on the pan and add dilution water until the combined weight of the bottle, sediment, cap and bottle equals 250 grams plus the rounded average gram weight of the containers obtained in step V-C-2-b above. For example if the average rounded weight of the centrifuge bottles was 35.0 grams, water would be added to the centrifuge bottle containing 50 grams of sediment until the combined weight of the bottle, cap, sediment and water was 285.00 ± .05 grams.

3. Mixing elutriates.

a. Centrifuge bottles containing the appropriate weights of water and sediment are placed in the elutriate mixing apparatus and rotated end over end for 30 minutes at 12 rpm per minute.

b. After samples have mixed for 30 minutes, re-weigh all centrifuge bottles to 0.01 grams prior to transferring them to the centrifuge. Record weights. All bottles must be within ± 0.20 grams of each other. If necessary, add sufficient SJVDP water with a pipet to bottles containing weights below this range.

FAILURE TO BRING ALL BOTTLES WITHIN ± 0.2 GRAMS PRIOR TO CENTRIFUGATION MAY RESULT IN ROTOR IMBALANCE AND DAMAGE TO THE CENTRIFUGE.

4. Centrifuging elutriates.

   a. Transfer bottles to centrifuge buckets. Position of bottles in the rotor is not important if all bottles are within the ± 0.02 gram range.

      (1) Bottles must be centrifuged in pairs and placed in opposite buckets in the rotor. If an odd number of bottles are to be centrifuged, prepare a blank bottle that weighs within ± 0.20 grams of the opposite bottle.

   b. Samples are centrifuged at 5,000 rpm (7000 x G) for 15 min. at 4 °C.

      (1) Check that temperature displayed is 4 ± 2 °C.

      (2) Set SPEED thumb-wheel switch to 18.5 min. (adding 3.5 minutes to the 15 min. centrifuge time allows centrifuge to attain the set speed).

      (3) Set BRAKE thumb-wheel switch to 2.

      (4) Set ACCELERATION thumb-wheel switch to 1.

      (5) Press Start/Stop button.

5. Removing elutriate from centrifuge bottles.

   a. The overlying water from each centrifuge bottle containing sediment from the same site is poured through a clean 50 mesh stainless steel standard sieve into a clean 3.0 l. glass bottle and mixed.

   b. Sub-samples of the elutriate are obtained from the 3.0 l. glass bottle and are stored in appropriate containers in the dark at 4°C.

6. Elutriate sub-sampling and analyses.

   a. Chemical characterization of the elutriate sample may include the following: pH, total water hardness, alkalinity, conductivity, ammonia, dissolved oxygen, and turbidity.

   b. A 500 mL sample of the elutriate will be placed in 500 mL teflon-lined bottle for metal analysis. These samples will be acidified to pH 1.7-2.0 using Baker instant analyzed acid. About 0.5 mL of acid in 500 mL of elutriate sample should achieve this range in pH. The sample will be stored at 4°C until analysis for metals.

   c. The elutriate sample may need to be filtered before using in toxicity testing (e.g., Selenastrum capricornutum or Ames testing).

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GUIDE FOR DESIGNING BIOLOGICAL TESTS WITH SEDIMENTS

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1.0 Scope

1.1 As contamination of freshwater, estuarine, and marine ecosystems continues to be reduced through the implementation of regulations governing both point and non-point source discharges, there is a growing emphasis and concern regarding historical inputs and their influence on water and sediment quality. Many locations in urban areas exhibit significant sediment contamination which poses a continual and long-term threat to the health of benthic communities and other species inhabiting these areas (NOAA, 1988). Benthic communities are an important component of many food chains leading to humans and it is becoming increasingly important to identify contaminated sites to properly manage remediation and resource use.

1.2 Biological tests with sediments are an efficient means for evaluating sediment contamination because they provide information complementary to chemical characterizations and ecological surveys (Chapman, 1988). Acute sediment toxicity tests can be used as screening tools in the early phase of an assessment hierarchy that ultimately could include chemical measurements or bioaccumulation and chronic effects tests. Sediment tests have been applied in both marine and freshwater environments (Swartz 1987; Chapman, 1988; Lamberson and Swartz, 1988). Sediment tests have been used for dredge material permitting, site ranking for remediation,

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recovery studies following management actions, and trend monitoring. A particularly important application is in establishing contaminant-specific effects and the processes controlling contaminant bioavailability.

2.0 Application

2.1 This document provides general interpretative guidance on the selection, application and interpretation of biological tests with sediments. As such, it serves as a preface to other ASTM documents describing: methods for sediment collection, storage and manipulation (ASTM E 1391); toxicity tests with marine (ASTM E 1367) and freshwater organisms (ASTM E 1383); and bioaccumulation studies. This guide serves as an introduction and summary of sediment testing; it is not meant, however, to provide specific guidance on test methods. Rather, its intent is to provide information necessary to:

2.1.1 Select a sediment exposure strategy that is appropriate to the assessment need of the toxicity test. For example, a suspended phase exposure is relevant to evaluation of dredged sediments for disposal at a dispersive aquatic site.

2.1.2 Select the test organism and biological endpoints that are appropriate to the desired exposure and aquatic resources at risk. For example, the potential for water quality problems and subsequent effects on oyster beds may dictate the use of sediment elutriate exposures with bivalve larvae.

o Establish an experimental design consistent with the objectives of the sediment evaluation. The use of appropriate controls is particularly important here.

o Determine which statistical procedures should be applied to the analysis of the data, and define the limits of applicability of the resultant analyses in the data interpretation.

3.0 Organization (To be drafted)

4.0 Hazard statement/Safety precautions

4.1 Many substances may pose health risks to humans if adequate precautions are not taken. Information on toxicity to humans, recommended handling procedures, and chemical and physical properties of the test material should be studied before a test is begun and made aware to all personnel involved (6,7,8). Contact with test materials, overlying water and sediments should be minimized.

4.2 Many materials can adversely affect humans if precautions are inadequate. Skin contact with test materials and solutions should be minimized by such means as wearing appropriate protective gloves, laboratory coats, aprons, and safety glasses, and by using dip nets, sieves or tubes to remove test organisms from overlying water. When handling potentially hazardous sediments the proper handling procedures may include (a) sieving and distributing sediments under a ventilated hood or in an enclosed glove box, (b) enclosing and ventilating the water bath, and (c) use of respirators, aprons, safety glasses, and gloves. Field collected sediments may contain potentially toxic materials and should be treated with caution to minimize occupational exposure to workers. Worker safety should also be considered when working with spiked sediments containing various organic or inorganic compounds, compounds that are radiolabeled, and with materials that are, or are suspected of being, carcinogenic or teratogenic (7).

4.3 Careful consideration should be given to those chemicals which might biodegrade, biotransform to more toxic components, volatilize, combust, oxidize, or photolyze during the test period.

4.4 Health and safety precautions and applicable regulations for disposal of stock solutions, test organisms, sediments, and overlying water should be considered before beginning a test (ASTM Standard D 4447).

5.0 Applicable Documents

5.1 ASTM Documents

E 380 Standard for Metric Practice

D 1129 Definitions of Terms Relating to Water

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E 943 Standard Definitions of Terms Relating to Biological Effects and Environmental Fate

E 1367 Guide for Conducting Solid Phase 10-Day Static Sediment Toxicity Tests with Marine and Estuarine Infaunal Amphipods

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing.

E 1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates

6.0 Terminology

6.1 The words "must", "should", "may", "can", and "might" have very specific meanings in this guidance. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy a specific condition, unless the purpose of the test requires a different design. "Must" is only used in connection with the factors that apply directly to the acceptability of the test. "Should" is used to state that the specified conditions are recommended and ought to be met in most tests. Although a violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable", "is often desirable", and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to", "can" is used to mean "is (are) able to", and "might" is used to mean "could possibly". Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym of either "may" or "can".

6.2 sediment -- is used to denote a naturally occurring particulate material which has been transported and deposited at the bottom of a body of water. The term can also be applied to an artificially prepared substrate within which the test organisms can interact.

6.2.1 whole sediment -- is distinguished from elutriate, and resuspended sediments, in that the whole, intact sediment is used to expose the organisms, not a form or derivative of the sediment.

6.2.2 clean -- denotes a sediment (or water) that does not contain concentrations of test materials or xenobiotics which cause apparent stress to the test organisms or reduce their survival.

6.3 elutriate -- refers to the water or solvent used to elute contaminants from the sediment and is then used in aquatic exposures.

6.4 suspended -- is a slurry of sediment and water used to expose the organisms.

6.5 overlying water -- the water placed over the solid-phase of a sediment in the test chamber for the conduct of the biological test, and may also include the water used to manipulate the sediments.

6.6 interstitial water -- the water within a wet sediment that surrounds the sediment particles, expressed as the percent ratio of the weight of the water in the sediment to the weight of the wet sediment.

6.7 spiking -- the experimental addition of a test material such as a chemical or mixture of chemicals, sewage sludge, oil, particulate matter, or highly contaminated sediment to a clean negative control or reference sediment, such that the toxicity of the material added can be determined.

6.8 concentration -- the weight or volume of test material(s) associated with a weight or volume of test sample.

6.9 exposure -- is contact with a chemical or physical agent.

6.10 toxicity -- is the property of a material or combination of materials to adversely affect organisms.

6.11 bioaccumulation -- the net uptake of a material by an organism from its environment through direct exposure or ingestion.

6.12 Control sediment -- a sediment essentially free of contaminants (USEPA-COE 1990). Any contaminants in control sediment originates from the global spread of pollutants and does not reflect any substantial input from local or non-point sources (Lee et al. 1989). The comparison of the test sediment to the control sediment is a measure of any toxicity from the test sediment beyond inevitable background contamination (Lee et al. 1989). The control sediment is used to assess the acceptability of the test and provide evidence of the health and quality of the test animals (Nelson et al. 1990).

6.13 Reference sediment -- a sediment substantially free of contaminants (USEPA-COE 1990). The reference sediment may be used as an indicator of localized sediment conditions exclusive of the specific pollutant input of concern. Such sediment would be collected near the site of concern and would represent the background conditions resulting from any localized pollutant inputs as well as the global input (Lee et al. 1989). This is the manner in which reference sediment is used in the dredged material evaluations (EPA-COE 1990).

6.14 For definitions of other terms used in this practice, refer to Standards E 729, E 943, D 1129, E 1023, and E 1241. For an explanation of units and symbols, refer to Standard E 380.

7.0 Summary of Guide

7.1 This guide provides general guidance and objectives for conducting biological tests with sediments. Detailed technical information on the conduct and evaluation of specific sediment tests is included in other documents referenced in this guide.

7.2 Neither this guide nor any specific test methodology can adequately address the multitude of technical factors that must be considered when designing and conducting a specific investigation. Therefore, the intended use of this document is not to provide detailed guidance but rather to assist the investigator in developing technically sound and environmentally relevant biological tests that adequately address the questions being posed by a specific investigation.

8.0 Sediment Test Rationale (Significance and Use)

8.1 Contaminated sediments may have adverse effects on natural populations of aquatic organisms. Sediment dwelling organisms may be directly exposed to contaminants by the ingestion of sediments and by the uptake of sediment-associated contaminants from interstitial and overlying water. Contaminated sediments may directly affect water column species by serving as a source of contaminants to overlying waters or a sink for contaminants from overlying waters. Organisms may also be affected when contaminated sediments are suspended in the water column by natural or human activities. Water column species and non-aquatic species

may also be indirectly affected by contaminated sediments by the transfer of contaminants through aquatic-terrestrial food chains.

8.2 The test methodologies described herein may be used and adapted for incorporation in basic and applied research projects to further clarify the ecological effects of contaminated sediments. These same methods may also be used in the development and implementation of regulatory programs designed to prevent the contamination of sediments and manage sediments that are already contaminated.

8.3 Sediment tests with aquatic organisms can be used to quantify the acute and chronic toxicity and the bioavailability of new and presently used materials. In many cases, consideration of the adverse effects of sediment-associated contaminants is only one part of a complete hazard assessment of manufactured compounds that are intentionally released to the environment (e.g., pesticides, herbicides) and those released only inadvertently through the manufacturing process (e.g., through wastewater effluents).

8.4 Sediment tests can be used to develop dose-response relationships for individual toxicants by spiking clean sediments with varying concentrations of a test chemical and determining the concentration that elicits the target response in the test organism. In a similar fashion, sediment tests can be designed to determine the effects that the physical and chemical properties of sediments have on the bioavailability and toxicity of compounds.

8.5 Properly designed and conducted sediment tests can provide valuable information needed to make decisions regarding the management of contaminated sediments from hazardous waste sites and other contaminated areas. Biological tests with sediments can also be used to make defensible management decisions on the dredging and disposal of potentially contaminated sediments from rivers and harbors.

9.0 Sediment Test Types

9.1 Recent reviews have summarized methods for assessing the toxicity of marine [2,3] and freshwater [4,5] sediments to benthic organisms. Those methods are provided in Table 1 and Table 2, for marine and freshwater tests, respectively.

9.2 The selection of a specific toxicity test type is intimately related to the objectives of the sediment evaluation program. These assessments, whether they be for monitoring, regulatory, or research purposes, should be guided by a set of null hypotheses which define the appropriate exposure route and the endpoint of interest.

9.3 Organism exposure methods most commonly employ the whole sediment in the bedded phase, but suspended and elutriate phase exposures have also been used. More recently, methods have been developed to test pore waters directly and to prepare organic extracts for testing. The relationship between toxicity resulting from these latter exposures and what may be found in situ, however, is not well defined.

9.4 Programs seeking to characterize or rank sediments on a basin-wide or regional scale typically use whole sediment, solid phase exposures. Regulatory or permitting programs for dredged material disposal at a containment site should also evaluate this exposure route. Disposal at a dispersive site, or concerns over resuspension and transport of in-place sediments, would suggest use of suspended phase or elutriate exposures.

9.5 Methods have been developed to isolate and test the toxicity of elutriates (e.g., USEPA-COE 1977) or sediment interstitial water (e.g., Ankley et al. 1990) to aquatic organisms. The elutriate test was developed for assessing the potential acute effects of open-water disposal of dredged material. Tests with elutriate samples are used to estimate the water soluble constituents that may be released from sediment to the water column during

disposal operations (Shuba et al. 1977). Toxicity tests of the elutriate with water column organisms have generally indicated little toxicity is associated with the discharge material (Lamberson and Swartz 1988). However, elutriates have been reportedly more toxic than interstitial water samples (Giesy and Hoke 1989).

9.5.1 For many benthic invertebrates, the toxicity and bioaccumulation of sediment-associated contaminants such as metals, and non-ionic organic contaminants are correlated with the concentration of these chemicals in the interstitial water (Ankley et al. 1990 ammonia). The sediment interstitial water toxicity test was developed for assessing the potential in situ effects of contaminated sediment on aquatic organisms. Once the interstitial water (or elutriate) has been isolated from the whole sediment, the toxicity testing procedures would be similar to effluent toxicity testing with non-benthic species. If benthic species are used as test animals, they may be stressed by the absence of sediment (Lamberson and Swartz 1988).

9.5.2 Examination of organic extracts may have specific uses when whole sediments have a predetermined toxicity and cause-effect relationship. However, caution must be exercised in the use of organic extracts because the resultant contaminant interactions within a sediment matrix have not been determined.

9.6 Biological responses in sediment toxicity tests range from genotoxic effects to individual organism responses to alterations in community levels of organization. Because of its ease of interpretation, the response criterion that is most commonly employed has been lethality. This endpoint is generally insensitive to sediment contaminants unless appropriately sensitive species, such as amphipods, are used. The application of sublethal toxicity tests has been limited because of the uncertainty in relating these responses to ecologically relevant endpoints such as survival and population dynamics. Behavioral responses of infaunal organisms, such as emergence from the sediments are indicative of potential ecological effects because the animals may be subject to predation. Many biochemical and genetic endpoints, e.g., enzyme induction and chromosome aberration, are indicative of exposure to specific classes of chemicals, and are useful from that perspective. Sublethal tests which show the most promise are those using growth and reproduction as response parameters. These are relevant endpoints that can be used as predictors of potential population effects. Most of these tests, however, are still in development and are limited in their application. Tests combining lethality with growth and reproduction have been developed and routinely applied using freshwater and marine organisms.

9.7 The selection of the proper response parameter can also be predicated on the goals of the evaluation program, but the choice is often based on available resources, time, and test methods. Sublethal endpoints are generally the preferred responses, but they are more difficult to interpret and/or the data are more costly to generate. Sediment screening programs commonly use simple reliable testes, e.g. amphipod mortality, bacterial bioluminescence or sea urchin fertilization. The latter two tests are conducted on either pore waters or organic extracts. In depth evaluations of single sediments, as in U.S. Army Corps of Engineers dredging evaluations, are more likely to involve a more complex suite of tests including life cycle scale responses or long-term bioaccumulation studies. Specific sublethal responses such as genotoxicity or enzyme induction may be used to identify contaminant-specific exposures.

10.0 Test Organisms

10.1 Once the exposure routes and endpoints of interest have been established, there are several criteria that need to be considered when selecting the appropriate test species (Shuba 1981, Swartz 1987). Ideally, the test species should:

- have a toxicological (sediment) database demonstrating sensitivity to a range of toxicants,

- o be readily available through field collection or culture,
- o be easily maintained in the laboratory,
- o be ecologically or economically important,
- o have a broad geographical distribution,
- o be indigenous to the site being evaluated or closely related to an inhabitant,
- o be tolerant to a broad range of sediment geochemical characteristics (e.g., organic carbon and grain size), and
- o be capatible with selected exposures and endpoints.

Of these criteria, demonstrated sensitivity to contaminants, ecological relevance, and tolerance to varying sediment geochemical characteristics are the most important. The use of indigenous species that are ecologically important and easily collected is often very straightforward, however, many indigenous species at a contaminated site may be insensitive to contaminants. These might present a greater concern relative to their bioaccumulation potential. With the exception of some amphipods, few test organisms have broad sediment or water toxicity databases. Additionally, many organisms can be maintained in the laboratory long enough for acclimation to test conditions, but very few are easily cultured. Widespread toxicity testing will require cultured organisms or the use of standard source populations which can be transported without experiencing excessive mortality.

10.2 Sensitivity is related to the degree of contact between the sediment and the organism. Feeding habits including the type of food and feeding rate will control the dose of contaminant from sediment (Adams 1987). Infaunal deposit-feeding organisms can receive a dose of sediment contaminants from three sources: interstitial water, whole sediment, and overlying water. Benthic invertebrates may selectively consume particles with higher organic carbon and higher contaminant concentrations. Organisms in direct contact with sediment may also accumulate contaminants by direct adsorption to the body wall or exoskeleton, or by absorption through the integument (Knezovich et al. 1987). Thus, estimates of bioavailability will be more complex for epibenthic animals that inhabit both the sediment and the water column. Some benthic organism are exposed primarily by detrital feeding (Boese 1988 sab). Detrital feeders may not receive most of their body burden directly from interstitial water. For certain higher Kow compounds, uptake by the gut can exceed uptake across the gill (Landrum 1989, Boese et al. 1990). However, for many benthic invertebrates, the toxicity and bioaccumulation of sediment-associated contaminants such as metals, kepone, fluorathene, and organochlorines are highly correlated with the concentration of these chemicals in the interstitial water (Ankley et al. 1990).

