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Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates

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Foreword

Sediment contamination is a widespread environmental problem that can potentially pose a threat to a variety of aquatic ecosystems. Sediment functions as a reservoir for common contaminants such as pesticides, herbicides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons, and metals such as lead, mercury, and arsenic. In-place contaminated sediment can result in depauparate benthic communities, while disposal of contaminated dredge material can potentially exert adverse effects on both pelagic and benthic systems. Historically, assessment of sediment quality has been limited to chemical characterizations. The United States Environmental Protection Agency (USEPA) is developing methodologies to calculate chemical-specific sediment quality criteria for use in the Agency's regulatory programs. However, quantifying contaminant concentrations alone cannot always provide enough information to adequately evaluate potential adverse effects that arise from interactions among chemicals, or that result from time-dependent availability of sediment-associated contaminants to aquatic organisms. Because relationships between concentrations of contaminants in sediment and bioavailability are not fully understood, determination of contaminated sediment effects on aquatic organisms may require the use of controlled toxicity and bioaccumulation tests.

As part of USEPA's Contaminated Sediment Management Strategy, all Agency programs have agreed to use the same methods to determine whether sediments have the potential to affect aquatic ecosystems. More than ten federal statutes provide authority to many USEPA program offices to address the problem of contaminated sediment. The sediment test methods in this manual will be used by USEPA to make decisions under a range of statutory authorities concerning such issues as: dredged material disposal, registration of pesticides and toxic substances, Superfund site assessment, and assessment and cleanup of hazardous waste treatment, storage, and disposal facilities. The use of uniform sediment testing procedures by USEPA programs is expected to increase data accuracy and precision, facilitate test replication, increase the comparative value of test results, and, ultimately, increase the efficiency of regulatory processes requiring sediment tests.

For additional guidance on the technical considerations in the manual, please contact Teresa Norberg-King, USEPA, Duluth, MN.

Abstract

Procedures are described for testing freshwater organisms in the laboratory to evaluate the toxicity or bioaccumulation of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory. Toxicity methods are outlined for two organisms, the amphipod *Hyalella azteca* and the midge *Chironomus tentans*. The toxicity tests are conducted for 10 d in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and test organisms are fed during the toxicity tests. The endpoint in the toxicity test with *H. azteca* is survival and the endpoints in the toxicity test with *C. tentans* are survival and growth. Procedures are primarily described for testing freshwater sediments; however, estuarine sediments (up to 15‰ salinity) can also be tested with *H. azteca*. Guidance for conducting 28-d bioaccumulation tests with the oligochaete *Lumbriculus variegatus* is provided in this manual. Overlying water is renewed daily and test organisms are not fed during bioaccumulation tests. Methods are also described for determining bioaccumulation kinetics of different classes of compounds during 28-d exposures with *L. variegatus*.

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Acknowledgments

This document is a general purpose testing manual for freshwater sediments. The approaches have also been described in ASTM (1994a) ASTM (1994b), Ankley et al. (1993), Phipps et al. (1993), Brooke et al. (1993), Call et al. (1993a), Call et al. (1993b), Lee et al. (1994), and USEPA (1994a).

This manual reflects the consensus of the Freshwater Sediment Toxicity Assessment Committee and the U.S. Environmental Protection Agency (USEPA) Program Offices. Members of the Freshwater Sediment Toxicity Assessment Committee are G.T. Ankley, USEPA, Duluth, MN; D.A. Benoit, USEPA, Duluth, MN; G.A. Burton, Wright State University, Dayton, OH; F.J. Dwyer, National Biological Survey (NBS; formerly U.S. Fish and Wildlife Service), Columbia, MO; I.E. Greer, NBS, Columbia, MO; R.A. Hoke, SAIC, Hackensack, NJ; C.G. Ingersoll, NBS, Columbia, MO; P. Kosian, USEPA, Duluth, MN; P.F. Landrum, NOAA, Ann Arbor, MI; J.M. Lazorchak, USEPA, Cincinnati, OH; T.J. Norberg King, USEPA, Duluth, MN; and P.V. Winger, NBS, Athens, GA.

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Section 1
Introduction

1.1 Significance of Use

1.1.1 Sediment provides habitat for many aquatic organisms and is a major repository for many of the more persistent chemicals that are introduced into surface waters. In the aquatic environment, most anthropogenic chemicals and waste materials including toxic organic and inorganic chemicals eventually accumulate in sediment. Mounting evidence exists of environmental degradation in areas where USEPA Water Quality Criteria (WQC; Stephan et al., 1985) are not exceeded, yet organisms in or near sediments are adversely affected (Chapman, 1989). The WQC were developed to protect organisms in the water column and were not intended to protect organisms in sediment. Concentrations of contaminants in sediment may be several orders of magnitude higher than in the overlying water; however, bulk sediment concentrations have not been strongly correlated to bioavailability (Burton, 1991). Partitioning or sorption of a compound between water and sediment may depend on many factors including: aqueous solubility, pH, redox, affinity for sediment organic carbon and dissolved organic carbon, grain size of the sediment, sediment mineral constituents (oxides of iron, manganese, and aluminum), and the quantity of acid volatile sulfides in sediment (Di Toro et al., 1990, 1991). Although certain chemicals are highly sorbed to sediment, these compounds may still be available to the biota. Contaminated sediments may be directly toxic to aquatic life or can be a source of contaminants for bioaccumulation in the food chain.

1.1.2 Assessments of sediment quality have commonly included sediment chemical analyses and surveys of benthic community structure. Determination of sediment contaminant concentrations on a dry weight basis alone offers little insight into predicting adverse biological effects because bioavailability may be limited by the intricate partitioning factors mentioned above. Likewise, benthic community surveys may be inadequate because they sometimes fail to discriminate between effects of contaminants and those that result from unrelated non-contaminant factors, including water-quality fluctuations, physical parameters, and biotic interactions. In order to obtain a direct measure of sediment toxicity or bioaccumulation, laboratory tests have been developed in which surrogate organisms are exposed to sediments under controlled conditions. Sediment toxicity tests have evolved into effective tools providing direct, quantifiable evidence of biological consequences of sediment contamination that can only be inferred from chemical or benthic community analyses. The USEPA is developing a national inventory of contaminated sediment sites. This inventory will be used to develop a biennial report to Congress on sediment quality in the United States required under the Water Resources Development Act of 1992. The use of consistent sediment testing methods will provide high quality data needed for the national inventory and for regulatory programs to prevent, remediate, and manage contaminated sediment (Southerland et al., 1991).

1.1.3 The objective of a sediment test is to determine whether contaminants in sediment are harmful to or are bioaccumulated by benthic organisms. The tests can be used to measure interactive toxic effects of complex contaminant mixtures in sediment. Furthermore, knowledge of specific pathways of interactions among sediments and test organisms is not necessary in order to conduct the tests (Kemp and Swartz, 1988). Sediment tests can be used to (1) determine the relationship between toxic effects and bioavailability, (2) investigate interactions among contaminants, (3) compare the sensitivities of different organisms, (4) determine spatial and temporal distribution of contamination, (5) evaluate hazards of dredged material, (6) for measuring toxicity as part of product licensing or safety testing or chemical approval, (7) rank areas for clean up, and (8) set cleanup goals and estimate the effectiveness of remediation or management practices.

1.1.4 Results of toxicity tests on sediments spiked at different concentrations of contaminants can be used to establish cause and effect relationships between chemicals and biological responses. Results of toxicity tests with test materials spiked into sediments at different concentrations may be reported in terms of an LC50 (median lethal concentration), an EC50 (median effect concentration), an IC50 (inhibition concentration), or as a NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration). In some cases, results of bioaccumulation tests may also be reported in terms of a Biota-sediment Accumulation Factor (BSAF) (Ankley et al., 1992a; Ankley et al., 1992b).

1.1.5 Evaluating effect concentrations for chemicals in sediment requires knowledge of factors controlling their bioavailability. Similar concentrations of a chemical in

units of mass of chemical per mass of sediment dry weight often exhibit a range in toxicity in different sediments (Di Toro et al., 1990; Di Toro et al., 1991). Effect concentrations of chemicals in sediment have been correlated to interstitial water concentrations, and effect concentrations in interstitial water are often similar to effect concentrations in water-only exposures. The bioavailability of nonionic organic compounds in sediment is often inversely correlated with the organic carbon concentration. Whatever the route of exposure, these correlations of effect concentrations to interstitial water concentrations indicate that predicted or measured concentrations in interstitial water can be used to quantify the exposure concentration to an organism. Therefore, information on partitioning of chemicals between solid and liquid phases of sediment is useful for establishing effect concentrations (Di Toro et al., 1991).

1.1.6 Field surveys can be designed to provide either a qualitative reconnaissance of the distribution of sediment contamination or a quantitative statistical comparison of contamination among sites. Surveys of sediment toxicity or bioaccumulation are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlations may be improved and sampling costs may be reduced if subsamples are taken simultaneously for sediment tests, chemical analyses, and benthic community structure.

1.1.7 Table 1.1 lists several approaches the USEPA has considered for the assessment of sediment quality (USEPA, 1992c). These approaches include (1) equilibrium partitioning, (2) tissue residues, (3) interstitial water toxicity, (4) whole-sediment toxicity and sediment-spiking tests, (5) benthic community structure, and (6) Sediment Quality Triad and Effects Range Median (see Chapman, 1989 and USEPA, 1989a; USEPA, 1990a; USEPA, 1990b; USEPA, 1992b for a critique of these methods). The sediment assessment approaches listed in Table 1.1 can be classified as numeric (e.g., equilibrium partitioning), descriptive (e.g., whole-sediment toxicity tests), or a combination of numeric and descriptive approaches (e.g., Effects Range Median; USEPA, 1992c). Numeric methods can be used to derive chemical-specific sediment quality criteria (SQC). Descriptive methods such as toxicity tests with field-collected sediment cannot be used alone to develop numerical SQC for individual chemicals. Although each approach can be used to make site-specific decisions, no one single approach can adequately address sediment quality. Overall, an integration of several methods using the weight of evidence is the most desirable approach for assessing the effects of contaminants associated with sediment (Long and Morgan, 1991). Hazard evaluations integrating data from laboratory exposures, chemical analyses, and benthic community assessments provide strong complementary evidence of the degree of pollution-induced degradation in aquatic communities (Chapman et al., 1992; Burton, 1991).

1.2 Program Applicability

1.2.1 The USEPA has authority under a variety of statutes to manage contaminated sediment. Until recently, the USEPA has not addressed sediment quality except in relation to disposal of material removed during navigational dredging (Table 1.2). Southerland et al. (1992) outlined four goals of an USEPA management strategy for contaminated sediments: (1) in-place sediment should be protected from contamination to ensure beneficial uses of surface waters, (2) protection of in-place sediment should be achieved through pollution prevention and source control, (3) in-place remediation should be limited to locations where natural recovery will not occur in an acceptable period of time, and (4) consistent methods should be used to trigger regulatory decisions.

1.2.2 The Clean Water Act (CWA) is the single most important law dealing with environmental quality of surface waters in the United States. The goal of the CWA is to restore and maintain physical, chemical, and biological integrity of the nation's waters (Southerland et al., 1992). Federal and state monitoring programs traditionally have focused on evaluating water column problems caused by point-source dischargers. The USEPA is developing a national inventory of contaminated sediment sites. This inventory will be used to develop a biennial report to Congress on sediment quality in the United States required under the Water Resources Development Act of 1992. The use of consistent sediment testing methods will provide high quality data needed for the national inventory and for regulatory program to prevent, remediate, and manage contaminated sediment (Southerland et al., 1992).

1.2.3 The Office of Water (OW), the Office of Pesticide Programs (OPP), the Office of Pollution Prevention and Toxics (OPPT), the Office of Solid Waste (OSW), and the Office of Emergency and Remedial Response (OERR) are all committed to the principle of consistent tiered testing outlined in the Agency-wide Contaminated Sediment Strategy (Southerland et al., 1992). Agency-wide consistent testing is desirable because all USEPA programs will use similar methods to evaluate whether a sediment poses an ecological or human health risk, and comparable data would be produced. It will also provide the basis for uniform cross-program decision-making within the USEPA. Each program will, however retain the flexibility of deciding whether identified risks would trigger regulatory actions.

1.2.4 Tiered testing should include a hierarchy of tests with the tests in each successive tier becoming progressively more rigorous, complex, and costly (Southerland et al, 1992). Guidance needs to be developed to explain how information within each tier would trigger regulatory action. The guidance could be program specific, describing decisions based on a weight of evidence approach, a pass-fail approach, or comparison to a reference site depending on statutory and regulatory requirements. There are now two approaches for sediment

Table 1.1 Sediment Quality Assessment Procedures[1]

Method	Type			Approach
	Numeric	Descriptive	Combination	
Equilibrium Partitioning	•			A sediment quality value for a given contaminant is determined by calculating the sediment concentration of the contaminant that corresponds to an interstitial water concentration equivalent to the USEPA water-quality criterion for the contaminant.
Tissue Residues	•			Safe sediment concentrations of specific chemicals are established by determining the sediment chemical concentration that results in acceptable tissue residues.
Interstitial Water Toxicity	•	•	•	Toxicity of interstitial water is quantified and identification evaluation procedures are applied to identify and quantify chemical components responsible for sediment toxicity.
Benthic Community Structure		•		Environmental degradation is measured by evaluating alterations in benthic community structure.
Whole-sediment Toxicity and Sediment Spiking	•	•	•	Test organisms are exposed to sediments that may contain known or 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 endpoint. Dose-response relationships can be established by exposing test organisms to sediments that have been spiked with known amounts of chemicals or mixtures of chemicals.
Sediment Quality Triad	•	•	•	Sediment chemical contamination, sediment toxicity, and benthic community structure are measured on the same sediment sample. Correspondence between sediment chemistry, toxicity, and field effects is used to determine sediment concentrations that discriminate conditions of minimal, uncertain, and major biological effects.
Apparent Effects Threshold (AET)	•	•	•	The sediment concentration of a contaminant above which statistically significant biological effects (e.g., sediment toxicity) are always expected. AET values are empirically derived from paired field data for sediment chemistry and a range of biological effects indicators.

[1] Modified from USEPA (1992c)

testing used by USEPA: (1) the Office of Water-U.S. Army Corps of Engineers dredged material testing framework and (2) the OPPT ecological risk assessment tiered testing framework. Tier 1 of the dredged material testing framework consists of a review of existing chemical and biological data or an inventory of nearby sources. In Tier 2, chemical data are compared to water and sediment quality criteria. Tier 3 evaluations consist of acute toxicity and bioaccumulation testing, and a comparison of the results to a reference area. The OPP testing framework consists of acute toxicity testing in Tier 1, followed by chronic (early life stage) toxicity testing in Tier 2 and further chronic toxicity testing (full life cycle) in Tier 3. Tier 4 consists of field or mesocosm testing. A tiered testing framework has not yet been chosen for Agency-wide use, but some of the components have been identified to be standardized. These components are toxicity tests, bioaccumulation tests, chemical criteria, and other measurements that may have ecological significance including benthic community structure evaluation, colonization rate, and *in situ* sediment testing within a mesocosm (USEPA, 1992a).

1.3 Scope and Application

1.3.1 Procedures are described for testing freshwater organisms in the laboratory to evaluate the toxicity or bioaccumulation of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory. Toxicity methods are outlined for two organisms, the amphipod *Hyalella azteca* and the midge *Chironomus tentans*. The toxicity tests are conducted for 10 d in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and organisms are fed during the toxicity tests. The endpoint in the toxicity test with *H. azteca* is survival and the endpoints in the toxicity test with *C. tentans* are survival and growth. Procedures are primarily described for testing freshwater sediments; however, estuarine sediments (up to 15 ‰ salinity) can also be tested with *H. azteca*. Guidance for conducting 28-d bioaccumulation tests with the oligochaete *Lumbriculus variegatus* is provided in this manual. Overlying water is renewed daily and organisms are not fed during bioaccumulation tests. Methods are also described for determining bioaccumu-

Table 1.2 Statutory Needs for Sediment Quality Assessment[1]

Law[2]	Area of Need
CERCLA	• Assess need for remedial action with contaminated sediments; assess degree of cleanup required, disposition of sediments
CWA	• NPDES permitting, especially under Best Available Technology (BAT) in water-quality-limited water • Section 403(c) criteria for ocean discharges; mandatory additional requirements to protect marine environment • Section 301(g) waivers for publicly owned treatment works (POTWs) discharging to marine waters • Section 404 permits for dredge and fill activities (administered by the Corps of Engineers)
FIFRA	• Review uses of new and existing chemicals • Pesticide labeling and registration
MPRSA	• Permits for ocean dumping
NEPA	• Preparation of environmental impact statements for projects with surface water discharges
TSCA	• Section 5: Premanufacture notice reviews for new chemicals • Sections 4, 5, and 6: Reviews for existing chemicals
RCRA	• Assess suitability (and permit) on-land disposal or beneficial use of contaminated sediments considered "hazardous"

[1] Modified from Dickson et al. 1984, and Southerland et al. 1992.
[2] CERCLA Comprehensive Environmental Response, Compensation and Liability Act (Superfund).
CWA Clean Water Act.
FIFRA Federal Insecticide, Fungicide, and Rodenticide Act.
MPRSA Marine Protection, Resources and Sanctuary Act.
NEPA National Environmental Policy Act.
TSCA Toxic Substances Control Act.
RCRA Resource Conservation and Recovery Act.

lation kinetics of different classes of compounds during 28-d exposures with *L. variegatus*.

1.3.2 Additional research and methods development are now in progress to (1) develop standard chronic sediment toxicity tests (e.g., 28-d exposures with *H. azteca),* (2) refine formulated sediment, (3) refine sediment dilution procedures, (4) refine sediment Toxicity Identification Evaluation (TIE) procedures (Ankley and Thomas, 1992), (5) refine sediment spiking procedures, and (6) produce additional data on confirmation of responses in laboratory tests with natural populations of benthic organisms. This information will be described in future editions of this manual.

1.3.3 This methods manual serves as a companion to the marine sediment testing method manual (USEPA, 1994a).

1.3.4 Procedures described in this manual are based on the following documents: ASTM (1994a), Ankley et al. (1993), Phipps et al. (1993), Call et al. (1994), Lee et al. (1994), and USEPA (1993a). This manual outlines specific test methods for evaluating the toxicity of sediments with *H. azteca* and *C. tentans*. Because additional research is still needed on the standardization of bioaccumulation procedures with sediments, this manual outlines only general guidance on procedures for evaluating the bioaccumulation of contaminants in sediment with *L. variegatus*. Many of the critical issues necessary for interpretation of test results are the subject of continuing research including the influence of feeding on bioavailability, nutritional requirements of the test organisms, additional performance criteria for organism health, and confirmation of responses in laboratory tests with natural benthos populations. See Section 4 for additional details.

1.3.5 General procedures described in this manual might be useful for conducting tests with other aquatic organisms; however, modifications may be necessary. Altering the procedures described in this manual may alter bioavailability and produce results that are not directly comparable with results of acceptable procedures. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms (e.g., *Diporeia* spp., *Tubifex tubifex, Hexagenia* spp.). If tests are conducted with procedures different from those described in this manual, additional tests are required to determine comparability of results.

1.3.6 Methods have been described for culturing and testing indigenous species that may be as sensitive or more sensitive than the species recommended in this manual. However, the USEPA currently allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species. Where state regulations prohibit im-

portation or use of the recommended test species, permission should be requested from the appropriate regulatory agency before their using indigenous species.

1.3.7 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained with sediments or reference toxicants, to ensure that the species selected are at least as sensitive and appropriate as the recommended species.

1.3.8 Selection of Test Organisms

1.3.8.1 The choice of a test organism has a major influence on the relevance, success, and interpretation of a test. Test organism selection should be based on both environmental relevance and practical concerns (DeWitt et al., 1989, Swartz 1989). Ideally, a test organism should (1) have a toxicological database demonstrating relative sensitivity and discrimination to a range of contaminants of interest in sediment; (2) have a database for interlaboratory comparisons of procedures (e.g., round-robin studies); (3) be in direct contact with sediment; (4) be readily available through culture or from field collection; (5) be easily maintained in the laboratory; (6) be easily identified; (7) be ecologically or economically important; (8) have a broad geographical distribution, be indigenous (either present or historical) to the site being evaluated, or have a niche similar to organisms of concern (e.g., similar feeding guild or behavior to the indigenous organisms); (9) be tolerant of a broad range of sediment phsyico-chemical characteristics (e.g., grain size); and (10) be compatible with selected exposure methods and endpoints (Table 1.3, ASTM, 1993a). The method should also be (11) peer reviewed (e.g., journal articles, ASTM guides) and (12) confirmed with responses with natural populations of benthic organisms (Sections 1.3.8.8 and 1.3.9.6).

Table 1.3 Rating of Selection Criteria for Freshwater Sediment Toxicity Testing Organisms[1]

Criterion	*Hyalella azteca*	*Diporeia* spp.	*Chironomus tentans*	*Chironomus riparius*	*Lumbriculus variegatus*	*Tubifex tubifex*	*Hexagenia* spp.	*Mollusks*	*Daphnia* spp. and *Ceriodaphnia* spp.
Relative sensitivity toxicity database	+	-	+	-	+	-	-	-	-
Round-robin studies conducted	+	-	+	-	-	-	-	-	-
Contact with sediment	+	+	+	+	+	+	+	+	-
Laboratory culture	+	-	+	+	+	+	-	-	+
Taxonomic identification	+/-	+/-	+/-	+/-	+	+	+	+	+
Ecological importance	+	+	+	+	+	+	+	+	+
Geographical distribution	+	+/-	+	+	+	+	+	+	+/-
Sediment physico-chemical tolerance	+	+	+/-	+	+	+	-	+	NA
Response confirmed with benthos populations	+	+	+	+	+	+	+	-	+
Peer reviewed	+	+	+	+	+	+	+	-	+/-
Endpoints[2] monitored	S, G, M	S, B, A	S, G, E	S, G, E	B, S, R	S, R	S, G	B	S, G, R

[1] A "+" or "-" rating indicates a positive or negative attribute

[2] S = Survival, G = Growth, B = Bioaccumulation, A = Avoidance, R - Reproduction, M = Maturation, E = Emergence. NA = not applicable

1.3.8.2 Of these criteria (Table 1.3), a database demonstrating relative sensitivity to contaminants, contact with sediment, ease of culture in the laboratory, interlaboratory comparisons, tolerance to varying sediment phsyicochemical characteristics, and confirmation with responses of natural benthos populations were the primary criteria used for selecting *H. azteca, C. tentans,* and *L. variegatus* for the current edition of this manual. Many organisms that might be appropriate for sediment testing do not now meet these selection criteria because historically little emphasis has been placed on developing standardized testing procedures for benthic organisms. A similar database must be developed in order for other organisms to be included in future editions of this manual (e.g., mayflies (*Hexagenia* spp.), other midges *(C. riparius),* other amphipods (*Diporeia* spp.), cladocerans *(Daphnia magna, Ceriodaphnia dubia),* or mollusks).

1.3.8.3 An important consideration in the selection of specific species for test method development is the existence of information concerning relative sensitivity of the organisms both to single chemicals and complex mixtures. A number of studies have evaluated the sensitivity of *H. azteca, C. tentans* and *L. variegatus,* relative to one another, as well as other commonly tested freshwater species. For example, Ankley et al. (1991b) found *H. azteca* to be as, or slightly more, sensitive than *Ceriodaphnia dubia* to a variety of sediment elutriate and pore-water samples. In that study, *L. variegatus* were less sensitive to the samples than either the amphipod or the cladoceran. West et al. (1993) found the rank sensitivity of the three species to the lethal effects of copper-contaminated sediments could be ranked (from greatest to least): *H. azteca* > *C. tentans* > *L. variegatus.* In short-term (48 to 96 h) exposures, *L. variegatus* generally was less sensitive than *H. azteca, C. dubia,* or *Pimephales promelas* to cadmium, nickel, zinc, copper, and lead (Schubauer-Berigan et al., 1993). Of the latter three species, no one was consistently the most sensitive to all five metals.

Table 1.4 Water-only, 10-d LC50 (µg/L) Values for *Hyalella azteca, Chironomus tentans,* and *Lumbriculus variegatus*[1]

Chemical	*H. azteca*	*C. tentans*	*L. variegatus*
Copper	35	54	35
Zinc	73	1,125[2]	2,984
Cadmium	2.8[3]	NT[4]	158
Nickel	780	NT	12,160
Lead	<16	NT	794
p,p'-DDT	0.07	1.23	NT
p,p'-DDD	0.17	0.18	NT
p,p'-DDE	1.39	3.0	>3.3
Dieldrin	7.6	1.1	NT
Chlorpyrifos	0.086	0.07	NT

[1] Chemicals tested at ERL-Duluth in soft water—hardness 45 mg/L as $CaCO_3$ at pH 7.8 to 8.2 (Phipps et al., 1994).
[2] 50% mortality at highest concentration tested.
[3] 70% mortality at lowest concentration tested.
[4] NT = not tested.

1.3.8.3.1 In a study of contaminated Great Lakes sediment, *H. azteca, C. tentans,* and *C. riparius* were among the most sensitive and discriminatory of 24 organisms tested (Burton and Ingersoll, 1994; Ingersoll et al., 1993). Kemble et al. (1993) found the rank sensitivity of four species to metal-contaminated sediments to be (from greatest to least): *H. azteca* > *C. riparius* > *Oncorhynchus mykiss* (rainbow trout) > *Daphnia magna.* The relative sensitivity of the three endpoints evaluated in the *H. azteca* test with Clark Fork River sediments was (from greatest to least): length > sexual maturation > survival.

1.3.8.3.2 In 10-d water-only and whole-sediment tests, *H. azteca* and *C. tentans* were more sensitive than *D. magna* to fluoranthene (Suedel et al. 1993).

1.3.8.3.3 Ten-day, water-only tests also have been conducted with a number of chemicals using the three species described in this manual (Phipps et al., 1994; Table 1.4). All tests were flow-through exposures using a soft natural water (Lake Superior) with measured chemical concentrations that, other than the absence of sediment, were conducted under conditions (e.g., temperature, photoperiod, feeding) similar to those being described for the standard 10-d sediment test. In general, *H. azteca* was more sensitive to copper, zinc, cadmium, nickel and lead than either *C. tentans* or *L. variegatus.* Conversely, the midge was usually comparable to or more sensitive than the amphipod to the pesticides tested. *Lumbriculus variegatus* was not tested with several of the pesticides; however, in other studies with whole sediments contaminated by DDT and associated metabolites, and in short-term (96-h) experiments with organophosphate insecticides (diazinon, chlorpyrifos), *L. variegatus* has proven to be far less sensitive than either *H. azteca* or *C. tentans.* These results highlight two important points germane to the methods in this manual. First, neither of the two test species selected for estimating sediment toxicity *(H. azteca, C. tentans)* was consistently more sensitive to all chemicals, indicating the importance of using multiple test organisms when performing sediment assessments. Second, *L. variegatus* appears to be relatively insensitive to most of the test chemicals, which perhaps is a positive attribute for an organism used in bioaccumulation tests.

1.3.8.3.4 Using the data from Table 1.4, sensitivity of *H. azteca, C. tentans* and *L. variegatus* can be evaluated relative to other freshwater species. For this analysis, acute and chronic toxicity data from water quality criteria (WQC) documents for copper, zinc, cadmium, nickel, lead, DDT, dieldrin and chlorpyrifos, and toxicity information from the AQUIRE database (AQUIRE, 1992) for DDD and DDE, were compared to assay results for the three species (Phipps et al., 1994). The sensitivity of *H. azteca* to metals and pesticides, and *C. tentans* to pesticides was comparable to chronic toxicity data generated for other test species. This was not completely

unexpected given that the 10-d exposures used for these two species are likely more similar to chronic partial life-cycle tests than the 48- to 96-h exposures traditionally defined as acute in WQC documents. Interestingly, in some instances (e.g., dieldrin, chlorpyrifos), LC50 data generated for *H. azteca* or *C. tentans* were comparable to or lower than any reported for other freshwater species in the WQC documents. This observation likely is a function not only of the test species, but of the test conditions; many of the tests on which early WQC were based were static, rather than flow-through, and utilized unmeasured contaminant concentrations.

1.3.8.4 Relative species sensitivity frequently varies among contaminants; consequently, a battery of tests including organisms representing different trophic levels may be needed to assess sediment quality (Craig, 1984; Williams et al., 1986a; Long et al., 1990; Ingersoll et al., 1990; Burton and Ingersoll, 1994; USEPA, 1989b). For example, Reish (1988) reported the relative toxicity of six metals (As, Cd, Cr, Cu, Hg, and Zn) to crustaceans, polychaetes, pelecypods, and fishes and concluded that no single species or group of test organisms was the most sensitive to all of the metals.

1.3.8.5 The sensitivity of an organism to contaminants should be balanced with the concept of discrimination (Burton and Ingersoll, 1994). The response of a test organism should provide discrimination between different levels of contamination.

1.3.8.6 The sensitivity of an organism is related to the route of exposure and biochemical response to contaminants. Sediment-dwelling organisms can receive exposure via three primary sources: interstitial water, sediment particles, and overlying water. Food type, feeding rate, assimilation efficiency, and clearance rate will control the dose of contaminants from sediment. Benthic invertebrates often selectively consume different particle sizes (Harkey et al., 1994) or particles with higher organic carbon concentrations that may have higher contaminant concentrations. Grazers and other collector-gatherers that feed on aufwuchs and detritus may receive most of their body burden directly from materials attached to sediment or from actual sediment ingestion. In amphipods (Landrum, 1989) and clams (Boese et al., 1990) uptake through the gut can exceed uptake across the gills of certain hydrophobic compounds. Organisms in direct contact with sediment may also accumulate contaminants by direct adsorption to the body wall or by absorption through the integument (Knezovich et al., 1987).

1.3.8.7 Despite the potential complexities in estimating the dose that an animal receives from sediment, the toxicity and bioaccumulation of many contaminants in sediment such as Kepone®, fluoranthene, organochlorines, and metals have been correlated with either the concentration of these chemicals in interstitial water or in the case of nonionic organic chemicals, concentrations in sediment on an organic carbon normalized basis (Di Toro et al., 1990; Di Toro et al., 1991). The relative importance of whole sediment and interstitial water routes of exposure depends on the test organism and the specific contaminant (Knezovich et al., 1987). Because benthic communities contain a diversity of organisms, many combinations of exposure routes may be important. Therefore, behavior and feeding habits of a test organism can influence its ability to accumulate contaminants from sediment and should be considered when selecting test organisms for sediment testing.

1.3.8.8 The use of *H. azteca* and *C. tentans* in laboratory toxicity studies has been confirmed with natural benthos populations.

1.3.8.8.1 Chironomids were not found in sediment samples that decreased growth of *C. tentans* by 30% or more in 10-d laboratory toxicity tests (Giesy et al., 1988). Wentsel et al. (1977a, 1977b, 1978) reported a correlation between effects on *C. tentans* in laboratory tests and the abundance of *C. tentans* in metal-contaminated sediments.

1.3.8.8.2 Benthic community evaluations and laboratory tests with *H. azteca* both provided evidence of metal-induced degradation of aquatic communities in the Clark Fork River (Canfield et al., 1994). Total abundance of benthic organisms did not follow a consistent pattern when compared to metals in sediment samples. The number of chironomid genera was higher at stations that showed reduced growth or sexual maturation of *H. azteca* in laboratory sediment tests and had higher concentrations of metals in sediment.

1.3.8.8.3 The results from laboratory sediment toxicity tests were compared to colonization of artificial substrates exposed *in situ* to contaminated Great Lakes sediment (Burton and Ingersoll, 1994). Survival or growth of *H. azteca* and *C. tentans* in 10- to 28-d laboratory exposures were negatively correlated to percent chironomids and percent tolerant taxa colonizing artificial substrates in the field. Scklekat et al. (1994) reported generally good agreement between sediment tests with *H. azteca* and benthic community responses in the Anacostia River, Washington, D.C.

1.3.8.8.4 Sediment toxicity to amphipods in 10-d toxicity tests, field contamination, and field abundance of benthic amphipods were examined along a sediment contamination gradient of DDT (Swartz et al., 1994). Survival of *Eohaustorius estuarius*, *Rhepoxynius abronius*, and *H. azteca* in laboratory toxicity tests was positively correlated to abundance of amphipods in the field and along with the survival of *H. azteca*, was negatively correlated to DDT concentrations. The threshold for 10-d sediment toxicity in laboratory studies was about 300 µg DDT (+metabolites)/g organic carbon. The threshold for abundance of amphipods in the field was about 100 µg DDT (+metabolites)/g organic carbon. Therefore, correlations between toxicity, contamination, and field populations indicate that acute sediment toxicity tests can provide reliable evidence of biologically adverse sediment con-

tamination in the field, but may be underprotective of chronic effects.

1.3.9 Selection of Organisms for Sediment Bioaccumulation Testing

1.3.9.1 Several studies have demonstrated that hydrophobic organic compounds are bioaccumulated from sediment by freshwater infaunal organisms including larval insects *(C. tentans*, Adams et al., 1985; Adams, 1987; *Hexagenia limbata*, Gobas et al., 1989), oligochaetes *(Tubifex tubifex* and *Limnodrilus hoffmeisteri;* Oliver, 1984, Oliver, 1987; Connell et al., 1988), and by marine organisms (polychaetes, *Nephtys incisa;* mollusks, *Mercenaria mercenaria, Yoldia limatula;* Lake et al., 1990). Consumers of these benthic organisms may bioaccumulate or biomagnify contaminants. Therefore, in addition to sediment toxicity, it may be important to examine the uptake of chemicals by aquatic organisms from contaminated sediments.

1.3.9.2 Various species of organisms have been suggested for use in studies of chemical bioaccumulation from aquatic sediments. Several criteria should be considered before a species is adopted for routine use in these types of studies (Ankley et al., 1992a; Call et al., 1994). These criteria include (1) availability of organisms throughout the year, (2) known chemical exposure history, (3) adequate tissue mass for chemical analyses, (4) ease of handling, (5) tolerance of a wide range of sediment phsyico-chemical characteristics (e.g., particle size), (6) low sensitivity to contaminants associated with sediment (e.g., metals, organics), (7) amenability to long-term exposures without adding food, (8) and ability to accurately reflect concentrations of contaminants in field-exposed organisms (e.g., exposure is realistic). With these criteria in mind, the advantages and disadvantages of several potential freshwater taxa for bioaccumulation testing are discussed below.

1.3.9.3 Freshwater clams provide an adequate tissue mass, are easily handled, and can be used in long-term exposures. However, few non-exotic freshwater species are available for testing. Exposure of clams is uncertain because of valve closure. Furthermore, clams are filter feeders and may accumulate lower concentrations of contaminants compared to detritivores (Lake et al., 1990). Chironomids can be readily cultured, are easy to handle, and reflect appropriate routes of exposure. However, their rapid life-cycle makes it difficult to perform long-term exposures with hydrophobic compounds; also, chironomids can readily biotransform organic compounds such as benzo[a]pyrene (Harkey et al., 1994). Larval mayflies reflect appropriate routes of exposure, have adequate tissue mass for residue analysis, and can be used in long-term tests. However, mayflies cannot be continuously cultured in the laboratory and consequently are not always available for testing. Furthermore, the background concentrations of contaminants and health of field-collected individuals may be uncertain. Amphipods (e.g., *H. azteca)* can be cultured in the laboratory, are easy to handle, and reflect appropriate routes of exposure. However, their size may be insufficient for residue analysis and *H. azteca* are sensitive to contaminants in sediment. Fish (e.g., fathead minnows) provide an adequate tissue mass, are readily available, are easy to handle, and can be used in long-term exposures. However, the route of exposure is not appropriate for evaluating the bioavailability of sediment-associated contaminants to benthic organisms.

1.3.9.4 Oligochaetes are infaunal benthic organisms that meet many of the test criteria listed above. Certain oligochaete species are easily handled and cultured, provide reasonable biomass for residue analyses, and are tolerant of varying sediment physical and chemical characteristics. Oligochaetes are exposed to contaminants via all appropriate routes of exposure including pore water and ingestion of sediment particles. Oligochaetes need not be fed during long-term bioaccumulation exposures (Phipps et al., 1993). Various oligochaete species have been used in toxicity and bioaccumulation evaluations (Chapman et al., 1982a, Chapman et al., 1982b; Wiederholm, 1987; Kielty et al., 1988a, Kielty et al., 1988b; Phipps et al., 1993), and field populations have been used as indicators of the pollution of aquatic sediments (Brinkhurst, 1980; Spencer, 1980; Oliver, 1984; Lauritsen, 1985; Robbins et al., 1989; Ankley et al., 1992b; E.L. Brunson, NBS, Columbia, MO, unpublished data).

1.3.9.5 *Lumbriculus variegatus* does not biotransform PAHs (Harkey et al., 1994b).

1.3.9.6 The response of *L. variegatus* in laboratory bioaccumulation studies has been confirmed with natural populations of oligochaetes.

1.3.9.6.1 Total PCB concentrations in laboratory-exposed *L. variegatus* were similar to concentrations measured in field-collected oligochaetes from the same sites (Ankley et al., 1992b). PCB homologue patterns also were similar between laboratory-exposed and field-collected oligochaetes. The more highly chlorinated PCBs tended to have greater bioaccumulation in the field-collected organisms. In contrast, total PCBs in laboratory-exposed *(Pimephales promelas)* and field-collected *(Ictalurus melas)* fish revealed poor agreement in bioaccumulation relative to the sediment concentrations at the same sites.

1.3.9.6.2 Bioaccumulation of laboratory-exposed *L. variegatus* and field-collected oligochaetes from the same sites were also compared (E.L. Brunson, NBS, Columbia, MO, unpublished data). Select PAH and DDT peak concentrations were similar in field-collected oligochaetes and *L. variegatus* exposed for 28 d in the laboratory.

1.4 Performance-based Criteria

1.4.1 USEPA's Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing

chemical analytical standards (Williams, 1993). Performance-based methods were defined by EMMC as a monitoring approach that permits the use of appropriate methods that meet pre-established demonstrated performance standards (Section 9.2).

1.4.2 The USEPA Office of Water, Office of Science and Technology, and Office of Research and Development held a workshop on September 16-18, 1992 in Washington, DC to provide an opportunity for experts in the field of sediment toxicology and staff from USEPA's Regional and Headquarters program offices to discuss the development of standard freshwater and marine sediment testing procedures (USEPA, 1992a and Appendix A). Workgroup participants reached a consensus on several culturing and testing methods. In developing guidance for culturing freshwater test organisms to be included in the USEPA methods manual for sediment tests, it was agreed that no single method should be required to culture organisms. However, the consensus at the workshop was that since the success of a test depends on the health of the cultures, having healthy test organisms of known quality and age for testing was the key consideration. A performance-based criteria approach was selected as the preferred method through which individual laboratories should evaluate culture methods rather than by control-based criteria. This method was chosen to allow each laboratory to optimize culture methods and minimize effects of test organism health on the reliability and comparability of test results. See Tables 11.3, 12.3, and 13.4 for a listing of performance criteria for culturing and testing.

Section 2
Summary of Method

2.1 Method Description and Experimental Design

2.1.1 Method Description

2.1.1.1 This manual describes procedures for testing freshwater organisms in the laboratory to evaluate the toxicity or bioaccumulation of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory. Toxicity methods are outlined for two organisms, the amphipod *Hyalella azteca* and the midge *Chironomus tentans*. The toxicity tests are conducted for 10 d in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and test organisms are fed during the toxicity tests. The endpoint in the toxicity test with *H. azteca* is survival and the endpoints in the toxicity test with *C. tentans* are survival and growth. Procedures are primarily described for testing freshwater sediments; however, estuarine sediments (up to 15 ‰ salinity) can also be tested with *H. azteca*. Guidance for conducting 28-d bioaccumulation tests with the oligochaete *Lumbriculus variegatus* is provided in this manual. The overlying water is renewed daily and the test organisms are not fed during bioaccumulation tests. This guidance describes are also described for determining bioaccumulation kinetics of different classes of compounds during 28-d exposures with *L. variegatus*.

2.1.2 Experimental Design

The following section is a general summary of experimental design. See Section 14 for additional detail.

2.1.2.1 Control and Reference Sediment

2.1.2.1.1 Sediment tests include a control sediment (sometimes called a negative control). A control sediment is a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test and is not necessarily collected near the site of concern. Any contaminants in control sediment are thought to originate from the global spread of pollutants and do not reflect any substantial input from local or nonpoint sources (Lee et al., 1994). A control sediment provides a measure of test acceptability, evidence of test organism health, and a basis for interpreting data obtained from the test sediments. A reference sediment is collected near an area of concern and is used to assess sediment conditions exclusive of material(s) of interest. Testing a reference sediment provides a site-specific basis for evaluating toxicity.

2.1.2.1.2 Natural geomorphological and physicochemical characteristics such as sediment texture may influence the response of test organisms (DeWitt et al., 1988). The physicochemical characteristics of test sediment must be within the tolerance limits of the test organism. Ideally, the limits of a test organism should be determined in advance; however, controls for factors including grain size and organic carbon can be evaluated if the limits are exceeded in a test sediment. See Section 10.1 for information on physicochemical requirements of test organisms. If the physicochemical characteristics of a test sediment exceed the tolerance limits of the test organism it may be desirable to include a control sediment that encompasses those characteristics. The effects of sediment characteristics on the results of sediment tests may be able to be addressed with regression equations (DeWitt et al., 1988; Ankley et al., 1994a). The use of formulated sediment can also be used to evaluate physicochemical characteristics of sediment on test organisms (Walsh et al., 1991; Suedel and Rodgers, 1994).

2.1.2.2 The experimental design depends on the purpose of the study. Variables that need to be considered include the number and type of control sediments, the number of treatments and replicates, and water-quality characteristics. For instance, the purpose of the study might be to determine a specific endpoint such as an LC50 and may include a control sediment, a positive control, a solvent control, and several concentrations of sediment spiked with a chemical. A useful summary of field sampling design is presented by Green (1979). See Section 14 for additional guidance on experimental design and statistics.

2.1.2.3 If the purpose of the study is to conduct a reconnaissance field survey to identify contaminated sites for further investigation, the experimental design might include only one sample from each site to allow for maximum spatial coverage. The lack of replication at a site usually precludes statistical comparisons (e.g., ANOVA), but these surveys can be used to identify contaminated sites for further study or may be evaluated using regression techniques (Sokal and Rohlf, 1981; Steel and Torrie, 1980).

2.1.2.4 In other instances, the purpose of the study might be to conduct a quantitative sediment survey to determine statistically significant differences between effects among control and test sediments from several sites. The number of replicates/site should be based on the need for sensitivity or power (Section 14). In a quantitative survey, replicates (separate samples from different grabs collected at the same site) would need to be taken at each site. Chemical and physical characterizations of each of these grabs would be required for each of these replicates used in sediment testing. Separate subsamples might be used to determine within-sample variability or to compare test procedures (e.g., comparative sensitivity among test organisms), but these subsamples cannot be considered to be true field replicates for statistical comparisons among sites (ASTM, 1994a).

2.1.2.5 Sediments often exhibit high spatial and temporal variability (Stemmer et al., 1990a). Therefore, replicate samples may need to be collected to determine variance in sediment characteristics. Sediment should be collected with as little disruption as possible; however, subsampling, compositing, or homogenization of sediment samples may be necessary for some experimental designs.

2.1.2.6 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 contaminated in a reconnaissance survey. Comparisons can be made in both space and time. In pre-dredging studies, a sampling design can be prepared to assess the contamination of samples representative of the project area to be dredged. Such a design should include subsampling cores taken to the project depth.

2.1.2.7 The primary focus of the physical and experimental test design, and statistical analysis of the data, is the experimental unit. The experimental unit is defined as the smallest physical entity to which treatments can be independently assigned (Steel and Torrie, 1980) and to which air and water exchange between test chambers are kept to a minimum. As the number of test chambers/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 (Section 14). Because of factors that might affect results within test chambers and results of a test, all test chambers should be treated as similarly as possible. Treatments should be randomly assigned to individual test chamber locations. Assignment of test organisms to test chambers should be non-biased.

2.2 Types of Tests

2.2.1 Toxicity methods are outlined for two organisms, the amphipod *H. azteca* (Section 11) and the midge *C. tentans* (Section 12). This manual primarily describes methods for testing freshwater sediments; however, the methods described can also be used for testing *H. azteca* in estuarine sediments (up to 15 ‰ salinity).

2.2.2 Guidance for conducting 28-d bioaccumulation tests with the oligochaete *L. variegatus* is described in Section 13. Methods are also described for determining bioaccumulation kinetics of different classes of compounds during 28-d exposures with *L. variegatus*.

2.3 Test Endpoints

2.3.1 The endpoints measured in the toxicity tests are survival or growth (the growth endpoint is optional in the *H. azteca* test). Endpoints measured in bioaccumulation tests are tissue concentrations of contaminants and for some types of studies, lipid content. Behavior of test organisms should be qualitatively observed daily in all tests (e.g., avoidance of sediment).

Section 3
Definitions

3.1 Terms

The following terms were defined in Lee (1980), NRC (1989), USEPA (1989c), USEPA-USCOE (1991), USEPA-USCOE (1994), Lee et al. (1994), ASTM (1993b), or ASTM (1994a).

3.1.1 Technical Terms

3.1.1.1 ***Sediment.*** Particulate material that usually lies below water. Formulated particulate material that is intended to lie below water in a test.

3.1.1.2 ***Contaminated sediment.*** Sediment containing chemical substances at concentrations that pose a known or suspected threat to environmental or human health.

3.1.1.3 ***Whole sediment.*** Sediment and associated pore water that have had minimal manipulation. The term bulk sediment has been used synonymously with whole sediment.

3.1.1.4 ***Control sediment.*** A sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test. Any contaminants in control sediment may originate from the global spread of pollutants and does not reflect any substantial input from local or nonpoint sources. Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination.

3.1.1.5 ***Reference sediment.*** A whole sediment near an area of concern used to assess sediment conditions exclusive of material(s) of interest. 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 global pollutant input. This is the manner in which reference sediment is used in dredge material evaluations.

3.1.1.6 ***Interstitial water or pore water.*** Water occupying space between sediment or soil particles.

3.1.1.7 ***Spiked sediment.*** A sediment to which a material has been added for experimental purposes.

3.1.1.8 ***Reference toxicity test.*** A test with a high-grade reference material conducted in conjunction with sediment tests to determine possible changes in condition of the test organisms. Deviations outside an established normal range indicate a change in the condition of the test organism population. Reference-toxicity tests are most often performed in the absence of sediment.

3.1.1.9 ***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.

3.1.1.10 ***Overlying water.*** The water placed over sediment in a test chamber during a test.

3.1.1.11 ***Concentration.*** The ratio of weight or volume of test material(s) to the weight or volume of sediment.

3.1.1.12 ***No-observable-effect concentration (NOEC).*** The highest concentration of a toxicant to which organisms are exposed in a test that causes no observable adverse effect on the test organisms (i.e., the highest concentration of a toxicant in which the value for the observed response is not statistically significant different from the controls).

3.1.1.13 ***Lowest-observable-effect concentration (LOEC).*** The lowest concentration of a toxicant to which organisms are exposed in a test that causes an adverse effect on the test organisms (i.e., where a significant difference exists between the value for the observed response and that for the controls).

3.1.1.14 ***Lethal concentration (LC).*** The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observable adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

3.1.1.15 ***Effect concentration (EC).*** The toxicant concentration that would cause an effect in a given percent of the test population. Identical to LC when the observable adverse effect is death. For example, the EC50 is the concentration of toxicant that would cause death in 50% of the test population.

3.1.1.16 ***Inhibition concentration (IC).*** The toxicant concentration that would cause a given percent reduction in a non-quantal measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction.

3.1.1.17 ***Biota-sediment accumulation factor (BSAF).*** The ratio of tissue residue to source concentration (e.g., sediment at steady state normalized to lipid and sediment organic carbon).

3.1.1.18 ***Bioaccumulation.*** The net accumulation of a substance by an organism as a result of uptake from all environmental sources.

3.1.1.19 ***Bioaccumulation factor.*** Ratio of tissue residue to contaminant source concentration at steady-state.

3.1.1.20 ***Bioaccumulation potential.*** Qualitative assessment of whether a contaminant is bioavailable.

3.1.1.21 ***Bioconcentration.*** The net assimilation of a substance by an aquatic organism as a result of uptake directly from aqueous solution.

3.1.1.22 ***Bioconcentration factor (BCF).*** Ratio of tissue residue to water contaminant concentration at steady-state.

3.1.1.23 ***Depuration.*** Loss of a substance from an organism as a result of any active (e.g., metabolic breakdown) or passive process when the organism is placed into an uncontaminated environment. Contrast with Elimination.

3.1.1.24 ***Elimination.*** General term for the loss of a substance from an organism that occurs by any active or passive means. The term is applicable either in a contaminated environment (e.g., occurring simultaneously with uptake) or in a clean environment. Contrast with Depuration.

3.1.1.25 k_1. Uptake rate coefficient from the aqueous phase, with units of g-water x g-tissue^{-1} x time^{-1}. Contrast with k_s.

3.1.1.26 k_s. Sediment uptake rate coefficient from the sediment phase, with units of g-sediment x g-tissue^{-1} x time^{-1}. Contrast with k_1.

3.1.1.27 k_2. Elimination rate constant, with units of time^{-1}.

3.1.1.28 ***Kinetic Bioaccumulation Model.*** Any model that uses uptake and/or elimination rates to predict tissue residues.

3.1.1.29 K_{oc}. Organic carbon-water partitioning coefficient.

3.1.1.30 K_{ow}. Octanol-water partitioning coefficient.

3.1.1.31 ***Steady-state.*** An equilibrium or "constant" tissue residue resulting from the balance of the flux of compound into and out of the organism. Operationally determined by no statistically significant difference in tissue residue concentrations from three consecutive sampling periods.

3.1.2 Grammatical Terms

3.1.2.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this manual.

3.1.2.2 "Must" is used to express an absolute requirement, that is, to state that a test ought to be designed to satisfy the specified conditions, 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 a test.

3.1.2.3 "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.

3.1.2.4 Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors.

3.1.2.5 "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."

Section 4
Interferences

4.1 General Introduction

4.1.1 Interferences are characteristics of a sediment or sediment test system that can potentially affect test organism survival aside from those related to sediment-associated contaminants. These interferences can potentially confound interpretation of test results in two ways: (1) toxicity is observed in the test when contamination is not present, or there is more toxicity than expected; and (2) no toxicity or bioaccumulation is observed when contaminants are present at elevated concentrations, or there is less toxicity or bioaccumulation than expected.

4.1.2 There are three categories of interfering factors: those characteristics of sediments affecting survival independent of chemical concentration (i.e., non-contaminant factors); changes in chemical bioavailability as a function of sediment manipulation or storage; and the presence of indigenous organisms. Although test procedures and test organism selection criteria were developed to minimize these interferences, this section describes the nature of these interferences.

4.1.3 Because of the heterogeneity of natural sediments, extrapolation from laboratory studies to the field can sometimes be difficult (Table 4.1; Burton, 1991). Sediment collection, handling, and storage may alter bioavailability and concentration by changing the physical, chemical, or biological characteristics of the sediment. Maintaining the integrity of a field-collected sediment during removal, transport, mixing, storage, and testing is extremely difficult and may complicate the interpretation of effects. Direct comparisons of organisms exposed in the laboratory and in the field would be useful to verify laboratory results. However, spiked sediment may not be representative of contaminated sediment in the field. Mixing time (Stemmer et al., 1990a) and aging (Word et al., 1987; Landrum, 1989; Landrum and Faust, 1992) of spiked sediment can affect responses of organisms.

4.1.3.1 Laboratory sediment testing with field-collected sediments may be useful in estimating cumulative effects and interactions of multiple contaminants in a sample. Tests with field samples usually cannot discriminate between effects of individual chemicals. Most sediment samples contain a complex matrix of inorganic and organic contaminants with many unidentified compounds. The use of Toxicity Identification Evaluations (TIE) in conjunction with sediment tests with spiked chemicals may provide evidence of causal relationships and can be applied to many chemicals of concern (Ankley and Thomas, 1992; Adams et al., 1985). Sedi-

Table 4.1 Advantages and Disadvantages for Use of Sediment Tests[1]

Advantages
• Measure bioavailable fraction of contaminant(s).
• Provide a direct measure of benthic effects, assuming no field adaptation or amelioration of effects.
• Limited special equipment is required.
• Methods are rapid and inexpensive.
• Legal and scientific precedence exists for use; ASTM standard guides are available.
• Measure unique information relative to chemical analyses or benthic community analyses.
• Tests with spiked chemicals provide data on cause-effect relationships.
• Sediment-toxicity tests can be applied to all chemicals of concern.
• Tests applied to field samples reflect cumulative effects of contaminants and contaminant interactions.
• Toxicity tests are amenable to confirmation with natural benthos populations.
Disadvantages
• Sediment collection, handling, and storage may alter bioavailability.
• Spiked sediment may not be representative of field contaminated sediment.
• Natural geochemical characteristics of sediment may affect the response of test organisms.
• Indigenous animals may be present in field-collected sediments.
• Route of exposure may be uncertain and data generated in sediment toxicity tests may be difficult to interpret if factors controlling the bioavailability of contaminants in sediment are unknown.
• Tests applied to field samples may not discriminate effects of individual chemicals.
• Few comparisons have been made of methods or species.
• Only a few chronic methods for measuring sublethal effects have been developed or extensively evaluated.
• Laboratory tests have inherent limitations in predicting ecological effects.

[1] Modified from Swartz (1989)

ment spiking can also be used to investigate additive, antagonistic, or synergistic effects of specific contaminant mixtures in a sediment sample (Swartz et al., 1988).

4.1.4 Methods that measure sublethal effects are either not available or have not been routinely used to evaluate sediment toxicity (Craig, 1984; Dillon and Gibson, 1986; Ingersoll and Nelson, 1990; Ingersoll, 1991; Burton et al., 1992). Most assessments of contaminated sediment rely on short-term-lethality testing methods (e.g., ≤10 d; USEPA-USCOE, 1977; USEPA-USCOE, 1991). Short-term-lethality tests are useful in identifying "hot spots" of sediment contamination but may not be sensitive enough to evaluate moderately contaminated areas. However, sediment quality assessments using sublethal responses of benthic organisms such as effects on growth and reproduction have been used to successfully evaluate moderately contaminated areas (Scott, 1989). Additional methods development of chronic sediment testing procedures and culturing of infaunal organisms with a variety of feeding habits including suspension and deposit feeders is needed.

4.1.5 Despite the interferences discussed in this section, existing sediment testing methods can be used to provide a rapid and direct measure of effects of contaminants on benthic communities. Laboratory tests with field-collected sediment can also be used to determine temporal, horizontal, or vertical distribution of contaminants in sediment. Most tests can be completed within two to four weeks. Legal and scientific precedents exist for use of toxicity and bioaccumulation tests in regulatory decision-making (e.g., USEPA, 1986a). Furthermore, sediment tests with complex contaminant mixtures are important tools for making decisions about the extent of remedial action for contaminated aquatic sites and for evaluating the success of remediation activities.

4.2 Non-Contaminant Factors

4.2.1 Results of sediment tests can be used to predict effects that may occur with aquatic organisms in the field as a result of exposure under comparable conditions. Yet motile organisms might avoid exposure in the field. Photoinduced toxicity caused by ultraviolet (UV) light, may be important for some compounds associated with sediment (e.g., polycyclic aromatic hydrocarbons (PAHs); Davenport and Spacie, 1991; Ankley et al., 1994b). Fluorescent light does not contain UV light, but natural sunlight does. Lighting can therefore affect toxicological responses and is an important experimental variable for photoactivated chemicals. However, lighting typically used to conduct laboratory tests does not include the appropriate spectrum of ultraviolet radiation to photoactivate compounds (Oris and Giesy, 1985), and thus laboratory tests may not account for toxicity expressed by this mode of action.

4.2.2 Natural geomorphological and physicochemical characteristics such as sediment texture may influence the response of test organisms (DeWitt et al., 1988). The physicochemical characteristics of test sediment need to be within the tolerance limits of the test organism. Ideally, the limits of the test organism should be determined in advance; however, control samples reflecting differences in factors such as grain size and organic carbon can be evaluated if the limits are exceeded in the test sediment (Section 10.1). The effects of sediment characteristics can also be addressed with regression equations (DeWitt et al., 1988; Ankley et al., 1994a). The use of formulated sediment can also be used to evaluate physicochemical characteristics of sediment on test organisms (Walsh et al., 1991; Suedel and Rodgers, 1994).

4.2.3 Interferences of tests with each specific species are described in Tables 11.3, 12.3, and 13.4.

4.3 Changes in Bioavailability

4.3.1 Sediment toxicity tests are meant to serve as an indicator of contaminant-related toxicity that might be expected under field or natural conditions. Although the tests are not designed to simulate natural conditions, in some cases contaminant availability in laboratory toxicity test may be different from what it is representative of in-place sediments in the field.

4.3.2 Sediment collection, handling, and storage may alter contaminant bioavailability and concentration by changing the physical, chemical, or biological characteristics of the sediment. These manipulation processes are generally thought to increase availability of organic compounds because of disruption of the equilibrium with organic carbon in the pore water/particle system. Similarly, oxidation of anaerobic sediments increases the availability of certain metals (Di Toro et al., 1990). Because the availability of contaminants may be a function of the degree of manipulation, this manual recommends that handling, storage, and preparation of the sediment for actual testings be as consistent as possible. If sieving is performed, it is done primarily to remove predatory organisms and large debris. This manipulation most likely results in a worst-case condition of heightened bioavailability yet eliminates predation as a factor that might confound test results. When sediments are sieved, it may be desirable to take samples before and after sieving (e.g., pore-water metals or DOC, AVS, TOC) to document the influence of sieving on sediment chemistry. USEPA does not recommend sieving sediments on a routine basis.

4.3.3 Testing sediments at temperatures different from the field might affect contaminant solubility, partitioning coefficients, or other physical and chemical characteristics. Interaction between sediment and overlying water and the ratio of sediment to overlying water may influence bioavailability (Stemmer et al., 1990b).

4.3.4 The addition of food, water, or solvents to the test chambers might obscure the bioavailability of contaminants in sediment or might provide a substrate for bacterial or fungal growth. Without addition of food, the test organisms may starve during exposures (Ankley et al., 1994). However, the addition of food may alter the availability of the contaminants in the sediment (Wiederholm et al., 1987, Harkey et al., 1994) depending on the amount of food added, its composition (e.g., TOC), and the chemical(s) of interest.

4.3.5 Depletion of aqueous and sediment-sorbed contaminants resulting from uptake by an organism or test chamber may also influence availability. In most cases, the organism is a minor sink for contaminants relative to the sediment. However, within the burrow of an organism, sediment desorption kinetics may limit uptake rates. Within minutes to hours, a major portion of the total chemical may be inaccessible to the organisms because of depletion of available residues. The desorption of a particular compound from sediment may range from easily reversible (labile; within minutes) to irreversible (non-labile; within days or months; Karickhoff and Morris, 1985). Interparticle diffusion or advection and the quality and quantity of sediment organic carbon can also affect sorption kinetics.

4.3.6 The route of exposure may be uncertain, and data from sediment tests may be difficult to interpret if factors controlling the bioavailability of contaminants in sediment are unknown. Bulk-sediment chemical concentrations may be normalized to factors other than dry weight. For example, concentrations of nonionic organic compounds might be normalized to sediment organic-carbon content (USEPA, 1992c) and certain metals normalized to acid volatile sulfides (Di Toro et al., 1990). Even with the appropriate normalizing factors, determination of toxic effects from ingestion of sediment or from dissolved chemicals in the interstitial water can still be difficult (Lamberson and Swartz, 1988).

4.4 Presence of Indigenous Organisms

4.4.1 Indigenous organisms may be present in field-collected sediments. An abundance of the same organism or organisms taxonomically similar to the test organism in the sediment sample may make interpretation of treatment effects difficult. For example, growth of amphipods, midges, or mayflies may be reduced if high numbers of oligochaetes are in a sediment sample (Reynoldson et al., 1994). Previous investigators have inhibited the biological activity of sediment with sieving, heat, mercuric chloride, antibiotics, or gamma irradiation (ASTM, 1994b; K.E. Day Environment Canada, Burlington, Ontario, personal communication). However, further research is needed to determine effects on contaminant bioavailability or other modifications of sediments from treatments such as those used to remove or destroy indigenous organisms.

Section 5
Health, Safety, and Waste Management

5.1 General Precautions

5.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management and includes (1) the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal written health and safety plan, which is provided to each laboratory staff member, (3) an ongoing training program on laboratory safety, and (4) regular safety inspections.

5.1.2 This manual addresses procedures that may involve hazardous materials, operations, and equipment, and it does not purport to address all of the safety problems associated with their use. It is the responsibility of the user to establish appropriate safety and health practices, and determine the applicability of regulatory limitations before use. While some safety considerations are included in this manual, it is beyond the scope of this manual to encompass all safety requirements necessary to conduct sediment tests.

5.1.3 Collection and use of sediments may involve substantial risks to personal safety and health. Contaminants in field-collected sediment may include carcinogens, mutagens, and other potentially toxic compounds. Inasmuch as sediment testing is often begun before chemical analyses can be completed, worker contact with sediment needs to be minimized by (1) using gloves, laboratory coats, safety glasses, face shields, and respirators as appropriate, (2) manipulating sediments under a ventilated hood or in an enclosed glove box, and (3) enclosing and ventilating the exposure system. Personnel collecting sediment samples and conducting tests should take all safety precautions necessary for the prevention of bodily injury and illness that might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation because of lack of oxygen or presence of noxious gases.

5.1.4 Before sample collection and laboratory work, personnel should determine that all required safety equipment and materials have been obtained and are in good condition.

5.2 Safety Equipment

5.2.1 Personal Safety Gear

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

5.2.2 Laboratory Safety Equipment

5.2.2.1 Each laboratory should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

5.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

5.3 General Laboratory and Field Operations

5.3.1 Special handling and precautionary guidance in Material Safety Data Sheets should be followed for reagents and other chemicals purchased from supply houses.

5.3.2 Work with some sediments may require compliance with rules pertaining to the handling of hazardous materials. Personnel collecting samples and performing tests should not work alone.

5.3.3 It is advisable to wash exposed parts of the body with soap and water immediately after collecting or manipulating sediment samples.

5.3.4 Strong acids and volatile organic solvents should be used in a fume hood or under an exhaust canopy over the work area.

5.3.5 An acidic solution should not be mixed with a hypochlorite solution because hazardous vapors might be produced.

5.3.6 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.

5.3.7 Use of ground-fault systems and leak detectors is strongly recommended to help prevent electrical shocks. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories should not be used. Ground-fault interrupters should be installed in all "wet" laboratories where electrical equipment is used.