10.3 The marine tests cover a broad spectrum of taxa and feeding types including crustaceans, bivalves, polychaetes and fish. Tests using amphipods have received a great deal of attention because field surveys have shown them to be absent from contaminated sites. This sensitivity has led to the development of routine methods using the burrowing amphipod Rhepoxynius abronius. This ten day acute toxicity test has recently been adapted for use with other amphipod species and has been established as a standard method by ASTM. Since 1977, the U.S. Army Corps of Engineers dredging permit program has routinely required tests with three species: a bivalve, a polychaete and a fish or shrimp, incorporating organisms which burrow into the sediment and those inhabiting the water column. Broad applications of these protocols reveal that these tests are not as sensitive as those with amphipods and the latter recently have been recommended for permit programs.

10.4 Sediment tests in freshwater utilize a number of different species. Whole sediment tests with the amphipod Hyalella azteca generally start with juvenile animals and are conducted for up to four weeks until reproductive maturation (Nelson et al. 1990). Although a direct measurement of amphipod reproduction is appealing, the quantitative isolation of young amphipods from sediment is difficult because of their small size (<2mm). Indirect measures of reproduction, such as time to reproductive maturation, or the number of eggs or young carried in the marsupium are more easily quantified than the number of young produced. Moreover, the total number of young produced during the exposure may reflect not only a direct effect on reproduction, but may also be affected by a reduction in adult survival (Ingersoll and Nelson 1990).

Tests with Chironomus tentus are generally started with 2nd instar larvae (10-14 d old) and continued for 10 to 17 d until the 4th instar; larval survival or growth is the measure of toxicity (Nelson et al. 1990). Exposures of C. tentans that started with 1st instar larvae or that measured adult emergence have met with only limited success [39, Nebeker et al. 1988 BECT). Whole sediment testing procedures with C. riparius are started with 1 to 3 day old larvae and continued through pupation and adult emergence (Nelson et al. 1990). Midge exposures started with older larvae may underestimate midge sensitivity to toxicants. For instance, 1st instar C. tentans larvae were 6 to 27 times more sensitive than 4th instar larvae to acute copper exposure [34,39], and 1st instar C. riparius larvae were 127 times more sensitive than 2nd instar larvae to acute cadmium exposure [44].

Sediment toxicity tests with mayflies and cladocerans are generally conducted for up to 10 days (Bahnick et al. 1981, Nebeker et al. 1984, Giesy et al. 1990 ETC 9:2). Survival and molting frequency are the toxicity endpoints monitored in the mayfly tests and survival, growth, and reproduction are monitored in the cladoceran tests. While cladocerans are not in direct contact with the sediment, they are frequently in contact with the sediment surface and are likely exposed to both water soluble and particulate bound contaminants in the overlying water and surface sediment (Stemmer and Burton 1990 ASTM). Cladocerans are also one of the more sensitive groups of organisms used in toxicity testing.

The most frequently described sediment test methods for oligochaetes are acute toxicity testing procedures (e.g., Keilty et al. 1988 AECT 17:95-101). Wiederholm et al. (1987) describe methods for conducting up to 500 day oligochaete exposures with growth and reproduction as the toxicity endpoint. Recently, Reynoldson et al. (in prep.) describe a 28 d test started with sexually mature Tubifex tubifex. In this shorter test, effects on growth and reproduction can be monitored and the duration of the exposure makes the test more useful for routine sediment toxicity assessments with oligochaetes. Oligochaetes have complex life cycles and reproductive strategies and as a result laboratory culturing requirements have prohibited their use in toxicity testing (Dillion and Gibson 1985).

10.5 Because of the database that has been developed with existing tests, it is recommended that, for whole sediment exposures, either phoxocephalid or ampeliscid amphipods be used in marine tests. For freshwater applications, hyalellid amphipods, midge larvae, or mayfly larvae would be appropriate. As new methods are developed, it will be important to establish each method's sensitivity relative to a benchmark procedure for comparative purposes (Chapman 1988). The marine benchmark should be the Rhepoxynius abronius ten day acute test and the freshwater benchmark should be the Hyalella azteca. Although sublethal tests with whole sediments are rare, aggressive attempts should be made to develop tests using growth and reproduction endpoints with marine and freshwater amphipods.

10.6 Multispecies/microcosm tests can also be used to evaluate potential ecosystem responses to contaminated sediments. However, results from multispecies or microcosm tests are more difficult to interpret due to interactions and limited reference literature (13, Prater and Hoke 1980).

11.0 Experimental design considerations

11.1 Sample methods

11.1.1 Purpose of the study--the probable source and type of contamination and the objectives of the study, should be evaluated before developing the sampling regime. The number of samples taken and method of sampling may vary depending on the objectives of the study (8,13,11,1).

11.1.2. The number of replicate samples taken at a site should be determined based on a preliminary survey of sediment variability at the site. The mean and standard deviation of the replicates can be used to calculate a minimum number of replicates (13,1).

11.1.3 In general, both toxicity and bioaccumulation tests require at least two exposures - a control and one or more test treatments. The experimental unit for each test is the exposure chamber. Typically a sediment sample is split into four or more test chambers. Individual observations obtained from within an individual chamber should not be used as replicate observations. Replicate chambers for a particular sediment provide an estimate of the variability within the test system and are not sediment sample replicates.

11.1.4 There are several acceptable methods of sampling sediments, e.g. corers and grabs or dredges. Grabs or dredges (e.g., Ponar or Eckman) are appropriate when sediments are known to be unstratified with respect to the contaminants of concern. If the contaminants are in strata or if their accumulation rates are of interest, one of several core samplers should be used. Pb^{210} or Cs^{137} dating can be performed on cores to identify the thickness of the mixed layer (1,3). See ASTM 1391 for additional details.

11.2 Sample handling and preservation are discussed in ASTM E 1391 and depend on the type of chemical characterization that will be performed. The use of clean sampling devices and sample containers is essential to ensure the accurate determination of sediment contamination (13,1).

11.3 Physical and chemical characterization of sediments may include loss on ignition, percent water, grain size, total organic carbon, total phosphorus, nitrogen forms, trace metals and organic compounds, pH, total volatile solids, biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, pE, total inorganic carbon, acid volatile sulfides, and ammonia (8,11,1).

11.4 Overlying Water -- Besides being available in adequate supply, overlying water used in toxicity tests, and water used to hold organisms before testing, should be acceptable to test species and uniform in quality. To be acceptable to the test species, the water must allow satisfactory survival and growth, without showing signs of disease or apparent stress, such as discoloration, or unusual behavior.

11.4.1 Natural overlying water should be uncontaminated and of constant quality and should meet the specifications established in ASTM E 729. Water should be characterized in accordance with ASTM E 729 at least twice each year, and more often if (a) such measurements have not been determined semiannually for at least two years, or (b) if surface water is used.

11.4.2 A natural overlying water is considered to be of uniform quality if the 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 units. Natural overlying 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 minimize fluctuations in quality and the possibility of contamination and maximize the concentration of dissolved oxygen and to help ensure low concentrations of sulfide and iron. Chlorinated water should not used for, or in the preparation of, overlying water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals and dechlorination is often incomplete.

11.4.3 For certain applications the experimental design might require use of water from the test sediment collection site.

11.4.4 Reconstituted water is prepared by adding specified amounts of reagent grade chemicals to high quality distilled or deionized water (see ASTM E 729). Acceptable water can be prepared using deionization, distillation, or reverse-osmosis units. Conductivity, pH, hardness and alkalinity should be measured on each batch of reconstituted water. If the water is prepared from a surface water, total organic carbon or chemical oxygen demand should be measured on each batch. Filtration through sand, rock, bag, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low. The reconstituted water should be intensively aerated before use, except that buffered soft fresh waters should be aerated before, but not after, addition of buffers. Problems have been encountered with some species in some fresh reconstituted waters, but these problems can be overcome by the aging the reconstituted water for one or more weeks.

11.5 Test Design

11.5.1 Materials used to construct test chambers may include glass, stainless steel, silicone and plastics that have been properly prepared and tested for toxicity (ASTM E 1367, E 1383).

11.5.2 The use of site water or reconstituted water in toxicity tests may depend on the type of test to be performed and the time lapse between sample collection and test initiation. 11.5.3 Static sediment toxicity tests are the simplest to perform and have been commonly used. In such tests, water overlying the sediment is not changed during the test period, but may be added to replace that which has evaporated. Since changes in water quality may affect the availability of contaminants to the test organisms, static exposures are more appropriate for acute tests (7-10 days).

11.5.4 Flow-through exposure chambers are suggested for use in chronic tests or with larger animals. Since water is renewed on a continual basis, fewer water quality changes are likely due to the buildup of waste products or interactions between the sediment and overlying water.

11.5.5 General water quality (variables such as pH, dissolved oxygen, ammonia, and temperature) in the test chambers should meet culture and maintenance requirements for the test organisms. These parameters should be monitored and recorded on a frequency appropriate to the test length. For example, if the test duration is only a few days, daily monitoring should be performed. However, if the test will continue for weeks or months, measurements may be reduced to every other day or every few days.

11.5.6 The depth of sediment in test chambers may vary depending on the organism being tested, its size and degree of burrowing activity, and its sediment processing rate. The latter should be determined prior to the beginning of a sediment toxicity test (13).

11.5.7 Control and/or reference sediments should be used in each sediment test. A standard reference sediment is a well characterized sediment containing a known amount of a specific pollutant (13) which may be prepared by spiking in the laboratory with an appropriate compound, e.g. organic or metal compound. The

standard reference sediment is useful as an indicator of test organism variability among seasons or sample sites. Its use also facilitates interlaboratory comparisons.

11.5.8 Test temperature should be chosen based on conditions of particular interest, or to match the conditions at the sample site. In either case, the choice of temperature and test organism should be compatible (ASTM 1984). Suggested test temperatures may range from 7-33 • C and should correspond to the average spring-summer temperature of the study area (ASTM 1984).

11.5.9 Dissolved oxygen should be maintained between 40% and 100% saturation.

11.5.10 Light quality and daylength are important because of their impacts on both chemical degradation and organism health. Light should be provided from cool-white fluorescent lamps at an intensity appropriate for the test species (ASTM 1984).

11.5.11 The photoperiod can be selected to mimic that experienced at the sample site, or to simulate a particular season. Suggested periods of daylight and darkness include 16 h light/8 h dark, 14 h light/10 h dark, 12 h light/12 h dark (13, ASTM 1984).

11.5.12 Whether or not test organisms should be fed during the test depends on test duration and the type of test organism in use. The addition of food can complicate the interpretation of test results because it adds new particulate material, and the food may interact in unknown ways with contaminants in the sediments (13). For acute tests ($\leq$ 1 week) and many infaunal organisms which process sediments directly, enough sediment has generally been provided to ensure adequate nutrition and feeding may not be necessary. If the organisms are fish or filter feeders, food may be required, especially during long tests.

11.6 Chemical analysis of test water, sediment and organisms

11.6.1 Test water and sediments should be analyzed for contaminants of concern if the objectives of the study are to determine the sources and concentrations of contaminants. If the test is designed to assess toxicity only, then identification of sources of toxicity are not necessary.

11.6.2 Analyses of specific contaminants in tissues of the test organisms are needed if bioaccumulation or bioconcentration is of interest. If measurement of organic chemicals, metals or other contaminants is desireable, appropriate preservation methods should be followed when samples are collected.

12.0 Data interpretation

12.1 Bioaccumulation of contaminants or toxic effects such as mortality from sediment or sediment extract exposure are important to the individuals of a particular species however, the ecological significance of those data are difficult to predict (ref 12). Toxic effects observed in laboratory exposures may not reflect affects on natural populations. However, bioaccumulation of a contaminant above a certain level or a toxicity response higher (or lower) when compared to that same response in a population of organisms exposed to a control sediment is undesirable.

12.2 The calculation procedure(s) and interpretation of the results should be appropriate to the experimental design. Procedures used to calculate results of tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (a) the advantages and disadvantages of various alternative procedures, and (b) appropriate preliminary tests, such as those for outliers and for heterogeneity.

12.3 When samples from field sites are independently replicated, site effects (bioaccumulation and toxicity endpoints) can be statistically compared by t-tests, analysis of variance (ANOVA) or regression analysis. Analysis of variance is used to determine whether any of the observed differences among the concentrations (or samples) are statistically significant. This is a test of the null hypothesis that no differences exist in effects observed among test concentrations (or samples) and controls. If the F-test is not statistically significant ($P>0.05$), it can be concluded that the effects observed in the test material treatments (or field sites) were not large enough to be detected as statistically significant by the experimental design and hypothesis test used. Non-rejection does not mean that the null hypothesis is true. The NOEC based on this end point is then taken to be the highest test concentration tested (33,34). The amount of effect that occurred at this concentration should be considered.

12.3.1 All exposure concentration effects (or field sites) can be compared with the control effects by using mean separation techniques such as those explained by Chew orthogonal contrasts (35), Fisher's methods, Dunnett's procedure or Williams' method. The lowest concentration for which the difference in observed effect exceeds the statistical significant difference is defined as the LOEC for that end point. The highest concentration for which the difference in effect is not greater than the statistical significant difference is defined as the NOEC for that end point (33).

12.4 In cases where sediment dilution series toxicity studies are conducted the LC50 or EC50 and its 95% confidence limits should be calculated (when appropriate) on the basis of (a) the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and (b) the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LC or ECs are calculated, their 95% confidence limits should also be calculated (see ASTM E 729).

12.4.1 Most toxicity tests produce quantal data, that is, counts of the number of responses in two mutually exclusive categories, such as alive or dead. A variety of methods (32) can be used to calculate an LC50 or EC50 and 95% confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or effected is between zero and 100. The most widely used are the probit, moving average, Spearman-Karber and Litchfield-Wilcoxon methods. The method used should appropriately take into account the number of test organisms per chamber. The binomial test can also be used to obtain statistically sound information about the LC50 or EC50 even when less than two concentrations kill or affect between zero and 100 percent. The binomial test provides a range within which the LC50 or EC50 should lie.

Table 1. Marine sediment toxicity tests.

TAXA	EXPOSURE	REFERENCE
Mortality		
Larval fish	El[a]	S7
Amphipods, bivalves polychaetes, cumaceans	So[b]	S18
Amphipods	So	S19,21,22,59,60 and more
Fish bivalves	Su[c]	S23
Fish	So, Su	S24
Shrimp, polychaetes	So	S25,26
Shrimp So, Su	S27,28	
Copepods, amphipods, isopods, shrimp	So, Su	C17
Amphipods, mysids, bivalves fish	So, Su	Rogerson et al.
Mysids, amphipods	Su	Gentile et al. 85,87,S&R
Phytoplankton	El	C15
Fish	El	C22, S7
Shrimp, mussel, crab, tunicate, lobster	Su	S31
Avoidance/behavior		
Echinoderm, lobster, crab, shrimp, bivalve, amphipod	So	S9
Amphipod	So	S10
Crab, shrimp, fish, bivalve, polychaete	So	S11
Fish	So	S12
Bivalves So	S13,14,16	
Polychaetes	So	S15, Olla 1989
Growth/reproduction/life cycle		
Fish	Su	S4
Bivalve Su	S5	
Mysids Su	S17, G&al 85,87	
Amphipods	Su	S&R
Nematodes	So	S32
Polychaetes	So,Su	S4,61
Copepods	So	C34
Sea urchin	El	C32
Polychaetes	So, Su	Johns et al
Pathology		
Fish	So	S58, C30
Bivalves, polychaetes, amphipods	So, Su	Yevich et al.
Oyster, fish	Su, So	Gardner et al.

Table 1. Marine sediment toxicity test. (continued)

Physiology		
Oligochaetes	El	C10
Shrimp, polychaetes	Su	C35
Fish	Su	C36
Polychaetes	So, Su	Johns etal
Chromosome damage		
Fish	El	S3,57,C9
Polychaetes	Su	Pesch et al.
Bacterial activity		
Bacteria El	C12	
Bacteria El	C13	
Community recolonization		
Macrobenthos	So	S34,35,36
Macrobenthos	So	S37
Macrobenthos	So	S38
Macrobenthos	So	S33
Macrobenthos	So	S39

[a]El - elutriate, extract, pore water exposure
[b]So - solid-phase sediment exposure
[c]Su - suspended sediment exposure

Table 2. Freshwater sediment toxicity tests.

TAXA	EXPOSURE	REFERENCE
Mortality		
Cladocerans, insect larvae, isopods, fish, Lirceus?	So	L1,2,3,4
Insect larvae, cladocerans, amphipods, fish	So, El	L5
Cladocerans, insect larvae	So	L6,15,16,17
Cladocerans	So, Su	L7
Cladocera, amphipods, insect larvae	So	L8,9
Oligochaetes	So	L10,11,12
Amphipods, insect larvae	So	L13
Cladocerans	So	L14,20
Insect larvae	So	L18
Growth/reproduction		
Insect larvae	So	L18
Fish, cladocerans, bacteria, Paratanytarsus	So, El	L19
Insect larve, amphipods, cladocerans	So	L9
Insect larvae	So	L21,22
Nematodes	El	L29
Physiology		
Oligochaetes	El	L23,24
Genetic damage		
Fish	El	L25,26,27,28
Nematodes	El	L29
Bacterial activity		
Bacteria El	Giesy et al.	

PERFORMING TOXICITY TESTS WITH THE MICROTOX[R] MODEL 500

I. GENERAL:

This document describes procedures for performing toxicity tests with the Microtox[R] Model 500. The instrument measures the light output of luminescent bacteria (supplied by the manufacturer) before and after they are challenged by dilutions of a sample of unknown toxicity. A Reagent Blank containing no toxicant is used to normalize the responses of the four sample test concentrations during data reduction. The degree of light loss resulting from metabolic inhibition in the test organisms indicates the toxicity of the sample and is used to determine a dose-response curve from which the effective concentration of the sample is found. Measured light readings are transmitted via a RS232 interface to a personal computer which estimates an Effective Concentration using a data reduction program written in BASIC[R].

NOTE: Because individual users will select different computers and Microbics[R] periodically updates its data reduction software, this document cannot provide detailed instructions for interfacing the instrument with the computer or reducing data with the program supplied with the instrument. Consult latest software guide for specific information.

II. REQUIRED REAGENTS

A. Microtox[R] Reconstitution Solution.

1. Distilled water, (may be stored indefinitely at room temperature).

B. Microtox[R] Osmotic Adjustment Solution (MOAS).

1. MOAS is a solution of de-ionized water containing 22% NaCl (220 ppt) used during the standard bioassay procedure to osmotically adjust the sample, thereby preventing cells from lysing. Generally, one part of MOAS is added to 10 parts of sample. The assay is normally run at 2% NaCl. MOAS may be stored indefinitely at room temperature.