5.3.8 All containers should be adequately labeled to identify their contents.

5.3.9 Good housekeeping contributes to safety and reliable results.

5.4 Disease Prevention

5.4.1 Personnel handling samples that are known or suspected to contain human wastes should be given the opportunity to be immunized against hepatitis B, tetanus, typhoid fever, and polio.

5.5 Safety Manuals

5.5.1 For further guidance on safe practices when handling sediment samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals including USEPA (1986b) and Walters and Jameson (1984).

5.6 Pollution Prevention, Waste Management, and Sample Disposal

5.6.1 It is the laboratory's responsibility to comply with the federal, state, and local regulations governing the waste management, particularly hazardous waste identification rules and land disposal restrictions, and to protect the air, water and land by minimizing and controlling all releases from fume hoods and bench operations. Also, compliance is required with any sewage discharge permits and regulations. For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

5.6.2 Guidelines for the handling and disposal of hazardous materials should be strictly followed. The federal government has published regulations for the management of hazardous waste and has given the states the option of either adopting those regulations or developing their own. If states develop their own regulations, they are required to be at least as stringent as the federal regulations. As a handler of hazardous materials, it is your responsibility to know and comply with the pertinent regulations applicable in the state in which you are operating. Refer to The Bureau of National Affairs Inc., (1986) for the citations of the federal requirements.

Section 6
Facilities, Equipment, and Supplies

6.1 General

6.1.1 Before a sediment test is conducted in any test facility, it is desirable to conduct a "non-toxicant" test with each potential test species, in which all test chambers contain a control sediment (sometimes called the negative control), and clean overlying water for each organism to be tested. Survival, growth, or reproduction of the test organism will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable species-specific control numbers. Evaluations may also be made on the magnitude of between-chamber variance in a test.

6.2 Facilities

6.2.1 The facility must include separate areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding and culture chambers should not be in a room in which sediment tests are conducted, where stock solutions or sediments are prepared, or equipment is cleaned. Test chambers may be placed in a temperature-controlled recirculating water bath or a constant-temperature area. An enclosed test system is desirable to provide ventilation during tests to limit exposure of laboratory personnel to volatile substances.

6.2.2 Light of the quality and luminance normally obtained in the laboratory is adequate (about 500 to 1000 lux using wide-spectrum fluorescent lights; e.g., cool-white or daylight) for culturing and testing. Lux is the unit selected for reporting luminance in this manual. Multiply units of lux by 0.093 to convert to units of foot candles. Multiply units of lux by 6.91×10^{-3} to convert to units of $\mu E/m^2/s^1$ (assuming an average wavelength of 550 nm ($\mu mol^{-2}\ s^{-1} = W\ m \times \lambda(nm) \times 8.36 \times 10^{-3}$) (ASTM, 1994c). Luminance should be measured at the surface of the water. A uniform photoperiod of 16L:8D can be achieved in the laboratory or in an environmental chamber using automatic timers.

6.2.3 During phases of rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light or pedestrian traffic.

6.2.4 The test facility should be well ventilated and free of fumes. Air used for aeration should be free of oil and fumes. Filters to remove oil, water, and bacteria are desirable. Oil-free air pumps should be used where possible. Particulates can be removed from the air using filters such as BALSTON® Grade BX or equivalent (Balston, Inc., Lexington, MA), and oil and other organic vapors can be removed using activated carbon filters (e.g., BALSTON®, C-1 filter, or equivalent). Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories or sample handling areas is not circulated to culture or testing rooms, or that air from testing rooms does not contaminate culture rooms. Air pressure differentials between rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely fitting doors.

6.3 Equipment and Supplies

6.3.1 Equipment and supplies that contact stock solutions, sediments, or overlying water should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and supplies 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 high-density plastic containers may be used for holding and culture chambers, and in the water-supply system. These materials should be washed in detergent, acid rinsed, and soaked in flowing water for a week or more before use. Cast-iron pipe should not be used in water-supply systems 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 a 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.

6.3.2 New lots of plastic products should be tested for toxicity by exposing organisms to them under ordinary test conditions before general use.

6.3.3 General Equipment

6.3.3.1 Environmental chamber or equivalent facility with photoperiod and temperature control (20 to 25°C).

6.3.3.2 Water purification system capable of producing at least 1 mega-ohm water (USEPA, 1993a).

6.3.3.3 Analytical balance capable of accurately weighing to 0.01 mg.

6.3.3.4 Reference weights, Class S—for documenting the performance of the analytical balance(s). The balance(s) should be checked with reference weights that are at the upper and lower ends of the range of the weighings made when the balance is used. A balance should be checked at the beginning of each series of weighings, periodically (such as every tenth weight) during a long series of weighings, and after taking the last weight of a series.

6.3.3.5 Volumetric flasks and graduated cylinders—Class A, borosilicate glass or nontoxic plastic labware, 10 to 1000 mL for making test solutions.

6.3.3.6 Volumetric pipets—Class A, 1 to 100 mL.

6.3.3.7 Serological pipets—1 to 10 mL, graduated.

6.3.3.8 Pipet bulbs and fillers—PROPIPET® or equivalent.

6.3.3.9 Droppers, and glass tubing with fire polished edges, 4 to 6 mm ID—for transferring test organisms.

6.3.3.10 Wash bottles—for rinsing small glassware, instrument electrodes and probes.

6.3.3.11 Glass or electronic thermometers—for measuring water temperature.

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

6.3.3.13 Dissolved oxygen (DO), pH/selective ion, and specific conductivity meters and probes for routine physical and chemical measurements are needed. Unless a test is being conducted to specifically measure the effect of DO or conductivity, a portable field-grade instrument is acceptable.

6.3.3.14 See Table 6.1 for a list of additional equipment and supplies.

6.3.4 Water-delivery System

6.3.4.1 The water-delivery system used in water-renewal testing can be one of several designs. The system should be capable of delivering water to each replicate test chamber. Mount and Brungs (1967) diluters have been successfully modified for sediment testing. Other diluter systems have also been useful (Ingersoll and Nelson, 1990; Maki, 1977; Benoit et al., 1993; Zumwalt et al., 1994). The water-delivery system should be calibrated before the test by determining the flow rate of the overlying water. The general operation of the system should be visually checked daily throughout the length 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%.

6.3.4.2 The overlying water can be replaced manually (e.g., siphoning); however, manual systems take more time to maintain during a test. In addition, automated systems generally result in less suspension of sediment compared to manual renewal.

6.3.5 Test Chambers

6.3.5.1 Test chambers may be constructed in several ways and of various materials, depending on the experimental design and the contaminants of interest. Clear silicone adhesives, suitable for aquaria, sorb some organic compounds that might be difficult to remove. Therefore, as little adhesive as possible should be in contact with the test material. Extra beads of adhesive 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 held at least 48 h in overlying water before use in a test.

6.3.5.2 Test chambers for specific tests are described in Sections 11, 12, and 13.

6.3.6 Cleaning

6.3.6.1 All non-disposable sample containers, test chambers, and other equipment that have come in contact with sediment should be washed after use in the manner described below to remove surface contaminants.

1. Soak 15 min in tap water, and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

Table 6.1 Equipment and Supplies for Culturing and Testing Specific Test Organisms

A. Biological Supplies

Brood stock of test organisms
Active dry yeast (HA)
Cerophyl® (dried cereal leaves; HA)
Trout food pellets (HA)
Tetrafin® goldfish food (CT)
Trout starter (LV)
Helisoma sp. snails (optional; LV)
Algae (e.g., *Selenastrum capricornutum, Chlorella;* CT)
Diatoms (e.g., *Navicula* sp; HA)

B. Glassware

Culture chambers
Test chambers (300-mL high-form lipless beaker; HA and CT)
Test chambers (15.8- x 29.3- x 11.7-cm, W x L x H; LV)
Juvenile holding beakers (e.g., 1 L; HA)
Crystallizing dishes or beakers (200- to 300-mL; CT)
Erlenmeyer flasks (250 and 500 mL; CT)
Larval rearing chambers (e.g., 19-L capacity; CT)
1/4" glass tubing (for aspirating flask; CT)
Glass bowls (20-cm diameter; LV)
Glass vials (10 mL; LV)
Wide-bore pipets (4 to 6 mm ID)
Glass disposable pipets
Burettes (for hardness and alkalinity determinations)
Graduated cylinders (assorted sizes, 10 mL to 2 L)

C. Instruments and Equipment

Dissecting microscope
Stainless-steel sieves (e.g., U.S. Standard No. 25, 30 35, 40, 50 mesh)
Delivery system for overlying water (See Appendix B for a listing of equipment needed for water delivery systems)
Photoperiod timers
Light meter
Temperature controllers
Thermometer
Continuous recording thermometers
Dissolved oxygen meter
pH meter
Ion-specific meter
Ammonia electrode (or ammonia test kit)
Specific-conductance meter
Drying oven
Desiccator
Balance (0.01 mg sensitivity)

C. Instruments and Equipment

Blender
Refrigerator
Freezer
Light box
Hemacytometer (HA)
Paper shredder, cutter, or scissors (CT, LV)
Tissue homogenizer (LV)
Electric drill with stainless steel auger (diameter 7.6 cm, overall length 38 cm, auger bit length 25.4 cm (Section 8.3)

D. Miscellaneous

Ventilation system for test chambers
Air supply and airstones (oil free and regulated)
Cotton surgical gauze or cheese cloth (HA)
Stainless-steel screen (no. 60 mesh, for test chambers)
Glass hole-cutting bits
Silicon adhesive caulking
Plastic mesh (110 µm mesh opening; Nytex® 110; HA)
Aluminum-weighing pans
Fluorescent-light bulbs
Nalgene bottles (500 mL and 1000 mL for food preparation and storage)
Deionized water
Airline tubing
White plastic dish pan
"Coiled-web material" (3-M, St. Paul, MN; HA)
White paper toweling (for substrate; CT)
Brown paper toweling (for substrate; LV)
Screening material (e.g., Nitex® (110 mesh), window screen, or panty hose; CT)
Water squirt bottle
Dissecting probes (LV)
Dental picks (LV)
Shallow pans (plastic (light-colored), glass, stainless steel)

E. Chemicals

Detergent (non-phosphate)
Acetone (reagent grade)
Hexane (reagent grade)
Hydrochloric acid (reagent grade)
Chloroform (LV)
Methanol (LV)
Copper Sulfate
Potassium Chloride
Reagents for reconstituting water
Formalin (or Notox®)
Sucrose

HA = *Hyalella azteca*
CT = *Chironomus tentans*
LV = *Lumbriculus variegatus*

6.3.6.2 All test chambers and equipment should be thoroughly rinsed with the dilution water immediately before use in a test.

6.3.6.3 Many organic solvents leave a film that is insoluble in water. A dichromate-sulfuric acid cleaning solution can be used in place of both the organic solvent and the acid (see ASTM, 1988a), but the solution might attack silicone adhesive and leave chromium residues on glass. A alternative to use of dichromate-sulfuric acid could be to heat glassware for 8 h at 450°C.

Section 7
Water, Formulated Sediment, Reagents, and Standards

7.1 Water

7.1.1 Requirements

7.1.1.1 Water used to test and culture organisms should be uniform in quality. Acceptable water should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (e.g., discoloration, unusual behavior). If problems are observed in the culturing or testing of organisms, it is desirable to evaluate the characteristics of the water. See USEPA (1993a) and ASTM (1994a) for a recommended list of chemical analyses of the water supply.

7.1.2 Source

7.1.2.1 A natural water is considered to be of uniform quality if monthly ranges of the hardness, alkalinity, and specific conductance are less than 10% of their respective averages and if the monthly range of pH is less than 0.4. Natural waters should be obtained from an uncontaminated well or spring, if possible, or from a surface-water source. If surface water is used, the intake should be positioned to (1) minimize fluctuations in quality and contamination, (2) maximize the concentration of dissolved oxygen, and (3) ensure low concentrations of sulfide and iron. Municipal-water supplies may be variable and may contain unacceptably high concentrations of materials such as copper, lead, zinc, fluoride, chlorine, or chloramines. Chlorinated water should not be used for culturing or testing because residual chlorine and chlorine-produced oxidants are toxic to many aquatic organisms. Use of tap water is discouraged unless it is dechlorinated and passed through a deionizer and carbon filter (USEPA, 1993a).

7.1.2.2 For site-specific investigations, it is desirable to have the water-quality characteristics of the overlying water as similar as possible to the site water. For certain applications the experimental design might require use of water from the site where sediment is collected.

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

7.1.2.4 Water might need aeration using air stones, surface aerators, or column aerators. 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. Excessive aeration may reduce hardness and alkalinity of hard water (e.g., 280 mg/L hardness as $CaCO_3$; E.L. Brunson, NBS, Columbia, MO, personal communication). The concentration of dissolved oxygen in source water should be between 90 to 100% saturation to help ensure that dissolved oxygen concentrations are acceptable in test chambers.

7.1.3 Reconstituted Water

7.1.3.1 Ideally, reconstituted water should be prepared by adding specified amounts of reagent-grade chemicals to high-purity distilled or deionized water (ASTM, 1988a; USEPA, 1993a). In some applications, acceptable high-purity water can be prepared using deionization, distillation, or reverse-osmosis units (Section 6.3.3.2; USEPA, 1993a). In some applications, test water can be prepared by diluting natural water with deionized water (Kemble et al., 1993).

7.1.3.2 Deionized water should be obtained from a system capable of producing at least 1 mega-ohm water. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a mixed-bed water treatment system.

7.1.3.3 Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured on each batch of reconstituted water. The reconstituted water should be aerated before use to adjust pH and dissolved oxygen to the acceptable ranges (e.g., Section 7.1.3.4.1). USEPA (1993a) recommends using a batch of reconstituted water for two weeks.

7.1.3.4 Reconstituted Fresh Water

7.1.3.4.1 To prepare 100 L of reconstituted fresh water, use the reagent grade chemicals as follows:

1. Place about 75 L of deionized water in a properly cleaned container.

2. Add 5 g of $CaSO_4$ and 5 g of $CaCl_2$ to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 min or until the salts dissolve.

3. Add 3 g of $MgSO_4$, 9.6 g $NaHCO_3$, and 0.4 g KCl to a second 2-L aliquot of deionized water and mix on a stir plate for 30 min.

4. Pour the two 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.

5. Aerate the mixture for at least 24 h before use.

6. The water quality of the reconstituted water should be approximately the following: hardness, 90 to 100 mg/L as $CaCO_3$, alkalinity 50 to 70 mg/L as $CaCO_3$, conductivity 330 to 360 µS/cm, and pH 7.8 to 8.2.

7.1.3.4.2 This reconstituted fresh water was developed by USEPA EMSL-Cincinnati (J.M. Lazorchak, USEPA, Cincinnati, OH, personal communication) and has been used successfully in round-robin testing with *H. azteca, C. tentans,* and *C. riparius* (Section 15). This reconstituted water has a higher proportion of chloride to sulfate compared to the reconstituted waters described in ASTM (1988a) and USEPA (1993a). Variable success has been reported using USEPA or ASTM reconstituted waters (USEPA, 1993a) with *H. azteca.* Research is ongoing to develop additional types of reconstituted waters suitable for these test organisms.

7.1.3.5 Synthetic Seawater

7.1.3.5.1 Reconstituted salt water can be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX®, INSTANT OCEAN®, or equivalent to deionized water.

7.1.3.5.2 A synthetic seawater formulation called GP2 is prepared with reagent grade chemicals that can be diluted with deionized water to the desired salinity (USEPA, 1994c).

7.1.3.5.3 Ingersoll et al. (1992) describe procedures for culturing *H. azteca* at salinities up to 15 ‰. Reconstituted salt water was prepared by adding INSTANT OCEAN® salts to a 25:75 (v/v) mixture of freshwater (hardness 283 mg/L as $CaCO_3$) and deionized water that was held at least two weeks before use. Synthetic seawater was conditioned by adding 6.2 mL of Frit-zyme® #9 nitrifying bacteria (*Nitromonas* sp. and *Nitrobacter* sp.; Fritz Chemical Company, Dallas, TX) to each liter of water. The cultures were maintained by using static renewal procedures; 25% of the culture water was replaced weekly. *Hyalella azteca* have been used to evaluate the toxicity of estuarine sediments up to 15 ‰ salinity (Nebeker and Miller, 1988; Roach et al., 1992; Winger et al., 1993).

7.2 Formulated Sediment

7.2.1 General Requirements

7.2.1.1 Formulated sediments are mixtures of materials that mimic natural sediments. Formulated sediments have not been routinely applied to evaluate sediment contamination. A primary use of formulated sediment could be as a control sediment. Formulated sediments allow for standardization of sediment testing or as a basis for conducting sediment research. Formulated sediment provides a basis by which any testing program can assess the acceptability of their procedures and facilities. In addition, formulated sediment provides a consistent measure evaluating performance-based criteria necessary for test acceptability. The use of formulated sediment eliminates interferences caused by the presence of indigenous organisms. For toxicity tests with sediments spiked with specific chemicals, the use of a formulated sediment eliminates or controls the variation in sediment physico-chemical characteristics and provides a consistent method for evaluating the fate of chemicals in sediment. However, additional research is needed before formulated sediments are used routinely for sediment spiking procedures (e.g., identifying standardized and representative sources of organic carbon).

7.2.1.2 A formulated sediment should (1) support the survival, growth, or reproduction of a variety of benthic invertebrates, (2) provide consistent acceptable biological endpoints for a variety of species, and (3) the materials used in formulation of the sediment should have consistent characteristics. Consistent material characteristics include (1) consistency of materials from batch to batch, (2) contaminant concentrations below concentrations of concern, and (3) availability to all individuals and facilities.

7.2.1.3 Physico-chemical characteristics that might be considered when evaluating the appropriateness of a formulated sediment include percent sand, percent clay, percent silt, organic carbon content, cation exchange capacity (CEC), oxidation reduction potential (redox), pH, and carbon:nitrogen:phosphorus ratios.

7.2.2 Sources of Materials

7.2.2.1 A variety of methods describe procedures for making formulated sediments. These procedures often use similar constituents; however, they often include either a component or a formulation step that would result in variation from test facility to test facility. In addition, none of the procedures have been subjected to standardization and consensus approval or round-robin (ring) testing.

7.2.2.2 Most formulated sediments include sand and clay/silt that meet certain specifications; however, some may be quite different. For example, three sources of clay and silt include Attagel® 50, ASP® 400, and ASP®

400P. Table 7.1 summarizes the characteristics of these materials. The percentage of clay ranges from 56.5 to 88.5 and silt ranges from 11.5 to 43.5. These characteristics should be evaluated when considering the materials to use in a formulated sediment.

Table 7.1 Characteristics of Three Sources of Clays and Silts Used in Formulated Sediments

Characteristic	Attagel® 50	ASP® 400	ASP® 400P
% Sand	0.0	0.01	0.0
% Clay	88.50	68.49	56.50
% Silt	11.50	31.50	43.50
Soil class	Clay	Clay	Silty clay

Note: Table 7.3 is list of suppliers.

7.2.2.3 A critical component of formulated sediment is the source of organic carbon. Many procedures have used peat as the source of organic carbon. Other sources of organic carbon listed in Table 7.2 have been evaluated including humus, potting soil, maple leaves, composted cow manure, rabbit chow, cereal leaves, chlorella, trout chow, Tetramin® and Tetrafin®. Only peat, humus, potting soil, and composted cow manure have been used successfully without fouling the overlying water. The other sources of organic carbon (Table 7.2) cause dissolved oxygen concentrations to fall to unacceptable levels (F.J. Dwyer, NBS, Columbia, MO, personal communication). If appropriate conditioning procedures can be determined these other sources of organic carbon may be acceptable. An important consideration in the selection of an organic carbon source is the ratio of carbon:nitrogen:phosphorus. As demonstrated in Table 7.2, percentage carbon ranged from 30 to 47, nitrogen ranged from 3 to 45 mg/g, and phosphorus ranged from below detection to 11 µg/g for several different carbon sources. These characteristics should be evaluated when considering the materials to use in a formulated sediment.

Table 7.2. Carbon, Nitrogen, Phosphorus Levels for Various Sources of Organic Carbon[1]

Organic carbon Source	Carbon (%)	Nitrogen (mg/g)	Phosphorus (µg/g)
Peat	47	4	0.4
Maple leaves 1	42	6	1.3
Maple leaves 2	47	3	1.7
Cow manure	30	11	8.2
Rabbit chow	40	18	0.2
Humic acid	40	3	—
Cereal leaves	47	4	0.4
Chlorella	40	41	5.7
Trout chow	43	36	11
Tetramin®	37	45	9.6
Tetrafin®	36	29	8.6

[1] F.J. Dwyer, NBS, Columbia, MO, personal communication

7.2.3 Procedure

7.2.3.1 A summary of procedures that have been used to formulate sediment are listed below. Suppliers of various components are listed in Table 7.3.

1. Walsh et al. (1981): (1) Wash sand (Mystic White No 85, 45, and 18—New England Silica Inc) and sieve into three grain sizes: coarse (500 to 1500 µm); medium (250 to 499 µm); and fine (63 to 249 µm). (2) Clay and silt were obtained from Engelhard Corp.; (3) Peat moss is milled and sieved through an 840 µm screen. (4) Constituents are mixed dry in the following quantities: coarse sand (0.6%); medium sand (8.7%); fine sand (69.2%); silt (10.2%); clay (6.4%); and organic matter (4.9%).

2. Clements, W.H. (Colorado State University, Ft. Collins, CO, personal communication): (1) Rinse peat moss then soak for 5 d in deionized water renewing water daily. (2) After acclimation for 5 d remove all water and spread out to dry. (3) Grind moss and sieve using the following sieve sizes: 1.18 mm (discard these particles); 1.00 mm (average size 1.09 mm); 0.85 mm (average size 0.925); 0.60 (average size 0.725); 0.425 mm (average size 0.5125 mm); retainer (average size 0.2125 mm). (4) Use a mixture of sizes that provides an average particle size of 840 µm. (5) Wash sand (Mystic white #45) and dry. (6) Clay and silt are obtained using ASP 400 (Englehard Corp). (7) Constituents are mixed dry in the following quantities: sand (1242 g); silt and clay (219 g); dolomite (7.5 g); peat moss (31.5 g); and humic acid (0.15 g). (8) Sediment is mixed for an hour on a rolling mill and stored dry until ready for use.

3. Hanes et al. (1991): (1) Sieve sand and retain two particle sizes (90 to 180 um and 180 to 250 um) which are mixed in a ratio of 2:1. (2) Potting soil is dried for 24 h at room temperature and sieved through a 1-mm screen. Clay is commercially available sculptors clay. (3) Determine percent moisture of clay and soil after drying for 24 h at 60 to 100°C. (correct for percent moisture when mixing materials). (4) Constituents are mixed by weight in the following ratios: sand mixture (42%); clay (42%); and soil (16%). (5) After mixing, autoclave in a foil-covered container for 20 min. (6) Mixture can be stored indefinitely if kept covered after autoclaving.

4. Naylor (1993): (1) Sand is acid-washed and sieved to obtain a 40 to 100 mm size. (2) Clay is kaolin light. (3) Peat moss is ground and sieved using a 2-mm screen (peat moss which is allowed to dry out will not rehydrate and will float on the water surface). (4) Adjust for the use of moist peat moss by determining moisture content (dry 5 samples of peat at 60°C until constant weight is achieved). (5) Constituents are mixed by weight in the following percentages: sand (69%); kaolin (20%); peat (10% [adjust for moisture

Table 7.3 Sources of Components Used in Formulated Sediments

Component	Sources
Sand	• Mystic White #18, #45, #85, #90—New England Silica, Inc., South Windsor, CT
	• Product No. 33094, BDH Chemical, Ltd., Poole, England
Kaolinite	• ASP 400, ASP 400P, ASP 600, ASP 900—Englehard Corporation, Edison, NJ
	• Product No. 33059, BDH Chemical, Ltd., Poole, England
Montmorillonite	• W.D. Johns, Source Clays, University of Missouri, Columbia, MO
Clay	• Lewiscraft Sculptor's Clay, available in hobby and artist supply stores
Humus	• Sims Bark Co., Inc., Tuscumbia, AL
Peat	• D.L. Browning Co., Mather, WI
	• Joseph Bentley, Ltd., Barrow-on-Humber, South Humberside, England
	• Mellinger's, North Lima, OH
Potting soil	• Zehr's No Name Potting Soil, Mississauga, Ontario
Humic acid	• Aldrich Chemical Co, Milwaukee, WI
Cow manure	• A.H. Hoffman, Inc., Landisville, PA
Dolomite	• Ward's Natural Science Establishment, Inc., Rochester, NY
	• Southern Agri-minerals Corp., Hartford, AL

content]); and $CaCO_3$ (1%). (6) Mix for 2 h in a soil shaker and store in sealed containers.

5. Suedel and Rodgers (1994): (1) Sand (Mystic White #18 and 90) is sieved to provide three different size fractions: coarse (2.0 to 0.5 mm), medium (0.5 to 0.25 mm) and fine (0.25 to 0.05 mm). (2) Silt (ASP 400), clay (ASP 600 and 900), montmorillonite clay, and dolomite are ashed at 550°C. for 1 h to remove organic matter. (3) Humus is dried (70°C) and milled to 2.0 mm. (4) Dolomite is added as 1% of the silt requirement. (5) Materials are aged for 7 d in flowing water before mixing. (6) Constituents are mixed to mimic the desired characteristics of the sediment of concern.

7.2.3.2 The procedure for formulating a sediment is a combination of methods outlined in Section 7.2.3.1. The characteristics of this formulation would be sand 77%, silt/clay 17%. The organic matter would depend on the source of organic carbon. This approach could be modified to mimic specific characteristics of a sediment. If a formulated sediment is to be used as a control sediment, the physico-chemical characteristics of the formulated sediment should be within the tolerance limits of the test organism.

1. Wash sand, sieve, and retain the following two size groups: medium (0.5 to 0.25 mm) and fine (0.25 to 0.05 mm). Sand should be mixed at a ratio of 2:1, fine:medium.

2. Clay and silt fractions are obtained using ASP® 400. Other clays or silts (e.g., Attagel® 50, ASP® 400P, ASP® 600, ASP® 900, montmorillonite) might be used if specific characteristics are required.

3. Organic matter (peat, humus, cow manure) should be dried, milled, and passed through a 0.84 mm sieve.

4. Either $CaMg(CO_3)_2$ or $CaCO_3$ should be added to buffer the sediments.

5. All constituents are mixed on a percent dry weight basis. Mix in the following ratios: sand (77%); silt/clay (17%); organic matter (5%); buffer (1%).

7.3 Reagents

7.3.1 Data sheets should be followed for reagents and other chemicals purchased from supply houses. The test material(s) should be at least reagent grade, unless a test on formulation commercial product, technical-grade, or use-grade material is specifically needed. Reagent containers should be dated when received from the supplier, and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

7.4 Standards

7.4.1 Appropriate standard methods for chemical and physical analyses should be used when possible. For those measurements for which standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources.

Section 8
Sample Collection, Storage, Manipulation, and Characterization

8.1 Collection

8.1.1 Before the preparation or collection of sediment, a procedure should be established for the handling of sediments that might contain unknown quantities of toxic contaminants (Section 5).

8.1.2 Sediments are spatially and temporally variable (Stemmer et al., 1990a). Replicate samples should be collected to determine variance in sediment characteristics. Sediment should be collected with as little disruption as possible; however, subsampling, compositing, or homogenization of sediment samples may be necessary for some experimental designs. Sampling may cause loss of sediment integrity, change in chemical speciation, or disruption of chemical equilibrium (ASTM, 1994b). A benthic grab or core should be used rather than a dredge to minimize disruption of the sediment sample. Sediment should be collected from a depth that will represent expected exposure. For example, oligochaetes may burrow 4 to 15 cm into sediment. Samples collected for evaluations of dredged material should include all sediment to project depth. Surveys of the toxicity of surficial sediment are often based on cores of the upper 2 cm sediment depth.

8.1.3 Exposure to direct sunlight during collection should be minimized, especially if the sediment contains photolytic compounds. Sediment samples should be cooled to 4°C in the field before shipment (ASTM, 1994a). Dry ice can be used to cool samples in the field; however, sediments should never be frozen. Monitors can be used to measure temperature during shipping (e.g., TempTale Temperature Monitoring and Recording System, Sensitech, Inc., Beverly, MA).

8.1.4 For additional information on sediment collection and shipment see ASTM (1994b).

8.2 Storage

8.2.1 Manipulation or storage can alter bioavailability of contaminants in sediment (Burton and Ingersoll, 1994); however, the alterations that occur may not substantially affect toxicity. Storage of sediment samples for several months at 4°C did not result in significant changes in chemistry or toxicity (T. Dillon and H. Tatem, USCOE, Vicksburg, MS, personal communication; G.T. Ankley and D. DeFoe, USEPA, Duluth, MN, unpublished data); however, others have demonstrated changes in spiked sediment within days to weeks (e.g., Burton, 1991; Stemmer et al., 1990a). Sediments primarily contaminated with nonionic, nonvolatile organics will probably change little during storage because of their relative resistance to biodegradation and sorption to solids. However, metals and metalloids may be affected by changing redox, oxidation, or microbial metabolism (such as with arsenic, selenium, mercury, lead, and tin; all of which are methylated by a number of bacteria and fungi). Metal-contaminated sediments may need to be tested relatively soon after collection with as little manipulation as possible (Burton and Ingersoll, 1994).

8.2.2 Given that the contaminants of concern and the influencing sediment characteristics are not always known *a priori*, it is desirable to hold sediments in the dark at 4°C and start tests soon after collection from the field. Recommended sediment holding time ranges from less than two (ASTM, 1994a) to less than eight weeks (USEPA-USCOE, 1994). If whole-sediment tests are started after two weeks of collection, it may be desirable to conduct additional characterizations of sediment to evaluate possible effects of storage on sediment. For example, concentrations of contaminants of concern could be measured in pore water within two weeks from sediment collection and at the start of the sediment test (Kemble et al., 1993). Ingersoll et al. (1993) recommend conducting a toxicity test with pore water within two weeks from sediment collection and at the start of the sediment test. Freezing and longer storage might further change sediment properties such as grain size or contaminant partitioning and should be avoided (ASTM, 1994b; Schuytema et al., 1989; K.E. Day Environment Canada, Burlington, Ontario, personal communication). Sediment should be stored with no air over the sealed samples (no head space) at 4°C before the start of a test (Shuba et al., 1978; ASTM, 1994b). Sediment may be stored in containers constructed of suitable materials as outlined in Section 6. It is desirable to avoid contact with metals, including stainless steel and brass sieving screens, and some plastics.

8.3 Manipulation

8.3.1 Homogenization

8.3.1.1 Samples tend to settle during shipment. As a result, water above the sediment should not be discarded but should be mixed back into the sediment during homogenization. Sediment samples should not be sieved to remove indigenous organisms unless there is a good reason to believe indigenous organisms may influence the response of the test organism. However, large indigenous organisms and large debris can be removed using forceps. Reynoldson et al. (1994) observed reduced growth of amphipods, midges, and mayflies in sediments with elevated numbers of oligochaetes and recommended sieving sediments suspected to have high numbers of indigenous oligochaetes. If sediments must be sieved, it may be desirable to analyze samples before and after sieving (e.g., pore-water metals, DOC, AVS, TOC) to document the influence of sieving on sediment chemistry.