C. Microtox[R] Diluent.

1. Diluent is 2% NaCl (20 ppt) used for diluting the sample and reagent. Diluent may be stored indefinitely at room temperature.

D. Microtox[R] Reagent.

1. Reagent is a freeze-dried culture of a specially developed strain of the marine bacterium Photobacterium phoshoreum. Reagent has a shelf-life of one year when stored in a freezer at -20°C. SELF-DEFROSTING FREEZERS SHOULD NOT BE USED FOR LONG TERM STORAGE OF THE REAGENT. Self defrosting freezers periodically warm to prevent frost accumulation. Periodic warming of the reagent may decrease storage time and viability of the cultures.

III. REQUIRED EQUIPMENT.

A. Microtox® disposable cuvettes

B. Pipettors and pipettor tips

1. 1, 10 µL (white tips)

2. 1, 250 µL (blue tips)

3. 1, 500 µL (blue tips)

4. 1, Oxford 1000 µL P-7000 Micropipettor (optional).

5. 1, Oxford/Nichiryo Model 8100 syringe dispenser and 15.0 mL syringes.

C. Microbics Microtox® Model 500

D. Microbics data capture and reduction program

E. Micro-computer with one serial port capable of running Micro Soft Basic or BasicA software.

IV. INSTRUMENT PREPARATION.

A. To preform a single standard bioassay, place clean, unused cuvettes in Reagent Well and in the incubator block wells in rows A and B.

B. Pipette 1 mL Reconstitution Solution into the cuvette in the Reagent Well

C. Pipette 500 µL Diluent into each cuvette in wells B1 through B5

D. Pipette 1 mL Diluent into each cuvette in wells A1 through A4

NOTE: To perform more than one assay at a time repeat steps A-D with additional cuvettes placed in rows C and D and E and F.

V. SAMPLE PREPARATION.

NOTE: A PRIMARY DILUTION OF THE SAMPLE MAY BE NECESSARY. REMEMBER TO ACCOUNT FOR THE ADDITIONAL DILUTION IN DATA REDUCTION. (FOR DATA OF OPTIMUM VALUE, TRY TO BRACKET THE EC_{50} WITH THE DILUTIONS)

A. Pipette 250 µL MOAS into the cuvette in well A5.

B. Add 2.5 mL of sample (or Phenol Standard 90 mg/L) to cuvette A5, then mix by aspirating and ejecting the sample, using the 500 µL micropipettor.

C. Transfer 1.0 mL from A5 to A4, and mix two to three times as described above A4 using the 500 µL micropipettor.

D. Transfer 1.0 mL from A4 to A3, and mix A3 as described above using the 500 µL micropipettor.

E. Transfer 1.0 mL from A3 to A2, and mix A2 as described above using the 500 µL micropipettor.

F. Wait 5 minutes for solutions to come to controlled temperature.

VI. REAGENT PREPARATION.

A. Just prior to reagent reconstitution, remove vial of Microtox[R] Reagent from the freezer. Remove the seal and the stopper.

1. If the reagent pellet is not seated on the bottom of the vial, tap and shake the vial until the pellet is seated.

B. Take the cuvette of reconstitution solution from the Reagent Well. Place the lip of the cuvette on top of the reagent vial. Then, as QUICKLY as possible, DUMP the reconstitution solution into the reagent vial. Swirl the reagent into the reagent cuvette, put the cuvette back in the reagent well.

C. Mix the reconstituted reagent 20 times by aspirating and ejecting the solution with a new tip on the 500 μL micropipettor.

D. Pipette 10 μL reconstituted reagent into the cuvette in wells B1 through B5.

NOTE: When transferring the 10 μL of reagent into a cuvette, leave both cuvettes in the wells. Place the pipette tip under the surface of the liquid, but DO NOT REST THE PIPETTE TIP ON THE BOTTOM OF THE CUVETTE.

SUGGESTION: Rest the 10 μL pipette tip against the cuvette's inside rim. Slide the tip of the pipet down until the ridge on the pipette tip touches the rim of the cuvette. Stop there. The tip is in a good position for removing liquid from the cuvette.

E. Mix the reagent in row B by aspirating and ejecting two to three times using a 250 μL micropipettor.

F. Wait 15 minutes for reagent to stabilize.

NOTE: The reconstituted reagent is viable for approximately 2 hours.

VII. STANDARD ASSAY PROCEDURE.

A. Take the cuvette from well B1, and place it in the turret well.

B. Press the SET button.

1. Wait for the Ready Green Light to illuminate on the front panel of the unit. DO NOT PRESS THE SET BUTTON AGAIN FOR THIS ASSAY.

C. Read the initial (I_o) light levels of the prepared cuvettes.

1. Place the cuvettes in the turret in the following order: B1, B2, B3, B4, B5 and press the read button after each.

a. The light reading for each cuvette will be displayed on the computer screen in the appropriate sample number column.

D. Make the following 500μL transfers, mixing each sample by aspirating and ejecting 2-3 times after each transfer using the 500 μL micropipettor: A1 to B1, A2 to B2, A3 to B3, A4 to B4, A5 to B5.

1. The final assay concentrations are approximately: 5.6, 11.3, 22.5 and 45 percent of the sample being used. For example if the sample was originally a 100% sample (undiluted) the resulting tested concentrations would be 5.5, 11.3, 22, and 45%. However if the sample being tested was originally diluted 1:1 before the dilutions were made to perform the assay, the tested concentrations would be 2.25, 5.65, 11, and 22.6%.

E. When the final transfer and mixing is complete, HIT THE RETURN KEY.

1. Hitting the return key tells the computer to record how long the transfers took to accomplish and display the elapsing time.

2. When TIME1 is elapsed the program will prompt you to transfer each curvet (starting with B1 through B5) to the turret and push the READ button. The prompt is the word "enter" which is displayed under each successive concentration. The program spaces the prompts to accommodate for the time it took to make the initial volume transfers between the A cuvettes and the B cuvettes.

3. When TIME2 has elapsed the program will again prompt you to transfer the cuvettes starting with A1 through B5 to the turret and press the READ button.

F. After the last reading is taken, the program will store data in the previously named data file with either the TIME1 or TIME2 "dot designator" to differentiate between the two sets of light readings.

VIII. REDUCING STORED DATA WITH Microtox^R DATA REDUCTION SOFTWARE.

A. Refer to the appropriate version of the Microtox^R guide for detailed instruction on using the data collection and reduction program.

IX. MICROTOX 100% ASSAY PROCEDURE.

NOTE: The standard Microtox^R test procedure tests four sample dilutions, of which the highest possible concentration is about 49.5%. Some Microtox users have a need to test samples without dilution. At the present time, the automated data capture program cannot be used with the 100% assay. Because the data capture program does not store the light readings it is necessary to manually record the individual I_o and I_t readings. The recorded data can be used to generate an EC value using the data reduction program using the "Enter data from the keyboard" option at the main menu.

This procedure differs from the standard assay in that initial light (I_o) readings are not recorded for each sample. To calculate an EC value using the data reduction program, it is necessary to input the I_o reading obtained for the sample A1 as the "theoretical" I_o reading for each sample (A1 through A5).

A. Instrument and sample preparation.

1. Add 1.0 mL of Reconstitution Solution to a cuvette in reagent well.

2. Add 1.0 mL diluent to cuvettes A1, A2, A3, and A4.

3. Add 50 mg AR grade NaCl to A5.

4. Add 2.5 mL sample to A5, and mix A5 until the NaCl is dissolved. Discard 500 µL from A5.

5. Transfer 1.0 mL from A5 to A4 and mix A4.

6. Transfer 1.0 mL from A4 to A3, and mix A3.

7. Transfer 1.0 mL from A3 to A2, and mix A2.

8. Discard 1 mL from A2.

9. Set timer to 5 minutes to allow solutions to temperature equilibrate.

B. Reagent preparation and assay procedure.

1. Reconstitute a vial of Microtox® Reagent and mix 20 times with the 500 µL pipettor.

2. Start timer set for 5 minutes (after the initial 5 minute temperature equilibration period has passed). Transfer 10 µL of reconstituted reagent to A1, A2, A3, A4, and A5.

   a. If using the 10 µL pipettor to transfer reagent to the cuvettes, use pre-cooled tips and discard each tip between transfers to prevent contaminating Reagent stock.

   b. If using the Oxford/Nichiryo model 8100 multiple pipettor, use a syringe which has been pre-cooled by placing in a refrigerator or held in a beaker containing ice (pre-cooling the syringe minimizes warming of reagent while it is in the dispenser).

3. Mix each cuvette from A1 through A5 using a 500 µL pipettor. Record the time required to complete the transfers of the Reagent to the cuvettes and divide the time in seconds by 5. The resulting time will be the interval between each reading at the five and fifteen minute readings. Normally, the time between transfers is accounted for by the automated program.

4. At five minutes after beginning of reagent transfer, place the A1 cuvette in the turret and press the SET button.

5. After the instrument is set, press the read button with A1 in the turret and record the value.

6. Read the remaining samples at the appropriate interval by placing each in the turret and pressing the READ button. Record each value for subsequent data reduction.

7. Beginning at 15 minutes, re-read the samples starting with A1 proceeding through A5 allowing for the appropriate time interval between readings as calculated in step 2 (above). Record all values for data reduction.

C. Data reduction of 100% assay.

1. Refer to the appropriate version of the Microtox^{R} guide for detailed instruction on using the data collection and reduction program as it applies to the 100% assay.

X. COLOR CORRECTION PROCEDURE.

NOTE: Colored aqueous samples, particularly those colored red or brown may cause non-specific reductions in light level when analyzed according to the standard Microtox assay procedure. These light level reductions cannot be distinguished from those caused by toxicants in the standard toxicity assay. The following procedure, utilizing a special Color Correction Cuvette, measures the amount of color interference in a given sample. The measurement is then used to correct the results obtained for the sample. Reconstituted Reagent left over from the standard toxicity assay may be used for this procedure even if it is several hours old.

The color correction procedure is necessary only when appreciable color is visible in a diluted sample near the EC_{50} concentration.

A. Sample Preparation.

1. If the sample is turbid, centrifuge at 10,000 x G for 15 minutes.

2. Perform standard assay, and determine the EC_{50}. If the EC_{50} concentration has no visible color, the color correction is not required.

3. If color is appreciable at the EC_{50} concentration, make a sample dilution (min. vol. 2 mL.) close to the EC_{50} concentration. (example EC_{50} = 7.5%, make a 5% or 10% sample)

4. Add 2.0 mL of the diluted sample to cuvette in A4.

5. If more than one sample is to be run at this time, repeat steps 3 and 4 above for each sample using B and C wells for storage. The sequence of sample reading should be "least color" first to "most color" last.

B. Instrument Preparation.

1. Add 1.0 mL Diluent to cuvette in A2.

2. Add 2.0 mL Diluent to cuvette in A5 and OUTER CHAMBER of the Color Correction Cuvette in A1.

3. Wait 5 minutes.

C. Color Correction Procedure.

1. Transfer 50 μL reagent to A2 and mix using 500 μL pipettor.

2. Using a glass Pasteur pipette, transfer diluted reagent to center chamber of the Color Correction Cuvette until the reagent is at diluent level.

3. Place Color Correction Cuvette in turret. Wait 5 minutes. Press SET button.

4. Wait for ready green light then press the READ button. Record the first blank light level reading (B_0).

CAUTION: Do not move or rotate the Color Correction Cuvette until the entire color correction procedure has been completed.

5. Immediately remove and discard diluent from outside chamber of the Color Correction Cuvette, using a glass Pasteur pipette.

6. Transfer entire volume of prepared diluted sample (from A4) to outside chamber of Color Correction Cuvette using the Pasteur pipette.

7. Five minutes after the B_0 reading (Time 0) press READ and record the light level IT.

8. Remove and discard sample from outside chamber of Color Correction Cuvette using an aspirator. If you have additional colored samples, read their light levels at this time, repeating steps 6 and 7 (above) for each sample. Time the readings at 5 minute intervals, T_0 + 10, T_0 +15, T_0 + 20, etc...

9. Transfer entire volume of diluent in A5 to outside chamber of Color Correction Cuvette using a Pasteur pipette.

10. Wait 5 minutes from sample reading, press READ button. Record second blank (B_t) light level reading.

D. Tabulating and reducing color correction data.

1. Consult the appropriate version of the Microtox[R] guide for detailed instruction on using the data collection and reduction program to correct for sample color.

Daphnia magna Elutriate Testing Experimental Design

Static acute toxicity tests will be conducted with Daphnia magna and sediment elutriates. The daphnids will be exposed for 48 hours to full strength (100%) elutriates and 50%, 25%, and 12.5% dilutions of the 100% elutriates, and to a dilution water control without replication. Dilutions will be prepared a fresh water.

Ten Daphnia magna (<24 hours old) will be placed into each 250 mL test beaker in 200 mL of test solutions. Adult daphnids will be isolated from laboratory cultures on Day -1 of the test. Young daphnids (<24-hours old) will be removed from the cultures on Day 0 and placed into a culture water box. Ten of the young daphnids will be removed from the culture water box with smooth glass tubes (large bore) and placed directly into each 250-mL test beaker in the order of dilution water controls, 12.5%, 25%, 50%, and 100% sediment elutriates. The test temperature will be maintained at 20°C with a temperature-controlled waterbath. The photoperiod for the tests will be 16:8 (light:darkness) with a light intensity of about 50 fc. The daphnids will not be fed during the tests.

The pH, total hardness, alkalinity, conductivity, ammonia, dissolved oxygen, turbidity, chloride, and sulfate will be determined on the fresh water, and on each 100% elutriate (except sulfate) sample. On Day 0 of the test, pH, dissolved oxygen, and conductivity will be measured in the 100% elutriate samples before dilutions are made. Dissolved oxygen in the 100% elutriate samples will be adjusted at this time if necessary. At the end of each test, pH, dissolved oxygen, and conductivity will be measured in the 100%, 25%, and 0% elutriate treatments.

Survival of the daphnids will be recorded in all treatments at 24 and 48 hours. The lack of mobility in response to prodding with a blunt probe during 5 seconds of observation will be used as criteria to determine death.

I. Type of test.
- A. 48-hour acute toxicity test.
- B. Toxicants.
  1. Sediment elutriates.
- C. Test conditions.
  1. Fresh water dilutions of the sediment elutriates.
  2. Temperature: 20°C
  3. No Feed.
  4. Dilution water quality.
     a. Fresh water 134 mg/L total hardness as $CaCO_3$, alkalinity 65 mg/L $CaCO_3$, sulfate 72 mg/L, pH 7.3, conductivity 245 µmhos.
  5. Photoperiod of 16:8 (light:dark) with light intensity of about 50 fc.
  6. Ten daphnids per test chamber, no replication.

II. Test description.
- A. Treatments.
  1. Freshwater control.
  2. 100% sediment elutriate.
  3. 50% sediment elutriate.
  4. 25% sediment elutriate.
  5. 12.5% sediment elutriate.
- B. Treatments will not be replicated.
- C. 200-mL test solution per 250-mL test beaker.
- D. 375-mL sediment elutriate per test.

III. Pre-test Preparation.
- A. Regulate water bath temperature to 20°C.
- B. Run Microtox on sediment elutriates if storage time exceeds two weeks.

IV. Water quality monitoring.
   A. pH, total hardness, alkalinity, conductivity, ammonia, dissolved oxygen, turbidity, chloride, and sulfate on fresh water.
   B. pH, alkalinity, total hardness, conductivity, oxygen, chloride, ammonia, and turbidity on each elutriate sample.
   C. pH, dissolved oxygen, and conductivity on the elutriate samples on Day 0 of the test before dilutions are made.
   D. pH, dissolved oxygen, and conductivity in the 100%, 25%, and 0% elutriate dilutions at the end of the test.
   E. Temperature will be monitored daily in the test waterbaths.

V. Test stocking regime.
   A. Ten $\leq$ 24-hour old Daphnia magna per test beaker.
   B. Count groups of 5 daphnids directly into 250-mL test beakers until there is a total of 10 per beaker.

VI. Biological Sampling.
   A. Survival will be recorded in all treatments at 24 and 48 hours.
   B. Lack of mobility in response to prodding with a blunt probe during 5 seconds of observation will be used as criteria to determine death.

STANDARD GUIDE FOR CONDUCTING SEDIMENT TOXICITY TESTS WITH FRESHWATER INVERTEBRATES[1]

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1. Scope

1.1 This guide describes procedures for obtaining laboratory data to evaluate adverse effects of contaminants associated with whole sediment on freshwater organisms. The methods are designed to assess the toxic effects on invertebrate survival, growth, or reproduction, from short (for example, 10 days) or long-term tests, in static or flow-through water systems. Sediments to be tested may be collected from field sites or spiked with known compounds in the laboratory. Test procedures are described for three species, (1) Hyalella azteca, (2) Chironomus tentans, and (3) Chironomus riparius. Methods described in this document should also be useful for conducting sediment toxicity tests with other aquatic species, although modifications may be necessary.

1.2 Modification of these procedures might be justified by special needs. Results of tests conducted using unusual procedures are not likely to be comparable to results using this guide. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment toxicity tests with freshwater organisms.

1.3 The results from field collected sediments used in toxicity tests to determine a spatial or temporal distribution of sediment toxicity may be reported in terms of the biological effects on survival, growth, or reproduction (see Section 16, Calculation of Results). In addition, these procedures are applicable to most sediments or chemicals added to sediment. Materials either adhering to sediment particles or dissolved in interstitial water can be tested. With appropriate modifications these procedures can be used to conduct sediment toxicity tests when factors such as temperature, dissolved oxygen, pH, and sediment characteristics (for example, particle size, organic carbon content, total solids) are of interest, or when there is a need to test such materials such as sewage sludge, oils and particulate matter. These methods might also be useful for conducting bioaccumulation tests.

1.4 Results of toxicity tests with test materials experimentally added to sediments may be reported in terms of an LC50 (median lethal concentration), and sometimes an EC50 (median effect concentration). Results of tests may be reported in terms of an NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration).

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[1] This test method is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E-47.03 on Sediment Toxicity.

1.5 This guide is arranged as follows:

1.6 This guide addresses procedures which may involve hazardous materials, operations, and equipment, and it does not purport to address all of the safety problems associated with its use. It is the responsibility of the user to establish appropriate safety and health practices, and determine the applicability of regulatory limitations prior to use. While some safety considerations are included in this document, it is beyond the scope of this document to encompass all safety requirements necessary to conduct sediment toxicity tests. Precautionary statements are given in Section 7.