8.3.1.2 If sediment is collected from multiple field samples, the sediment can be pooled and mixed using stirring or a rolling mill, feed mixer, or other suitable apparatus (see ASTM, 1994b). Homogenization of sediment can be accomplished using a modified 30-cm bench-top drill press (Dayton Model 3Z993) or a variable-speed hand-held drill outfitted with a stainless-steel auger (diameter 7.6 cm, overall length 38 cm, auger bit length 25.4 cm; Part No. 800707, Augers Unlimited, Exton, PA; Kemble et al., 1994). These procedures could also be used to mix test sediment with a control sediment in dilution experiments.

8.3.2 Sediment Spiking

8.3.2.1 Test sediment can be prepared by manipulating the properties of a control sediment. Additional research is needed before formulated sediments are used routinely for sediment spiking procedures (e.g., identifying standardized and representative sources of organic carbon). Mixing time (Stemmer et al., 1990a) and aging (Word et al., 1987; Landrum, 1989; Landrum and Faust, 1992) of spiked sediment can affect responses. Many studies with spiked sediment are often started only a few days after the chemical has been added to the sediment. This short time period may not be long enough for sediments to equilibrate with the spiked chemicals. Consistent spiking procedures should be followed in order to make interlaboratory comparisons. It is recommended that spiked sediment be aged at least one month before starting a test; however equilibration for some chemicals may not be achieved for long periods of time.

8.3.2.1.1 The cause of sediment toxicity and the magnitude of interactive effects of contaminants can be estimated by spiking a sediment with chemicals or complex waste mixtures (Lamberson and Swartz, 1992). Sediments spiked with a range of concentrations can be used to generate either point estimates (e.g., LC50) or a minimum concentration at which effects are observed (lowest-observable-effect concentration; LOEC). Results of tests may be reported in terms of a BSAF (Ankley et al., 1992b). The influence of sediment physico-chemical characteristics on chemical toxicity can also be determined with sediment-spiking studies (Adams et al., 1985).

8.3.2.2 The test material(s) should be at least reagent grade, unless a test on formulation commercial product, technical-grade, or use-grade material is specifically needed. Before a test is started, the following should be known about the test material: (1) the identity and concentration of major ingredients and impurities, (2) water solubility in test water, (3) estimated toxicity to the test organism and to humans, (4) if the test concentration(s) are to be measured, the precision and bias of the analytical method at the planned concentration(s) of the test material, and (5) recommended handling and disposal procedures.

8.3.2.2.1 Organic compounds have been added in the dry form or coated on the inside walls of the container (Ditsworth et al., 1990). Metals are generally added in an aqueous solution (ASTM, 1994b; Carlson et al., 1991; Di Toro et al., 1990). If an organic solvent is used, the solvent in the sediment should be at a concentration that does not affect the test organism. Concentrations of the chemical in the pore water and in the whole sediment should be monitored at the beginning and the end of a test.

8.3.2.3 Use of a solvent other than water should be avoided if possible. Addition of organic solvents may dramatically influence the concentration of dissolved organic carbon in pore water (G.T. Ankley, USEPA, Duluth, MN, personal communication). If an organic solvent must be used, both a solvent-control and a negative-control sediment must be included in a test. The solvent control must contain the highest concentration of solvent present and must be from the same batch used to make the stock solution (see ASTM, 1988a). The same concentration of solvent should be used in all treatments. If an organic solvent is used as a carrier, it may be possible to perform successive washes of sediment to remove most of the solvent while leaving the compound of study (Harkey et al., 1994).

8.3.2.4 If the concentration of solvent is not the same in all test solutions that contain test material, 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.

8.3.2.4.1 If the test contains both a negative control and a solvent control, the survival, growth, or reproduction of the organisms tested should be compared. If a statistically significant difference 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 calculating 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 calculating the results (ASTM, 1992).

8.3.2.5 Test Concentration(s) for Laboratory Spiked Sediments

8.3.2.5.1 If a test is intended to generate an LC50, the selected 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 with the same or a similar test organism. 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. Results from water-only tests could be used to establish concentrations to be tested in a whole-sediment test based on predicted pore-water concentrations (Di Toro et al., 1991).

8.3.2.5.2 Bulk-sediment chemical concentrations might be normalized to factors other than dry weight. For example, concentrations of nonpolar organic compounds might be normalized to sediment organic-carbon content, and simultaneously extracted metals might be normalized to acid volatile sulfides (Di Toro et al, 1990; Di Toro et al., 1991).

8.3.2.5.3 In some situations it might be necessary to only determine whether a specific concentration of test material is toxic to the test organism, or whether adverse effects occur 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 an LC50.

8.3.2.6 Addition of test material(s) to sediment may be accomplished using various methods, such as a (1) rolling mill, (2) feed mixer, or (3) hand mixing (ASTM, 1994b). Modifications of the mixing techniques might be necessary to allow time for a test material to equilibrate with the sediment. Mixing time of spiked sediment should be limited from minutes to a few hours, and temperature should be kept low to minimize potential changes in the physico-chemical and microbial characteristics of the sediment (ASTM, 1994b). Duration of contact between the chemical and sediment can affect partitioning and bioavailability (Word et al., 1987). Care should be taken to ensure that the chemical is thoroughly and evenly distributed in the sediment. Analyses of sediment subsamples are advisable to determine the degree of mixing homogeneity (Ditsworth et al., 1990). Moreover, results from sediment-spiking studies should be compared to the response of test organisms to chemical concentrations in natural sediments (Lamberson and Swartz, 1992).

8.4 Characterization

8.4.1 All sediments should be characterized and at least the following determined: pH and ammonia of the pore water, organic carbon content (total organic carbon, TOC), particle size distribution (percent sand, silt, clay), and percent water content (ASTM, 1994a; Plumb, 1981).

8.4.2 Other analyses on sediments might include biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, total inorganic carbon, total volatile solids, acid volatile sulfides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, as well as interstitial water analyses for various physico-chemical parameters.

8.4.3 Macrobenthos may be evaluated by subsampling the field-collected sediment. If direct comparisons are to be made, subsamples for toxicity testing should be collected from the same sample for analysis of sediment physical and chemical characterizations. Qualitative descriptions of the sediment may include color, texture, presence of macrophytes or animals. Monitoring the odor of sediment samples should be avoided because of potential hazardous volatile contaminants.

8.4.4 Analytical Methodology

8.4.4.1 Chemical and physical data should be obtained using appropriate standard methods whenever possible. For those measurements for which standard methods do not exist or are not sensitive enough, methods should be obtained from other reliable sources.

8.4.4.2 The precision, accuracy, and bias of each analytical method used should be determined in the appropriate matrix: that is, sediment, water, tissue. Reagent blanks and analytical standards should be analyzed, and recoveries should be calculated.

8.4.4.3 Concentration of spiked test material(s) in sediment, interstitial water, and overlying water should be measured as often as practical during a 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 a test. Measurement of test material(s) degradation products might also be desirable.

8.4.4.4 Separate chambers should be set up at the start of a test and destructively sampled during and at the end of the test to monitor sediment chemistry. Test organisms might be added to these extra chambers depending on the objective of the study.

8.4.4.5 Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from about 1 to 2 cm above the sediment surface in the test chamber. Overlying water samples should not con-

tain any surface debris, any material from the sides of the test chamber, or any sediment.

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

8.4.4.7 A variety of procedures have been used to isolate interstitial water including centrifugation, filtration, pressure, or by using an interstitial water sampler; however, centrifugation without filtration is the recommended procedure (Ankley and Schubauer-Berigan, 1994). Filtration may reduce concentrations of materials in interstitial water (Schults et al., 1992). Care should be taken to ensure that contaminants do not transform, degrade, or volatilize during isolation or storage of the interstitial water sample.

Section 9
Quality Assurance and Quality Control

9.1 Introduction

9.1.1 Developing and maintaining a laboratory quality assurance (QA) program requires an ongoing commitment by laboratory management and also includes the following: (1) appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program, (2) preparation of a Quality Assurance Project Plan with Data Quality Objectives, (3) preparation of written descriptions of laboratory Standard Operating Procedures (SOPs) for test organism culturing, testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, and (4) provision of adequate, qualified technical staff and suitable space and equipment to assure reliable data. Additional guidance for QA can be obtained in USEPA (1989d).

9.1.2 QA practices within a testing laboratory should address all activities that affect the quality of the final data, such as (1) sediment sampling and handling, (2) the source and condition of the test organisms, (3) condition and operation of equipment, (4) test conditions, (5) instrument calibration, (6) replication, (7) use of reference toxicants, (8) record keeping, and (9) data evaluation.

9.1.3 Quality control (QC) practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to testing see FDA (1978), USEPA (1979a), USEPA (1980a), USEPA (1980b), USEPA (1993a), USEPA (1994b), USEPA (1994c), DeWoskin (1984), and Taylor (1987).

9.2 Performance-based Criteria

9.2.1 USEPA Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards for chemical analytical methods (Williams, 1993). Performance-based methods were defined by EMMC as a monitoring approach that permits the use of appropriate methods that meet pre-established demonstrated performance standards. Minimum required elements of performance, such as precision, reproducibility, bias, sensitivity, and detection limits should be specified, and the method should be demonstrated to meet the performance standards.

9.2.2 Participants at a September 1992 USEPA sediment toxicity workshop arrived at a consensus on several culturing and testing methods for freshwater organisms (Appendix A, Section S.4). In developing guidance for culturing test organisms to be included in this manual for sediment tests, it was generally agreed that no single method must be used to culture organisms. Success of a test relies on the health of the culture from which organisms are taken for testing. Having healthy organisms of known quality and age for testing is the key consideration relative to culture methods. Therefore, a performance-based criteria approach is the preferred method through which individual laboratories should evaluate culture health rather than using control-based criteria. Performance-based criteria were chosen to allow each laboratory to optimize culture methods while providing organisms that produce reliable and comparable test results. See Tables 11.3, 12.3, and 13.4 for a listing of performance criteria for culturing and testing.

9.3 Facilities, Equipment, and Test Chambers

9.3.1 Separate areas for test organism culturing and testing must be provided to avoid loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into test organism culturing or sediment testing areas, and from sediment testing laboratories and sample preparation areas into culture rooms.

9.3.2 Equipment for temperature control should be adequate to maintain recommended test-water temperatures. Recommended materials should be used in the fabricating of the test equipment that comes in contact with the sediment or overlying water.

9.3.3 Before a sediment test is conducted in a new facility, a "non-contaminant" test should be conducted in which all test chambers contain a control sediment and

overlying water. This information is used to demonstrate that the facility, control sediment, water, and handling procedures provide acceptable responses of test organisms (Section 9.14).

9.4 Test Organisms

9.4.1 The organisms should appear healthy, behave normally, feed well, and have low mortality in cultures, during holding (e.g., <20% for 48 h before the start of a test), and in test controls. The species of test organisms should be positively identified to species.

9.5 Water

9.5.1 The quality of water used for organism culturing and testing is extremely important. Overlying water used in testing and water used in culturing organisms should be uniform in quality. Acceptable water should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (e.g., discoloration, unusual behavior). See Section 7 for additional details.

9.6 Sample Collection and Storage

9.6.1 Sample holding times and temperatures should conform to conditions described in Section 8.

9.7 Test Conditions

9.7.1 It is desirable to measure temperature continuously in at least one chamber during each test. Temperatures should be maintained within the limits specified for each test. Dissolved oxygen, alkalinity, water hardness, conductivity, ammonia, and pH should be checked as prescribed in Sections 11.3, 12.3, and 13.3.

9.8 Quality of Test Organisms

9.8.1 Monthly reference-toxicity tests should be conducted on all test organisms using procedures outlined in Section 9.16. If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test must be evaluated using a reference toxicant. Physiological measurements such as lipid content might also provide useful information regarding the health of the cultures.

9.8.2 The quality of test organisms obtained from an outside source must be verified by conducting a reference-toxicity test concurrently with the sediment test. The supplier should provide data with the shipment describing the history of the sensitivity of organisms from the same source culture. If the supplier has not conducted five reference toxicity tests with the test organism, it is the responsibility of the testing laboratory to conduct these five reference toxicity tests before starting a sediment test (Section 9.14.1).

9.8.3 The supplier should also certify the species identification of the test organisms and provide the taxonomic references or name(s) of the taxonomic expert(s) consulted.

9.9 Quality of Food

9.9.1 Problems with the nutritional suitability of the food will be reflected in the survival, growth, or reproduction of the test organisms in cultures or in sediment tests.

9.9.2 Food used to culture organisms used in bioaccumulation tests must be analyzed for compounds to be measured in the bioaccumulation tests.

9.10 Test Acceptability

9.10.1 For the test results to be acceptable, survival at 10 d must equal or exceed 80% for *H. azteca* and 70% for *C. tentans* in the control sediment. Numbers of *L. variegatus* should not be reduced in test sediments relative to the control sediment and organisms should burrow into the test sediment. Avoidance of test sediment by *L. variegatus* will decrease bioaccumulation. See Table 11.3, 12.3, and 13.4 for additional requirements for acceptability of the tests.

9.10.2 An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of a test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications should be noted when reporting data from a test.

9.11 Analytical Methods

9.11.1 All routine chemical and physical analyses for culture and testing water, food, and sediment should include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a; USEPA, 1979b; USEPA, 1993a; USEPA, 1994b).

9.11.2 Reagent containers should be dated when received from the supplier, and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

9.12 Calibration and Standardization

9.12.1 Instruments used for routine measurements of chemical and physical characteristics such as pH, dissolved oxygen, temperature, and conductivity should be calibrated before use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1; USEPA, 1979b). Calibration data should be recorded in a permanent log.

9.12.2 A known-quality water should be included in the analyses of each batch of water samples (e.g., water hardness, alkalinity, conductivity).

9.13 Replication and Test Sensitivity

9.13.1 The sensitivity of sediment tests will depend in part on the number of replicates/treatment, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of a test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

9.14 Demonstrating Acceptable Performance

9.14.1 It is the responsibility of a laboratory to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs sediment tests (see Section 9.16). Intralaboratory precision, expressed as a coefficient of variation (CV) of the range in response for each type of test to be used in a laboratory, should be determined by performing five or more tests with different batches of test organisms using the same reference toxicant at the same concentrations with the same test conditions (e.g., the same test duration, type of water, age of test organisms, feeding) and the same data analysis methods. This should be done to gain experience for the toxicity tests and as a point of reference for future testing. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical (Section 15).

9.14.2 Before conducting tests with contaminated sediment, the laboratory should demonstrate its ability to conduct tests by conducting five exposures in control sediment as outlined in Table 11.1, 12.1, or 13.1. It is recommended that these five exposures with control sediment be conducted concurrently with the five reference toxicity tests described in Section 9.14.1.

9.14.3 Laboratories should demonstrate that their personnel are able to recover an average of at least 90% of the organisms from whole sediment. For example, test organisms could be added to control sediment or test sediments and recovery could be determined after 1 h (Tomasovic et al., 1994).

9.15 Documenting Ongoing Laboratory Performance

9.15.1 Satisfactory laboratory performance on a continuing basis is demonstrated by conducting monthly water-only 96-h reference-toxicity tests with each test organism. For a given test organism, successive tests should be performed with the same reference toxicant at the same concentrations in the same type of water using the same data analysis method (Section 15).

9.15.2 Outliers, which are data falling outside the control limits and trends of increasing or decreasing sensitivity are readily identified. If the reference toxicity datum from a given test falls outside the "expected" range (e.g., ± 2 SD), the sensitivity of the organisms and the credibility of the test results are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

9.15.3 A sediment test may be acceptable if specified conditions of a reference toxicity test fall outside the expected ranges (Section 9.10.2). Specifically, a sediment test should not automatically be judged unacceptable if the LC50 for a given reference toxicity test falls outside the expected range or if mortality in the control of the reference toxicity test exceeds 10%. All the performance criteria outlined in Tables 11.3, 12.3, and 13.4 must be considered when determining the acceptability of a sediment test. The acceptability of the sediment test would depend on the experience and judgment of the investigator and the regulatory authority.

9.15.4 Performance should improve with experience, and the control limits should gradually narrow, as the statistics stabilize. However, control limits of ± 2 SD, by definition, will be exceeded 5% of the time, regardless of how well a laboratory performs. For this reason, good laboratories that develop very narrow control limits may be penalized if a test result that falls just outside the control limits is rejected *de facto*. The width of the control limits should be considered in decisions regarding rejection of data (Section 15).

9.16 Reference Toxicants

9.16.1 Ideally, reference-toxicity tests should be conducted in conjunction with sediment tests to determine possible changes in condition of a test organism (Lee, 1980). Water-only reference-toxicity tests should be conducted monthly. Deviations outside an established normal range may indicate a change in the condition of the test organism population. Results of reference-toxicity tests also enable interlaboratory comparisons of test organism sensitivity.

9.16.2 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), and copper sulfate ($CuSO_4$) are suitable for use. No one reference toxicant can be used to measure the condition of test organisms with respect to another toxicant with a different mode of action (Lee, 1980). However, it may be unrealistic to test more than one or two reference toxicants routinely. KCl has been used successfully in round-robin water-only exposures with *H. azteca* and *C. tentans* (Section 15).

9.16.3 Test conditions for conducting reference-toxicity tests with *H. azteca, C. tentans,* and *L. variegatus* are outlined in Tables 9.1 and 9.2. Reference-toxicity tests can be conducted using one organism/chamber or multiple organisms in each chamber. Some laboratories

have observed low control survival when more than one midge/chamber is tested in water-only exposures.

9.17 Record Keeping

9.17.1 Proper record keeping is important. A complete file should be maintained for each individual sediment test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the sediment test(s); chemical analysis data on the sample(s); control data sheets for reference toxicants; detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions used; and results of reference toxicant tests. Laboratory data should be recorded immediately to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests. For additional detail see Section 14.

Table 9.1 Recommended Test Conditions for Conducting Reference-Toxicity Tests with One Organism/Chamber

	Parameter	Conditions
1.	Test type:	Water-only test
2.	Dilution series:	Control and at least 5 test concentrations (0.5 dilution factor)
3.	Toxicant:	NaCl, KCl, Cd, or Cu
4.	Temperature:	23 ± 1°C
5.	Light quality:	Wide-spectrum fluorescent lights
6.	Illuminance:	About 500 to 1000 lux
7.	Photoperiod:	16L:8D
8.	Renewal of water:	None
9.	Age of organisms:	*H. azteca:* 7- to 14-d old *C. tentans:* third instar larvae[1] *L. variegatus:* adults
10.	Test chamber:	30-mL plastic cups (covered with glass or plastic)
11.	Volume of water:	20 mL
12.	Number of organisms/chamber:	1
13.	Number of replicate chambers/treatment:	10 minimum
14.	Feeding:	*H. azteca:* 0.1 mL YCT (1800 mg/L stock) on Day 0 and 2 *C. tentans:* 0.25 mL Tetrafin® (4 g/L stock) on Day 0 and 2 *L. variegatus:* not fed
15.	Substrate:	*H. azteca:* Nitex® screen (110 mesh) *C. tentans:* sand (monolayer) *L. variegatus:* no substrate
16.	Aeration:	None
17.	Dilution water:	Culture water, well water, surface water, site water, or reconstituted water
18.	Test chamber cleaning:	None
19.	Water quality:	Hardness, alkalinity, conductivity, dissolved oxygen, and pH at the beginning and end of a test. Temperature daily
20.	Test duration:	96 h
21.	Endpoint:	Survival (LC50)
22.	Test acceptability:	90% control survival

[1] Age requirement: All animals must be third instar or younger with at least 50% of the organisms at third instar.

Table 9.2 Recommended Test Conditions for Conducting Reference-Toxicity Tests with More Than One Organism/Chamber

	Parameter	Conditions
1.	Test type:	Water-only test
2.	Dilution series:	Control and at least 5 test concentrations (0.5 dilution factor)
3.	Toxicant:	NaCl, KCl, Cd, or Cu
4.	Temperature:	23 ± 1°C
5.	Light quality:	Wide-spectrum fluorescent lights
6.	Illuminance:	About 500 to 1000 lux
7.	Photoperiod:	16L:8D
8.	Renewal of water:	None
9.	Age of organisms:	*H. azteca:* 7- to 14-d old *C. tentans:* third instar *L. variegatus:* adults
10.	Test chamber:	250-mL glass beaker (covered with glass or plastic)
11.	Volume of water:	100 mL (minimum)
12.	Number of organisms/chamber:	10 minimum
13.	Number of replicate chambers/treatment:	3 minimum
14.	Feeding:	*H. azteca:* 0.5 mL YCT (1800 mg/L stock) on Day 0 and 2 *C. tentans:* 1.25 mL Tetrafin® (4 g/L stock) on Day 0 and 2 *L. variegatus:* not fed
15.	Substrate:	*H. azteca:* Nitex® screen (110 mesh) *C. tentans:* sand (monolayer) *L. variegatus:* no substrate
16.	Aeration:	None
17.	Dilution water:	Culture water, well water, surface water, site water or reconstituted water
18.	Test chamber cleaning:	None
19.	Water quality:	Hardness, alkalinity, conductivity, dissolved oxygen, and pH at the beginning and end of a test. Temperature daily
20.	Test duration:	96 h
21.	Endpoint:	Survival (LC50)
22.	Test acceptability:	90% control survival

[1] Age requirement: All animals must be third instar or younger with at least 50% of the organisms at third instar.

Section 10
Collection, Culturing, and Maintaining Test Organisms

10.1 Life Histories

10.1.1 Hyalella azteca

10.1.1.1 *Hyalella azteca* inhabit permanent lakes, ponds, and streams throughout North and South America (de March, 1981; Pennak, 1989). Occurrence of *H. azteca* is most common in warm (20 to 30°C for much of the summer) mesotrophic or eutrophic lakes that support aquatic plants. These amphipods are also found in ponds, sloughs, marshes, rivers, ditches, streams, and springs, but in lower numbers. *Hyalella azteca* have achieved densities of >10,000 m^2 in preferred habitats (de March, 1981).

10.1.1.2 *Hyalella azteca* are epibenthic detritivores that burrow into the sediment surface. Hargrave (1970) reported that *H. azteca* selectively ingest bacteria and algae. The behavior and feeding habits of *H. azteca* make them excellent test organisms for sediment assessments.

10.1.1.3 Reproduction by *H. azteca* is sexual. The adult males are larger than females and have larger second gnathopods (de March, 1981). Males pair with females by grasping the females (amplexus) with their gnathopods while on the backs of the females. After feeding together for 1 to 7 d the female is ready to molt and the two organisms separate for a short time while the female sheds her old exoskeleton. Once the exoskeleton is shed, the two organisms reunite and copulation occurs. The male places sperm near the marsupium of the female and her pleopods sweep the sperm into the marsupium. The organisms again separate and the female releases eggs from her oviducts into the marsupium where they are fertilized. *Hyalella azteca* average about 18 eggs/brood (Pennak, 1989) with larger organisms having more eggs (Cooper, 1965).

10.1.1.4 The developing embryos and newly hatched young are kept in the marsupium until the next molt. At 24 to 28°C, hatching ranges from 5 to 10 d after fertilization (Embody, 1911; Bovee, 1950; Cooper, 1965). The time between molts for females is 7 to 8 d at 26 to 28°C (Bovee, 1950). Therefore, about the time embryos hatch, the female molts and releases the young. *Hyalella azteca* average 15 broods in 152 d (Pennak, 1989). Pairing of the sexes is simultaneous with embryo incubation of the previous brood in the marsupium. *Hyalella azteca* have a minimum of nine instars (Geisler, 1944). There are 5 to 8 pre-reproductive instars (Cooper, 1965) and an indefinite number of post-reproductive instars. The first five instars form the juvenile stage of development, instar stages 6 and 7 form the adolescent stage when sexes can be differentiated, instar stage 8 is the nuptial stage, and all later instars are the adult stages of development (Pennak, 1989).

10.1.1.5 *Hyalella azteca* have been successfully cultured at illuminance of about 500 to 1000 lux (Ingersoll and Nelson, 1990; Ankley et al., 1991a; Ankley et al., 1991b). *Hyalella azteca* avoid bright light, preferring to hide under litter and feed during the day.

10.1.1.6 Temperatures tolerated by *H. azteca* range from 0 to 33°C (Embody, 1911; Bovee, 1949; Sprague, 1963). At temperatures less than 10°C the organisms rest and are immobile (de March, 1977; de March, 1978). At temperatures of 10 to 18°C, reproduction can occur. Juveniles grow more slowly at colder temperatures and become larger adults. Smaller adults with higher reproduction are typical when organisms are grown at 18 to 28°C. The highest rates of reproduction occur at 26 to 28°C (de March, 1978) while lethality occurs at 33 to 37°C (Bovee, 1949; Sprague, 1963).

10.1.1.7 *Hyalella azteca* are found in waters of widely varying types. *Hyalella azteca* can inhabit saline waters up to 29 ‰; however, their distribution in these saline waters has been correlated to water hardness (Ingersoll et al., 1992). *Hyalella azteca* inhabit water with high Mg concentrations at conductivities up to 22,000 µS/cm, but only up to 12,000 µS/cm in Na-dominated waters (Ingersoll et al., 1992). De March (1981) reported *H. azteca* were not collected from locations where calcium was less than 7 mg/L. *Hyalella azteca* have been cultured in water with a salinity up to 15 ‰ in reconstituted salt water (Ingersoll et al., 1992; Winger and Lasier, 1993). In laboratory studies, Sprague (1963) reported a 24-h LC50 for dissolved oxygen at 20°C of 0.7 mg/L. Pennak and Rosine (1976) reported similar findings. Nebeker et al. (1992) reported 48-h and 30-d LC50s for *H. azteca* of less than 0.3 mg/L dissolved oxygen. Weight and reproduction of *H. azteca* were reduced after 30-d exposure to 1.2 mg/L dissolved oxygen.

10.1.1.8 *Hyalella azteca* tolerate a wide range of substrates. Ingersoll and Nelson (1990) and Ingersoll et al. (1993) reported that *H. azteca* tolerated sediments ranging from more than 90% silt- and clay-sized particles to 100% sand-sized particles without detrimental effects on either survival or growth. *Hyalella azteca* tolerated a wide range in grain size and organic matter in 10-d tests with formulated sediment (Suedel and Rodgers, 1994). Ankley et al. (1994a) evaluated the effects of natural sediment physico-chemical characteristics on the results of 10-d laboratory toxicity tests with *H. azteca, C. tentans,* and *L. variegatus.* Tests were conducted with and without the addition of exogenous food. Survival of organisms was decreased in tests without added food. Physico-chemical sediment characteristics including grain size and TOC were not significantly correlated to the response of *H. azteca* in either fed or unfed tests.

10.1.2 Chironomus tentans

10.1.2.1 *Chironomus tentans* have a holarctic distribution (Townsend et al., 1981) and are commonly found in eutrophic ponds and lakes (Flannagan, 1971; Driver, 1977). Midge larvae are important in the diet of fish and waterfowl (Sadler, 1935; Siegfried, 1973; Driver et al., 1974; McLarney et al., 1974). Larvae of *C. tentans* usually penetrate a few cm into sediment. In both lotic and lentic habitats with soft bottoms, about 95% of the chironomid larvae occur in the upper 10 cm of substrates, and very few larvae are found below 40 cm (Townsend et al., 1981). Larvae were found under the following conditions in British Columbia lakes by Topping (1971): particle size <0.15 mm to 2.0 mm, temperature 0 to 23.3°C, dissolved oxygen 0.22 to 8.23 mg/L, pH 8.0 to 9.2, conductivity 481 to 4,136 µmhos/cm, and sediment organic carbon 1.9 to 15.5%. Larvae were absent from lakes if hydrogen sulfide concentration in overlying water exceeded 0.3 mg/L. Abundance of larvae was positively correlated with conductivity, pH, amount of food, percentages of particles in the 0.59 to 1.98 mm size range, and concentrations of Na, K, Mg, Cl, SO_4, and dissolved oxygen. Others (e.g., Curry, 1962; Oliver, 1971) have reported a temperature range of 0 to 35°C and a pH range of 7 to 10.

10.1.2.2 *Chironomus tentans* are aquatic during the larval and pupal stages. The life-cycle of *C. tentans* can be divided into four distinct stages: (1) an egg stage, (2) a larval stage, consisting of four instars, (3) a pupal stage, and (4) an adult stage. Mating behavior has been described by Sadler (1935) and others (ASTM, 1994a). Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. The male has paired genital claspers on the posterior tip of the abdomen (Townsend et al., 1981). The adult female weighs about twice as much as the male, with about 30% of the female weight contributed by the eggs. After mating, adult females oviposit a single transparent, gelatinous egg mass directly into the water. At ERL-D, the females oviposit eggs within 24 h after emergence. An egg mass contains about 2,300 eggs (Sadler, 1935) and will hatch in 2 to 4 d at 23°C. Under optimal conditions larvae will pupate and emerge as adults after about 21 d at 23°C. Larvae begin to construct tubes (or cases) on the second or third day after hatching. The cases lengthen and enlarge as the larvae grow with the addition of small particles bound together with threads from the mouths of larvae (Sadler, 1935). The larvae draw food particles inside the tubes and also feed in the immediate vicinity of either end of the open-ended tubes with their caudal extremities anchored within the tube. The four larval stages are followed by a black-colored pupal stage (lasting about 3 d) and emergence to a terrestrial adult (imago) stage. The adult stage lasts for 3 to 5 d, during which time the adults mate during flight and the females oviposit their egg masses (2 to 3 d post-emergence; Sadler, 1935).

10.1.2.3 *Chironomus tentans* tolerate a wide range of substrates. Survival or growth of *C. tentans* was not reduced over a wide range in sediment grain sized in 10-d tests with formulated sediment; however, survival was reduced in artificial sediments below 0.91% organic matter when organisms were not fed (Suedel and Rodgers, 1994). Ankley et al. (1994a) evaluated the effects of natural sediment physico-chemical characteristics on the results of 10-d laboratory toxicity tests with *H. azteca, C. tentans,* and *L. variegatus.* Tests were conducted with and without the addition of exogenous food. Survival and growth of organisms was decreased in tests without added food. Physico-chemical sediment characteristics including grain size and TOC were not significantly correlated to survival of *C. tentans* in tests in which organisms were fed. However, linear modeling indicated growth of *C. tentans* was influenced by grain size distribution of the test sediments (growth slightly increased in coarser sediment).

10.1.3 Lumbriculus variegatus

10.1.3.1 *Lumbriculus variegatus* inhabit a variety of sediment types throughout the United States and Europe (Chekanovskaya, 1962; Cook, 1969; Spencer, 1980; Brinkhurst, 1986). *Lumbriculus variegatus* typically tunnel in the upper aerobic zone of sediments of reservoirs, rivers, lakes, ponds, and marshes. When not tunneling, they bury their anterior portion in sediment and undulate their posterior portion in overlying water for respiratory exchange.

10.1.3.2 Adults of *L. variegatus* can reach a length of 40 to 90 mm, diameter of 1.0 to 1.5 mm, and wet weight of 5 to 12 mg (Call et al., 1991; Phipps et al., 1993). Lipid content is about 1.0% (wet weight, Ankley et al., 1992b). *Lumbriculus variegatus* most commonly reproduce asexually, although sexual reproduction has been reported (Chekanovskaya, 1962). Newly hatched worms have not been observed in cultures (Call et al., 1991; Phipps et al., 1993. Cultures consist of adults of various sizes. Populations of laboratory cultures double (number of organisms) every 10 to 14 d at 20°C (Phipps et al., 1993).

10.1.3.3 *Lumbriculus variegatus* tolerate a wide range of substrates. Ankley et al. (1994a) evaluated the effects of natural sediment physico-chemical characteristics on the results of 10-d laboratory toxicity tests with *H. azteca, C. tentans,* and *L. variegatus.* Tests were conducted with and without the addition of exogenous food. Survival and reproduction of organisms was decreased in tests without added food. Physico-chemical sediment characteristics including grain size and TOC were not significantly correlated to reproduction or growth of *L. variegatus* in either fed or unfed tests.

10.2 General Culturing Procedures

10.2.1 Acceptability of a culturing procedure is based in part on performance of organisms in culture and in the sediment test (Section 1.4 and 9.2). No single technique for culturing test organisms is required. What may work well for one laboratory may not work as well for another laboratory. While a variety of culturing procedures are outlined in Section 10.3 for *H. azteca,* in Section 10.4 for *C. tentans,* and in Section 10.5 for *L. variegatus,* organisms must meet the test acceptability requirements listed in Tables 11.3, 12.3, or 13.4.

10.2.2 All organisms in a test must be from the same source. Organisms may be obtained from laboratory cultures, from commercial, or government sources (Table 10.1). The test organism used should be identified using an appropriate taxonomic key, and verification should be documented. Obtaining organisms from wild populations should be avoided unless organisms are cultured through several generations in the laboratory. In addition, the ability of the wild population of sexually reproducing organisms to cross-breed with the existing laboratory population must be determined. Sensitivity of the wild population to select contaminants (e.g., Table 1.4) should also be documented.

Table 10.1 Sources of Test Organisms

Source	Species
U.S. Environmental Protection Agency Environmental Research Laboratory-Duluth 6201 Congdon Boulevard Duluth, MN 55804 Teresa Norberg-King (218/720-5500)	*H. azteca* *C. tentans* *L. variegatus*
U.S. Environmental Protection Agency Environmental Monitoring System Laboratory 3411 Church Street Cincinnati, OH 45244 Jim Lazorchak (513/569-7076)	*H. azteca* *C. tentans*
Midwest Science Center National Biological Survey 4200 New Haven Road Columbia, MO 65201 Eugene Greer (314/875-5399)	*H. azteca* *C. tentans* *L. variegatus*
Great Lakes Environmental Research Laboratory, NOAA 2205 Commonwealth Boulevard Ann Arbor, MI 48105-1593 Peter Landrum (313/741-2276)	*L. variegatus*
Wright State University Department of Biological Sciences Dayton, OH 45435 Allen Burton (513/873-2201)	*H. azteca* *C. tentans* *L. variegatus*
Michigan State University Department of Fisheries and Wildlife No. 13 Natural Resources Building East Lansing, MI 48824-1222 John Giesy (517/353-2000)	*H. azteca* *C. tentans* *L. variegatus*

10.2.3 Test organisms obtained from commercial sources should be shipped in well-oxygenated water in insulated containers to maintain temperature during shipment. Temperature and dissolved oxygen of the water in the shipping containers should be measured on arrival to determine if the organisms might have been subjected to low dissolved oxygen or temperature fluctuations. The temperature of the shipped water should be gradually adjusted to the desired culture temperature at a rate not exceeding 2°C per 24 h. Additional reference-toxicity testing is required if organisms are not cultured at the testing laboratory (Section 9.16).

10.2.4 A group of organisms should not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed (e.g., >20% mortality for 48 h before the start of a test). If the organisms fail to meet these criteria, the entire batch should be discarded and a new batch should be obtained. All organisms should be as uniform as possible in age and life stage. 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.

10.2.5 *H. azteca, C. tentans,* and *L. variegatus* can be cultured in a variety of waters. Water of a quality sufficient to culture fathead minnows (*Pimephales promelas*) or cladocerans will generally be adequate.

10.2.5.1 Variable success has been reported using reconstituted waters described in ASTM (1988a) or USEPA (1993a) to culture or test *H. azteca* (USEPA. 1992). However, the reconstituted water described in Section 7.1.3.4 has been used to successfully culture *H. azteca* (J.M. Lazorchak, USEPA, Cincinnati, OH, personal communication). The reconstituted water described in Section 7.1.3.4 has a higher proportion of chloride to sulfate compared to the reconstituted waters described in ASTM (1988a) and USEPA (1993a). *H. azteca* can be cultured and tested at salinities up to 15 ‰ (Ingersoll et al., 1992; Winger et al., 1993).

10.2.5.2 Organisms can be cultured using either static or renewal procedures. Renewal of water is recommended to limit loss of the culture organisms from a drop in dissolved oxygen or a buildup of waste products. In renewal systems, there should be at least one volume addition/d of culture water to each chamber. In static systems, the overlying water volume should be changed at least weekly by siphoning down to a level just above the substrate and slowly adding fresh water. Extra care should be taken to ensure that proper water quality is

maintained in static systems. For example, aeration is needed in static systems to maintain dissolved oxygen at >40% of saturation.

10.2.5.3 A recirculating system using an under-gravel filter has been used to culture amphipods and midges (P.V. Winger, NBS, Athens, GA, personal communication). The approach for using a recirculating system to culture organisms has been described by New et al. (1974), Crandall et al. (1981), and Rottmann and Campton (1989). Under-gravel filters can be purchased from aquarium suppliers and consist of an elevated plate with holes that fit on the bottom of an aquarium. The plate has a standpipe to which a pump can be attached. Gravel or an artificial substrate (e.g., plastic balls or multi-plate substrates) are placed on the plate. The substrates provide surface area for microorganisms that use nitrogenous compounds. A simple example of a recirculating system is two aquaria positioned one above the other with a total volume of 120 L. The bottom aquarium contains the under-gravel filter system, gravel, or artificial substrate, and a submersible pump. The top aquarium is used for culture of animals and has a hole in the bottom with a standpipe for returning overflow water to the bottom aquarium. Water lost to evaporation is replaced weekly, and water is replaced at one- to two-month intervals. Cultures fed foods such as Tetramin® should include limestone gravel to help avoid depression in pH. Recirculating systems require less maintenance than static systems.

10.2.6 Cultures should be maintained at 23°C with a 16L:8D photoperiod at a illuminance of about 500 to 1000 lux (ASTM, (1994a) and Appendix A). Cultures should be observed daily. Water temperature should be measured daily or continuously, and dissolved oxygen should be measured weekly. Reference-toxicity tests should be conducted at least monthly. If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test must be evaluated using a reference toxicant. Culture water hardness, alkalinity, ammonia, and pH should be measured at least quarterly and the day before the start of a sediment test. If reconstituted water is used to culture organisms, water quality should be measured on each batch of reconstituted water. Culture procedures should be evaluated and adjusted as appropriate to restore or maintain the health of the culture.

10.3 Culturing Procedures for *Hyalella azteca*

10.3.1 The culturing procedures described below are based on methods described in USEPA (1993), Ankley et al. (1994a), Call et al. (1994), Tomasovic et al. (1994), Greer (1993), Ingersoll and Nelson (1990), ASTM (1993a) and Appendix A. The culturing procedure must produce 7- to 14-d old amphipods to start a sediment test (Table 11.3). A narrower age range of organisms used to start a test may be desirable when growth is measured as an endpoint. Amphipods within a range of 1- to 2-d old will be more uniform in size than organisms within a range of 7-d old.

10.3.2 The following procedure described by Call et al. (1994) and USEPA (1993) can be used to obtain known-age amphipods to start a test. Mature amphipods (50 organisms ≥30-d old at 23°C) are held in 2-L glass beakers containing 1 L of aerated culture water and cotton gauze as a substrate. Cotton gauze should be soaked in water for 24 h before use and should be renewed on a weekly basis. Amphipods are fed 10 mL of a the yeast-Cerophyl®-trout chow (YCT) mixture (Appendix C), 10 mL of the green algae *Selenastrum capricornutum* (about 35 x 10^6 cells/mL), and 10 mL of the diatom, *Navicula* spp. (1.0 x 10^9 cells/mL) on Monday. Five mL of each food is added to cultures on Wednesdays and Fridays.

10.3.2.1 Water in the culture chambers is changed weekly. Survival of adults and juveniles and production of young amphipods should be measured at this time. The contents of the culture chambers are poured into a translucent white plastic or white enamel pan. After the adults are removed, the remaining amphipods will range in age from <1- to 7-d old. Young amphipods are transferred with a pipet into a 1-L beaker containing culture water and are held for one week before starting a toxicity test. Presoaked cotton gauze is placed in the beakers, and organisms are fed 10 mL of YCT and 10 mL of green algae, and 10 mL of diatoms with renewal of water, and 5 mL of each food on Wednesdays and Fridays (Appendix C). Survival of young amphipods should be >80% during this one week holding period. Records should be kept on the number of surviving adults, number of breeding pairs, and young production and survival. This information can be used to develop control charts which are useful in determining if cultures are maintaining a vigorous reproductive rate indicative of culture health. Some of the adult amphipods can be expected to die in the culture chambers, but mortality greater than about 50% should be cause for concern. Reproductive rates in culture chambers containing 60 adults can be as high as 500 young per week. A decrease in reproductive rate may be caused by a change in water quality, temperature, food quality, or brood stock health. Adult females will continue to reproduce for several months; however, young production gradually decreases after about three months.

10.3.3 A second procedure for obtaining known-age amphipods is described by Borgmann et al. (1989). Known-age amphipods are cultured in 2.5-L chambers containing about 1 L of culture water and between 5 and 25 adult *H. azteca*. Each chamber contains pieces of cotton gauze presoaked in culture water. Once a week the test organisms are isolated from the gauze and collected using a sieve. Amphipods are then rinsed into petri dishes where the young and adults are sorted. The adults are returned to the culture chambers containing fresh water and food.

10.3.4 A third procedure for obtaining known-age amphipods is described by Greer (1993) and Tomasovic et al. (1994). Mass cultures of mixed-age amphipods are maintained in 80-L glass aquaria containing about 50 L of water (Ingersoll and Nelson, 1990). Tetramin® is added to each culture chamber receiving daily water renewals to provide about 20 g dry solids/50 L of water twice weekly in an 80-L culture chamber. Additional Tetramin® is added when most of the Tetramin® has been consumed. Laboratories using static systems should develop lower feeding rates specific to their systems. Each culture chamber has a substrate of maple leaves and artificial substrates (six 20-cm diameter sections per 80-L aquaria of "coiled-web material"; 3-M, St. Paul, MN). Before use, leaves are soaked in water for about 30 d. The leaves are then flushed with water to remove residuals of naturally occurring tannic acid before placement in the cultures.

10.3.4.1 To obtain known-age amphipods, a U.S. Standard Sieve #25 (710 µm mesh) is placed underwater in a chamber containing mixed-age amphipods. A #25 sieve will retain mature amphipods, and immature amphipods will pass through the mesh. Two or three pieces of artificial substrate (3-M coiled-web material) or a mass of leaves with the associated mixed-age amphipods are quickly placed into the sieve. The sieve is brought to the top of the water in the culture chamber keeping all but about 1 cm of the sieve under water. The artificial substrates or leaves are then shaken under water several times to dislodge the attached amphipods. The artificial substrates or leaves are taken out of the sieve and placed back in the culture chamber. The sieve is agitated in the water to rinse the smaller amphipods back into the culture chamber. The larger amphipods remaining in the sieve are transferred with a pipet into a dish and then placed into a shallow glass pan (e.g., pie pan) where immature amphipods are removed. The remaining mature amphipods are transferred using a pipet into a second #25 sieve which is held in a glass pan containing culture water.

10.3.4.2 The mature amphipods are left in the sieve in the pan overnight to collect any newborn amphipods that are released. After 24 h, the sieve is moved up and down several times to rinse the newborn amphipods (<24-h old) into the surrounding water in the pan. The sieve is removed from the pan, and the mature amphipods are placed back into their culture chamber or placed in a second pan containing culture water if additional organisms are needed for testing. The newborn amphipods are moved with a pipet and placed in a culture chamber with flowing water during a grow-out period. The newborn amphipods should be counted to determine if adequate numbers have been collected for the test.

10.3.4.3 Isolation of about 1500 (750 pairs) adults in amplexus provided about 800 newborn amphipods in 24 h and required about six man-hours of time. Isolation of about 4000 mixed-age adults (some in amplexus and others not in amplexus) provided about 800 newborn amphipods in 24 h and required less than one man-hour of time. The newborn amphipods should be held for 6 to 13 d to provide 7- to 14-d old organisms to start a test. A few maple leaves and a small amount of Tetramin® is placed into the grow-out culture chamber to provide food.

10.3.5 Laboratories that use mixed-age amphipods for testing must demonstrate that the procedure used to isolate amphipods will produce test organisms that are 7- to 14-d old. For example, amphipods passing through a U.S. Standard #35 sieve (500 µm), but stopped by a #45 sieve (355 µm) averaged 1.54 mm (SD 0.09) in length (P.V. Winger, NBS, Athens, Ga, unpublished data). The mean length of these sieved organisms corresponds to that of 6-d old amphipods (Figure 10.1). After holding for 3 d before testing to eliminate organisms injured during sieving, these amphipods were about 9-d old (length 1.84 mm, SD 0.11) at the start of a toxicity test.

10.3.5.1 Ingersoll and Nelson (1990) and ASTM (1994a) describe the following procedure for obtaining mixed-age amphipods of a similar size to start a test. Smaller amphipods are isolated from larger amphipods using a stack of U.S. Standard sieves: #30 (600 µm), #40 (425 µm), and #60 (250 µm). Sieves should be held under water to isolate the amphipods. Amphipods may float on the surface of the water if they are exposed to air. Artificial substrate or leaves are placed in the #30 sieve. Culture water is rinsed through the sieves and small amphipods stopped by the #60 sieve are washed into a collecting pan. Larger amphipods in the #30 and #40 sieves are returned to the culture chamber. The smaller amphipods are then placed in 1-L beakers containing culture water and food (about 200 amphipods per beaker) with gentle aeration.

10.3.5.2 Amphipods should be held and fed at a rate similar to the mass cultures for least 2 d before the start of a test to eliminate animals injured during handling.

10.3.6 See Section 10.2.6 for procedures used to evaluate the health of cultures.

10.4 Culturing Procedures for *Chironomus tentans*

10.4.1 The culturing methods described below are based on methods described in USEPA (1993), Ankley et al. (1994a), Call et al. (1994), Greer (1993), ASTM (1994a), and Appendix A. Sediment tests must be started with third instar larvae (at least 50% of the larvae must be third instar with the remaining larvae second instar; Table 12.3). At a temperature of 23°C, larvae should develop to the third instar by 9 to 11 d after hatching (about 11 to 13 d post-oviposition). The instar of midges at the start of a test must be determined using head capsule width (Table 10.2). It is also desirable to monitor the weight or length of midges at the start of a sediment test.

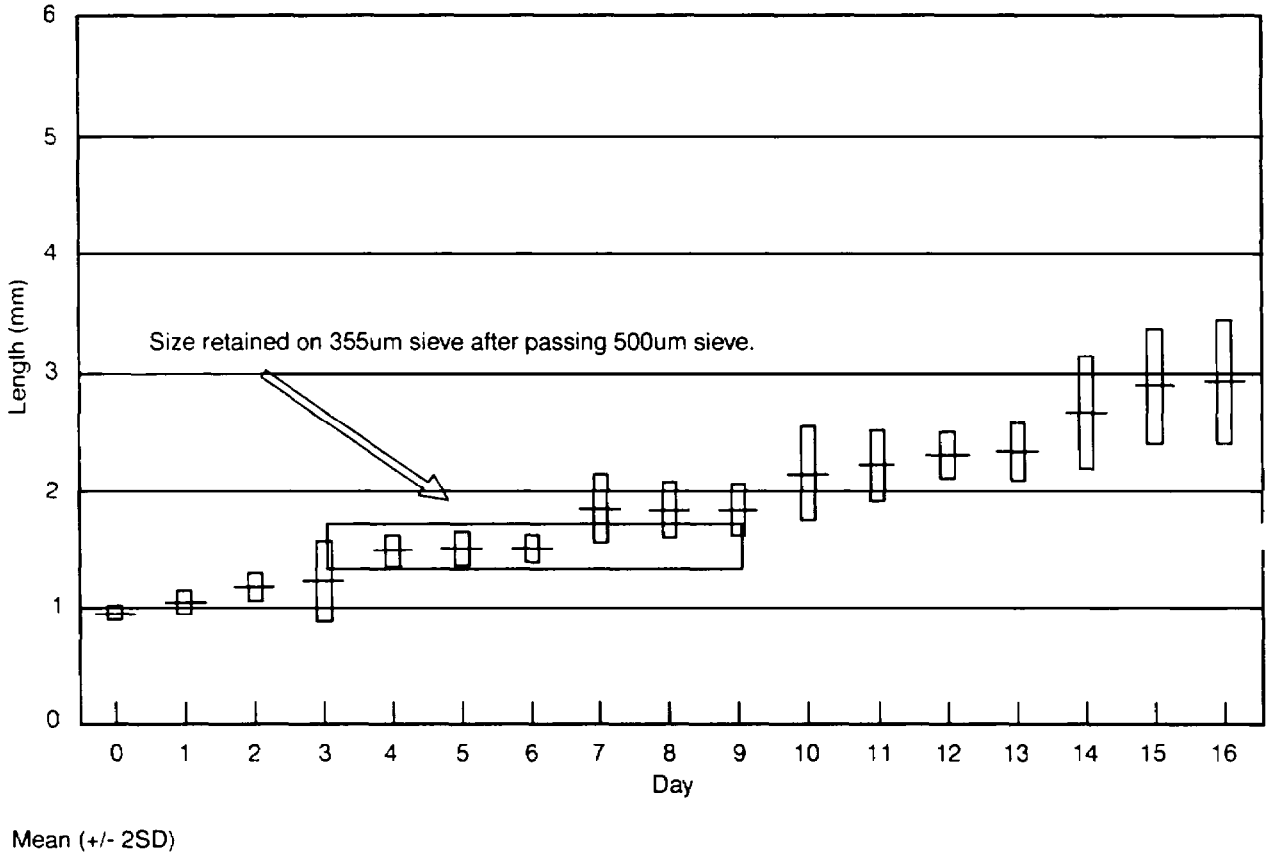


Figure 10.1 Length and relative age of *Hyalella azteca* collected by sieving in comparison with length of known-age organisms. P.V. Winger, NBS, Athens, GA, unpublished data.

Table 10.2 *Chironomus tentans* Instar and Head Capsule Widths[a]

Instar	Days after hatching	Mean (mm)	Range (mm)
First	1 to 4.4	0.10	0.09 to 0.13
Second	4.4 to 8.5	0.20	0.18 to 0.23
Third	8.5 to 12.5	0.38	0.33 to 0.45
Fourth	≥12.5	0.67	0.63 to 0.71

[a] T.J. Norberg-King, USEPA, Duluth, MN, unpublished data.

10.4.2 Recent research has indicated that the third instar *C. tentans* were frequently referred to as the second instar in previous literature (T.J. Norberg-King, USEPA, Duluth, MN, unpublished data). When *C. tentans* larvae were measured daily, the *C. tentans* raised at 22 to 24°C were third instar, not second instar, by 9 to 11 d after hatching.

10.4.3 Both silica sand and shredded paper toweling have been used as substrates to culture *C. tentans*. Either substrate may be used if a healthy culture can be maintained. Greer (1993) used sand or paper toweling to culture midges; however, sand was preferred due to the ease in removing larvae for testing. Sources of sand are listed in Section 7.

10.4.3.1 Paper towels are prepared according to a procedure adapted from Batac-Catalan and White (1982). Plain white kitchen paper towels are cut into strips. Cut toweling is loosely packed into a 2-L beaker, submersed in acetone, covered and placed in a fume hood, and soaked overnight to solubilize organic contaminants. The acetone is drained completely, and deionized water is added, brought to a boil, and stirred to drive off any remaining acetone vapors. This process is repeated two more times. Finally, the toweling is rinsed three times with cold deionized water. A mass of the toweling sufficient to fill a 150-mL beaker is placed into a blender containing 1 L of deionized water, and blended for 30 sec or until the strips are broken apart in the form of a pulp. The pulp is then sieved using a 710 µm sieve and rinsed well with deionized water to remove the shortest fibers.

10.4.3.2 Dry shredded paper toweling loosely packed into a 2-L beaker will provide sufficient substrate for about ten 19-L chambers (USEPA, 1993). The shredded toweling placed in a 150-mL beaker produces enough substrate for one 19-L chamber. Additional substrate can be frozen in deionized water for later use.

10.4.4 Five egg masses will provide a sufficient number of organisms to start a new culture chamber. Egg masses should be held at 23°C in a glass beaker or crystallizing

dish containing about 100 to 150 mL of culture water (temperature change should not exceed 2°C per d). Food is not added until the embryos start to hatch (in about 2 to 4 days at 23°C) to reduce the risk of oxygen depletion. A small amount of green algae (e.g., a thin green layer) is added to the water when embryos start to hatch. When most of the larvae have left the egg mass, 150 to 200 larvae should be placed into a culture chamber. Crowding of larvae will reduce growth. Larvae that have formed cases can be transferred to aquaria or culture chambers using a gentle stream of water from a squeeze bottle. See Section 10.4.5.1 or 10.4.6.1 for a description of feeding rates. Larvae should reach the third instar by about 10 d after median hatch (about 12 to 14 d after the time the eggs were laid).

10.4.5 *Chironomus tentans* are cultured in soft water at the USEPA laboratory in Duluth in glass aquaria (19.0-L capacity, 36- x 21- x 26-cm high). A water volume of about 6 to 8 L in these flow-through chambers can be maintained by drilling an overflow hole in one end 11 cm from the bottom. The top of the aquarium is covered with a mesh material to trap emergent adults. Pantyhose with the elasticized waist is positioned around the chamber top and the legs are cut off. Fiberglass-window screen glued to a glass-strip (about 2- to 3-cm wide) rectangle placed on top of each aquarium has also been used by Call et al. (1994). About 200 to 300 mL of sand is placed in each chamber.

10.4.5.1 Tetrafin® food is added to each culture chamber to provide a final food concentration of about 0.04 mg dry solids/mL of culture water. A stock suspension of the solids is prepared in culture water such that a total volume of 5.0 mL of food suspension is added daily to each culture chamber. For example, if a culture chamber volume is 8 L, 300 mg of food would be added daily by adding 5 mL of a 56-g/L stock suspension. The stock suspension should be well mixed immediately before removing an aliquot for feeding. Each batch of food should be refrigerated and can be used for up to two weeks (Appendix C). Laboratories using static systems should develop lower feeding rates specific to their systems.

10.4.6 *Chironomus tentans* are cultured by Greer (1993) in Rubbermaid® 5.7-L polyethylene cylindrical containers. The containers are modified by cutting a semicircle into the lid 17.75 cm across by 12.5 cm. Stainless-steel screen (20 mesh/0.4 cm) is cut to size and melted to the plastic lid. The screen provides air exchange, retains emerging adults, and is a convenient way to observe the culture. Two holes about 0.05 cm in diameter are drilled through the uncut portion of the lid to provide access for an air line and to introduce food. The food access hole is closed with a No. 00 stopper. Greer (1993) cultures midges under static conditions with moderate aeration, and about 90% of the water is replaced weekly. Each 5.7-L culture chamber contains about 3 L of water and about 25 mL of fine sand. Eight to 10 chambers are used to maintain the culture.

10.4.6.1 Midges in each chamber are fed 2 mL/d of a 100 g/L Tetrafin® suspension on Tuesday, Wednesday, Thursday, Friday, and Sunday. A 2-mL chlorella suspension (deactivated "Algae-Feast® *Chlorella*", Earthrise Co., Callpatria, CA) is added to each chamber on Saturday and on Monday. The chlorella suspension is prepared by adding 5 g of dry chlorella powder/L of water. The mixture should be refrigerated and can be used for up to two weeks.

10.4.6.2 The water should be replaced more often if animals appear stressed (e.g., at surface or pale color at the second instar) or if the water is cloudy. Water is replaced by first removing emergent adults with an aspirator. Any growth on the sides of the chamber should be brushed off before water is removed. Care should be taken not to pour or siphon out the larvae when removing the water. Larvae will typically stay near the bottom; however, a small mesh sieve or nylon net can be used to catch any larvae that float out. After the chambers have been cleaned, temperature-adjusted culture water is poured back into each chamber. The water should be added quickly to stir up the larvae. Using this procedure, the approximate size, number, and the general health of the culture can be observed.

10.4.7 Adult emergence will begin about three weeks after hatching at 23°C. Once adults begin to emerge, they can be gently siphoned into a dry aspirator flask on a daily basis. An aspirator can be made using a 250- or 500-mL Erlenmeyer flask, a two-hole stopper, some short sections of 0.25 inch glass tubing, and Tygon® tubing for collecting and providing suction (Figure 10.2). Adults should be aspirated with short inhalations to avoid injuring the organisms. The mouth piece on the aspirator should be replaced or disinfected between use. Sex ratio of the adults should be checked to ensure that a sufficient number of males are available for mating and fertilization. One male may fertilize more than one female. However, a ratio of one female to three males ensures good fertilization.

10.4.7.1 A reproduction and oviposit chamber may be prepared in several different ways (Figure 10.2). Culture water (about 50 to 75 mL) can be added to the aspiration flask in which the adults were collected (Figure 10.2; Batac-Catalan and White, 1982). ERL-Duluth (USEPA, 1993) uses a 500-mL collecting flask with a length of Nitex® screen positioned vertically and extending into the culture water (Figure 10.2). The Nitex® screen is used by the females to position themselves just above the water during oviposition. The two-hole stopper and tubing of the aspirator should be replaced by screened material or a cotton plug for good air exchange in the oviposition chamber.

10.4.7.2 Greer (1993) used an oviposition box to hold emergent adults. The box is constructed of a 5.7-L chamber with a 20-cm tall cylindrical chamber on top. The top chamber is constructed of stainless steel screen (35 mesh/2.54 cm) melted onto a plastic lid with a 17.75 cm hole. A 5-cm hole is cut into the side of the bottom

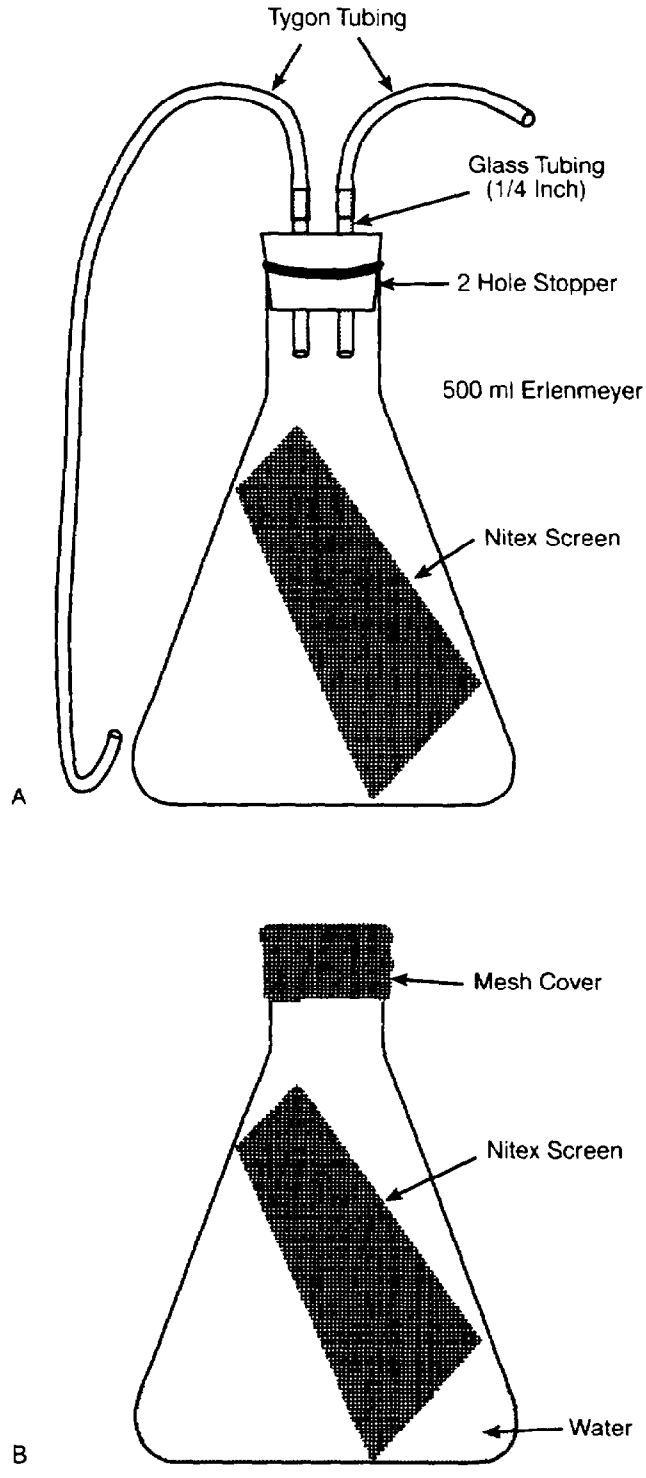


Figure 10.2 Aspirator chamber (A) and reproduction and oviposit chamber (B) for adult midges.

chamber and a #11 stopper is used to close the hole. Egg masses are removed by first sliding a piece of plexiglass between the top and bottom chambers. Adult midges are then aspirated from the bottom chamber. The top chamber with plexiglass is removed from the bottom chamber and a forceps is used to remove the egg masses. The top chamber is put back on top of the bottom chamber, the plexiglass is removed, and the aspirated adults are released from the aspirator into the chamber through the 5-cm hole.

10.4.8 About two to three weeks before the start of a test, at least 3 to 5 egg masses should be isolated for hatching using procedures outlined in Section 10.4.3.

10.4.9 Records should be kept on the time to first emergence and the success of emergence for each culture chamber. It is also desirable to monitor growth and head capsule width periodically in the cultures. See Section 10.2.6 for additional detail on procedures for evaluating the health of the cultures.

10.5 Culturing Procedures for *Lumbriculus variegatus*

10.5.1 The culturing procedures described below are based on methods described in Phipps et al. (1993), USEPA (1993), Call et al. (1994), E.L. Brunson (NBS, Columbia, MO, unpublished data), and Appendix A. Bioaccumulation tests are started with adult organisms.

10.5.2 *Lumbriculus variegatus* are generally cultured with daily renewal of water (57- to 80-L aquaria containing 45 to 50 L of water).

10.5.3 Paper towels can be used as a substrate for culturing *L. variegatus* (Phipps et al., 1993). Substrate is prepared by cutting unbleached brown paper towels into strips either with a paper shredder or with a scissors. Cut toweling is loosely packed into a 2-L beaker, submersed in acetone, covered and placed in a fume hood, and soaked overnight to solubilize organic contaminants. The acetone is discarded, and the towels are rinsed several times with deionized water. Deionized water is added, brought to a boil, and stirred three times to drive off the acetone vapors. This is repeated two more times. The strips are conditioned for at least one week by placing 4 L of strips into an aquarium equipped with two water lines each having a flow capacity of about 100 mL/min. One line is placed below and one line is placed above the towel mass. Glass weights (several 2.5-cm x 25.4-cm glass strips standing on edge and glued on both ends to glass strips about 50 cm in length) can be placed on the strips to prevent floating. This approach creates a uniform water flow throughout the strips and minimizes fouling. Following conditioning, the strips are removed and evenly placed on the bottom of the culture chamber. Glass weights keep the strips in place. Conditioned strips can be frozen in deionized water for later use. The substrate is renewed with conditioned towels when thin or bare areas appear. The substrate in the chamber will generally last for about two months.