2. **Applicable Documents**

2.1 ASTM Standards:

E 380 Standard for Metric Practice[2]

E 729 Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[3]

E 943 Standard Definitions of Terms Relating to Biological Effects and Environmental Fate3

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses3

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes3

D 1129 Definitions of Terms Relating to Water[4]

D 4387 Guide for Selecting Grab Sampling Devices for Collecting Benthic Macroinvertebrates[3]

D 4447 Guide for Disposal of Laboratory Chemicals and Samples[3]

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[2] Annual Book of ASTM Standards, Vol 14.02.

[3] Annual Book of ASTM Standards, Vol 11.04.

[4] Annual Book of ASTM Standards, Vol 11.01.

D 4823 Guide for Core Sampling Submerged, Unconsolidated Sediments[5]

E 1367 Standard Guide for Conducting Solid Phase 10-Day Static Sediment Toxicity Tests with Marine and Estuarine Infaunal Amphipods

E 1391 Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing

E XXX Guide for Designing Sediment Toxicity Tests (Draft 4)

3. **Terminology**

3.1 The words "must", "should", "may", "can", and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with the factors that directly relate to the acceptability of the test (see Section 15). "Should" is used to state that the 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. Terms such as "is desirable", "is often desirable", and "might be desirable" are used in connection with less important factors. "May" is used to mean "is(are) allowed to", "can" is used to mean "is(are) able to", and "might" is used to mean "could possibly". Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can".

3.2 Descriptions of Terms Specific to this Guide:

3.2.1 sediment---a naturally occurring particulate material which has been transported and deposited at the bottom of a body of water, or an experimentally prepared substrate within which the test organisms can interact.

3.2.2 whole sediment---distinguished from elutriates, and resuspended sediments, in that the whole, intact sediment is used to expose the organisms, not a form or derivative of the sediment.

3.2.3 clean---denotes a sediment or water that does not contain concentrations of test materials which cause apparent stress to the test organisms or reduce their survival.

3.2.4 overlying water---the water placed over the whole sediment in the test chamber for the conduct of the toxicity test, and may also include the water used to manipulate the sediments.

3.2.5 interstitial water---the water within a wet sediment that surrounds the sediment particles, expressed as the percent ratio of the weight of the water in the sediment to the weight of the wet sediment.

3.2.6 spiking---the experimental addition of a test material such as a chemical or mixture of chemicals, sewage sludge, oil, particulate matter, or highly contaminated sediment to a clean negative control or reference sediment, such that the toxicity of the material added can be determined. After the test material is added, which may involve a solvent carrier, the sediment is thoroughly mixed to evenly distribute the test material throughout the sediment.

3.2.7 concentration---the ratio of weight or volume of test material(s) to the weight or volume of sediment.

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[5] Annual Book of ASTM Standards, Vol 11.02.

3.3 For definitions of other terms used in this guide, refer to Standards E 729, E 943, E 1023, E 1241 and D 1129. For an explanation of units and symbols, refer to Standard E 380.

4. **Summary of Guide**

4.1 The toxicity of contaminated whole sediments is assessed during continuous exposure of aquatic organisms, using either static or flow-through exposure systems. Sediments tested may either be collected from field sites or spiked with a known compound(s). A negative control sediment or a reference sediment is used to (a) give a measure of the acceptability of the test, (b) provide evidence of the health and relative quality of the test organisms, (c) determine the suitability of the overlying water, test conditions, food, handling procedures, and (d) provide a basis for interpreting data obtained from the test sediments. A reference sediment is collected from the field in a clean area and represents the test sediments in sediment characteristics (for example, TOC, particles size, pH). Specified data are obtained to determine the toxic effects on survival, growth, or reproduction, from short (for example, 10 days), or long-term exposures to aquatic invertebrates.

5. **Significance and Use**

5.1 Protection of a species requires averting detrimental contaminant related effects on the survival, growth, reproduction, health, and uses of the individuals of that species (1). Sediment toxicity tests provide information concerning the bioavailability of contaminants associated with sediments to aquatic organisms. Invertebrates occupy an essential niche in aquatic ecosystems and are an important food source for fish, wildlife, and larger invertebrates. A major change in the availability of invertebrates as either a food source, or as organisms functioning properly in trophic energy transfer and nutrient cycling, could have serious adverse ecological effects on the entire aquatic system.

5.2 Results from sediment toxicity tests might be an important consideration when assessing the hazards of materials on aquatic organisms (see Guide E 1023) or when deriving sediment quality concentrations for aquatic organisms (2).

5.3 Information might also be obtained on accumulation of contaminants associated with sediments by analysis of animal tissues for the contaminant(s) being monitored.

5.4 The sediment toxicity test might be used to determine the temporal or spatial distribution of sediment toxicity. Test methods can be used to detect horizontal and vertical gradients in toxicity.

5.5 Results of sediment toxicity tests with test materials experimentally added to sediments could be used to compare the sensitivities of different species, the toxicity of different test materials, and to study the effects of various environmental factors or results of such tests. Results of sediment toxicity tests are useful for studying biological availability of test materials, and structure-activity relationships.

5.6 Results of sediment toxicity tests can be used to predict effects likely to occur with aquatic organisms in field situations as a result of exposure under comparable conditions, except that (a) motile organisms might avoid exposure and (b) toxicity to benthic organisms can be dependent on sediment physical characteristics, dynamics of equilibrium partitioning, and the route of exposure.

5.6.1 Field surveys can be designed to provide either a qualitative reconnaissance of the distribution of sediment toxicity or a quantitative statistical comparison of toxicity among sites.

5.6.2 Sediment toxicity surveys are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic conditions. Statistical correlation can be improved and costs reduced if subsamples

for sediment toxicity tests, geochemical analyses, and benthic community structure are taken simultaneously from the same grab of the same site.

5.7 Sediment toxicity tests can be an important tool for making decisions regarding the extent of remedial action needed for contaminated aquatic sites.

6. **Interferences**

6.1 Limitations to the methods described in this guide might arise and thereby influence sediment toxicity test results and complicate data interpretation. The following factors should be considered when testing whole sediments:

6.1.1 Alteration of field samples in preparation for laboratory testing (for example, sieving).

6.1.1.1 Maintaining the integrity of the sediment environment during its removal, transport, and testing in the laboratory is extremely difficult. The sediment environment is composed of a myriad of microenvironments, redox gradients and other interacting physiochemical and biological processes. Many of these characteristics influence sediment toxicity and bioavailability to benthic and planktonic organisms, microbial degradation, and chemical sorption. Any disruption of this environment complicates interpretations of treatment effects, causative factors, and in situ comparisons.

6.1.1.2 Sediments tested at temperatures other than what they are collected might affect contaminant solubility, partitioning coefficients, and other physical and chemical characteristics.

6.1.2 Interaction between sediment and overlying water and the influences of the ratio of sediment to overlying water.

6.1.3 Interaction among chemicals present in the sediment.

6.1.4 Use of laboratory spiked sediment that might not be representative of contaminants associated with sediments in the field.

6.1.5 Maintenance of acceptable quality of overlying water.

6.1.6 Addition of food (3) or solvents to the test chambers might obscure the adverse influence of contaminants associated with sediment, provide an organic substrate for bacterial or fungal growth, and might affect water quality characteristics(4).

6.1.7 Resuspension of sediment during the toxicity test.

6.1.8 Natural geochemical properties of test sediment collected from the field might not be within the tolerance limits of the test species,

6.1.9 Recovery of test organisms from the sediment,

6.1.10 Field collected sediments may contain indigenous organisms including (a) predators, (b) the same or closely related species to that being tested, and (c) microorganisms (for example, bacteria and molds) and algae species that might grow in or on the sediment and test chamber surfaces.

6.1.11 Test material concentrations might be reduced in the overlying water in flow-through testing, and compounds such as ammonia might increase during testing.

6.2 Static tests might not be applicable with materials that are highly volatile or rapidly transform biologically or chemically. The dynamics of test material partitioning between solid and dissolved phases at the start of the test should therefore be considered, especially in relation to assumptions of chemical equilibria.

7. **Safety Precautions**

7.1 Many substances pose health risks to humans if adequate precautions are not taken. Information on toxicity to humans, recommended handling procedures, and chemical and physical properties of the test material should be studied before a test is begun and made aware to all personnel involved (5,6,7,8). Contact with st materials, overlying water and sediments should be minimized.

7.1.1 Many materials can adversely affect humans if precautions are inadequate. Skin contact with test materials and solutions should be minimized by such means as wearing appropriate protective gloves, laboratory coats, aprons, and safety glasses, and by using dip nets, sieves or tubes to remove test organisms from overlying water. When handling hazardous sediments the proper handling procedures might include (a) sieving and distributing sediments under a ventilated hood or in an enclosed glove box, (b) enclosing and ventilating the toxicity testing water bath, and (c) use of respirators, aprons, safety glasses, and gloves. Field collected sediments might contain toxic materials and should be treated with caution to minimize occupational exposure to workers. Worker safety should also be considered when working with spiked sediments containing organics or inorganic contaminants, those that are radio-labeled, and with materials that are, or are suspected of being, carcinogenic

7.2 Careful consideration should be given to those chemicals which might biodegrade, transform to more toxic components, volatilize, oxidize, or photolyze during the test period.

7.3 For tests involving spiked sediments with known test materials, removal or degradation of test material before disposal of stock solutions, overlying water, and sediments is sometimes desirable.

7.4 Health and safety precautions and applicable regulations for disposal of stock solutions, test organisms, sediments, and overlying water should be considered before beginning a test (ASTM D 4447).

7.5 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame such as a pilot light is present.

7.6 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.7 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

7.8 Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks.

8. **Apparatus**

8.1 Facilities -- The facility should include constant temperature areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. Test chambers may be placed in a temperature controlled recirculating water bath or a constant-temperature area. Air used for aeration should be free of fumes, oil, and water. Filters to remove oil, water, and bacteria are desirable. Air filtration through a 0.22 μm bacterial filter or other suitable system may be used. The test facility should be well ventilated and free of fumes. Enclosures might be desirable to ventilate test chambers.

8.1.2 If a photoperiod other than continuous light is used, a timing device should be used to provide a light:darkness cycle. A 15- to 30-minute transition period (9) when lights go on and off it might be desirable to

reduce the possibility of test organisms being stressed by instantaneous illumination; a transition period when lights go off might also be desirable.

8.2 Construction Materials -- Equipment and facilities that contact stock solutions, test solutions, sediment and overlying water, into which test organisms will be placed, should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and facilities that contact sediment or water should be chosen to minimize sorption of test materials from water. Glass, type 316 stainless steel, nylon, high density polyethylene, polycarbonate and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Concrete and rigid (unplasticized) plastics may be used for holding, acclimation, and culture tanks, and in the water-supply system, but these materials should be soaked, preferably in flowing water, for a week or more before use (10). Cast-iron pipe should probably not be used in freshwater-supply system because colloidal iron will be added to the overlying water and strainers will be needed to remove rust particles. Copper, brass, lead, galvanized metal, and natural rubber should not contact overlying water or stock solutions before or during the test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect survival, growth, or reproduction of the test organisms.

8.3 Water Delivery System -- The water delivery system used in flow-through testing can be one of several designs. The system should be capable of delivering water to each replicate test chamber. Several designs of diluter systems are currently in use; **Mount** and **Brungs** (11) diluters have been successfully modified for sediment testing and other diluter systems have also been useful according to **Ingersoll** and **Nelson** (4) and **Maki** (12). Various metering systems, using different combinations of siphons, pumps, solenoids, valves, etc., have been used successfully to control the flow rates of overlying water.

8.3.1 The metering system should be calibrated before the test by determining the flow rate of the overlying water through each test chamber. The general operation of the metering system should be visually checked daily throughout the conduct of the test. If necessary the water delivery system should be adjusted during the test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%.

8.4 Test Chambers

8.4.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. However, screens, cups, etc., may be used to create two or more compartments within each chamber. Therefore, the overlying water can flow from one compartment to another within a test chamber but, by definition, cannot flow from one chamber to another. All test chambers and compartments if used, in a sediment toxicity test, must be identical. For the static tests, cover watch glasses may be used to fit over the top of the test chambers such that an aeration tip is accommodated.

8.4.2 Test chambers may be constructed in several ways of various materials, depending on the experimental design and the contaminants of interest. Clear silicone adhesives, suitable for aquaria, sorb some organic compounds which might be difficult to remove. Therefore, as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed, they should be on the outside of the test chambers rather than on the inside. To leach potentially toxic compounds from the adhesive, all new test chambers constructed

using silicone adhesives should be acclimated at least 48 hours in overlying water used in the sediment toxicity test.

8.4.3 Species-specific information on test chambers is given in each appendix (see Species Specific Appendices).

8.5 Cleaning -- Test chambers, water delivery systems, equipment used to prepare and store overlying water, and stock solutions, should be cleaned before use. New items should be washed in the following manner: (a) detergent wash, (b) water rinse, (c) water-miscible organic solvent wash, (d) water rinse, (e) acid wash (such as 10% concentrated hydrochloric acid), and (f) rinsed at least twice with distilled, deionized, or overlying water. Test chambers should be rinsed with overlying water just before use.

8.5.1 Many organic solvents leave a film that is insoluble in water. A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid (see ASTM E 729), but the solution might attack silicone adhesive and leave chromium residues on glass.

8.5.2 Upon completion of a test, all items to be used again should be immediately (a) emptied of sediment and overlying water (and properly disposed), (b) rinsed with water, (c) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), and (d) rinsed at least twice with distilled, deionized, or overlying water.

8.6 Acceptability -- Before a toxicity test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain a negative control or reference sediment, and overlying water with no added test material. Survival, growth, or reproduction of the test species will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable species-specific control numbers. The magnitude of the within-chamber and between-chamber variance should also be determined.

9. **Overlying Water**

9.1 Requirements -- Besides being available in adequate supply, overlying water used in toxicity tests, and water used to hold organisms before testing, should be acceptable to test species and uniform in quality. To be acceptable to the test species, the water must allow satisfactory survival and growth, without showing signs of disease or apparent stress, such as discoloration, or unusual behavior.

9.2 Source

9.2.1. Natural overlying water should be uncontaminated and of constant quality and should meet the following specifications as established in ASTM E 729. The values stated help to ensure that test organisms are not apparently stressed during holding, acclimation, and testing, and that test results are not unnecessarily affected by water quality characteristics:

Particulate matter	<5 mg/L
TOC	<5 mg/L
COD	<5 mg/L
Residual chlorine	<11 μg/L

9.2.1.2 A natural overlying water is considered to be of uniform quality if the 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 unit. Natural overlying 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

minimize fluctuations in quality and the possibility of contamination and maximize the concentration of dissolved oxygen and to help ensure low concentrations of sulfide and iron. Municipal water supplies often contain unacceptably high concentrations of copper, lead, zinc, fluoride, chlorine or chloramines, and quality is often variable (13). Chlorinated water should not be used for, or in the preparation of, overlying water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals (14). Dechlorinated water should only be used as a last resort, because dechlorination is often incomplete.

9.2.2 For certain applications the experimental design might require use of water from the test sediment collection site.

9.2.3 Reconstituted water is prepared by adding specified amounts of reagent grade[6] chemicals to high quality distilled or deionized water (see ASTM E 729). Acceptable water can be prepared using deionization, distillation, or reverse-osmosis units. Conductivity, pH, hardness and alkalinity should be measured on each batch of reconstituted water. If the water is prepared from a surface water, total organic carbon or chemical oxygen demand should be measured on each batch. Filtration through sand, rock, bag, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low. The reconstituted water should be intensively aerated before use, except that buffered soft fresh waters should be aerated before, but not after, addition of buffers. Problems have been encountered with some species in some fresh reconstituted waters, but these problems can be overcome by aging the reconstituted water for one or more weeks.

9.3 Characterization -- The following items should be measured at least twice each year, and more often if (a) such measurements have not been determined semiannually for at least two years, or (b) if surface water is used:

9.3.1 pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, chloride, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, sodium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc, hardness, alkalinity, and conductivity (see ASTM E 729).

9.3.2 For each method used the detection limit should be below (a) the concentration in the overlying water, or (b) the lowest concentration that has been shown to adversely affect the test species (14).

9.3.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (15) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 μm or less.

9.3.4 Water might need intense aeration using air stones, surface aerators, or column aerators (16,17,18). Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in

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[6] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

water should be between 90% and 100% saturation (19) to help ensure that dissolved oxygen concentrations are acceptable in test chambers.

10. **Sediment Characterization**

10.1 General -- Before the preparation or collection of sediment an approved written procedure should be prepared for the handling of sediments which might contain unknown quantities of toxic contaminants (see Section 7, Safety Precautions). All sediments should be characterized and at least the following determined: pH, organic carbon content (total organic carbon TOC) or total volatile sulfides, particle size distribution (percent sand, silt, clay), and percent water content (20,21). Other analyses on sediments might include biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, pE, total inorganic carbon, total volatile solids, acid volatile sulfides, total ammonia, metals, organosilicones, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and interstitial water analysis. Macrobenthos may be determined by a subsample of the field collected sediment. Toxicological results might provide information directing a more intensive analysis. Sediment toxicity testing procedures are detailed in Section 13, Procedures.

10.2 Negative Control and Reference Sediment -- A negative control sediment or a reference sediment is used to (a) give a measure of the acceptability of the test, (b) provide evidence of the health and relative quality of the test organisms, (c) determine the suitability of the overlying water, test conditions, food, handling procedures, and (d) provide a basis for interpreting data obtained from the test sediments. Every test requires a negative sediment control (sediment known to be non-toxic to, and within the geochemical requirements of the test species) or a reference sediment. A reference sediment should be collected from the field in a clean area and represent the test sediment in sediment characteristics (for example, TOC, particles size, pH). This provides a site-specific basis for comparison of toxic and non-toxic conditions. The same overlying water, conditions, procedures, and organisms should be used as in the other treatments, except that none of the test material(s) being tested, or contaminated field collected sediments, is added to the negative control or reference sediment test chambers.

10.2.1 If a field sediment has properties such as, grain size and organic content which might exceed the tolerance range of the test species, it is desirable to include a reference sediment for these characteristics.

10.3 Field Collected Test Sediment

10.3.1 Collection (see Section 7, Safety Precautions). A benthic grab or core should be used rather than a dredge to minimize disruption of the sample (see ASTM Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and ASTM Standard Guide D 4387). If the sediment is obtained with a grab, it is preferable to collect a sediment sample from the upper 2 cm. This operation is facilitated if the grab can be opened from the top so that the undisturbed sediment surface is exposed. The sample should be transferred to a clean (see Section 8.5, Cleaning) sample container. If the contaminants associated with sediments include compounds that readily photolyze, minimize direct sunlight during collection. All sediment samples should be cooled to 4°C ±2°C in the field.

10.3.2 Storage. Sediment samples should be stored at 4°C ±2°C and for no longer than two weeks before the start of the test. Freezing and longer storage might change sediment properties and should be avoided (see ASTM Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing). Sediment may be stored in containers constructed of suitable quality as outlined in Section 8.2, Construction Materials. It is desirable to avoid contact with metals, including stainless steel and brass sieving

screens, and some plastics. The samples should be thoroughly mixed and may be wet-press sieved through a suitably sized sieve to remove large particles and indigenous organisms, especially predators. Sediment may be diluted and mixed 1:1 with overlying water to facilitate sieving (22), see Section 6, Interferences.