10.5.4 Oligochaetes probably obtain nourishment from ingesting the organic matter in the substrate (Pennak, 1989). *Lumbriculus variegatus* in each of the culture chambers are fed a 10-mL suspension of 6 g of trout starter 3 times/week. The particles will temporarily disperse on the surface film, break through the surface tension, and settle out over the substrate. Laboratories using static systems should develop lower feeding rates specific to their systems. Food and substrate used to culture oligochaetes should be analyzed for compounds to be evaluated in bioaccumulation tests. If the concentration of the test compound is above the detection level and the food is not measured, the test may be invalidated. Recent studies in other laboratories, for example, have indicated elevated concentrations of PCBs in substrate and/or food used for culturing the oligochaete (J. Amato, AScI Corporation, Duluth, MN, personal communication).

10.5.5 Phipps et al. (1993) recommend starting a new culture with 500 to 1000 worms. Conditioned paper toweling should be added when the substrate in a culture chamber is thin.

10.5.6 On the day before the start of a test, oligochaetes can be isolated by transferring substrate from the cultures into a beaker using a fine mesh net. Additional organisms can be removed using a glass pipet (20 cm long, 5 mm i.d.) (Phipps et al., 1993). Water can be slowly trickled into the beaker. The oligochaetes will form a mass and most of the remaining substrate will be flushed from the beaker. On the day the test is started, organisms can be placed in glass or stainless-steel pans. A gentle stream of water from the pipet can be used to spread out clusters of oligochaetes. The remaining substrate can be siphoned from the pan by allowing the worms to reform in a cluster on the bottom of the pan. For bioaccumulation tests, aliquots of worms to be added to each test chamber can be transferred using a blunt dissecting needle or dental pick. Excess water can be removed during transfer by touching the mass of oligochaetes to the edge of the pan. The mass of oligochaetes is then placed in a tared weigh boat, quickly weighed, and immediately introduced into the appropriate test chamber. Organisms should not be blotted with a paper towel to remove excess water.

10.5.7 The culture population generally doubles (number of organisms) in about 10 to 14 d. See Section 10.2.6 for additional detail on procedures for evaluating the health of the cultures.

Section 11
Test Method 100.1
Hyalella azteca 10-d Survival Test for Sediments

11.1 Introduction

11.1.1 *Hyalella azteca* (Saussure) have many desirable characteristics of an ideal sediment toxicity testing organism including relative sensitivity to contaminants associated with sediment, short generation time, contact with sediment, ease of culture in the laboratory, and tolerance to varying physico-chemical characteristics of sediment. Their response has been evaluated in interlaboratory studies and has been confirmed with natural benthos populations. Many investigators have successfully used *H. azteca* to evaluate the toxicity of freshwater sediments (e.g., Nebeker et al., 1984a; Borgmann and Munwar, 1989; Ingersoll and Nelson, 1990; Ankley et al., 1991a, Ankley et al., 1991b; Burton et al., 1989; Winger and Lasier, 1993; Kemble et al., 1994). *H. azteca* has been used for a variety of sediment assessments (Ankley et al., 1991; West et al., 1993; Hoke et al., 1994; West et al., 1994; and Hoke et al., 1994). *Hyalella azteca* can also be used to evaluate the toxicity of estuarine sediments (up to 15 ‰ salinity; Nebeker and Miller, 1988; Roach et al., 1992, Winger et al., 1993). Endpoints typically monitored in sediment toxicity tests with *H. azteca* include survival and growth.

11.1.2 A specific test method for conducting a 10-d sediment toxicity test is described in Section 11.2 for *H. azteca*. Methods outlined in Appendix A and the literature cited in Table A.2 were used for developing test method 100.1. Results of tests using procedures different from the procedures described in Section 11.2 may not be comparable, and these different procedures may alter contaminant bioavailability. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in this manual, additional tests are required to determine comparability of results (Section 1.3).

11.2 Recommended Test Method for Conducting a 10-d Sediment Toxicity Test with *Hyalella azteca*

11.2.1 Recommended conditions for conducting a 10-d sediment toxicity test with *H. azteca* are summarized in Table 11.1. A general activity schedule is outlined in Table 11.2. Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Section 14). The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. When variability remains constant, the sensitivity of a test increases as the number of replicates increase.

11.2.2 The recommended 10-d sediment toxicity test with *H. azteca* must be conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux (Table 11.1). Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten 7- to 14-d old amphipods are used to start a test. The number of replicates/treatment depends on the objective of the test. Eight replicates are recommended for routine testing (Section 14). Amphipods in each test chamber are fed 1.5 mL of YCT food daily (Appendix C). Each chamber receives 2 volume additions/d of overlying water. Water renewals may be manual or automated, and Appendix B describes water-renewal systems that can be used to deliver overlying water. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected. Requirements for test acceptability are summarized in Table 11.3.

11.3 General Procedures

11.3.1 ***Sediment into Test Chambers:*** The day before the sediment test is started (Day -1) each sediment should be thoroughly mixed and added to the test chambers (Section 8.3.1). Sediment should be visually inspected to judge the degree of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components. If a quantitative measure of homogeneity is required, replicate subsamples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size.

11.3.1.1 Each test chamber should contain the same amount of sediment, determined either by volume or by

Table 11.1 Test Conditions for Conducting a 10-d Sediment Toxicity Test with *Hyalella azteca*

	Parameter	Conditions
1.	Test type:	Whole-sediment toxicity test with renewal of overlying water
2.	Temperature:	23 ± 1°C
3.	Light quality:	Wide-spectrum fluorescent lights
4.	Illuminance:	About 500 to 1000 lux
5.	Photoperiod:	16L:8D
6.	Test chamber:	300-mL high-form lipless beaker
7.	Sediment volume:	100 mL
8.	Overlying water volume:	175 mL
9.	Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10.	Age of organisms:	7- to 14-d old at the start of the test
11.	Number of organisms/chamber:	10
12.	Number of replicate chambers/treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing (see Section 14)
13.	Feeding:	YCT food, fed 1.5 mL daily to each test chamber
14.	Aeration:	None, unless dissolved oxygen in overlying water drops below 40% of saturation
15.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
16.	Test chamber cleaning:	If screens become clogged during a test, gently brush the *outside* of the screen (Appendix B)
17.	Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily
18.	Test duration:	10 d
19.	Endpoints:	Survival (growth optional)
20.	Test acceptability:	Minimum mean control survival of 80% and performance-based criteria specifications outlined in Table 11.3

Table 11.2 General Activity Schedule for Conducting a Sediment Toxicity Test with *Hyalella azteca* [1]

Day	Activity
-7	Separate known-age amphipods from the cultures and place in holding chambers. Begin preparing food for the test.
-6 to -2	Feed and observe isolated amphipods, monitor water quality (e.g., temperature and dissolved oxygen).
-1	Feed and observe isolated amphipods, monitor water quality. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer 10 7- to 14-day old amphipods into each test chamber. Release organisms under the surface of the water. Add 1.5 mL of YCT into each test chamber. Archive 20 test organisms for weight or length determination. Observe behavior of test organisms.
1 to 8	Add 1.5 mL of YCT food to each test chamber. Measure temperature and dissolved oxygen. Observe behavior of test organisms.
9	Same as Day 1. Measure total water quality.
10	Measure temperature and dissolved oxygen. End the test by collecting the amphipods with a sieve. Count survivors and set aside organisms for weight or length measurements.

[1] Modified from Call et al., 1994

Table 11.3 Test Acceptability Requirements for a 10-d Sediment Toxicity Test with *Hyalella azteca*

A. It is recommended for conducting a 10-d test with *Hyalella azteca* that the following performance criteria be met:

1. Age of *H. azteca* at the start of the test must be between 7- to 14-d old.
2. Average survival of *H. azteca* in the control sediment must be greater than or equal to 80% at the end of the test.
3. Hardness, alkalinity, pH, and ammonia in the overlying water within a treatment should not vary by more than 50% during the test.

B. Performance-based criteria for culturing *H. azteca* include

1. Laboratories should perform monthly 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms. If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test must be evaluated using a reference toxicant (Section 9.16).
2. Laboratories should track parental survival in the cultures and record this information using control charts if known-age cultures are maintained. Records should also be kept on the frequency of restarting cultures and the age of brood organisms.
3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly and the day before the start of a sediment test: pH, hardness, alkalinity, and ammonia. Dissolved oxygen should be measured weekly. Temperature should be recorded daily.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.
5. Physiological measurements such as lipid content might provide useful information regarding the health of the cultures.

C. Additional requirements:

1. All organisms in a test must be from the same source.
2. It is desirable to start tests soon after collection of sediment from the field (see Section 8.2 for additional detail).
3. All test chambers (and compartments) should be identical and should contain the same amount of sediment and overlying water.
4. Negative-control sediment and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms.
5. Test organisms must be cultured and tested at 23°C.
6. 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.
7. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

weight. Overlying water is added to the chambers in a manner that minimizes suspension of sediment. This can be accomplished by gently pouring water along the sides of the chambers or by pouring water onto a baffle (e.g., a circular piece of Teflon® with a handle attached) placed above the sediment to dissipate the force of the water. Renewal of overlying water is started on Day -1. A test begins when the organisms are added to the test chambers (Day 0).

11.3.2 ***Renewal of overlying water:*** Renewal of overlying water is required during a test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%. Mount and Brungs (1967) diluters have been modified for sediment testing, and other automated water delivery systems have also been used (Maki, 1977; Ingersoll and Nelson, 1990; Benoit et al., 1993; Zumwalt et al., 1994). The water-delivery system should be calibrated before a test is started to verify that the system is functioning properly. Renewal of overlying water is started on Day -1 before the addition of test organisms or food on Day 0. Appendix B describes water-renewal systems that can be used for conducting sediment tests.

11.3.2.1 In water-renewal tests with one to four volume additions of overlying water/d, water-quality characteristics generally remain similar to the inflowing water (Ingersoll and Nelson, 1990; Ankley et al., 1993); however, in static tests, water quality may change profoundly during the exposure (Shuba et al., 1978). For example, in static whole-sediment tests, the alkalinity, hardness, and conductivity of overlying water more than doubled in several treatments during a four-week exposure (Ingersoll and Nelson, 1990). Additionally, concentrations of metabolic products (e.g., ammonia) may also increase during static exposures, and these compounds can either be directly toxic to the test organisms or may contribute to the toxicity of the contaminants in the sediment. Furthermore, changes in water-quality characteristics such as hardness may influence the toxicity of many inorganic (Gauss et al., 1985) and organic (Mayer and Ellersieck, 1986) contaminants. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediment generally receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water.

11.3.3 ***Acclimation:*** Test organisms must be cultured and tested at 23°C. Ideally, test organisms should be cultured in the same water that will be used in testing. However, acclimation of test organisms to the test water is not required.

11.3.4 ***Placing Organisms in Test Chambers:*** Test organisms should be handled as little as possible. Amphipods should be introduced into the overlying water below the air-water interface. Test organisms can be pipetted directly into overlying water (Ankley et al., 1993). Alternatively, test organisms can be placed into 30-mL counting cups that are floated in the test chambers for 15 min before organisms are introduced into the overlying water (Ingersoll and Nelson, 1990). Length or weight should be measured on a subset of at least 20 organisms used to start the test.

11.3.5 ***Monitoring a Test:*** All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

11.3.5.1 ***Measurement of Overlying Water-quality Characteristics:*** Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Hardness, alkalinity, pH, conductivity, and ammonia in the overlying water with a treatment should not vary by more than 50% during a test.

11.3.5.1.1 Dissolved oxygen should be measured daily and should be between 40 and 100% saturation (ASTM, 1988a). If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Aeration can be used to maintain dissolved oxygen in the overlying water above 40% saturation. Dissolved oxygen and pH can be measure directly in the overlying water with a probe.

11.3.5.1.2 Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath or the exposure chamber should be continuously monitored. 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.

11.3.6 ***Feeding:*** Without addition of food, the test organisms may starve during exposures. However, the addition of the food may alter the availability of the contaminants in the sediment (Wiederholm et al., 1987; Harkey et al., 1994). Furthermore, if too much food is added to the test chamber or if the mortality of test organisms is high, fungal or bacterial growth may develop on the sediment surface. Therefore, the amount of food added to the test chambers is kept to a minimum.

11.3.6.1 Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 40% of saturation during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration (ASTM, 1994a). If feeding is suspended in one treatment, it should be suspended in all treatments. Detailed records of feeding rates and the appearance of the sediment surface should be made daily.

11.3.7 ***Ending a Test:*** Any of the surviving amphipods in the water column or on the surface of the sediment can be pipetted from the beaker before sieving the sediment. Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. Ankley et al. (1994a) recommend using a #25 sieve (710 µm mesh) to remove amphipods from sediment. Alternatively, Kemble et al. (1994) recommend sieving sediment using the following procedure: (1) pour about half of the overlying water through a #50 (300 µm) U.S. Standard mesh sieve, (2) swirl the remaining water to suspend the upper 1 cm of sediment, (3) pour this slurry through the #50 mesh sieve and wash the contents of the sieve into an examination pan, (4) rinse the coarser sediment remaining in the test chamber through a #40 (425 µm) mesh sieve and wash the contents of this second sieve into a second examination pan. Surviving test organisms can be removed from the two pans and preserved in 8% sugar formalin solution for growth measurements (Ingersoll and Nelson, 1990). NoTox® (Earth Safe Industries, Belle Mead, NJ) can be used as a substitute for formalin (Unger et al., 1993).

11.3.7.1 A consistent amount of time should be taken to examine sieved material for recovery of test organisms (e.g., 10 min/replicate). Laboratories should demonstrate that their personnel are able to recover an average of at least 90% of the organisms from whole sediment. For example, test organisms could be added to control or test sediments, and recovery could be determined after 1 h (Tomasovic et al., 1994).

11.3.8 ***Test Data:*** Survival is the primary endpoint recorded at the end of the 10-d sediment toxicity test with *H. azteca*. Measuring growth is optional; however, growth of amphipods may be a more sensitive toxicity endpoint compared to survival (Burton and Ingersoll, 1994; Kemble et al., 1994). The duration of the 10-d test started with 7- to 14-d old amphipods is not long enough to determine sexual maturation or reproductive effects.

11.3.8.1 Amphipod body length (±0.1 mm) can be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface. Ingersoll and Nelson (1990) describe the use of a digitizing system and microscope to measure lengths of *H. azteca*. Antennal segment number can also be used to estimate length or weight of amphipods (E.L. Brunson, NBS, Columbia, MO, personal communication). Wet or dry weight measurements have also been used to estimate growth of *H. azteca* (ASTM, 1994a). Dry weight of amphipods should be determined by pooling all living organisms from a replicate and drying the sample at about 60 to 90°C to a constant weight. The sample is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg to obtain mean weight per surviving organism per replicate.

11.4 Interpretation of Results

11.4.1 Section 14 describes general information for interpretation of test results. The following sections describe species-specific information that is useful in helping to interpret the results of sediment toxicity tests with *H. azteca*.

11.4.2 ***Age Sensitivity:*** The sensitivity of *H. azteca* appears to be relatively similar up to at least 24- to 26-d old organisms (Collyard et al., 1994). For example, the toxicity of diazinon, Cu, Cd, and Zn was similar in 96-h water-only exposures starting with 0- to 2-d old organisms through 24- to 26-d old organisms (Figure 11.1). The toxicity of alkylphenol ethoxylate (a surfactant) tended to increase with age. In general, this suggests that tests started with 7- to 14-d old amphipods would be representative of the sensitivity of *H. azteca* up to at least the adult life stage.

11.4.3 ***Grain Size:*** *Hyalella azteca* are tolerant of a wide range of substrates. Physico-chemical characteristics (e.g., grain size or TOC) of sediment were not significantly correlated to the response of *H. azteca* in toxicity tests in which organisms were fed (Section 10.1.1.8; Ankley et al., 1994a).

11.4.4 ***Isolating Organisms at the End of a Test:*** Quantitative recovery of young amphipods (e.g., 0- to 7-d old) is difficult given their small size (Figure 11.2, Tomasovic et al., 1994). Recovery of older and larger amphipods (e.g., 21-d old) is much easier. This was a primary reason for deciding to start 10-d tests with 7- to 14-d old amphipods (organisms are 17- to 24-d old at the end of the 10-d test).

11.4.5 ***Influence of Indigenous Organisms:*** Survival of *H. azteca* in 28-d tests was not reduced in the presence of oligochaetes in sediment samples (Reynoldson et al., 1994). However, growth of amphipods was reduced when high numbers of oligochaetes were placed in a sample. Therefore, it is important to determine the number and biomass of indigenous organisms in field-collected sediment in order to better interpret growth data (Reynoldson et al., 1994). Furthermore, presence of predators may also influence the response of test organisms in sediment (Ingersoll and Nelson, 1990).

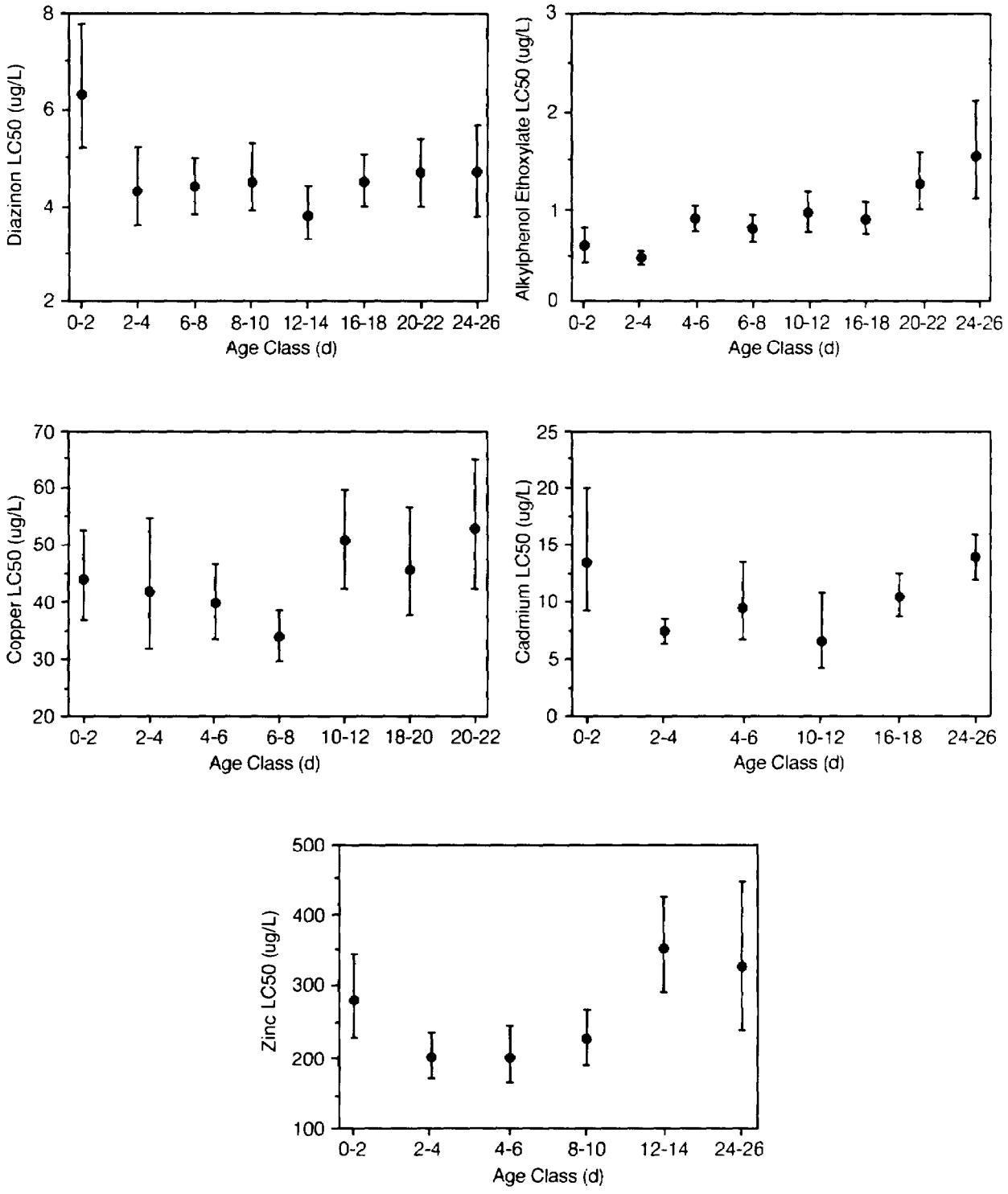


Figure 11.1 Lifestage sensitivity of *Hyalella azteca* in 96-h water-only exposures.

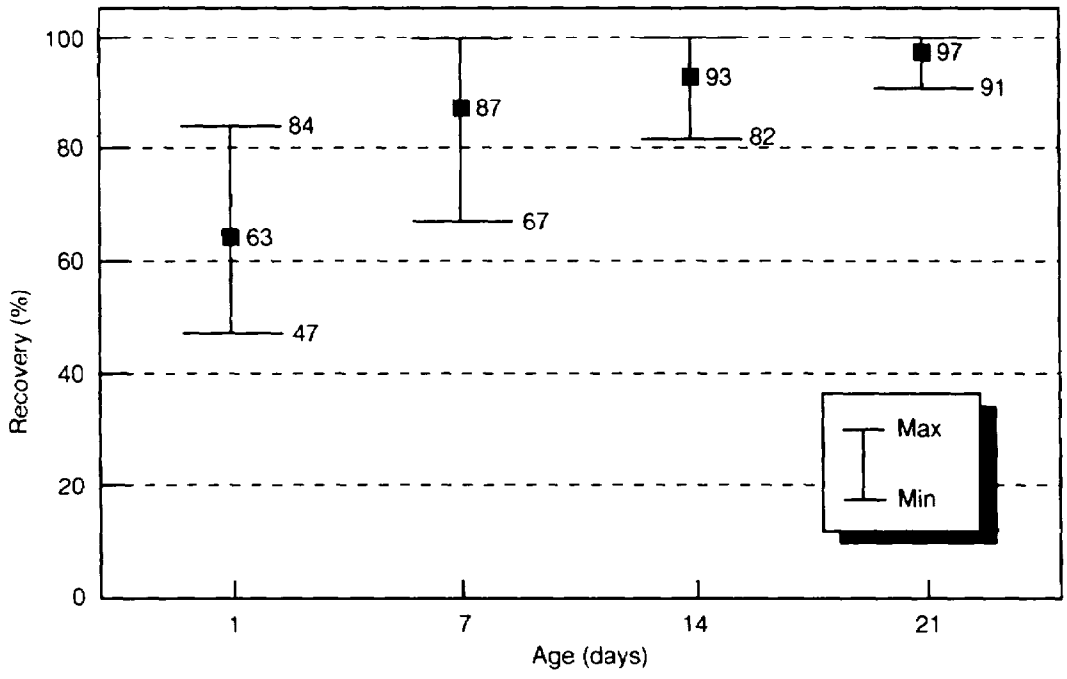


Figure 11.2 **Average recovery of different age *Hyalella azteca* from sediment by 7 individuals.**

Section 12
Test Method 100.2
Chironomus tentans 10-d Survival and Growth Test for Sediments

12.1 Introduction

12.1.1 *Chironomus tentans* (Fabricius) have many desirable characteristics of an ideal sediment toxicity testing organism including relative sensitivity to contaminants associated with sediment, contact with sediment, ease of culture in the laboratory, tolerance to varying physico-chemical characteristics of sediment, and short generation time. Their response has been evaluated in interlaboratory studies and has been confirmed with natural benthos populations. Many investigators have successfully used *C. tentans* to evaluate the toxicity of freshwater sediments (e.g., Wentsel et al., 1977; Nebeker et al., 1984a; Nebeker et al., 1988; Adams et al., 1985; Giesy et al., 1988; Hoke et al., 1990; West et al., 1993; Ankley et al., 1993; Ankley et al., 1994a; Ankley et al.,1994b). *C. tentans* has been used for a variety of sediment assessments (West et al., 1993; Hoke et al., 1994; West et al., 1994; and Ankley et al., 1994c). Endpoints typically monitored in sediment toxicity tests with *C. tentans* include survival and growth (ASTM, 1994a).

12.1.2 A specific test method for conducting a 10-d sediment toxicity test is described in Section 12.2 for *C. tentans*. Methods outlined in Appendix A and the literature cited in Table A.3 were used for developing test method 100.2. Results of tests using procedures different from the procedures described in Section 12.2 may not be comparable and these different procedures may alter contaminant bioavailability. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in this manual, additional tests are required to determine comparability of results (Section 1.3).

12.2 Recommended Test Method for Conducting a 10-d Sediment Toxicity Test with *Chironomus tentans*

12.2.1 Recommended conditions for conducting a 10-d sediment toxicity test with *C. tentans* are summarized in Table 12.1. A general activity schedule is outlined in Table 12.2. Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Section 14). The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. When variability remains constant, the sensitivity of a test increases as the number of replicates increases.

12.2.2 The recommended 10-d sediment toxicity test with *C. tentans* must be conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux (Table 12.1). Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten third-instar midges are used to start a test. All organisms must be third instar or younger with at least 50% of the organisms at third instar. The number of replicates/treatment depends on the objective of the test. Eight replicates are recommended for routine testing (see Section 14). Midges in each test chamber are fed 1.5 mL of a 4-g/L Tetrafin® suspension daily. Each chamber receives 2 volume additions/d of overlying water. Water renewals may be manual or automated, and Appendix B describes water-renewal systems that can be used to deliver overlying water. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected. Requirements for test acceptability are summarized in Table 12.3.

12.3 General Procedures

12.3.1 ***Sediment into Test Chambers:*** The day before the sediment test is started (Day -1) each sediment should be thoroughly mixed and added to the test chambers (Section 8.3.1). Sediment should be visually inspected to judge the extent of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components. If a quantitative measure of homogeneity is required, replicate subsamples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size.

Table 12.1 Recommended Test Conditions for Conducting a 10-d Sediment Toxicity Test with *Chironomus tentans*

	Parameter	Conditions
1.	Test type:	Whole-sediment toxicity test with renewal of overlying water
2.	Temperature:	23 ± 1°C
3.	Light quality:	Wide-spectrum fluorescent lights
4.	Illuminance:	About 500 to 1000 lux
5.	Photoperiod:	16L:8D
6.	Test chamber:	high-form lipless beaker
7.	Sediment volume:	100 mL
8.	Overlying water volume:	175 mL
9.	Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10.	Age of organisms:	Third instar larvae (All organisms must be third instar or younger with at least 50% of the organisms at third instar)
11.	Number of organisms/ chamber:	10
12.	Number of replicate chambers/treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing (see Section 14)
13.	Feeding:	Tetrafin® goldfish food, fed 1.5 mL daily to each test chamber (1.5 mL contains 4.0 mg of dry solids)
14.	Aeration:	None, unless dissolved oxygen in overlying water drops below 40% of saturation
15.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
16.	Test chamber cleaning:	If screens become clogged during a test; gently brush the *outside* of the screen (Appendix B)
17.	Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily
18.	Test duration:	10 d
19.	Endpoints:	Survival and growth (dry weight)
20.	Test acceptability:	Minimum mean control survival of 70% and mean weight per surviving control organism of 0.6 mg. Performance-based criteria specifications outlined in Table 12.3

12.3.1.1 Each test chamber should contain the same amount of sediment, determined either by volume or by weight. Overlying water is added to the chambers in a manner that minimizes suspension of sediment. This can be accomplished by gently pouring water along the sides of the chambers or by pouring water onto a baffle (e.g., a circular piece of Teflon with a handle attached) placed above the sediment to dissipate the force of the water. Renewal of overlying water is started on Day -1. A test begins when the organisms are added to the test chambers (Day 0).

12.3.2 ***Renewal of overlying water:*** Renewal of overlying water is required during a test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%. Mount and Brungs (1967) diluters have been modified for sediment testing, and other automated water delivery systems have also been used (Maki, 1977; Ingersoll and Nelson, 1990; Benoit et al., 1993; Zumwalt et al., 1994). Each water-delivery system should be calibrated before a test is started to verify that the system is functioning properly. Renewal of overlying water is started on Day -1 before the addition of test organisms or food on Day 0. Appendix B describes water-renewal systems that can be used for conducting sediment tests.

12.3.2.1 In water-renewal tests with one to four volume additions of overlying water/d, water-quality characteristics generally remain similar to the inflowing water (Ingersoll and Nelson, 1990; Ankley et al., 1993); however, in static tests, water quality may change profoundly during the exposure (Shuba et al., 1978). For example, in static whole-sediment tests, the alkalinity, hardness, and conductivity of overlying water more than doubled in several treatments during a four-week exposure (Ingersoll and Nelson, 1990). Additionally, concentrations of metabolic products (e.g., ammonia) may also increase during static exposures, and these compounds can either be directly toxic to the test organisms or may

Table 12.2 General Activity Schedule for Conducting a Sediment Toxicity Test with *Chironomus tentans*[1]

Day	Activity
-14	Isolate adults for production of egg masses.
-13	Place newly deposited egg masses into hatching dishes.
-12	A larval rearing chamber is prepared with new substrate.
-11	Examine egg masses for hatching success. If egg masses have hatched, transfer first instar larvae and any remaining unhatched embryos from the crystallizing dishes into the larval rearing chamber. Feed organisms.
-10	Same as Day -11.
-9 to -2	Feed and observe midges. Measure water quality (e.g., temperature and dissolved oxygen).
-1	Add food to each larval rearing chamber and measure temperature and dissolved oxygen. Add sediment into each test chamber, place chamber into exposure system, and start renewing overlying water.
0	Measure total water quality (temperature, pH, hardness, alkalinity, dissolved oxygen, conductivity, ammonia). Remove third-instar larvae from the culture chamber substrate. Add 1.5 mL of Tetrafin® (4.0 g/L) into each test chamber. Transfer 10 larvae into each test chamber. Release organisms under the surface of the water. Archive 20 test organisms for instar determination and weight or length determination. Observe behavior of test organisms.
1 to 8	Add 1.5 mL of food to each test chamber. Measure temperature and dissolved oxygen. Observe behavior of test organisms.
9	Same as Day 1. Measure total water quality.
10	Measure temperature and dissolved oxygen. End the test by collecting the midges with a sieve. Measure weight or length of surviving larvae.

[1] Modified from Call et al., 1994

contribute to the toxicity of the contaminants in the sediment. Furthermore, changes in water-quality characteristics such as hardness may influence the toxicity of many inorganic (Gauss et al., 1985) and organic (Mayer and Ellersieck, 1986) contaminants. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediment generally receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water.

12.3.3 ***Acclimation:*** Test organisms must be cultured and tested at 23°C. Ideally, test organisms should be cultured in the same water that will be used in testing. However, acclimation of test organisms to the test water is not required.

12.3.4 ***Placing Organisms in Test Chambers:*** Test organisms should be handled as little as possible. Midges should be introduced into the overlying water below the air-water interface. Test organisms can be pipetted directly into overlying water (Ankley et al., 1993). Alternatively, test organisms can be placed into 30-mL counting cups that are floated in the test chambers for 15 min before organisms are introduced into the overlying water (Ingersoll and Nelson, 1990). Length or weight should be measured on a subset of at least 20 organisms used to start the test. Head capsule width of midges must be measured on this subset of test organisms to determine the instar used to start the test (Table 10.2).

12.3.5 ***Monitoring a Test:*** All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

12.3.5.1 *Measurement of Overlying Water-quality Characteristics:* Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Hardness, alkalinity, pH, conductivity, and ammonia in the overlying water within a treatment should not vary by more than 50% during a test.