10.3.3 If the experimental design prescribes not sieving a field collected sediment, obvious large predators or other large organisms should be removed by using forceps. If sediment is to be collected from multiple field samples and pooled to meet technical objectives, the sediment should be thoroughly homogenized by stirring, or with the aid of a rolling mill, feed mixer, or other suitable apparatus (see ASTM Proposed Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing).

10.3.4 Additional samples may be taken from the same grab for other kinds of sediment analyses (see 10.1). Qualitative descriptions of the sediment may include color, texture, presence of macrophytes, animals, tracks, and burrows. Monitoring the odor of sediment samples should be avoided because of hazardous volatile contaminants (see Section 7, Safety Precautions).

10.3.5 The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. The limits for the test species should be determined experimentally in advance (see 10.2). Controls for such factors as particle size distribution, organic carbon content, pH, etc., should be run if the limits are exceeded in the test sediments (23).

10.4 Laboratory Spiked Sediment -- Test sediment can also be prepared in the laboratory by manipulating the properties of the negative control or the reference sediment. This can include adding chemicals or complex waste mixtures (see Section 1.4) (24). The toxicity of substances either dissolved in the interstitial water or adsorbed to sediment particles can be determined experimentally.

10.4.1 The test material(s) should be reagent grade[7] or better, unless a test on formulation commercial product (25), or technical-grade or use-grade material is specifically needed. Before a test is started, the following should be known about the test material (a) the identity and concentration of major ingredients and impurities, (b) water solubility in test water, (c) estimated toxicity to the test species and to humans, (d) precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentration(s) are to be measured, and (e) recommended handling and disposal procedures. The toxicity of the test material in sediments may be quite different from the toxicity in water borne exposures.

10.4.2 Stock Solution(s). Test material(s) to be tested in sediment should be dissolved in a solvent to form a stock solution that is then added to the sediment. The maximum concentration of the solvent in the sediment should be at a concentration that does not affect the test species. The concentration and stability of the chemical in the stock solution should be determined before beginning the test. If the chemical(s) is subject to photolysis, the stock solution should be shielded from the light both before and during the process of mixing into the sediment. If a solvent other than water is necessary (the preferred solvent is water), it should be one which can

[7] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

be driven off (for example, evaporated) leaving only the test chemical on the sediments. Concentrations of the chemical in the water and sediment should be monitored before the test begins.

10.4.3 If a solvent other than water is used, both a sediment solvent control, and a sediment negative control or reference sediment must be included in the test. The solvent control must contain the highest concentration of solvent present and must use solvent from the same batch used to make the stock solution (see ASTM E 729). The same concentration of solvent should be used in all treatments.

10.4.3.1 Triethylene glycol is often a good organic solvetn for preparing stock solutions because of its low toxicity to aquatic animals, low volatility, and ability to disslove many organic chemicals. Other water-miscible organic solvents, such as methanol, ethanol or acetone may be used, but they might affect total organic carbon levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microoorganisms (see Section 6, Interferences). Acetone is highly volatile and might leave the system more readily than methanol or ethanol. A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form and toxicity of the test material.

10.4.4 If the concentration of solvent is not the same in all test solutions that contain test material, either (a) a solvent test should be conducted to determine whether survival, growth, or reproduction of the test organisms is related to the concentration of the solvent over the range used in the toxicity test, or (b) such a solvent test already conducted using the same overlying water and test species. If survival, growth, or reproduction is found to be related to the concentration of solvent, a sediment toxicity test with that species in that amount of solvent is unacceptable if any treatment contained a concentration of solvent in that range.

10.4.4.1 If the test contains both a negative control and a solvent control, the survival, growth, or reproduction of the organisms tested in the two controls should be compared (see ASTM E 1241). If a statistically significant difference in either survival, growth, or reproduction is detected between the two controls, only the solvent control may be used for meeting the acceptability of the test and as the basis for calculation of results. The negative control might provide additional information on the general health of the organisms tested. If no statistically significant difference is detected, the data from both controls should be used for meeting the acceptability of the test and as the basis for calculation of results (see ASTM E 1241, Section 9.2.4.3).

10.4.5 Test Concentration(s) for Laboratory Spiked Sediments.

10.4.5.1 If the test is intended to allow calculation of an LC50, the test concentrations should bracket the predicted LC50. The prediction might be based on the results of a test on the same or a similar test material on the same or a similar species. The LC50 of a particular compound may vary depending on physical and chemical sediment characteristics. If a useful prediction is not available, it is desirable to conduct a range-finding test in which the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of ten.

10.4.5.2 If necessary, concentrations above aqueous solubility can be used, as indigenous organisms are at times exposed to concentrations above solubility in the real world (see ASTM E 729).

10.4.5.3 Bulk sediment chemical concentrations might be normalized to factors other than dry weight. For example, concentrations of non-polar organic compounds might be normalized to sediment organic carbon content, and metals normalized to acid volatile sulfides.

10.4.5.4 In some situations (for example, regulatory) it might be necessary to only determine (a) whether a specific concentration of test material is toxic to the test species, or (b) whether the LC50 is above or below a

specific concentration. When there is interest in a particular concentration, it might only be necessary to test that concentration and not to determine the LC50.

10.4.6 Addition of test material(s) to sediment may be accomplished using various methods, such as a (a) rolling mill, (b) feed mixer, or (c) hand mixing, (see ASTM Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing).

10.4.6.1 Modifications of the mixing techniques might be necessary to allow time for a test material to equilibrate with the sediment. If tests are repeated, mixing conditions such as duration and temperature of mixing, and time of mixing before the test starts, should be kept constant. Care should be taken to ensure that a test material added to sediment is thoroughly and evenly distributed within the sediment. If necessary, subsamples of the sediment within a mixing container can be analyzed to determine degree of mixing and homogeneity.

11. **Test Organisms**

11.1 Species -- Whenever possible and appropriate, tests should be conducted with species listed in the Appendices. Use of these species is encouraged to increase comparability of results. The source and type of sediment being tested or the type of test to be implemented might dictate selection of a particular species. The species used should be selected based on (a) availability, (b) sensitivity to a test material(s), and (c) tolerance to ecological conditions such as temperature, grain size, and ease of handling in the laboratory. The species used should be identified using an appropriate taxonomic key.

11.2 Age -- All organisms should be as uniform as possible in age and size class. The age or size class for a particular test species should be chosen so that sensitivity to test materials is not affected by state of maturity, reproduction, or other intrinsic life-cycle factors (see Species Specific Appendices).

11.3 Source -- All organisms in a test must be from the same source. Organisms may be obtained from (a) laboratory cultures, (b) commercial, state or federal institutions, or (c) natural populations from clean areas. Laboratory cultures of test species can provide organisms whose history, age, and quality are known. Local and state agencies might require collecting permits.

11.4 Quality -- Analysis of the test organisms for the test material(s) is desirable, as it might be present in the environment, and other chemicals to which major exposure might have occurred.

11.5 Brood Stock -- Brood stock should be cared for properly so as not to be unnecessarily stressed (see Species Specific Appendices). To maintain organisms in good condition and avoid unnecessary stress, they should not be crowded and should not be subjected to rapid changes in temperature or water quality characteristics.

11.6 Handling -- Test organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and as quickly as possible. Organisms should be introduced into solutions beneath the air-water interface. Any organisms that touch dry surfaces, are dropped, or injured during handling should be discarded.

12. **Experimental Design**

12.1 Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and test organisms per treatment, and water quality characteristics, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 16,

Calculation of Results). A test intended to allow calculation of a specific endpoint such as an LC50 should consist of a negative control sediment, a solvent control(s), a reference sediment, and several test sediments (see Section 10, Sediment Characterization).

12.2 The object of a qualitative reconnaissance survey is to identify sites of toxic conditions that warrant further study. It is often conducted in areas where little is known about contamination patterns. To allow for maximum spatial coverage, the survey design might include only one sample from each site. The lack of replication usually precludes statistical comparisons, but identification of samples for further study is possible, where survival, growth, or reproduction differ from the negative control or reference sediment. A useful summary of field sampling design is presented by Green (26).

12.2.1 The object of a quantitative statistical comparison is to test for statistically significant differences in effects (see Section 13.12, Biological Data) among negative control or reference sediments and test sediments from several sites. The number of replicates needed per site is a function of the need for sensitivity or power. Replicates (for example, separate samples from different grabs taken at the same site) should be taken at each site in the survey. Separate subsamples from the same grab might be used to test for within-grab variability, or split samples of composited sediment from one or more grabs might be used for comparisons of test procedures (such as comparative sensitivity among test species), but these subsamples should not be considered to be true replicates for statistical comparisons among sites.

12.2.2 Site locations might be distributed along a known pollution gradient, in relation to the boundary of a disposal site, or at sites identified as being toxic in a reconnaissance survey. Comparisons can be made in both space and time (see Calculation of Results, Section 16). In pre-dredging studies, a sampling design can be prepared to assess the toxicity of samples representative of the project area to be dredged. Such a design should include subsampling cores taken to the project depth.

12.3 Laboratory Experiments. The primary focus of the physical and experimental test design, and statistical analysis of the data, is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned (27). Because overlying water or air can not flow from one test chamber to another the test chamber is the experimental unit (see Section 8.4, Test Chambers). As the number of test chambers per treatment increases, the number of degrees of freedom increases, and, therefore, the width of the confidence interval on a point estimate, such as an LC50, decreases, and the power of a significance test increases (see Calculation of Results, Section 16). Because of factors that might affect results within test chambers and results of the test: (a) all test chambers should be treated as similarly as possible, such as temperature and lighting (unless these are the variables tested), and (b) each test chamber, including replicate test chambers, must be physically treated as a separate entity. Treatments must be randomly assigned to individual test chamber locations. Assignment of test organisms to test chambers must be randomized.

13. **Procedure**

13.1 Sediment into Test Chambers -- The day before the toxicity test is started (Day -1) each test sediment, reference sediment, and negative control sediment should be mixed and a sample added to the test chambers (4,24,28). Sediment depth in the test chamber is dependent on the experimental design and the test species (see Species Specific Appendices and Section 6.1.2). Each test chamber and replicates must contain the same amount of sediment, determined either by volume or weight.

13.1.1 The sediment aliquot in each test chamber should be settled by smoothing with a utensil constructed of a suitable material (see Section 8.2, Construction Materials). If beakers are used, bubbles can be removed by either tapping the test chamber against the palm of the hand or by displacement of bubbles with the utensil. After the sediment is placed in the test chambers, overlying water should be added. The overlying water should be gently poured along the side of the test chamber to prevent resuspension of the sediment.

13.2 Static Testing -- Overlying water should be added to the test chambers at the volume specified by the experimental design. Watch glasses should be used to cover the test chambers and overlying water gently aerated. Aeration can be provided to each test chamber through a 1-mL glass pipet that extends between the beaker spout and the watch glass cover to a depth not closer than 2 cm from the sediment surface. Air should be bubbled into the test chambers at a rate that does not cause turbulence or disturb the sediment surface. To allow any suspended sediments to settle, the test organisms should not be introduced into the test system for 12-24 hours. Water quality characteristics should be measured prior to the addition of the test organisms (see Section 13.11, Overlying Water Quality Measurements).

13.2.1 Water lost to evaporation or splattering should be replaced as needed with temperature acclimated de-ionized water or overlying water. The water quality of the overlying water in static sediment toxicity tests (water hardness, alkalinity, total dissolved solids, and dissolved oxygen) might be altered by the presence of sediment (4) or by the addition of food to the test chamber (3). These changes in water quality characteristics might influence the availability of contaminants to the test organisms (see Section 6, Interferences).

13.3 Flow-Through Testing -- The water-delivery system should be turned on before a test is started to verify that the system is functioning properly. The water flow to each test chamber should not differ by more than 10% (see Section 8.3.1). The total volume flow per hour for continuous flow diluters should be recorded.

13.3.1 After the sediment has been added (Day -1), overlying water is added to the test chambers (see Section 13.2 Static Testing). After aliquots are removed for water quality determinations (Day 0), overlying water flow is started prior to the addition of the test organisms and food (4).

13.4 Duration of Test -- The test begins when test organisms are first placed in the test chambers (Day 0) and continues for the duration specified in the experimental design for a specific test organism (see Species Specific Appendices).

13.5 Dissolved Oxygen -- The dissolved oxygen concentration in each test chamber should be measured in at least one test chamber in each treatment (a) at the beginning and end of the test and at least weekly (if possible) during the test, (b) whenever there is an interruption of the flow of air (static tests) or water (flow-through tests), and (c) whenever the behavior of the test organisms indicate that the dissolved oxygen concentration might be too low (for example, emergence from the sediment). A measured dissolved oxygen concentration should be > 40% and ≤ 100% saturation (E 729, Section 12.4.2).

13.6 Overlying Water Quality Measurements -- Conductivity, hardness, pH, and alkalinity should be measured in all treatments at the beginning and end of a short-term test, and at least weekly during a long-term test, using appropriate ASTM standards when possible.

13.7 Temperature -- Test temperature depends upon the species used (see Species Specific Appendices). Other temperatures may be used to study the effect of temperature on survival, growth, or reproduction of test

organisms, and contaminant related properties (for example, bioavailability). The daily mean test temperature must be within ± 1°C of the desired temperature. The instantaneous temperature must always be within ± 3°C of the desired temperature.

13.8 Feeding -- Recommended food, ration, method and frequency of feeding test organisms are contained in Species Specific Appendices. The food used should be analyzed for the test material and other possible contaminants. A batch of food may be used if it will support normal function. Detailed records on feeding rates and appearance of the sediment should be made daily.

13.9 Debris -- Any floating debris may be skimmed from the test chambers before test organisms are added. This can be accomplished with a piece of fine nylon screen or other suitable material. If more than 0.1 g of floating debris is removed, an analysis should be performed to determine the amount of chemical removed from the system (25).

13.10 Light -- For sediment toxicity tests various light:darkness regimes can be used depending on the species being tested (see Species Specific Appendices) and various experimental designs.

13.11 Acclimation -- Test organisms should be acclimated if they are cultured in water different from the overlying water or temperature (4) (see Species Specific Appendices).

13.12 Biological Data -- Effects indicating toxicity of test sediment include mortality and sublethal effects on growth, maturation, behavior, and reproduction. Test chambers should be observed at least daily. At the end of the exposure period, recovery of the test organisms from sediments should be accomplished following the methods outlined for each species (see Species Specific Appendices).

13.13 Other Measurements:

13.13.1 Field Sediment. Sediment samples should be collected from the same grab for analysis of sediment physical and chemical characterizations. A separate sample for benthic faunal analyses may be desirable (see ASTM D 4387).

13.13.2 Laboratory Spiked Sediments. At the beginning and at the end of the experiment, measurement of the concentration of the test material(s) in both stock solutions and sediment, is desirable. To monitor changes in sediment or interstitial water chemistry during the course of the experiment, separate sediment chemistry chambers should be set up and sampled at the start and end of the experiment. It is not necessary to add test organisms to these chambers at the beginning of the test, but for later sampling, test organisms should be added after the initial sample is taken.

13.13.2.1 Concentration of test material(s) in overlying water, interstitial water and sediment should be measured at several concentrations and as often as practical during the test. If possible, the concentration of the test material in overlying water, interstitial water and sediments should be measured at the start and end of the test. Measurement of test material(s) degradation products might also be desirable.

13.13.2.2 Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from a point midway between top, bottom and sides of the test chamber. Overlying water samples should not contain any surface scum, any material from the sides of the test chamber, or any sediment.

13.13.2.3 Measurement of test material(s) concentration in sediment at the end of a test can be taken by siphoning the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the sediment for chemical analysis.

13.13.2.4 Interstitial water can be sampled by using the water that (a) comes to the surface in a mixing apparatus, (b) is on the surface of the sediment after it settles, (c) is separated from the sediment particles by centrifuging a sediment sample, (d) is filtered through an apparatus to extract interstitial water, (e) has been pressed out of the sediment, or (f) by using an interstitial water sampler. Care should be taken to ensure that contaminants do not transform, degrade, or volatilize during the interstitial water sample preparation (see ASTM Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing).

14. **Analytical Methodology**

14.1 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources (29).

14.2 Concentrations should be measured for (a) contaminants in bulk sediment, (b) test material(s) in the interstitial water, (c) test material(s) in the overlying water, and (d) test material(s) in the stock solution. In addition, measurement of either the apparent dissolved or undissolved substances of the test material(s) is desirable. The apparent dissolved material is defined and determined as that which passes through a 0.45 μm membrane filter.

14.2.1 If samples of overlying water from test chambers, stock solutions, test sediment or interstitial water are not to be analyzed immediately, they should be handled and stored appropriately (30) (see Section 10, Sediments).

14.3 Methods used to analyze food or test organisms should be obtained from appropriate sources (31).

14.4 The precision and bias of each analytical method used should be determined in an appropriate matrix: that is, sediment, water, tissue. When appropriate, reagent blanks, recoveries, and standards should be included when samples are analyzed.

15. **Acceptability of Test**

15.1 A sediment toxicity test should be considered unacceptable if one or more of the following occurred, except, for example, if temperature was measured numerous times, a deviation of more than 3°C (see 13.6, Temperature) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature had been measured more often.

15.1.1 All test chambers (and compartments) were not identical (Section 8.4.1, 12.3).

15.1.2 The overlying water was not acceptable to the test organisms (Section 9.1).

15.1.3 Test organisms were not acclimated to the approriapte overlying water or temperature if they are cultured in water different from the overlying water or temperature.

15.1.4 The natural geochemical properties of test sediment collected from the field was not within the tolerance limits of the test species (Section 10.3.5).

15.1.5 Appropriate negative and solvent controls, or reference sediment, were not included in the test (Section 10.4.3).

15.1.6 The concentration of solvent in the range used affected survival, growth, or reproduction of the test organisms (Section 10.4.4).

15.1.7 All animals in the test population were not obtained from the same source, were not all of the same species, or were not of acceptable quality (Section 11.3).

15.1.8 Treatments were not randomly assigned to individual test chamber locations and the individual test organisms were not impartially or randomly assigned to test chambers or compartments (Section 12.3).

15.1.9 Each test chamber must contain the same amount of sediment, determined either by volume or weight.

15.1.10 Temperature, dissolved oxygen, and concentration of test material were not measured, or within the acceptable range (Section 13.7 and Species Specific Appendices).

15.1.11 The negative control or reference sediment organisms did not survive, grow or reproduce as required for the test species (see Species Specific Appendices).

15.1.12 Average survival in any negative control chamber is less than acceptable limits (see Species Specific Appendices).

16. **Calculation of Results**

16.1 The calculation procedure(s) and interpretation of the results should be appropriate to the experimental design. Procedures used to calculate results of toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (a) the advantages and disadvantages of various alternative procedures, and (b) appropriate preliminary tests, such as those for outliers and for heterogeneity.

16.2 For each set of data the LC50 or EC50 and its 95% confidence limits should be calculated (when appropriate) on the basis of (a) the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and (b) the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LC or ECs are calculated, their 95% confidence limits should also be calculated (see ASTM E 729).

16.3 Most toxicity tests produce quantal data, that is, counts of the number of responses in two mutually exclusive categories, such as alive or dead. A variety of methods (32) can be used to calculate an LC50 or EC50 and 95% confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or effected is between zero and 100, but the most widely used are the probit, moving average, Spearman-Karber and Litchfield-Wilcoxon methods. The method used should appropriately take into account the number of test organisms per chamber. The binomial test can also be used to obtain statistically sound information about the LC50 or EC50 even when less than two concentrations kill or affect between zero and 100 percent. The binomial test provides a range within which the LC50 or EC50 should lie.

16.4 When samples from field sites are independently replicated, the site effects can be statistically compared by t-tests, analysis of variance (ANOVA) or regression type analysis. Analysis of variance is used to determine whether any of the observed differences among the concentrations (or samples) are statistically significant. This is a test of the null hypothesis that no differences exist in the effects at all of the concentrations (or samples) and at the control. If the F-test is not statistically significant ($P>0.05$), it can be concluded that the effects observed in the test material treatments (or field sites) were not large enough to be detected as statistically significant by the experimental design and hypothesis test used. Non-rejection does not mean that the null hypothesis is true.

The NOEC based on this end point is then taken to be the highest test concentration tested (33,34). The amount of effect that occurred at this concentration should be considered.

16.4.1 All exposure concentration effects (or field sites) can be compared with the control effects by using mean separation techniques such as those explained by Chew (35) orthagonal contrasts, Fisher's methods, Dunnett's procedure or Williams' method. The lowest concentration for which the difference in observed effect exceeds the statistical significant difference is defined as the LOEC for that end point. The highest concentration for which the difference in effect is not greater than the statistical significant difference is defined as the NOEC for that end point (33).

17. **Documentation**

17.1 The record of the results of an acceptable sediment toxicity test should include the following information either directly or by reference to available documents.

17.1.1 Name of test and investigator(s), name and location of laboratory, and dates of start and end of test.

17.1.2 Source of negative control, reference or test sediment, method for collection, handling, shipping, storage and disposal of sediment.

17.1.3 Source of test material, lot number if applicable, composition (identities and concentrations of major ingredients and impurities if known), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

17.1.4 Source of overlying water, its chemical characteristics, and a description of any pretreatment, and results of any demonstration of the ability of a species to survive, grow or reproduce in the water.

17.1.5 Source, history and age of test organisms; source, history and age of brood stock, culture procedures; and source and date of collection of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, age, life-stage, means and ranges of weight and lengths, observed diseases or unusual appearance, treatments, holding and acclimation procedures.

17.1.6 Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency and ration.

17.1.7 Description of the experimental design and test chambers (and compartments), the depth and volume of sediment and overlying water in the chambers, lighting, number test chambers and number of test organisms per treatment, date and time test starts and ends, temperature measurements, dissolved oxygen concentration (as percent saturation) and any aeration used prior to initiating a test and during the conduct of a test.

17.1.8 Methods used for, and results (with standard deviations or confidence limits) of, physical and chemical analyses of sediment.

17.1.9 Definition(s) of the effects used to calculate LC50 or EC50s, biological endpoints for tests, and a summary of general observations of other effects.

17.1.10 A table of the biological data for each test chamber for each treatment including the control(s) in sufficient detail to allow independent statistical analysis.

17.1.11 Methods used for, and results of, statistical analyses of data.

17.1.12 Summary of general observations on other effects or symptoms.

17.1.13 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

17.1.14 Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

ANNEX X1. Hyalella azteca

X1.1 Significance - Hyalella azteca (Saussure), Amphipoda, has many desirable characteristics of a test species: short generation time, easily collected from natural sources or cultured in the laboratory in large numbers, and data on survival, growth, and reproduction can be obtained in toxicity tests (36). **Landrum and Scavia** (37), **Nebeker et al.** (22), and **Ingersoll and Nelson** (4) have successfully used H. azteca in sediment toxicity testing and have shown it to be a sensitive indicator of the presence of contaminants associated with sediments. **Ingersoll and Nelson** (4) report H. azteca to have a wide tolerance of sediment grain size. Sediment ranging from >90% silt- and clay-size particles to 100% sand-size particles did not reduce survival or growth in the laboratory.

X1.2 Life History and Life-Cycle - The life-cycle of H. azteca can be divided into three distinct stages according to **Cooper** (36): (1) an immature stage, consisting of the first 5 instars; (2) a juvenile stage, including instars 6 and 7; and (3) an adult stage, the 8th instar and older. The potential number of adult instars is large and growth is indeterminate such that old adults can be much larger than younger adults (38). **DeMarch** (39) indicates that juvenile H. azteca can complete a life-cycle in 27 days or longer depending on temperature.

X1.2.1 H. azteca is an epibenthic detritivore and will burrow in the sediment surface, and **Hargrave** (40) has demonstrated in laboratory experiments that H. azteca digests bacteria and algae from ingested sediment particles (< 65 μm), further illustrating sediment interactions by H. azteca.

X1.2.2 Sexual dimorphism occurs in H. azteca, the adult male is larger than females and has larger second gnathopods (41).

X1.2.3 **DeMarch** (41) indicates that the number of young produced per adult female is optimum at temperatures of 26-28 °C. Whereas, **Cooper** (36) and **Strong** (38) report that maximum brood size is more dependent on the size of the adult amphipods than on temperature.

X1.3 Obtaining Test Organisms - The following culture procedures are adapted from **deMarch** (41), **Nebeker et al.** (22), and **Ingersoll and Nelson** (4). H. azteca can be reared in 10- or 20-L aquaria under flowing water conditions with a 16:8 hour light:darkness photoperiod at 20±2°C, and about 500 foot-candles (5382 lux). For static cultures, the water should be gently aerated and about 25-30 percent of the water volume should be replaced weekly. In flow-through cultures, water delivery can be at a low rate (100 mL/min) (4).

X1.3.1 H. azteca can be cultured with a variety of foods. Dried maple, alder, birch or poplar leaves, presoaked for several days and tannins flushed out with water, then can be added weekly as the primary substrate and food. Rabbit pellets [8], ground cereal leaves [9], fish food flakes [10], frozen or newly hatched brine shrimp or heat-killed young Daphnia can be used to feed H. azteca. In addition, **Strong** (38) demonstrated success in

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[8] Purina Rabbit Chow, Purina Mills, Inc., 1401 Hanley, St. Louis, MO 63144.

[9] Cerophyl, Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.

[10] TetraMin Fish Food Flakes, TetraWerke, Dr. rer. nat. Ulrich Baensch GmbH, D-4520 Melle 1, W. Germany.

culturing H. azteca yielding the best survivorship and consistently the largest clutches by feeding the amphipods filamentous green algae (Oedogonium cardiacum) and homogenized rotting spinach ad libitum.

X1.3.2 To clean the culture tanks or reduce populations of animals, half of the leaf substrate containing a portion of the animals should be transferred to a sorting tray, discarding the remainder of the old contents and returning the leaf substrate and animals to the chamber. The number of amphipods should be reduced periodically as the population expands rapidly.

X1.4 Collection - H. azteca can be found in permanent lakes, ponds and streams throughout the entire American continent (41,42). Methods used by **Landrum and Scavia** (37) indicate that the amphipods can be collected from a natural freshwater source. **Pennak** (42) suggests using a dip-net to collect aquatic vegetation and bottom debris containing amphipods. Sites with stony bottoms might require collecting with forceps or the use a small aquarium net. Live specimens can be maintained in aquaria if they are well supplied with aquatic vegetation (42). Collection procedures for H. azteca by **deMarch** (41) indicate that rinsing aquatic vegetation is effective if a 200-550 μm mesh net is used to catch the amphipods. Up to 200 amphipods can be transported in a large plastic bag containing 1 L of water from the collection site, with the remainder of the bag filled with air or oxygen and then placed into a cooler (41). For verification and accurate identification of field collected H. azteca, it is important that mature males and females be used (42).

X1.5 Brood Stock - Brood stock can be obtained from the wild, another laboratory or a commercial source. H. azteca brought into the laboratory should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100% culture water. H. azteca should be acclimated to the culture temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached (41). Brood stock should be cultured so they are not unnecessarily stressed. To maintain H. azteca in good condition and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

X1.6 Handling - H. azteca should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the amphipods are not unnecessarily stressed. Amphipods should be introduced into solutions beneath the air-water interface (4). Any H. azteca that touch dry surfaces, are dropped, or injured during handling should be discarded. Removing animals from sieves may form air bubbles on body surfaces causing animals to float on the water surface. Any "floaters" should be gently placed into the water column using a probe. If the animals continue to float they should be removed and discarded.

X1.7 Age - Tests with H. azteca should be started with juvenile organisms, (second or third instar) about 2-3 mm in length (4,22). To obtain H. azteca for testing, amphipods should be separated from the leaf material by scooping up the leaves with clinging amphipods, and placing the leaves on a 5-10 mm mesh screen, which is placed over a collecting pan containing 2 cm of culture water. Culture water should be sprinkled on the leaves while turning and separating the leaves. Mixed age H. azteca should be washed from the leaves and drop through the screen into a collecting pan (22). To separate the juvenile amphipods from the larger adults a sieve stack (U.S. Standard) #30 (600 μm), #40 (425 μm), and a #60 (250 μm) can be used (4). Culture water should be rinsed through the sieves and juvenile animals retained by the #60 sieve are washed into a collecting pan while the larger animals in the top sieves (#30 and #40) are returned to the culture. The juvenile amphipods are then placed in 1-L beakers containing culture water (about 200 amphipods/beaker) and kept in the dark at the

temperature of the culture with gentle aeration. H. azteca can be isolated in the 1-L beakers up to 24 hours prior to the start of the sediment toxicity test.

X1.7.1 Borgmann (43) recommends collecting uniform aged young (< 1 week old) for experimental purposes using 2.5-L jars containing about 1 L of culture water and 5 - 25 adult H. azteca. The jars are placed in an incubator at 16 to 8 hour light to darkness photoperiod, about 500 foot-candles (5382 lux). Each jar contains pieces of pre-soaked (in culture water) cotton gauze as a substrate. Once a week the animals should be removed from the gauze and collected by filtration through a 275 μm nylon mesh screen, then rinsed into petri dishes where the young and adults are sorted. Fresh culture water and food should be placed in the jars and the adults returned. Each jar should receive 0.02 g of fish food flakes [10] or more if required by larger animals.

X1.8 Acclimation - If amphipods are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by **Ingersoll and Nelson** (4) is to first place animals for 2 h in a 50:50 mixture of culture water to overlying water, then for 2 h in a 25:75 mixture of culture water to overlying water, followed by a transfer into 100% overlying water. At this stage the amphipods are considered acclimated to the overlying water and are ready for immediate use. H. azteca can then be randomly selected from the acclimation water with a pipette and placed into counting beakers (for example, 30-mL) that can be floated in the test chambers before the amphipods are introduced into the exposure system (4).

X1.9 Toxicity Test Specifications

X1.9.1 Experimental Design - Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and amphipods per treatment, and water quality characteristics, should be based on the purpose of the test and the procedure used to calculate results. **Nebeker et al.** (22) recommend two or more replicate 20-L aquaria per treatment with 100 juvenile H. azteca placed in each aquarium. **Ingersoll and Nelson** (4) recommend four replicate 1-L beakers per treatment, with 20 H. azteca per replicate, for a total of 80 amphipods per treatment. Duration of the test can range from a ≤10 day short-term test to a long-term test >10 days and continuing up to 30 days (4,22). The number of young and adult survival (4,22), growth, and development (4) can be used as the biological endpoints. A test duration up to 30 days can add potential reproductive capacity as another biological endpoint, measuring effects on reproductive behavior, appearance of secondary sex characteristics, egg production, and number of young produced. Tests with H. azteca have been conducted at 20°C (4,22) and from 21-25°C (37), photoperiod 16 to 8 hour light to darkness, about 50 foot-candles (538 lux) (4).

X1.9.2 Static and Flow-through Tests - **Ingersoll and Nelson** (4) and **Nebeker et al.** (22) recommend using borosilicate glass 1-L beakers to expose the H. azteca to the test material. These exposure chambers contain about 800 mL overlying water and 200 mL (2 cm) test sediment, in both the static and flow-through water systems. For the static tests cover watch glasses may be used to fit over the top, such that an aeration tip fits through the beaker pour spout and the cover (4). **Nebeker et al.** (22) suggest for the static long-term test, using 20-L aquaria with 2 - 3 cm of test sediment on the bottom overlaid with 15 cm water. For flow-through testing, **Ingersoll and Nelson** (4) suggest using a 4 x 13 cm notch cut in the lip of the 1-L beaker. The notch should be covered with 0.33 mm U.S. Standard sieve size #50 screen, either made of stainless steel or polyethylene, using a silicone adhesive to attach the screen to the beaker.

X1.9.3 Initiation of a Test - Sediments should be homogenized and placed in the test chambers on the day prior to the addition of the test organisms (Day -1). Test chambers should be covered and overlying water aerated (4) or unaerated overnight but aerated for 30 minutes before H. azteca are added (22). The test begins when the juvenile H. azteca are introduced to the test chambers (Day 0). It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after amphipods are introduced to insure that animals are not trapped in the surface tension of the water (4). These "floaters" might not survive well and should be replaced with new animals (see X1.6).

X1.9.4 Feeding - **Ingersoll and Nelson** (4) recommend rabbit pellets [8] to be used as a food for H. azteca in short and long-term sediment toxicity tests, **Nebeker et al.** (22) suggest feeding rabbit pellets [8] in a 28 day test. The pellets should be ground and dispersed in deionized water. A flurocarbon plastic stir bar and a magnetic stir plate should be used to homogeneously resuspend the rabbit pellets [8] when aliquots are removed for feeding. If food collects on the sediment, a fungal or bacterial growth might start on the surface of the sediment, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen to 40% saturation might indicate that all of the food added in the water is not being consumed such that feeding might be suspended for the amount of time necessary to increase the dissolved oxygen concentration (4).

X1.9.4.1 In static tests **Nebeker et al.** (22) suggest a feeding regime twice weekly of 200 mg (0.5 mL dry volume) rabbit pellets [8] mixed in 100 mL distilled water for 100 juvenile H. azteca in a 20-L aquarium. **Nelson and Ingersoll** (4) recommend feeding H. azteca three times weekly 14 mg rabbit pellets [8] per feeding for 20 young amphipods in a 1-L beaker. Lower feeding levels for flow-through and static tests may be used for H. azteca: three times weekly 6 mg rabbit pellets per [8] feeding for the first week of the test, and 12 mg per feeding for the following weeks.

X1.9.4.2 For flow-through testing, prior to starting a test, 20 mg rabbit pellets [8] should be added to each test chamber, and three times a week each test chamber should be fed 20 mg per feeding for 20 young H. azteca during the exposure (4).

X1.10 Biological Data - During the conduct of the test, observations should be made to assess behavior (for example, "floaters", sediment avoidance) and reproductive activities (for example, amplexus). At the end of the test the H. azteca must be removed from the test chambers for survival (4,22), observable behavior, any noticeable reproduction (for example, amplexus, gravid females, young present) and growth (4). According to **Ingersoll and Nelson** (4) without material above the sediment surface, such as the leaves used in culturing, H. azteca burrow in the top 1 cm sediment surface or are found swimming in the water column. Many of the surviving amphipods can be pipeted from the water column before sieving the sediments. At the end of the test the sediment should be screened using a #35 (500 μm) U.S. Standard size sieve (22). **Ingersoll and Nelson** (4) recommend using a #50 (300 μm) U.S. Standard size screen cup first by swirling the overlying water to suspend the upper 1 cm of sediment and pouring that slurry into the cup. Next, a stack of sieves #25 and #40 U.S. Standard size should be used to sieve the bulk sediment in order to collect and count the live animals remaining in the sediment. The H. azteca are rinsed from the screens into collecting pans and pipeted from the rinse water (4). It might be difficult to recover young H. azteca due to their small size. Material retained in the collecting pans may be preserved in a sugar formalin mixture for examination at a later date (4). The preserved material may be inspected using a low power binocular microscope to search for H. azteca missed the last day of the test.

X1.10.1 For quantifying growth, H. azteca body length (±0.01 mm) should be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface (4). In addition, wet and dry weight measurements have been used to estimate growth for H. azteca (37).

X1.10.2 A H. azteca sediment toxicity test, independent of duration, is unacceptable if the average survival in any negative control chamber is less than 80% (see Section 15, Acceptability of Test).

ANNEX X2. Chironomus tentans

X2.1 Significance - Chironomus tentans Fabricius (Diptera: Chironomidae) has been used in sediment toxicity tests because it is a fairly large midge with a short generation time, is easily cultured in the laboratory, and the larvae have direct contact with the sediment by burrowing into sediment to build a case. C. tentans has been successfully used in sediment toxicity testing and is sensitive to many contaminants associated with sediments (22,25,44,45,46). The members of the genus are important in the diet of young and adult fish and surface feeding ducks (47).

X2.2 Life History and Life-Cycle - The classification of holometabolous insects, such as C. tentans, presents special difficulties because each life-stage often has different ecological requirements. Further detailed studies at the species level are needed to better understand the various physical, chemical, and biological factors that interact to produce a suitable environment for larval development (48). C. tentans has a holarctic distribution and is locally common in the mid-continental areas of North America (47,49,50). Sadler (51) describes the general biology of C. tentans. The larval stages often inhabit eutrophic lakes and ponds. Qualitative observations indicate larvae occur most frequently in fine sediment and detritus; however larvae reportedly inhabit sediments with particles ranging from <0.15 mm to 2.0 mm (52). Chironomid larvae usually penetrate a few centimeters into sediment. In both lotic and lentic habitats with soft bottoms, about 95% of the chironomid larvae occur in the upper 10 cm of substrate, very few larvae are found below 40 cm (48). Larvae are generally not found when hydrogen sulfide is greater than 0.3 mg/L (52). Larvae of C. tentans are found in the field at a temperature range between 0°C to 35°C, pH range between 7 to 10, conductivity range between 100-4000 $\mu S\ cm^{-1}$, sediment organic carbon range between 2 and 15 percent, and at dissolved oxygen concentrations as low as 1 mg/L (47,52,53). Sadler (51) reported that C. tentans will eat essentially any material of appropriate size.