12.3.5.1.1 Dissolved oxygen should be measured daily and should be between 40 and 100% saturation (ASTM, 1988a). If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Aeration can be used to maintain dissolved oxygen in the overlying water above 40% saturation. Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

12.3.5.1.2 Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath or the exposure

Table 12.3 Test Acceptability Requirements for a 10-d Sediment Toxicity Test with *Chironomus tentans*

A. It is recommended for conducting a 10-d test with *C. tentans* that the following performance criteria be met:

1. Tests must be started with third-instar and younger larvae. At least 50% of the larvae must be in the third instar at the start of the test.
2. Average survival of *C. tentans* in the control sediment must be greater than or equal to 70% at the end of the test.
3. Average size of *C. tentans* in the control sediment must be at least 0.6 mg at the end of the test.
4. Hardness, alkalinity, pH, and ammonia in the overlying water within a treatment should not vary by more than 50% during the test.

B. Performance-based criteria for culturing *C. tentans* include

1. Laboratories should perform monthly 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms. If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test must be evaluated using a reference toxicant (Section 9.16).
2. Laboratories should keep a record of time to first emergence for each culture and record this information using control charts. Records should also be kept on the frequency of restarting cultures.
3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly and the day before the start of a sediment test: pH, hardness, alkalinity, and ammonia. Dissolved oxygen should be measured weekly. Temperature should be recorded daily.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.
5. Physiological measurements such as lipid content might provide useful information regarding the health of the cultures.

C. Additional requirements:

1. All organisms in a test must be from the same source.
2. It is desirable to start tests soon after collection of sediment from the field (see Section 8.2 for additional detail).
3. All test chambers (and compartments) should be identical and should contain the same amount of sediment and overlying water.
4. Negative-control sediment and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms.
5. Test organisms must be cultured and tested at 23°C.
6. 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.
7. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

chamber should be continuously monitored. 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.

12.3.6 ***Feeding:*** Without addition of food, the test organisms may starve during exposures. However, the addition of the food may alter the availability of the contaminants in the sediment (Wiederholm et al., 1987; Harkey et al., 1994). Furthermore, if too much food is added to the test chamber or if the mortality of test organisms is high, fungal or bacterial growth may develop on the sediment surface. Therefore, the amount of food added to the test chambers is kept to a minimum.

12.3.6.1 Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 40% of saturation during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration (ASTM, 1994a). If feeding is suspended in one treatment, it should be suspended in all treatments. Detailed records of feeding rates and the appearance of the sediment surface should be made daily.

12.3.7 ***Ending a Test:*** Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. Ankley et al. (1994a) recommend using a #25 sieve (710 µm mesh) to remove midges from sediment. Alternatively, Kemble et al. (1994) recommend sieving sediment using the following procedure: (1) pour about half of the overlying water through a #50 (300 µm) U.S. Standard mesh sieve, (2) pour about half of the sediment through the #50 mesh sieve and wash the contents of the sieve into an examination pan, (3) rinse the coarser sediment remaining in the test

chamber through a #40 (425 µm) mesh sieve and wash the contents of this second sieve into a second examination pan. Surviving midges can then be isolated from these pans. See Section 12.3.8.1 and 12.3.8.2 for the procedures for measuring weight or length of midges.

12.3.7.1 A consistent amount of time should be taken to examine sieved material for recovery of test organisms (e.g., 10 min/replicate). Laboratories should demonstrate that their personnel are able to recover an average of at least 90% of the organisms from whole sediment. For example, test organisms could be added to control sediment and recovery could be determined after 1 h (Tomasovic et al., 1994).

12.3.8 ***Test Data:*** Dry weight and survival are the endpoints measured at the end of the 10-d sediment toxicity test with *C. tentans*. The duration of the 10-d test starting with third instar larvae is not long enough to determine emergence of adults. Average size of *C. tentans* in the control sediment must be at least 0.6 mg at the end of the test (Ankley et al., 1993; ASTM, 1994b; Section 15).

12.3.8.1 Head capsule width can be measured on surviving midges at the end of the test before dry weight is determined. Dry weight of midges should be determined by pooling all living larvae from a replicate and drying the sample at about 60 to 90°C to a constant weight. The sample is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg to obtain mean weights per surviving organism per replicate. Pupae or adult organisms must not be included in the sample to estimate dry weight.

12.3.8.2 Measurement of length is optional. Separate replicate beakers should be set up to sample lengths of midges at the end of an exposure. An 8% sugar formalin solution can be used to preserve samples for length measurements (Ingersoll and Nelson, 1990). NoTox® (Earth Safe Industries, Belle Mead, NJ) can be used as a substitute for formalin (Unger et al., 1993). Midge body length (±0.1 mm) can be measured from the anterior of the labrum to the posterior of the last abdominal segment (Smock, 1980). Kemble et al. (1994) photographed midges at magnification of 3.5x and measured the images using a computer-interfaced digitizing tablet. A digitizing system and microscope can also be used to measure length (Ingersoll and Nelson, 1990).

12.4 Interpretation of Results

12.4.1 Section 14 describes general information for interpretation of test results. The following sections describe species-specific information that is useful in helping to interpret the results of sediment toxicity tests with *C. tentans*.

12.4.2 ***Age Sensitivity:*** Midges are perceived to be relatively insensitive organisms in toxicity assessments (Ingersoll, 1994). This conclusion is based on the practice of conducting short-term tests with 4th instar larvae in water-only exposures, a procedure that may underestimate the sensitivity of midges to toxicants. The first and second instars of chironomids are more sensitive to contaminants than the third or fourth instars. For example, first instar *C. tentans* larvae were 6 to 27 times more sensitive than 4th instar larvae to acute copper exposure (Nebeker et al., 1984b; Gauss et al., 1985; Figure 12.1) and first instar *C. riparius* larvae were 127 times more sensitive than second instar larvae to acute cadmium exposure (Williams et al., 1986b; Figure 12.1). In chronic tests with first instar larvae, midges were often as sensitive as daphnids to inorganic and organic compounds (Ingersoll et al., 1990). Sediment tests should be started with uniform age and size midges because of the dramatic differences in sensitivity of midges by age. While third instar midges are not as sensitive as younger organisms, the larger larvae are easier to handle and isolate from sediment at the end of a test.

12.4.3 ***Grain Size:*** *Chironomus tentans* are tolerant of a wide range of substrates. Physicochemical characteristics (e.g., grain size or TOC) of sediment were not significantly correlated to the survival of *C. tentans* in toxicity tests in which organisms were fed. However, linear modeling indicated that growth of *C. tentans* may have been slightly influenced by grain size distribution of the test sediments (Section 10.1.2.3; Ankley et al., 1994a). Survival of *C. tentans* was reduced below 0.91% organic matter in 10-d tests with formulated sediment (Suedel and Rodgers, 1994); however these organisms did not receive a supplemental source of nutrition.

12.4.4 ***Isolating Organisms at the End of a Test:*** Quantitative recovery of larvae at the end of a 10-d sediment test should not be a problem. The larvae are red and typically greater than 5-mm long.

12.4.5 ***Influence of Indigenous Organisms:*** The influence of indigenous organisms on the response of *C. tentans* in sediment tests has not been reported. Survival of a closely related species, *C. riparius* was not reduced in the presence of oligochaetes in sediment samples (Reynoldson et al., 1994). However, growth of *C. riparius* was reduced when high numbers of oligochaetes were placed in a sample. Therefore, it is important to determine the number and biomass of indigenous organisms in field-collected sediment in order to better interpret growth data (Reynoldson et al., 1994). Furthermore, presence of predators may also influence the response of test organisms in sediment (Ingersoll and Nelson, 1990).

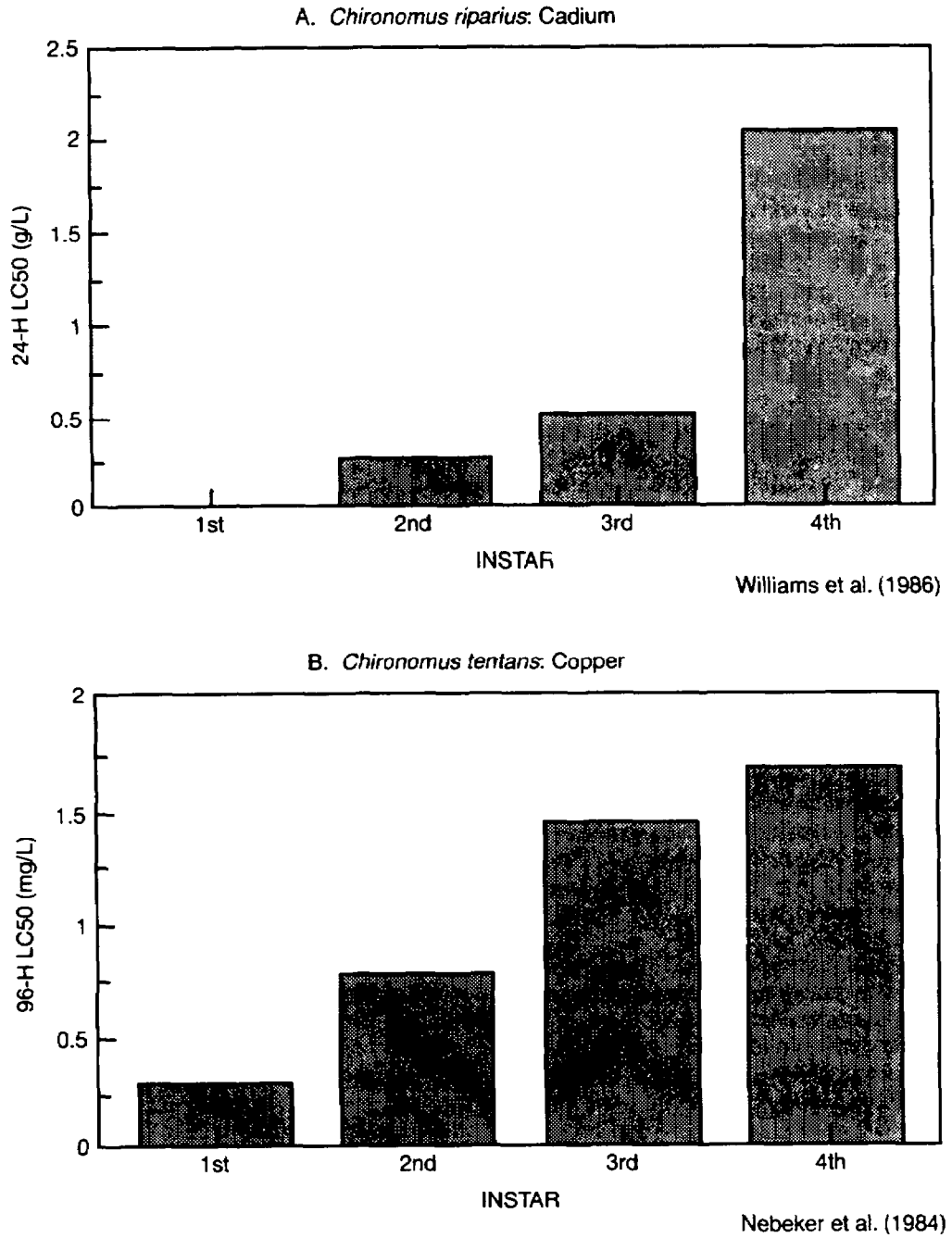


Figure 12.1 Lifestage sensitivity of chironomids.

Section 13
Test Method 100.3
Lumbriculus variegatus Bioaccumulation Test for Sediments

13.1 Introduction

13.1.1 *Lumbriculus variegatus* (Oligochaeta) have many desirable characteristics of an ideal sediment bioaccumulation testing organism including contact with sediment, ease of culture in the laboratory, and tolerance to varying physico-chemical characteristics of sediment. The response of *L. variegatus* in laboratory exposures has been confirmed with natural benthos populations. Many investigators have successfully used *L. variegatus* in toxicity or bioaccumulation tests. Toxicity studies have been conducted in water-only tests (Bailey and Liu, 1980; Hornig, 1980; Ewell et al., 1986; Nebeker et al., 1989; Ankley et al., 1991a, Ankley et al., 1991b), in effluent tests (Hornig, 1980), and in whole-sediment tests (Nebeker et al., 1989; Ankley et al., 1991a, Ankley et al., 1991b; Ankley et al., 1992a; Call et al., 1991; Carlson et al., 1991; Phipps et al., 1993; West et al., 1993). Several studies have reported the use of *L. variegatus* to examine bioaccumulation of chemicals from sediment (Schuytema et al., 1988; Nebeker et al., 1989; Ankley et al., 1991b; Call et al., 1991; Carlson et al., 1991; Ankley et al., 1993; Kukkonen and Landrum, 1994; and E.L. Brunson, NBS, Columbia, MO, unpublished data). However, interlaboratory studies have not yet been conducted with *L. variegatus*.

13.1.2 Additional research is needed on the standardization of bioaccumulation procedures with sediment. Therefore, Section 13.2 describes general guidance for conducting a 28-d sediment bioaccumulation test with *L. variegatus*. Methods outlined in Appendix A and the literature cited in Table A.4 were used for developing this general guidance. Results of tests using procedures different from the procedures described in Section 13.2 may not be comparable, and these different procedures may alter bioavailability. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in this manual, additional tests are required to determine comparability of results (Section 1.3).

13.2 Procedure for Conducting Sediment Bioaccumulation Tests with *Lumbriculus variegatus*

13.2.1 Recommended test conditions for conducting a 28-d sediment bioaccumulation test with *L. variegatus* are summarized in Table 13.1. Table 13.2 outlines procedures for conducting sediment toxicity tests with *L. variegatus*. A general activity schedule is outlined in Table 13.3. Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Section 14). The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. When variability remains constant, the sensitivity of a test increases as the number of replicates increases.

13.2.2 The recommended 28-d sediment bioaccumulation test with *L. variegatus* can be conducted with adult oligochaetes at 23°C with a 16L:8D photoperiod at a illuminance of about 500 to 1000 lux (Table 13.1). Test chambers can be 4 to 6 L that contain 1 to 2 L of sediment and 1 to 4 L of overlying water. The number of replicates/treatment depends on the objective of the test. Five replicates are recommended for routine testing (Section 14). To minimize depletion of sediment contaminants, the ratio of total organic carbon in sediment to dry weight of organisms should be about 50:1. A minimum of 1 g/replicate with up to 5 g/replicate should be tested. Oligochaetes are not fed during the test. Each chamber receives 2 volume additions/d of overlying water. Appendix B describes water-renewal systems that with minor modifications can be used to deliver overlying water. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected. Requirements for test acceptability are outlined in Table 13.4.

13.2.2.1 Before starting a 28-d sediment bioaccumulation test with *L. variegatus,* a toxicity screening test should be conducted for at least 4 d using procedures outlined in Table 13.2 (E.L. Brunson, NBS, Columbia,

Table 13.1 Recommended Test Conditions for Conducting a 28-d Sediment Bioaccumulation Test with *Lumbriculus variegatus*

Parameter	Conditions
1. Test type:	Whole-sediment bioaccumulation test with renewal of overlying water
2. Temperature:	23 ± 1°C
3. Light quality:	Wide-spectrum fluorescent lights
4. Illuminance:	About 500 to 1000 lux
5. Photoperiod:	16L:8D
6. Test chamber:	4- to 6-L aquaria with stainless steel screens or glass standpipes
7. Sediment volume:	1 L or more depending on TOC
8. Overlying water volume:	1 L or more depending on TOC
9. Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10. Age of test organisms:	Adults
11. Loading of organisms in chamber:	Ratio of total organic carbon in sediment to organism dry weight should be no less than 50:1. Minimum of 1 g/replicate. Preferably 5 g/replicate
12. Number of replicate chambers/treatment:	Depends on the objective of the test. Five replicates are recommended for routine testing (see Section 14)
13. Feeding:	None
14. Aeration:	None, unless dissolved oxygen in overlying water drops below 40% of saturation
15. Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
16. Test chamber cleaning:	If screens become clogged during the test, gently brush the *outside* of the screen (Appendix B)
17. Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily
18. Test duration:	28 d
19. Endpoint:	Bioaccumulation
20. Test acceptability:	Performance-based criteria specifications outlined in Table 13.4.

MO, unpublished data). The preliminary toxicity screening test is conducted at 23°C with a 16L:8D photoperiod at a illuminance of about 500 to 1000 lux. Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten adult oligochaetes/replicate are used to start a test. Four replicates are recommended for routine testing. Oligochaetes are not fed during the test. Each chamber receives 2 volume additions/d of overlying water. Appendix B describes water-renewal systems that can be used to deliver overlying water. Overlying water should be similar to the water to be used in the bioaccumulation test. Endpoints monitored at the end of a toxicity test are number of organisms and behavior. Numbers of *L. variegatus* in the toxicity screening test should not be significantly reduced in the test sediment relative to the control sediment. Test organisms should burrow into test sediment. Avoidance of test sediment by *L. variegatus* may decrease bioaccumulation.

13.3 General Procedures

13.3.1 ***Sediment into Test Chambers:*** The day before the sediment test is started (Day -1) each sediment should be thoroughly mixed and added to the test chambers (Section 8.3.1). Sediment should be visually inspected to judge the extent of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components. If a quantitative measure of homogeneity is required, replicate subsamples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size.

13.3.1.1 Each test chamber should contain the same amount of sediment, determined either by volume or by weight. Overlying water is added to the chambers in a manner that minimizes suspension of sediment. This can be accomplished by gently pouring water along the sides of the chambers or by pouring water onto a baffle (e.g., a circular piece of Teflon® with a handle attached) placed above the sediment to dissipate the force of the water. Renewal of overlying water is started on Day -1. A test begins when the organisms are added to the test chambers (Day 0).

13.3.2 ***Renewal of Overlying Water:*** Renewal of overlying water is recommended during a test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%. Mount and Brungs (1967) diluters have been modified for sediment testing, and other automated water delivery systems have also been used (Maki, 1977; Ingersoll and Nelson, 1990; Benoit et al., 1993; Zumwalt et al., 1994). Each water-delivery system should be calibrated before a test is started to verify that the system is functioning properly. Renewal of overlying water is started on Day

Table 13.2 Recommended Test Conditions for Conducting a Preliminary 4-d Sediment Toxicity Screening Test with *Lumbriculus variegatus*

Parameter	Conditions
1. Test type:	4-d Whole-sediment toxicity test with renewal of overlying water
2. Temperature:	23 ± °C
3. Light quality:	Wide-spectrum fluorescent lights
4. Illuminance:	about 500 to 1000 lux
5. Photoperiod:	16L:8D
6. Test chamber:	300-mL high-form lipless beaker
7. Sediment volume:	100 mL
8. Overlying water volume:	175 mL
9. Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10. Age of test organisms:	Adults
11. Number of organisms/chamber:	10
12. Number of replicate chambers/treatment:	4 minimum
13. Feeding:	None
14. Aeration:	None, unless dissolved oxygen in overlying water drops below 40% of saturation
15. Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
16. Test chamber cleaning:	If screens become clogged during the test, gently brush the *outside* of the screen
17. Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily
18. Test duration:	4 d (minimum; up to 10 d)
19. Endpoints:	Number of organisms and behavior. There should be no significant reduction in number of organisms in a test sediment relative to the control
20. Test acceptability:	Performance-based criteria specifications outlined in Table 13.4

-1 before the addition of test organisms or food on Day 0 (Appendix B).

13.3.2.1 In water-renewal tests with one to four volume additions of overlying water/d, water-quality characteristics generally remain similar to the inflowing water (Ingersoll and Nelson, 1990; Ankley et al., 1993); however, in static tests, water quality may change profoundly during the exposure (Shuba et al., 1978). For example, in static whole-sediment tests, the alkalinity, hardness, and conductivity of overlying water more than doubled in several treatments during a four-week exposure (Ingersoll and Nelson, 1990). Additionally, concentrations of metabolic products (e.g., ammonia) may also increase during static exposures, and these compounds can either be directly toxic to the test organisms or may contribute to the toxicity of the contaminants in the sediment. Furthermore, changes in water-quality characteristics such as hardness may influence the toxicity of many inorganic (Gauss et al., 1985) and organic (Mayer and Ellersieck, 1986) contaminants. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediment generally receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water.

13.3.3 ***Acclimation:*** Test organisms must be cultured and tested at 23°C. Ideally, test organisms should be cultured in the same water that will be used in testing. However, acclimation of test organisms to the test water is not required.

13.3.4 ***Placing Organisms in Test Chambers:*** Isolate oligochaetes for starting a test as described in Section 10.5.6.. A subset of *L. variegatus* at the start the test should be sampled to determine starting concentrations of contaminants of concern. Mean group weights should be measured on a subset of at least 100 organisms used to start the test. The ratio of total organic carbon in sediment to dry weight of organisms at the start of the test should be no less than 50:1.

13.3.4.1 Oligochaetes added to each replicate should not be blotted to remove excess water (Section 10.5.6). Oligochaetes can be added to each replicate at about 1.33x of the target stocking weight (E.L. Brunson, NBS, Columbia, MO, unpublished data). This additional 33% should account for the excess weight from water in the sample of nonblotted oligochaetes at the start of the test.

Table 13.3 General Activity Schedule for Conducting a 28-d Sediment Bioaccumulation Test with *Lumbriculus variegatus*

A. Conducting a 4-d Toxicity Screening Test (conducted before the 28-d bioaccumulation test)

Day	*Activity*
-1	Isolate worms for conducting toxicity screening test. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer 10 worms into each test chamber. Measure weight of a subset of 20 organisms used to start the test. Observe behavior of test organisms.
1-2	Measure temperature and dissolved oxygen. Observe behavior of test organisms.
3	Same as Day 1. Measure total water quality.
4	Measure temperature and dissolved oxygen. End the test by collecting the oligochaetes with a sieve and determine weight of survivors. Bioaccumulation tests should not be conducted with *L. variegatus* if a test sediment significantly reduces number of oligochaetes relative to the control sediment or if oligochaetes avoid the sediment.

B. Conducting a 28-d Bioaccumulation Test

Day	*Activity*
-1	Isolate worms for conducting bioaccumulation test. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer appropriate amount of worms (based on weight) into each test chamber. Sample a subset of worms used to start the test for residue analyses. Observe behavior of test organisms.
1-6	Measure temperature and dissolved oxygen. Observe behavior of test organisms.
7	Same as Day 1. Measure total water quality.
8-13	Same as Day 1
14	Same as Day 7
15-20	Same as Day 1
21	Same as Day 7
22-26	Same as Day 1
27	Same as Day 1. Measure total water quality.
28	Measure temperature and dissolved oxygen. End uptake by collecting the worms with a sieve. Separate any indigenous organisms from *L. variegatus*. Determine weight of survivors. Eliminate gut contents of surviving worms in water for 24 h.
29	Sample surviving worms after 24 h of elimination for chemical analysis.

13.3.5 ***Monitoring a Test:*** All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

13.3.5.1 ***Measurement of Overlying Water-quality Characteristics:*** Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Hardness, alkalinity, pH, conductivity, and ammonia in the overlying water within a treatment should not vary by more than 50% during a test.

13.3.5.1.1 Dissolved oxygen should be measured daily and should be between 40 and 100% saturation (ASTM, 1988a). If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Aeration can be used to maintain dissolved oxygen in the overlying water above 40% saturation. Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

13.3.5.1.2 Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath or the exposure chamber should be continuously monitored. 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.

Table 13.4 Test Acceptability Requirements for a 28-d Sediment Bioaccumulation Test with *Lumbriculus variegatus*

A. It is recommended for conducting a 28-d test with *L. variegatus* that the following performance criteria be met:

1. Numbers of *L. variegatus* in a 4-d toxicity screening test should not be significantly reduced in the test sediment relative to the control sediment.
2. Test organisms should burrow into test sediment. Avoidance of test sediment by *L. variegatus* may decrease bioaccumulation.
3. Hardness, alkalinity, pH, and ammonia in the overlying water within a treatment should not vary by more than 50% during the test.

B. Performance-based criteria for culturing *L. variegatus* include:

1. Laboratories should perform monthly 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms. If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test must be evaluated using a reference toxicant (Section 9.16).
2. Laboratories should monitor the frequency with which the population is doubling in the culture (number of organisms) and record this information using control charts (doubling rate would need to be estimated on a subset of animals from a mass culture). Records should also be kept on the frequency of restarting cultures.
3. Food used to culture organisms should be analyzed before the start of a test for compounds to be evaluated in the bioaccumulation test.
4. Laboratories should record the following water-quality characteristics of the cultures at least quarterly and the day before the start of a sediment test: pH, hardness, alkalinity, and ammonia. Dissolved oxygen should be measured weekly. Temperature should be recorded daily.
5. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.
6. Physiological measurements such as lipid content might provide useful information regarding the health of the cultures.

C. Additional requirements:

1. All organisms in a test must be from the same source.
2. It is desirable to start tests soon after collection of sediment from the field (see Section 8.2 for additional detail).
3. All test chambers (and compartments) should be identical and should contain the same amount of sediment and overlying water.
4. Negative-control sediment and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms.
5. Test organisms must be cultured and tested at 23°C.
6. 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.
7. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

13.3.6 ***Feeding:*** *Lumbriculus variegatus* should not be fed during a bioaccumulation test.

13.3.7 ***Ending a Test:*** Sediment at the end of the test can be sieved through a fine-meshed screen sufficiently small to retain the oligochaetes (e.g., U.S. Standard Sieve 35 (500 µm mesh) or 40 (425 µm mesh)). The sieved material should be quickly transferred to a shallow pan to keep oligochaetes from moving through the screen. Immobile organisms should be considered dead. Live oligochaetes are transferred to a 1-L beaker containing overlying water without sediment for 24 h to eliminate gut contents. Oligochaetes should not be placed in clean sediment to eliminate gut contents. Clean sediment can add 15 to 20% to the dry weight of the oligochaetes which would result in a dilution of contaminant concentrations on a dry weight basis (Peter Landrum, NOAA, Ann Arbor, MI, personal communication). The elimination beakers may need to be aerated to maintain dissolved oxygen above 40% of saturation. Worms will clear more than 90% of the gut contents in 24 h (Call et al., 1991). Following the 24-h elimination period, oligochaetes should be collected, placed in a tared weigh boat, blotted to remove excess water, and weighed to determine wet weight. Each sample should then be split into appropriate aliquots (e.g., metals, organics), placed in clean containers, and frozen for later analysis. Containers should be placed inside secondary freezer containers to minimize "freezer burn" or dehydration during storage.

13.3.7.1 Field-collected sediments may include indigenous oligochaetes. The behavior and appearance of indigenous oligochaetes are usually different from *L. variegatus*. It may be desirable to test extra chambers without the addition of *L. variegatus* to check for the presence of indigenous oligochaetes in field-collected sediment (Phipps et al., 1993). Bioaccumulation of con-

Table 13.5 Grams of *Lumbriculus variegatus* Tissue (Wet Weight) Required for Various Analytes at Selected Lower Limits of Detection

Analyte	Grams of Tissue 1.0	2.0	5.0
	Lower Limit of Detection (µg/g)		
PCBs			
PCB (total[1])	0.600	0.300	0.120
PCB (congener[2])			
Level of chlorination			
mono-trichloro	0.025	0.0125	0.005
tetra-hexachloro	0.050	0.025	0.010
hepta-octachloro	0.075	0.0375	0.015
nona-decachloro	0.125	0.0625	0.025
Organochlorine pesticides[1]			
p,p' DDE	0.050	0.025	0.010
p,p' - DDD	0.050	0.025	0.010
p,p' - DDT	0.050	0.025	0.010
o,p' - DDE	0.050	0.025	0.010
o,p' DDD	0.050	0.025	0.010
o,p' DDT	0.050	0.025	0.010
Alpha-chlordane	0.050	0.025	0.010
Gamma-chlordane	0.050	0.025	0.010
Dieldrin	0.050	0.025	0.010
Endrin	0.050	0.025	0.010
Heptachlorepoxide	0.050	0.025	0.010
Oxychlordane	0.050	0.025	0.010
Mirex	0.050	0.025	0.010
Trans - nonachlor	0.050	0.025	0.010
Toxaphene	0.600	0.300	0.120
PAHs[3]			
PAHs	0.012	0.006	0.002
Dioxins[4]			
TCDD	0.020 (ng/g)	0.010 (ng/g)	0.004 (ng/g)
Inorganic[4]			
Cadmium	0.005	0.0025	0.001
Copper	0.005	0.0025	0.001
Lead	0.005	0.0025	0.001
Zinc	0.005	0.0025	0.001

[1] Schmitt et al., 1990
[2] USEPA, 1990c
[3] Vassilaros et al., 1982
[4] USEPA, 1990d
[5] Schmitt and Finger, 1987

Table 13.6 Detection Limits (ng) of Individual PAHs by HPLC-FD[1]

Analyte	Detection Limit (ng)
Benzo(a)pyrene	0.01
Pyrene	0.03
Benzo(k)fluoranthene	0.03
Dibenz(a,h)anthracene	0.03
Anthracene	0.10
Benz(a)anthracene	0.10
Benzo(e)pyrene	0.10
Benzo(b)fluoranthene	0.10
Benzo(g,h,i)perylene	0.10
3-Methyleholanthrene	0.10

[1] Obana et al., 1981

taminants by indigenous oligochaetes exposed in the same chamber with introduced *L. variegatus* in a 28-d test has been evaluated (E.L. Bruson, NBS, Columbia, MO, personal communication). Peak concentrations of select PAHs and DDT were similar in the indigenous oligochaetes and *L. variegatus* exposed in the same chamber for 28 d.

13.3.7.2 Care should be taken to isolate at least the minimum amount of tissue mass from each replicate chamber needed for analytical chemistry.

13.3.8 ***Test Data:*** Tissue masses required for various analyses at selected lower limits of detection are listed in Table 13.5. Detection limits for individual PAHs in tissue are listed in Table 13.6. A minimum of 1 g/ replicate and preferably 5 g/replicate should be tested.

13.3.8.1 If an estimate of dry weight is needed, a subsample should be dried to a constant weight at about 60 to 90°C. The sample is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg. *Lumbriculus variegatus* typically contain about 1% lipid (dry weight).

13.3.8.2 Depending on specific study objectives, total lipids can be measured on a subsample of the total tissue mass of each thawed replicate sample. Gardner et al. (1985) describe procedures for measuring lipids in 1 mg of tissue. Different methods of lipid analysis can yield different results (Randall et al., 1991). The analytical method used for lipid analysis should be calibrated against the chloroform-methanol extraction method described by Folch et al. (1957) and Bligh and Dyer (1959).

13.3.8.2.1 A number of studies have demonstrated that lipids are the major storage site for organic contaminants in a variety of organisms (Roberts et al., 1977;

Oliver and Niimi, 1983; de Boer, 1988). Because of the importance of lipids, it may be desirable to normalize bioaccumulated concentrations of nonpolar organics to the tissue lipid concentration. Lipid concentration is one of the factors required in deriving the BSAF (Section 14). However, the difficulty with using this approach is that each lipid method generates different lipid concentrations (see Kates (1986) for discussion of lipid methodology). The differences in lipid concentrations directly translate to a similar variation in the lipid-normalized contaminant concentrations or BSAF.

13.3.8.2.2 For comparison of lipid-normalized tissue residues or BASFs, it is necessary to either promulgate a standard lipid technique or to intercalibrate the various techniques. Standardization of a single method is difficult because the lipid methodology is often intimately tied in with the extraction procedure for contaminant analysis. As an interim solution, the Bligh-Dyer lipid method (Bligh and Dyer, 1959) is recommended as a temporary "intercalibration standard" (Lee et al., 1994).