X2.2.1 The biology of C. tentans facilitates laboratory culture since larvae are tolerant of a wide spectrum of conditions and adults mate even when confined (47). The life-cycle of C. tentans can be divided into three distinct stages: (1) a larval stage, consisting of the 4 instars; (2) a pupal stage, and (3) an adult stage. Midge egg masses hatch in 2 or 3 days after deposition in water at 19-22°C. Larval growth occurs in four instars of about one week each. Under optimal conditions larvae will pupate and emerge as adults after 24-28 days at 20°C. Adults emerge from pupal cases over a period lasting several days. Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. Mating behavior has been described by Sadler (51) and others (54).

X2.3 Obtaining Test Organisms - The following is a description of culturing procedures adapted from Adams et al. (25), Nebeker et al. (22) and others (47,54). These procedures should not be considered definitive. What works in one laboratory sometimes works poorly in another laboratory. C. tentans can be reared in aquaria in static or flowing water with a 16 to 8 hour light to darkness photoperiod at 20-23°C, at about 50 foot-candles (538 lux). For static cultures the water should be gently aerated and about 25-30 percent of the water volume should be replaced weekly. Cultures should be maintained in an isolated area or room free of contamination and excessive disturbances. Adams et al. (25) recommends rearing midges in glass aquaria filled with water to a depth of 45 cm covered with nylon screen. The size of the aquaria may vary from a minimum of 3 L to a maximum of 19 L depending on the need for animals.

X2.3.1 Chironomus tentans require a substrate in which to construct a case. Shredded paper towels have been found to be well suited for this purpose. Strips cut from Scott[R] or Nibroc[R] brown paper towels should be soaked overnight in acetone to remove impurities and are then rinsed in three changes of culture water until the acetone is removed. A kitchen blender should be used to shred the rinsed towels into a pulp. Care must be taken to avoid over blending and possibly shortening the wood fibers in the pulp. The pulp should be rinsed twice with culture water to remove extremely small fibers and refrigerated until needed. The paper toweling pulp should be placed into the water of a culture chamber to a depth of 3 cm. One gram of dry fish food flakes [10] should be mixed in 10 mL of culture water with a kitchen blender and refrigerated. This suspension should be fed twice daily to the cultures for optimum growth. The amount given depends on the number and size of the larvae. If after feeding the culture water does not clear in 3 to 4 hours, the feeding level should be reduced. Overfeeding will lead to the growth of fungus in the aquaria and will necessitate more frequent water changes. Therefore, new cultures should receive 0.5 mL or less of this suspension per feeding. **Nebeker et al. (22)** suggest supplementing the fish food flakes [10] diet with ground cereal leaves [9].

X2.4 Brood Stock - Brood stock can be obtained from the wild, laboratory or a commercial source. When midges are brought into the laboratory, they should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100% culture water. Midges should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached. Brood stock should be cultured so they are not unnecessarily stressed. To maintain midges in good health and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

X2.5 Age - Test with C. tentans can be started with second instar larvae according to **Wentsel et al., (44), Adams et al. (25), Nebeker et al. (22)** and **Giesy (45)**. Tests started with first instar C. tentans larvae have met with limited success **(22)**. Twelve to 16 days before a test is begun, at least 3 freshly laid midge egg cases should be placed in a clean 20x40 cm glass or enameled rearing pan filled with water to a depth of 3 cm. Egg cases should be isolated by aspirating adults into a 250-mL Erlenmeyer flask in the morning. In late afternoon, about 20 mL of culture water should be added to the flask. Egg cases are deposited overnight and first instar larvae begin to hatch after about 3 days at 20°C. No substrate is added to the pan before hatching. Fish food flakes [10] should be added at a rate of 50 mg/day suspended in water. Fresh water should be added as needed to make up for evaporation. The larvae in the rearing pans are presumed to be 2nd instars on the 12th day from the time the eggs were laid (10 day old larvae). Most larvae will remain as 2nd instars through the 16th day (14 day old larvae). Larvae $\geq$16 days old should not be used to start a test. To maintain a supply of 2nd instar larvae for active toxicity testing, a rearing pan should be started every 4 days. Each pan can be expected to produce at least enough 2nd instar larvae for one sediment toxicity test.

X2.6 Handling - Midges should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly, so that the midges are not unnecessarily stressed. Larvae should be transferred with a 7-mm inner diameter glass pipet. Midges should be introduced into solutions beneath the air-water interface. Any midges that touch dry surfaces, are dropped, or injured during handling should be discarded.

X2.7 Acclimation - If the midges are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by **Ingersoll and Nelson** (4) is to first place animals for 2 h in a 50:50 mixture of culture water to overlying water, then for 2 h in a 25:75 mixture of culture water to overlying water, followed by a transfer into 100% overlying water. At this stage the midges are considered acclimated to the overlying water and are ready for immediate use. Midges should be randomly selected from the acclimation water with a pipette and placed into counting beakers, for example 30-mL, that can be floated in the test chambers before the midges are introduced into the exposure system (4).

X2.8 Toxicity Test Specifications

X2.8.1 Experimental Design - Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and midges per treatment, and water quality characteristics, should be based on the purpose of the test and the type of procedure that is to be used to calculate results. Tests with C. tentans have been conducted at 20-23°C (**22,25,44**). Cooler test temperature may reduce the growth of fungus on the sediment surface. Duration of the test can range from a $\leq$10 day test to >10 days and continuing up to 25 days (**22,25,44,45**). Larval survival, growth, or adult emergence can be monitored as biological endpoints.

X2.8.2 Static and Flow-through tests - **Wentsel et al.** (**44**) recommend using 20 C. tentans in each 2-L exposure beaker containing 2 cm of sediment and 1.5 L of overlying water in static testing. **Adams et al.** (**25**) use 3-L aquaria constructed of glass and silicone rubber for either static or flow-through testing. These test chambers measure 20.5 x 12.5 x 14.5 cm with a 12.5 x 44.5 cm piece of fine mesh stainless steel screen positioned on the upper end of one side. This overflow screen prevents the escape of larvae and maintains an overlying water volume of 2 L with 100 g of test sediment and 25 C. tentans larvae per chamber. **Nebeker et al.** (**22**) recommend 20-L aquaria with 100 C. tentans larvae and 2 to 3 cm of test sediment on the bottom with 15 cm of overlying water in static tests. If less sediment is available for testing, 4-L glass jars can be used, but proportionally fewer animals and less food should be used. **Adams et al.** (**25**) and **Giesy et al.** (**45**) also describe a method to expose midges individually to contaminated sediment in static tests. Up to 15 C. tentans are placed in separate 50-mL plastic centrifuge tubes. Each tube contains one midge, 7.5 g of sediment and 47 mL of water. For 24 hours after hatching, first instar midge larvae are often planktonic (**55**). If flow-through tests are started with first instar C. tentans larvae, water flow into the test chambers should not be started for at least 24 hours after larvae are added. This will allow time for larvae to settle onto the sediment surface.

X2.8.3 Initiation of a Test - Sediments should be homogenized and placed in the test chambers on the day before addition of test organisms (Day -1). Test chambers should be covered and overlying water aerated overnight. The test begins when midges are introduced to the test chambers (Day 0). Larvae must be collected from at least three separate egg cases to start a sediment toxicity test. It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after midges are introduced to insure that animals are not trapped in the surface tension of the water (4). These "floaters" do not survive well and should be replaced with healthy animals.

X2.8.4 Feeding - **Adams et al.** (**25**) recommend feeding animals in flow-through or static tests 50 mg fish food flakes [10] (dry weight, administered in a 0.5 mL suspension) daily to each 3-L test chamber containing 25 larvae. **Nebeker et al.** (**22**) suggest feeding animals in static tests a food mixture of 600 mg ground cereal leaves [9]

(1.5 mL dry volume) and 100 mg (0.3 mL dry volume) of finely crushed fish food flakes [10] in water and feeding this amount of food to the 100 C. tentans larvae in each 20-L test chamber at the start of the test (Day 0) and on Day 8. On day 14 they should be fed 800 mg (2.0 mL) ground cereal leaves [9] and 100 mg (0.3 mL) fish food flakes [10], and on day 18 they should be fed 1,000 mg (2.5 mL) ground cereal leaves [9] and 100 mg (0.3 mL) fish food flakes [10]. **Giesy et al. (45)** recommend feeding a 0.1 mL suspension of 0.06 g/mL goldfish food [11] daily to each individual midge in each centrifuge tube. If food collects on the sediment, a fungal or bacterial growth might start on the surface of the sediment, in which case feeding may be suspended for one or more days. A drop in dissolved oxygen to 40% saturation might indicate that all of the food added in the water is not being consumed such that feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration.

X2.8.5 Biological Data - Several endpoints can be monitored in midge sediment toxicity tests. During the test, emergence of larvae from the test sediment can be monitored. Additionally, data on larval survival, growth, and adult emergence can be obtained.

X2.8.5.1 Larval survival and growth can be assessed by ending the tests on Day 10 to Day 14 when larvae have reached the 3rd or 4th instar **(22,25,45)**. At this time, larvae can be removed from sediment using a #35 (500 μm) U.S. Standard size sieve **(4)**. The midges can be rinsed from the sieve into collecting pans and pipeted from the rinse water. Growth determinations using dry weight (dried at 60°C to a constant weight) is preferable to length. Growth can also be estimated by measuring head capsule width, and also be used to determine instar development.

X2.8.5.2 **Nebeker et al. (22)** suggest conducting adult C. tentans emergence sediment toxicity tests for 25 days when tests are started with second instar larvae. The adult emergence exposure chambers are covered by screen to retain emerging adults. The adult C. tentans should begin emerging after 20 days; the test should be continued for at least 5 days to count all the adults emerging and monitor delayed development. A small vacuum pump with a 10-mm diameter plastic line running through an Erlenmeyer flask trap is used to collect adults and make daily count of adults emerging. The screen cover is slowly lifted off the container and the adults are vacuumed from the screen and inside walls of the container. Percent adult emergence is generally less than 60% in these tests. Endpoints calculated in these adult emergence tests can include (1) percent emergence, (2) mean emergence time, or (3) day to first emergence. Egg hatching studies may also be conducted by covering the test chambers and confining the adults. Adults will emerge and lay eggs in these chambers. These egg masses can then be used to estimate effects of exposure on either the number of eggs produced or hatched.

X2.8.5.3 A C. tentans sediment toxicity test, independent of test duration, is unacceptable if the average survival in any negative control chamber is less than 70% (see Section 15, Acceptability of Test). (Note: a low percent emergence of adults might not be the result of low survival; larvae or pupae might not have completed development).

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[11] TetraFin Goldfish Food, TetraWerke, Dr. rer. nat. Ulrich Baensch GmbH, D-4520 Melle 1, W. Germany.

ANNEX X3. Chironomus riparius

X3.1 Significance - Chironomus riparius Meigen (Diptera: Chironomidae) has been used in sediment toxicity tests because it is a fairly large midge, has a short generation time, is easily cultured in the laboratory, and the larvae have direct contact with the sediment by burrowing into the sediment to build a case. C. riparius has been successfully used in sediment toxicity testing and is sensitive to many contaminants associated with sediments (4,56,57,58). The members of the genus are important in the diet of young and adult fish and surface feeding ducks (47).

X3.2 Life History and Life-Cycle - The classification of holometabolous insects, such as C. riparius, presents special difficulties because each life-stage often has different ecological requirements. Further detailed studies at the species level are needed to better understand the various physical, chemical, and biological factors that interact to produce a suitable habitat for larval development (47). The distribution of the family is world wide. Most of the species in the family are thermophilous and adapted to living in standing water, although species do occur in cold habitats and in running water (47). C. riparius is a non-biting midge. The tubiculous larvae frequently inhabits eutrophic lakes, ponds, and streams and reportedly live in mud-bottom littoral habitats to depths up to 1.0 meter (59). Qualitative observations indicate larvae inhabit gravel, limestone, marl, plants, and silt (53). Ingersoll and Nelson (4) report C. riparius to have a wide tolerance of sediment grain size. Sediment ranging from >90% silt- and clay-size particles to 100% sand-size particles did not reduce larval survival or growth in the laboratory. Larvae of C. riparius larvae reportedly occur in the field at a temperature range between 0°C to 33°C, pH range between 5 to 9, and at dissolved oxygen concentrations as low as 1 mg/L (53). C. riparius tubes are of the type characteristic of bottom-feeding chironomid larvae (59). Larvae frequently extend their anterior ends outside of their tubes feeding on the sediment surface (59). Credland (60) reported C. riparius will eat a variety of materials of the appropriate size.

X3.2.1 The biology of C. riparius facilitates laboratory culture since larvae are tolerant of a wide spectrum of conditions and adults mate even when confined (55,58,60). The life cycle of C. riparius can be divided into three distinct stages: (1) a larval stage, consisting of the 4 instars; (2) a pupal stage, and (3) an adult stage. Midge egg masses hatch in 2 or 3 days after deposition in water at 19-22°C. Larval growth occurs in four instars of about 4-7 days each. Under optimal conditions larvae will pupate and emerge as adults after 15 to 21 days at 20°C. Adults emerge from pupal cases over a period lasting several days. Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. Mating behavior has been described by Credland (60).

X3.3 Obtaining Test Organisms - The following is a description of culturing procedures adapted from Ingersoll and Nelson (4) and others (51,54,58,60). These procedures should not be considered definitive. What works in one laboratory sometimes works poorly in another laboratory. C. riparius can be reared in aquaria in either static or flowing water with a 16:8 hour light:darkness photoperiod at 20-22°C, at about 50 foot-candles (538 lux). For static cultures the water should be gently aerated and about 25-30 percent of the water volume should be replaced weekly. Cultures should be maintained in an isolated area or room free of contamination and excessive disturbances. Ingersoll and Nelson (4) recommend rearing C. riparius in 30 x 30 x 30-cm polyethylene containers covered with nylon screen. Each culture chamber contains 3 L of culture water. At least three egg cases should be used to start a new culture. To start a culture, 200-300 mg of ground cereal leaves [9] is added to

the culture chamber, additionally, green algae (Selenastrum capricornutum) (61) is added ad libitum to maintain a growth of algae in the water column and on the bottom of the culture chamber. Cultures should be fed about 3 mL of a suspension of commercial dog treats [12] (62) daily. This suspension should be prepared by heating and melting 15 g of dog treats [12] in 150 mL of culture water. After refrigeration, the oily layer which forms on the surface should be removed. The rest should be used to feed the cultures. This suspension contains about 100 mg dry solid/mL. Overfeeding will lead to the growth of fungus in the aquaria and will necessitate more frequent water changes. To obtain egg cases and larvae, adults should be left in the culture chamber to mate and deposit eggs. Egg cases adhere to the side of the culture chamber and can be removed with a sharp blade. These egg masses can then be placed in individual 100 mL beakers containing 50 mL of culture water; hatching should start in about 3 days at 20°C. While removal of adults by aspiration into a 250 mL flask before mating works well with C. tentans (see Appendix X2), this procedure has not been successful with C. riparius.

X3.4 Brood Stock - Brood stock can be obtained from the wild, another laboratory or a commercial source. When midges are brought into the laboratory, they should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100% culture water. Midges should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached. Brood stock should be cultured so they are not unnecessarily stressed. To maintain midges in good health and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

X3.5 Age - Tests with C. riparius can be started with either larvae less than 24-h old (4) or with three day old larvae (56,57). Freshly laid midge egg cases can be transferred from the culture into individual 100 mL beakers containing 50 mL of culture water. At 20°C larvae should begin to hatch within 3 days. Larvae must be collected from at least three separate egg cases to start a sediment toxicity test.

X3.6 Handling - Midges should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the midges are not unnecessarily stressed. First instar midges should be transferred with a 2 mm inner diameter glass pipet (eye dropper). Older larvae should be transferred with a 7 mm inner diameter glass pipet. Midges should be introduced into solutions beneath the air-water interface. Any midges that touch dry surfaces, are dropped, or injured during handling should be discarded.

X3.7 Acclimation - If the midges are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by **Ingersoll and Nelson** (4) is to first place animals for 2h in a 50:50 mixture of culture water to overlying water, then for 2 h in a 25:75 mixture of culture water to overlying water, followed by a transfer into 100% overlying water. At this stage the midges are considered acclimated to the overlying water and should be ready for immediate use. Midges should be randomly selected from the acclimation water with a pipette and placed into counting beakers (for example, 30-mL) that can be floated in the test chambers before the midges are introduced into the exposure system.

X3.8 Toxicity Test Specifications

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[12] Dog Kisses, The Hartz Mountain Corporation, Harrison, NJ 07029-9987.

X3.8.1 Experimental Design - Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and midges per treatment, and water quality characteristics, should be based on the purpose of the test and the type of procedure that is to be used to calculate results. **Ingersoll and Nelson** (4) recommend using 50 C. riparius in each 1-L exposure beaker containing 200 mL of sediment and 800 mL of overlying water in either static or flow-through testing. **Lee** (57) recommends using 13-L glass aquaria containing 130 C. riparius larvae, 2 L of sediment and 11 L of overlying water in static tests. Tests with C. riparius have been conducted at 20-22°C (**4,56,57**). Cooler test temperatures might reduce the growth of fungus on the sediment surface. Duration of the test can range from a $\leq$10 day test to >10 days and continuing up to 30 days (**4,56,57**). Larval survival, growth, or adult emergence can be monitored as biological endpoints.

X3.8.2 Static and Flow-through Tests - **Ingersoll and Nelson** (4) recommend that borosilicate glass 1-L beakers can be used to expose the C. riparius to the test material, in either static or flow-through tests. For the static tests, cover watch glasses may be used, such that an aeration line fits through the beaker pour spout and the cover. For flow-through testing, **Ingersoll and Nelson** (4) suggest using a 4 x 13 cm notch cut in the lip of the 1-L beaker. The notch should be covered with 0.33 mm U.S. Standard sieve size #50 screen, either made of stainless steel or polyethylene, using a silicone adhesive to attach the screen to the beaker. For 24 hours after hatching, first instar midge larvae are often planktonic (**55**). **Pittinger et al.** (56) suggest not running water through the diluter for at least 24 hours after larvae are added to the test chambers. This will allow time for larvae to settle onto the sediment surface.

X3.8.3 Initiation of a Test - Sediments are homogenized and placed in the test chambers the day before addition of test organisms (Day -1). Test chambers are then covered and overlying water is aerated overnight. The test begins when midges are introduced to the test chambers (Day 0). **Ingersoll and Nelson** (4) start sediment toxicity tests with 50 first instar C. riparius larvae per 1-L test chamber. **Pittinger et al.** (56) and **Lee** (57) suggest starting tests with 3 day old larvae (130 larvae per 13-L chamber (57)). It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after midges are introduced to insure that animals are not trapped in the surface tension of the water. These "floaters" do not survive well and should be replaced with healthy animals.

X3.8.4 Feeding - **Lee** (57) recommends feeding animals in a static system 200 mg fish food flakes [10] every other day to each 13-L test chamber containing 130 larvae. **Pittinger et al.** (56) suggest feeding animals in a static renewal system with trout food [13], dehydrated cereal leaves [9] (5:1 w/w) and commercial dog treats [12] daily to each test chamber containing 20 larvae. In flow-through and static toxicity tests, **Ingersoll and Nelson** (4) feed 50 C. riparius larvae in each 1-L test chamber a combination of ground cereal leaves [9] (suspended in water), a green algae (S. capricornutum) and commercial dog treats [12]. In flow-through sediment toxicity tests, 75 mg of ground cereal leaves [9], 30 mg of dog treats [12] and 6 x 10^7 S. capricornutum algal cells should be added to each 1-L test chamber the day test starts (day 0). From Day 1 to Day 6 of the test, 15 mg of ground cereal leaves [9] should be added to each test chamber; from Day 1 to Day 12, 30 mg of dog treats [12] should be added to each test chamber and from Day 13 to the end of the test, 15 mg of dog treats [12] should be added to each test

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[13] Purina Trout Chow, Purina Mills Inc., 1401 S. Hanley, St. Louis, MO 63144.

chamber; 6 x 10^7 S. capricornutum algal cells should be added to each test chamber daily. In static sediment toxicity tests, 10 mg of ground cereal leaves [9], 10 mg of dog treats [12] and 3 x 10^7 S. capricornutum algal cells should be added to each 1-L test chamber on Day 0. From Day 1 to Day 6 of the test, 10 mg of ground cereal leaves [9] and 3 x 10^7 algal cells should be added to each 1-L test chamber; for the first two weeks of the test, 10 mg of dog treats [12] should be added to each test chamber each Monday, Wednesday, and Friday and for the rest of the test 5 mg of dog treats [12] should be added to each test chamber each Monday, Wednesday and Friday; from Day 7 until the end of the test 3 x 10^7 algal cells should be added to each test chamber each Monday, Wednesday and Friday. Lower feeding levels for flow-through tests might be used for C. riparius daily: 6 x 10^7 S. capricornutum algal cells, 10 mg dog treats [12], and 10 mg ground cereal leaves [9] on Day 0 - 6. If food collects on the sediment, a fungal or bacterial growth might start on the surface of the sediment, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen to 40% saturation might indicate that all of the food added in the water is not being consumed such that feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration (4).

X3.8.5 Biological Data - Several endpoints can be monitored in midge sediment toxicity tests. During the test, emergence of larvae from the test sediment can be monitored. Additionally, data on larval survival, growth, and adult emergence can be obtained.

X3.8.5.1 Larval survival and growth can be assessed by ending the tests on Day 10 to Day 14 when larvae have reached the 3rd or 4th instar (4,25,45). At this time, larvae should be removed from sediment using a #35 (500 μm) U.S. Standard size sieve (4). The midges should be rinsed from the sieve into collecting pans and pipeted from the rinse water. Growth determination using dry weight (dried at 60°C to a constant weight) is preferable to length. Growth can also be estimated by measuring head capsule width, and also used to determine instar development.

X3.8.5.2 **Ingersoll and Nelson** (4), **Pittinger et al.** (56) and **Lee** (57) recommend conducting C. riparius sediment toxicity tests until the larvae pupate and emerge as adults. Cast pupal skins left by emerging adult C. riparius should be removed and recorded daily. These pupal skins remain on the water surface for over 24 hours after the emergence of the adult. The test should be ended after the animals have been exposed for up to 30 days, when about 70-95% of the control larvae should have completed metamorphosis into the adult form. Endpoints calculated in these adult emergence tests can include: (1) percent emergence, (2) mean emergence time, or (3) day to first emergence. Egg hatching studies may also be conducted by covering the test chambers and confining the adults. Adults will emerge and lay eggs in these chambers. These egg masses can then be used to estimate effects of exposure on either the number of eggs produced or hatched.

X3.8.5.3 A C. riparius sediment toxicity test, independent of duration, is unacceptable if the average survival in any negative control chamber is less than 70% (see Section 15, Acceptability of Test). (Note: a low percent adult emergence might not be the result of low survival; larvae or pupae might not have completed development).

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FATHEAD MINNOW WHOLE SEDIMENT TOXICITY TESTING

P. promelas culturing and test conditions are similar to the effluent test method guidance of the USEPA. Larvae (40 per treatment, 10 per beaker) less than 24h old are used in toxicity tests. The larvae are randomly added to 600 mL test beakers containing 62.5 mL sediment and 250 mL overlying water. In tests exceeding 48h exposure periods, the larvae are fed brine shrimp nauplii (0.1 mL, ~1050-1500 organisms) twice daily. Overlying waters are siphoned (80%) daily and replaced with fresh reconstituted, after removing larvae. the fish are weighed at time zero (subsample) and after 7 days in chronic tests using growth as the endpoint. Fish are removed on Day 7, placed in pre-weighed aluminum pans and dried at 105°C for 2 to 24 h. Dried larvae are weighed in groups of 10 on a Mettler balance. For the embryo-larval assays, test chambers (600 mL beakers) receive 50 mL of sediment and 200 mL of site water to provide a 1:4 ratio of sediment to water by volume. The water is slowly added to prevent sediment resuspension. Ten freshly-spawned embryos (less than 24h old) are added to each test chamber using a large bore pipette. Four replicate chambers are run per test concentration. A set of control chambers contain no sediment is run in quadruplicate concurrently with the exposure beakers. The water in the test beakers is gently and continuously aerated for the duration of the test. Approximately 80% of the dilution water in each test chamber is siphoned and renewed daily during the test period. Dead organisms are counted and removed daily. Organisms are considered dead when they become opaque and white. Test organisms are not fed during the test period. The temperature of the dilution water is maintained at 25°C, and a 16:8h light/dark photoperiod is used. The temperature, pH, and dissolved oxygen content is monitored daily, while the hardness, alkalinity, and conductivity of the test solution is measured at the beginning and end of the test, at a minimum. At the end of the test period, the surviving test organisms are counted, removed, and placed into a preservative until the larvae can be examined microscopically for terata and the lengths measured. The endpoints of test measured are 7 d survival, growth (as measured by length), and percent hatch.

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ASTM E1383. ANNEX X4. Daphnia and Ceriodaphnia sp.

X4.1 Significance - Daphnia magna and Ceriodaphnia dubia have many desirable characteristics as toxicity test organisms. They are easily cultured in the laboratory, have a short generation time, survival and reproduction data can be obtained in toxicity tests, and a large data base has developed regarding their sensitivity to toxicants. Nebeker et al. (1), Prater and Anderson (2), Giesy et al. (3), Malueg et al. (4) and Burton et al. (5) and others (6-15) have successfully used cladocerans in sediment testing and have shown them to be sensitive indicators of the presence of contaminants associated with sediments.

In whole sediment toxicity tests, cladocera behave as nonselective epibenthic zooplankton. The organisms are frequently observed on the sediment surface and are likely exposed to both water soluble and particulate bound contaminants in overlying water and surface sediments. These routes of exposure do not, however, mimic those of infaunal benthic invertebrates, which are exposed directly to sediment and interstitial water. One of the most important reasons for using cladocerans as toxicity test organisms is their importance in the food web of some systems (16-18). Also these assays have been useful at discriminating sediment contamination and allowing comparisons of relative sediment toxicity. Because they are not benthic organisms,

their responses may not be indicative of in situ benthic community effects.

X4.2 Life History and Life Cycle - Pennak (18) recognizes four distinct periods in the life history of a cladoceran: 1) egg, 2) juvenile, 3) adolescent, and 4) adult. Unstressed populations consist almost exclusively of females producing diploid parthenogenetic eggs which develop into female young. An adult Ceriodaphnia can produce from 4 to 15 parthenogenetic eggs in each brood whereas Daphnia can produce 5 to 25 or more eggs (19). Pennak (18) indicates that when a clutch of eggs is released into the brood chamber, segmentation begins promptly; the first juvenile instar is released into the surrounding water in approximately two days. There are only a few juvenile instars and the greatest growth occurs during these stages. The adolescent period is a single instar between the last juvenile instar and the first adult instar during which the first clutch of eggs reaches full development in the ovary. At the close of the adolescent instar, the animal molts and the first clutch of eggs is released into the brook chamber, while a second clutch is developing in the ovary. At the close of each adult instar, four successive events occur: 1) the young are released from the brood chamber to the outside environment, 2) molting occurs, with 3) an increase in size, and 4) there is release of a new clutch of eggs into the brood chamber.

When populations are under stress (e.g., low oxygen, crowding, starvation), males are produced from diploid parthenogenetic eggs. When males appear, females produce haploid eggs which require fertilization. Following fertilization, the eggs are enclosed by the ephippium and shed at the next molt. The embryos lie dormant until

suitable conditions arise upon which they become females producing diploid parthenogenetic eggs (20).

X4.3 Obtaining Test Organisms - The following culture procedures are adapted from Knight and Waller (21), while other appropriate methods include the U.S. Environmental Protection Agency (22,23) ASTM E729 and E1295. Following Knight and Waller's (21) methodology, D. magna and Ceriodaphnia dubia can be cultured in reconstituted hard water (160-180 mg/L $CaCO_3$) and fed a daily diet of a vitamin enriched Selenastrum capricornutum suspension. Cultures are maintained at 25°C ± 1°C with a light:dark cycle of 16:8 hours provided by overhead fluorescent lighting covered with opaque plastic to reduce light intensity to less than 20 lux. D. magna mass cultures are started by placing 10 neonates (less than 24 hours old) into one liter beakers containing 500 ml reconstituted hard water and 12 ml (approximately 240,000 algal cells/ml culture water) of S. capricornutum feeding suspension. Cultures are fed 12 ml initially and on day one, 25 mls (500,000 cells/ml culture water) on day two through four, and 25 to 50 mls (100,000 cells/ml culture water) on day five and thereafter. Using this culture method, D. magna typically will have first broods between days 6 and 8 with successive broods hatching every 36-48 hours thereafter. On days when hatches occur and young are not needed, adults are transferred to clean one liter beakers containing 300 ml hard water, 200 ml old culture water, and 50 ml of food. When young are needed for testing, the adults are isolated the night before by placing each adult into a separate 100 ml beaker containing 100 ml reconstituted hard water and 3 ml feeding suspension. Isolating

adults into smaller beakers allows one to easily remove individual young for testing. Neither first brood young nor young from females older than two weeks are used in toxicity testing or initiating new cultures. The Selenastrum capricornutum feeding suspension may also be supplemented with an approximate 6% by volume addition of a Cerophyl® preparation to the algal feeding suspension (Waller, personal communication). C. dubia mass cultures can be initiated by placing 20 neonates (less than 12 h old) into a 600 ml beaker containing 360 ml reconstituted hard water and 12 ml of S. capricornutum feeding suspension. Cultures are fed 12 ml initially and on days one and two, and then 18 mls thereafter. When three distinct sizes are noted (generally day 6) then the largest organisms are isolated in 100 ml beakers containing 60 ml of hard water and 2 ml feeding suspension. Less than 12 h old neonates from the next brood (third brood) are used in toxicity testing and initiating new mass cultures. Generally, first broods are produced on day four, second brood on day 5 and third brood on day 7. Isolated females generally produce between 10 and 16 neonates on their third brood (21).

The U.S. Environmental Protection Agency (23) recommends culturing D. magna in reconstituted hard water at 20°C with ambient light intensity of 50-100 ft c (10-20 $\mu E/m^2/s$, or 538-1076 lux), and a light:dark cycle of 16:8 hours. Culture vessels can be 3 L glass beakers containing 2.75 L reconstituted hard water and 30 D. magna. The D. magna can be fed on a daily diet of S. capricornutum (100,000 algal cells/ml culture water) or fed three times a week on a feeding suspension consisting of trout chow, alfalfa and yeast (TCY) (1.5 ml TCY/1000 ml culture water). This should supply approximately 300 young per week.

The U.S. Environmental Protection Agency (22) procedures for Ceriodaphnia cultures are as follows. Ceriodaphnia are cultured in moderately hard water (80-90 mg/L $CaCO_3$) at 25°C ± 1°C and receive a light:dark cycle of 16:8 hours. Mass cultures are maintained as "backup" organism reservoirs and individual organisms are cultured as the source of neonates for toxicity tests. Mass cultures can be initiated in 2-3 L beakers filled to three-fourths capacity with moderately hard water and 40-50 neonates per liter of medium. The stocked organisms should be transferred to fresh culture media twice weekly for two weeks. At each renewal, the adults are counted and the offspring and old medium discarded. The adults are discarded after two weeks and new mass cultures initiated with neonates. Mass cultures are fed daily at the rate of 7 ml of a yeast, Cerophyl, trout chow food preparation (YCT), and 7 ml of S. capricornutum concentrate (3.0-3.5 x 10^7 cells/ml). Individual C. dubia cultures are maintained in 30 ml plastic cups or beakers containing 15 ml of culture media. Cultures are fed daily at the rate of 0.1 m YCT and 0.1 ml algal concentrate per 15 ml media and are transferred to fresh media at least three times a week. Adults are used as sources of neonates until 14 days of age. Cultures properly maintained should produce at least 15 young per adult in three broods (seven days or less). Goulden and Henry (19) list two other fresh water algal species which can be used for cladoceran food: 1) Ankistrodesmus falcatus, and 2) Chlamydomonas reinhardtii. Winner (24) discusses the effects of four diets [Chlamydomonas reinhardtii, Selenastrum capricornutum, yeast-trout chow-Cerophyl (YTC), and YTC plus S. capricornutum] and two reconstituted waters on the vitality of five to six lifespan generations of C. dubia. His results indicate that healthy

populations can be maintained in reconstituted hard water containing only four salts as long as the food is nutritionally adequate and the water is reconstituted from an ultrapure base water.

X4.4 Brood Stock - D. magna and C. dubia starter cultures can be obtained from the Aquatic Biology Branch, Environmental Monitoring Systems Laboratory, USEPA, 3411 Church Street, Newtown, OH 45244. Animals received from an outside source should be acclimated gradually to new culture media over a period of 1-2 days.

X4.5 Background - The various decisions concerning experimental design, such as number of test chambers, number of treatments, animals per treatment and water quality characteristics, should be based on the purpose of the test and the procedure used to calculate results. See ASTM E729, E1295, E1297, and the preceding guide text for guidance. Nebeker et al. (25) recommended conducting 48 h sediment static tests in duplicate using 1 L beakers containing 200 ml of sediment and 800 ml of water (1:4 ratio). The sediment is allowed to settle overnight, followed by gentle aeration of overlying water for 30 minutes before introducing 15 D. magna per replicate. Malueg et al. (4) conducted recirculating sediment toxicity tests in a modified recycling device described by Prater and Anderson (2). The test chamber (23 cm long x 6.4 cm wide x 16 cm high) was positioned on a Plexiglass plate over two 4 - L jars. Twenty D. magna were placed in a vessel in the water column and 5 Hexagenia added to chamber sediment. Three to six replicates were used for each

control and test sediment. Seven day (three brood) toxicity tests for aqueous media using cladocerans have been conducted (1,26,27) and variations of these methods used to assess sediment toxicity (1,28).

X4.6 Handling - The cladocerans are delicate and should be handled as carefully and little as possible. They are transferred with a 5 mm bore pipet and released slowly beneath the water surface.

X4.7 Experimental Design for Acute Toxicity Tests - Sediments may be mixed, if appropriate for the study, by mixing with either a large plastic paddle, magnetic stirring bar or shaker table, before allocating to test chambers. See ASTM 1297 and 1391 for guidance. Whole sediment assays use a 1:4 ratio of sediment to water. Acute toxicity tests are conducted in triplicate using 250 or 100 ml beakers to which 30 ml of sediment (by weight) and 120 ml of reconstituted or site water are added (for 250 ml beakers). The weight of 30 ml of sediment is determined by initially calculating the average wet weight (grams) of five, 5 ml aliquots of sediment obtained using a 10 cc syringe. The average weight of 5 ml is divided by five to obtain the weight of 1 ml of sediment. The weight of 1 ml is multiplied by 30 ml to obtain the number of grams to be weighed into each test beaker. When a syringe cannot be used to dispense sediments, sediment weight is used rather than volume, weighing 30 grams (wet weight) into each test beaker. In addition, sediment dry weights are determined by weighing triplicate 3-5 ml aliquots of wet sediment, drying at 100-105°C for 24 hours and then reweighing the sediment. Percent dry

weight is calculated by dividing the dry sediment weight (grams) by the wet weight and multiplying by 100. Grams of dry weight per ml of wet sediment is determined by dividing the dry weight by the ml of wet sediment. Overlying water is gently added to each beaker, minimizing sediment resuspension. After a 1 to 2 hr settling period, ten test organisms are randomly added to each beaker. Test chambers should be inspected less than 2 h after the addition of test organisms to check for any "floaters." "Floaters" may not survive and are subjected to a different exposure, thus can be removed and replaced within the first two hours. Floating may be caused by the sediment sample and may be considered a treatment effect in some cases. However, responses tend to be variable and are seldom dose proportional. Surface films which entrap D. magna can be reduced by wiping the surface with cellulose filter paper prior to organism addition.

X4.8 Experimental Design for Short-term Chronic Toxicity Tests. Test initiation, test conditions and monitoring are as described in Section X4.7 and X4.9 with the following exceptions, and basically follow standard methods (22, ASTM E1295). Tests are conducted in 30 ml beakers using 5 ml (or grams) sediment and 20 ml overlying water in replicates of ten. One organism (D. magna less than 24 hr old or C. dubia less than 6 hr old) is randomly added to each beaker, after the settling period. At each 24 hr test interval, the adult is removed and placed in a beaker containing the control water, young are counted and discarded, and physicochemical measures made. Approximately 15 ml of overlying water is suctioned off and gently renewed. The culturing food (such as YCT or algal-Cerophyl® mixture) is then

added (0.1 ml) to each beaker. After feeding, the adult organism is returned to the test beaker. The test is terminated at 7 days and/or when at least 60% of the controls have produced their third brood.

X4.9 Monitoring Data - Test conditions and monitoring should follow standard methods (22,23). Test beakers are maintained at 25 ± 1°C and receive a 16:8 h light:dark cycle (20 lux). Dissolved oxygen and temperature are monitored at 0, 24 and 48 h. Dissolved oxygen should not be allowed to drop below 40% saturation. If it does, gentle bubbling should be used until adequate saturation is attained. The pH, hardness and alkalinity are monitored at 0 and 48 h. Survival numbers were recorded at 24 and 48 h. Death of a test animal is judged as a result of observing no movement upon gentle prodding. Tests are considered valid when control mortality is ≤ 10% (23). Control treatments consist of reconstituted water or reference site water, and a control and/or reference sediment with the overlying test water (reconstituted or reference site). See the preceding guide text for additional guidance on sediment characterization, controls, references, and data analyses.

The 7-day survival and reproduction test requires the daily counting of adult survivors and young production. Dissolved oxygen, temperature, and pH should be measured daily, before renewing overlying waters on two to three beakers in each treatment and control. Alkalinity and hardness are measured at test initiation and termination. For the test results to be acceptable controls must have 80% survival with C. dubia controls averaging 15 young and D. magna averaging 60 young per surviving female (22,26).

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