13.3.8.2.3 The potential advantages of Bligh-Dyer include its ability to extract neutral lipids not extracted by many other solvent systems and the wide use of this method (or the same solvent system) in biological and toxicological studies (e.g., Roberts et al., 1977; Oliver and Niimi, 1983; de Boer, 1988; Landrum, 1989). Because the technique is independent of any particular analytical extraction procedure, it will not change when the extraction technique is changed. Additionally, the method can be modified for small tissue sample sizes as long as the solvent ratios are maintained (Herbes and Allen, 1983; Gardner et al., 1985).

13.3.8.2.4 If the Bligh-Dyer method is not the primary lipid method used, the chosen lipid analysis method should be compared with Bligh-Dyer for each tissue type. The chosen lipid method can then be converted to "Bligh-Dyer" equivalents and the lipid-normalized tissue residues reported in "Bligh-Dyer equivalents." In the interim, it is suggested that extra tissue of each species be frozen for future lipid analysis in the event that a different technique proves more advantageous (Lee et al., 1994).

13.4 Interpretation of Results

13.4.1 Section 14 describes general information for interpretation of test results. The following sections describe species-specific information that is useful in helping to interpret the results of sediment bioaccumulation tests with *L. variegatus.*

13.4.2 ***Duration of Exposure:*** Because data from bioaccumulation tests often will be used in ecological or human health risk assessments, the procedures are designed to generate quantitative estimates of steady-state tissue residues. Eighty percent of steady-state is used as the general criterion (Lee et al., 1994). Because results from a single or few species often will be extrapolated to other species, the procedures are designed to maximize exposure to sediment-associated contaminants so as not to systematically underestimate residues in untested species.

13.4.2.1 A kinetic study can be conducted to estimate steady-state concentrations instead of conducting a 28-d bioaccumulation test (e.g., sample on Day 1, 3, 7, 14, 28; E.L. Brunson, NBS, Columbia, MO, unpublished data; USEPA-USCOE, 1991). A kinetic test conducted under the same test conditions outlined above, can be used when 80% of steady-state will not be obtained within 28 d or when more precise estimates of steady-state tissue residues are required. Exposures shorter than 28 d may be used to determine whether compounds are bioavailable (i.e., bioaccumulation potential).

13.4.2.2 DDT reportedly reached 90% of steady state by Day 14 of a 56 d exposure with *L. variegatus.* However, low molecular weight PAHs (e.g., acenaphthylene, fluorene, phenanthrene) generally peaked at Day 3 and tended to decline to Day 56 (E.L. Bruson, NBS, Columbia, MO, unpublished data). In general, concentrations of high molecular weight PAHs (e.g., benzo(b)fluoranthene, benzo(e)pyrene, indeno (1,2,3-c,d)pyrene) either peaked at Day 28 or continued to increase during the 56 d exposure.

13.4.3 ***Influence of Indigenous Organisms:*** Field-collected sediments may include indigenous oligochaetes. Phipps et al. (1993) recommend testing extra chambers without the addition of *L. variegatus* to check for the presence of indigenous oligochaetes in field-collected sediment.

13.4.4 ***Sediment Toxicity in Bioaccumulation Tests:*** Toxicity or altered behavior of organisms in a sample may not preclude use of bioaccumulation data; however, information on adverse effects of a sample should be included in the report.

13.4.4.1 ***Grain Size:*** *Lumbriculus variegatus* are tolerant of a wide range of substrates. Physicochemical characteristics (e.g., grain size) of sediment were not significantly correlated to the growth or reproduction of *L. variegatus* in 10-d toxicity tests (see Section 10.1.3.3; Ankley et al., 1994a).

13.4.4.2 ***Sediment Organic Carbon:*** Reduced growth of *L. variegatus* may result from exposure to sediments with low organic carbon concentrations (G.T. Ankley, USEPA, Duluth, MN, personal communication). Therefore, number of organisms and behavior in the 4-d toxicity screening test should be the criteria used to judge the acceptability of a bioaccumulation test.

Section 14
Data Recording, Data Analysis and Calculations, and Reporting

14.1 Data Recording

14.1.1 Quality assurance project plans with data quality objectives and standard operating procedures should be developed before starting a test. Procedures should be developed by each laboratory to verify and archive data.

14.1.2 A file should be maintained for each sediment test or group of tests on closely related samples (Section 9). This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the sediment test(s); chemical analysis data on the sample(s); control data sheets for reference toxicants; detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions used; and results of reference toxicant tests. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

14.1.3 Example data sheets are included in Appendix D.

14.2 Data Analysis

14.2.1 Statistical methods are used to make inferences about populations, based on samples from those populations. In most sediment toxicity and bioaccumulation tests, test organisms are exposed to contaminated sediment to estimate the response of the population of laboratory organisms. The organism response to these contaminated sediments is usually compared with the response to a control or reference sediment, or in some analyses of bioaccumulation test data, with a fixed standard such as an Food and Drug Administration (FDA) action level. In any toxicity or bioaccumulation test, summary statistics such as means and standard errors for response variables (e.g., survival, contaminant levels in tissue) should be provided for each treatment (e.g., pore-water concentration, sediment).

14.2.1.1 ***Types of Data.*** Two types of data can be obtained from sediment toxicity or bioaccumulation tests. The most common endpoint in toxicity testing is mortality, which is a dichotomous or categorical type of data. Other endpoints commonly encountered in sublethal evaluations are growth (e.g. in sediment toxicity tests conducted with amphipods and midges) and tissue concentrations (e.g. in sediment bioaccumulation tests conducted with oligochaetes or polychaetes and mollusks; USEPA, 1994a). These types of endpoints are representative of continuous data.

14.2.1.2 ***Sediment Testing Scenarios.*** Sediment tests are conducted to determine whether contaminants in sediment are harmful to or are bioaccumulated in benthic organisms. Sediment tests are commonly used in studies designed to (1) evaluate hazards of dredged material, (2) assess site contamination in the environment (e.g., to rank areas for cleanup), and (3) determine effects of specific contaminants, or combinations of contaminants, through the use of sediment spiking techniques. Each of these broad study designs has specific statistical design and analytical considerations, which are detailed below.

14.2.1.2.1 ***Dredged Material Hazard Evaluation.*** In these studies, n sites are compared individually to a reference sediment. The statistical procedures appropriate for these studies are generally pairwise comparisons. Additional information on toxicity testing of dredged material and analysis of data from dredged material hazard evaluations is available in USEPA-USCOE 1994.

14.2.1.2.2 ***Site Assessment of Field Contamination.*** Surveys of sediment toxicity or bioaccumulation often are included in more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlation can be improved and costs may be reduced if subsamples are taken simultaneously for sediment toxicity or bioaccumulation tests, chemical analyses, and benthic community structure determinations. There are several statistical approaches to field assessments, each with a specific purpose. If the objective is to compare the response or residue level at all sites individually to a control sediment, then the pairwise comparison approach described below is appropriate. If the objective is to compare among all sites in the study area, then a multiple comparison procedure that employs an experiment-wise error rate is appropriate. If the objective is to compare among groups of sites, then orthogonal contrasts are a useful data analysis technique.

14.2.1.2.3 ***Sediment-Spiking Experiments.*** Sediments spiked with known concentrations of contaminants can be used to establish cause and effect relationships between chemicals and biological responses. Results of toxicity tests with test materials spiked into sediments at different concentrations may be reported in terms of an LC50, EC50, IC50, NOEC, or LOEC. Results of bioaccumulation tests with either field or spiked samples may be reported in terms of a BSAF (biota-sediment accumulation factor, Ankley et al. 1992b). The statistical approach outlined above for spiked sediment toxicity tests also applies to the analysis of data from sediment dilution experiments or water-only reference toxicant tests.

14.2.2 The guidance outlined below on the analysis of sediment toxicity and bioaccumulation test data is adapted from a variety of sources including Lee et al. (1994), USEPA (1993a), USEPA (1993b), USEPA (1993c), and USEPA-USCOE (1994). The objectives of a sediment toxicity or bioaccumulation test are to quantify contaminant effects on or accumulation in test organisms exposed to natural or spiked sediments or dredged materials and to determine whether these effects are statistically different from those occurring in a control or reference sediment. Each experiment consists of at least two treatments: the control and one or more test treatment(s). The test treatment(s) consist(s) of the contaminated or potentially contaminated sediment(s). A control sediment is always required to ensure that no contamination is introduced during the experiment setup and that test organisms are healthy. A control sediment is used to judge the acceptability of the test. Some designs will also require a reference sediment that represents an environmental condition or potential treatment effect of interest.

14.2.2.1 ***Experimental Unit.*** During toxicity testing, each test chamber to which a single application of treatment is applied is an experimental unit. During bioaccumulation testing, however, the test organism may be the experimental unit if individual members of the test species are evaluated and they are large enough to provide sufficient biomass for chemical analysis. The important concept is that the treatment (sediment) is applied to each experimental unit as a discrete unit. Experimental units should be independent and should not differ systematically.

14.2.2.2 ***Replication.*** Replication is the assignment of a treatment to more than one experimental unit. The variation among replicates is a measure of the within-treatment variation and provides an estimate of within-treatment error for assessing the significance of observed differences between treatments.

14.2.2.3 ***Minimum Detectable Difference (MDD).*** As the minimum difference between treatments which the test is required or designed to detect decreases, the number of replicates required to meet a given significance level and power increases. Because no consensus currently exists on what constitutes a biologically acceptable MDD, the appropriate statistical minimum significant difference should be a data quality objective (DQO) established by the individual user (e.g., program considerations) based on their data requirements, the logistics and economics of test design, and the ultimate use of the sediment toxicity or bioaccumulation test results.

14.2.2.4 ***Minimum Number of Replicates.*** Four replicates per treatment or control are the absolute minimum number of replicates for a sediment toxicity test. However, USEPA recommends five replicates for marine testing (USEPA, 1994) or eight replicates for freshwater testing for each control or experimental treatment. It is always prudent to include as many replicates in the test design as are economically and logistically possible. A minimum of five replicates per treatment also is recommended for bioaccumulation testing. USEPA sediment toxicity testing methods recommend the use of 20 organisms per replicate for marine testing (USEPA, 1994a) or 10 organisms per replicate for freshwater testing. An increase in the number of organisms per replicate in all treatments, including the control, is allowable only if (1) test performance criteria for the recommended number of replicates are achieved and (2) it can be demonstrated that no change occurs in contaminant availability due to the increased organism loading.

14.2.2.5 ***Randomization.*** Randomization is the unbiased assignment of treatments within a test system and to the exposure chambers ensuring that no treatment is favored and that observations are independent. It is also important to (1) randomly select the organisms (but not the number of organisms) for assignment to the control and test treatments (e.g., a bias in the results may occur if all of the largest animals are placed in the same treatment), (2) randomize the allocation of sediment (e.g., do not take all the sediment in the top of a jar for the control and the bottom for spiking), and (3) randomize the location of exposure units.

14.2.2.6 ***Pseudoreplication.*** The appropriate assignment of treatments to the replicate exposure chambers is critical to the avoidance of a common error in design and analysis termed "pseudoreplication" (Hurlbert, 1984). Pseudoreplication occurs when inferential statistics are used to test for treatment effects even though the treatments are not replicated or the replicates are not statistically independent (Hurlbert, 1984). The simplest form of pseudoreplication is the treatment of subsamples of the experimental unit as true replicates. For example, two aquaria are prepared, one with control sediment, the other with test sediment, and 10 organisms are placed in each aquarium. Even if each organism is analyzed individually, the 10 organisms only replicate the biological response and do not replicate the treatment (i.e., sediment type). In this case, the experimental unit is the 10 organisms and each organism is a subsample. A less obvious form of pseudoreplication is the potential systematic error due to the physical segregation of exposure chambers by treatment. For example, if all the control exposure chambers are placed in one area of a

room and all the test exposure chambers are in another, spatial effects (e.g., different lighting, temperature) could bias the results for one set of treatments. Random physical intermixing of the exposure chambers or randomization of treatment location may be necessary to avoid this type of pseudoreplication. Pseudoreplication can be avoided or reduced by properly identifying the experimental unit, providing replicate experimental units for each treatment, and applying the treatments to each experimental unit in a manner that includes random physical intermixing (interspersion) and independence. However, avoiding pseudoreplication completely may be difficult or impossible given resource constraints.

14.2.2.7 ***Compositing Samples.*** Compositing is used primarily in bioaccumulation experiments when the biomass of an individual organism is insufficient for chemical analysis. Compositing consists of combining samples (e.g., organisms, sediment) and chemically analyzing the mixture rather than the individual samples. The chemical analysis of the mixture provides an estimate of the average concentration of the individual samples making up the composite. Compositing also may be used when the cost of analysis is high. Each organism or sediment sample added to the composite should be of equal size (i.e., wet weight) and the composite should be completely homogenized before taking a sample for chemical analysis. If compositing is performed in this manner, the value obtained from the analysis of the composite is the same as the average obtained from analyzing each individual sample (within any sampling and analytical errors). If true replicate composites (not subsample composites) are made, the variance of the replicates will be less than the variance of the individual samples, providing a more precise estimate of the mean value. This increases the power of a test between means of composites over a test between means of individuals or samples for a given number of samples analyzed. If compositing reduces the actual number of replicates, however, the power of the test will also be reduced. If composites are made of individuals or samples varying in size, the value of the composite and the mean of the individual organisms or sediment samples are no longer equivalent. The variance of the replicate composites will increase, decreasing the power of any test between means. In extreme cases, the variance of the composites can exceed the population variance (Tetra Tech, 1986). Therefore, it is important to keep the individuals or sediment samples comprising the composite equivalent in size. If sample sizes vary, consult the tables in Schaeffer and Janardan (1978) to determine if replicate composite variances will be higher than individual sample variances, which would make compositing inappropriate.

14.2.3 The purpose of a toxicity or bioaccumulation test is to determine if the biological response to a treatment sample differs from the response to a control sample. Figure 14.1 presents the possible outcomes and decisions that can be reached in a statistical test of such a hypothesis. The null hypothesis is that no difference exists among the mean control and treatment responses.

Decision	TR =Control	TR > Control
TR =Control	Correct 1 - α	Type II Error β
TR > Control	Type I Error α	Correct 1 - β (Power)

Treatment response (TR), Alpha (α) represents the probability of making a Type I statistical error (false positive); beta (β) represents the probability of making a Type II statistical error (false negative).

Figure 14.1 Treatment response for a Type I and Type II error.

The alternative hypothesis of greatest interest in sediment tests is that the treatments are toxic, or contain concentrations of bioaccumulatable compounds, relative to the control or reference sediment.

14.2.3.1 Statistical tests of hypotheses can be designed to control for the chances of making incorrect decisions. In Figure 14.1, alpha (α) represents the probability of making a Type I statistical error. A Type I statistical error in this testing situation results from the false conclusion that the treated sample is toxic or contains chemical residues not found in the control or reference sample. Beta (β) represents the probability of making a Type II statistical error, or the likelihood that one erroneously concludes there are no differences among the mean responses in the treatment, control or reference samples. Traditionally, acceptable values for α have ranged from 0.1 to 0.01 with 0.05 or 5% used most commonly. This choice should depend upon the consequences of making a Type I error. Historically, having chosen α, environmental researchers have ignored β and the associated power of the test (1-β).

14.2.3.2 Fairweather (1991) presents a review of the need for, and the practical implications of, conducting power analysis in environmental monitoring studies. This review also includes a comprehensive bibliography of recent publications on the need for, and use of, power analyses in environmental study design and data analysis. The consequences of a Type II statistical error in environmental studies should never be ignored and may in fact be the most important criterion to consider in experimental designs and data analyses that include statistical hypothesis testing. To paraphrase Fairweather (1991), "The commitment of time, energy and people to a false positive (a Type I error) will only continue until the mistake is discovered. In contrast, the cost of a false negative (a Type II error) will have both short- and long-term costs (e.g., ensuing environmental degradation and the eventual cost of its rectification)."

14.2.3.3 The critical components of the experimental design associated with the test of hypothesis outlined above are (1) the required MDD between the treatment and control or reference responses, (2) the variance among treatment and control replicate experimental units,

(3) the number of replicate units for the treatment and control samples, (4) the number of animals exposed within a replicate exposure chamber, and (5) the selected probabilities of Type I (α) and Type II (β) errors.

14.2.3.4 Sample size or number of replicates may be fixed due to cost or space considerations or may be varied to achieve *a priori* probabilities of α and β. The MDD should be established ahead of time based upon biological and program considerations. The investigator has little control of the variance among replicate exposure chambers. However, this variance component can be minimized by selecting test organisms that are as biologically similar as possible and maintaining test conditions within prescribed quality control (QC) limits.

14.2.3.5 The MDD is expressed as a percentage change from the mean control response. To test the equality of the control and treatment responses, a two-sample t-test with its associated assumptions is the appropriate parametric analysis. If the desired MDD, the number of replicates per treatment, the number of organisms per replicate and an estimate of typical among replicate variability, such as the coefficient of variation (CV) from a control sample, are available, it is possible to use a graphical approach as in Figure 14.2 to determine how likely it is that a 20% reduction will be detected in the treatment response relative to the control response. The CV is defined as 100% x (standard deviation divided by the mean). In a test design with 8 replicates per treatment and with an α level of 0.05, high power (i.e., >0.8) to detect a 20% reduction from the control mean occurs only if the CV is 15% or less (Figure 14.2). The choice of these variables also affects the power of the test. If 5 replicates are used per treatment (Figure 14.3), the CV needs to be 10% or lower to detect a 20% reduction in response relative to the control mean with a power of 90%.

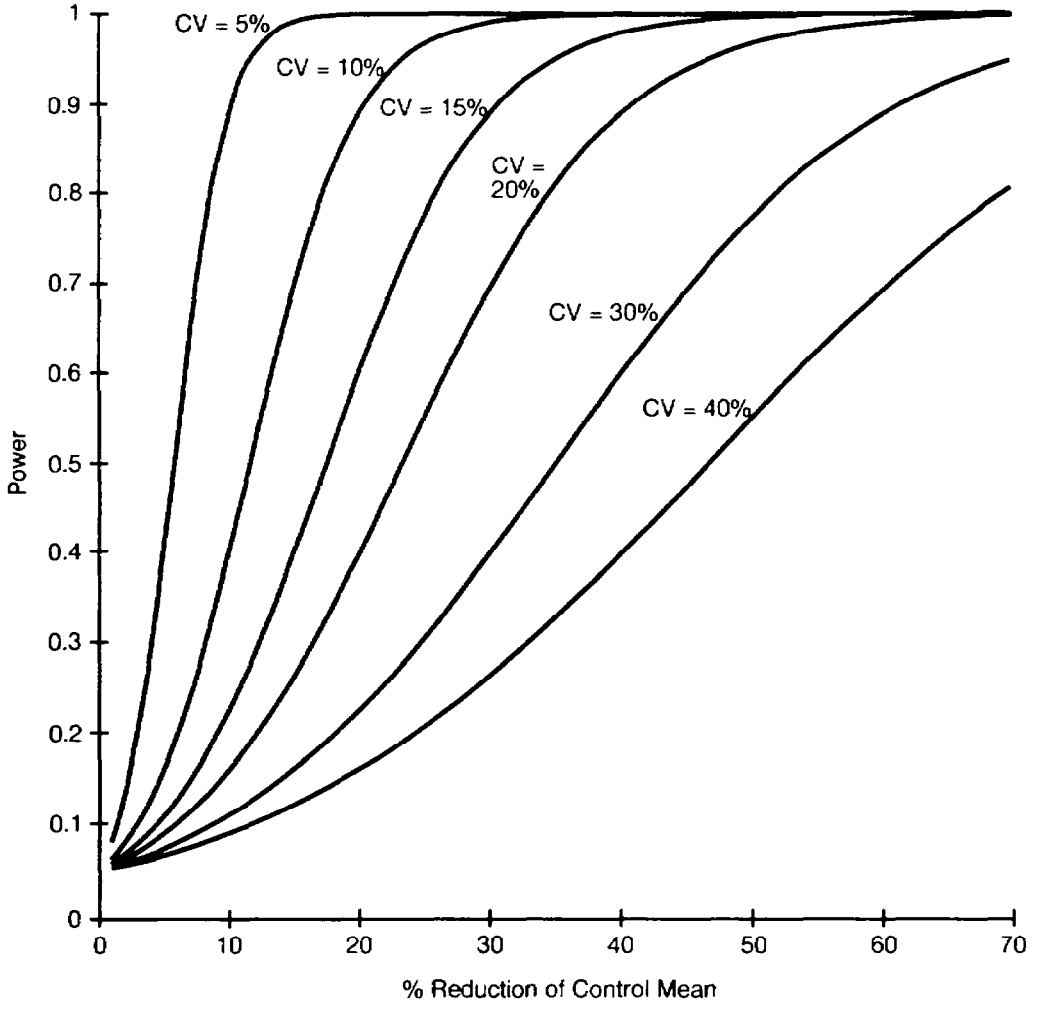


Figure 14.2 Power of the test vs. percent reduction in treatment response relative to the control mean at various CVs (8 replicates, alpha = 0.05 (one-tailed)).

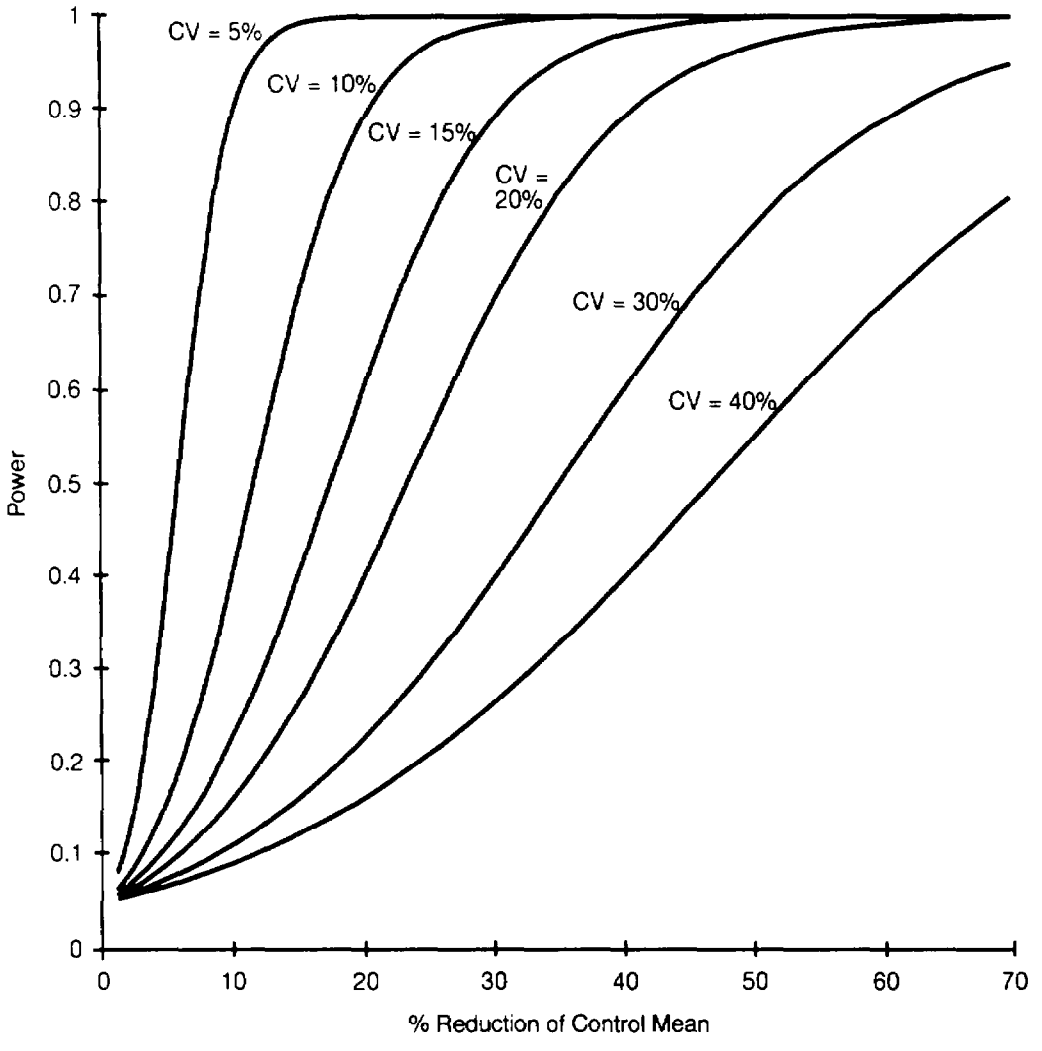


Figure 14.3 Power of the test vs. percent reduction in treatment response relative to the control mean at various CVs (5 replicates, alpha = 0.05 (one-tailed)).

14.2.3.6 Relaxing the α level of a statistical test increases the power of the test. Figure 14.4 duplicates Figure 14.2 except that α is 0.10 instead of 0.05. Selection of the appropriate α level of a test is a function of the costs associated with making Type I and II statistical errors. Evaluation of Figure 14.2 illustrates that with a CV of 15% and an α level of 0.05, there is an 80% probability (power) of detecting a 20% reduction in the mean treatment response relative to the control mean. However, if α is set at 0.10 (Figure 14.4) and the CV remains at 15%, then there is a 90% probability (power) of detecting a 20% reduction relative to the control mean. The latter example would be preferable if an environmentally conservative analysis and interpretation of the data is desirable.

14.2.3.7 Increasing the number of replicates per treatment will increase the power to detect a 20% reduction in treatment response relative to the control mean (Figure 14.5). Note, however, that for less than 8 replicates per treatment it is difficult to have high power (i.e., >0.80) unless the CV is less than 15%. If space or cost limit the number of replicates to fewer than 8 per treatment, then it may be necessary to find ways to reduce the among replicate variability and consequently the CV. Options that are available include selecting more uniform organisms to reduce biological variability or increasing the α level of the test. For CVs in the range of 30% to 40%, even eight replicates per treatment is inadequate to detect small reductions ($\leq$ 20%) in response relative to the control mean.

14.2.3.8 The effect of the choice of α and β on number of replicates for various CVs is illustrated in Figure 14.6 in which the combined total probability of Type I and Type II statistical errors is fixed and assumed to be 0.25. An α of 0.10 therefore establishes a β of 0.15. In Figure 14.6, if $\alpha = \beta = 0.125$, the number of replicates required to detect a difference of 20% relative to the control is at a minimum. As α or β decrease, the number of replicates required to detect the same 20% difference relative to the control increases. However, the curves are

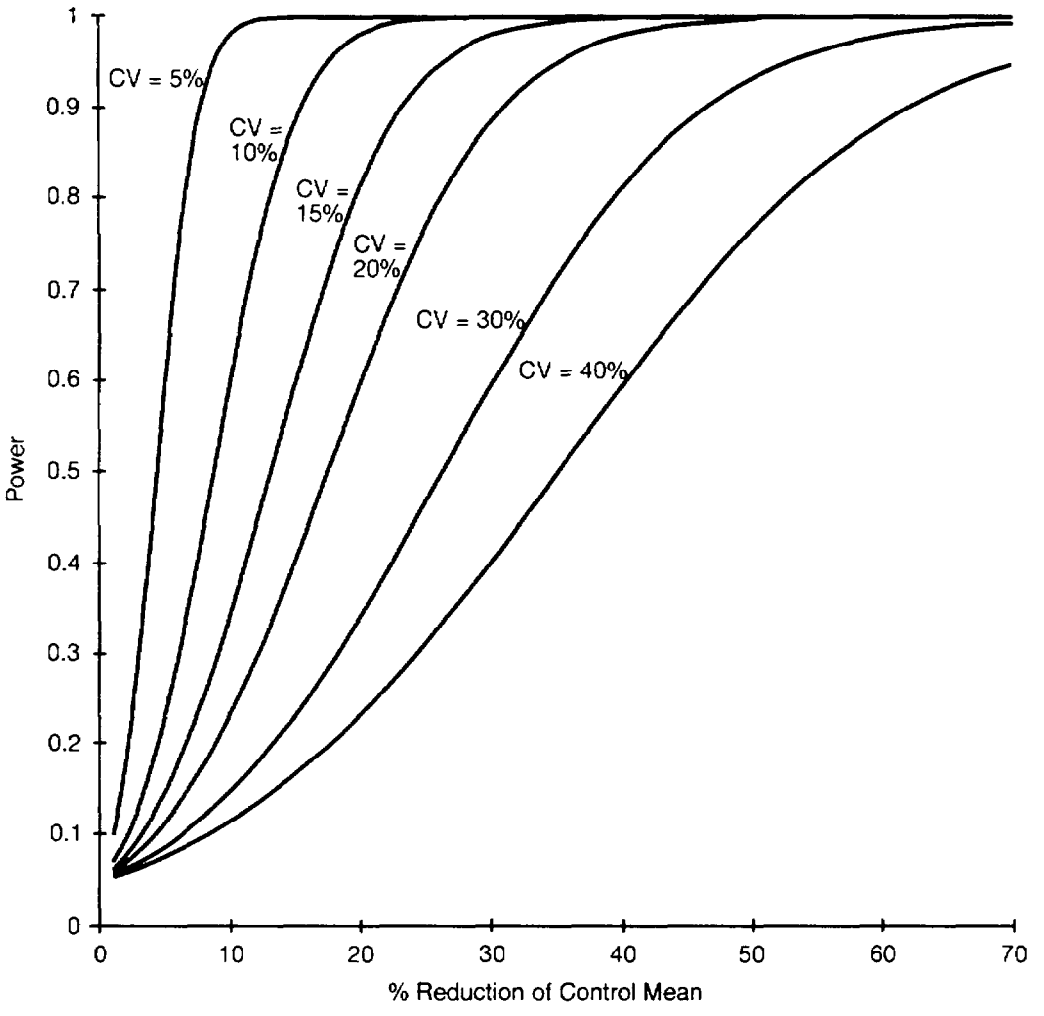


Figure 14.4 **Power of the test vs. percent reduction in treatment response relative to the control mean at various CVs (8 replicates, alpha = 0.10 (one-tailed)).**

relatively flat over the range of 0.05 to 0.20 and the curves are very dependent upon the choice of the combined total of $\alpha + \beta$. Limiting the total of $\alpha + \beta$ to 0.10 greatly increases the number of replicates necessary to detect a preselected percentage reduction in mean treatment response relative to the control mean.

14.2.4 Figure 14.7 outlines a decision tree for analysis of survival and growth data subjected to hypothesis testing. In the tests described herein, samples or observations refer to replicates of treatments. Sample size n is the number of replicates (i.e., exposure chambers) in an individual treatment, not the number of organisms in an exposure chamber. Overall sample size N is the combined total number of replicates in all treatments. The statistical methods discussed in this section are described in general statistics texts such as Steel and Torrie (1980), Sokal and Rohlf (1981), Dixon and Massey (1983), Zar (1984), and Snedecor and Cochran (1989). It is recommended that users of this manual have at least one of these texts and associated statistical tables on hand. A nonparametric statistics text such as Conover (1980) may also be helpful.

14.2.4.1 ***Mean.*** The sample mean ($\bar{x}$) is the average value, or $\sum x_i / n$, where

n = number of observations (replicates)

x_i = ith observation

$\sum x_i$ = every x summed = $x_1 + x_2 + x_3 + \ldots + x_n$

14.2.4.2 ***Standard Deviation.*** The sample standard deviation (s) is a measure of the variation of the data around the mean and is equivalent to $\sqrt{s^2}$. The sample variance, s^2, is given by the following "machine" or "calculation" formula:

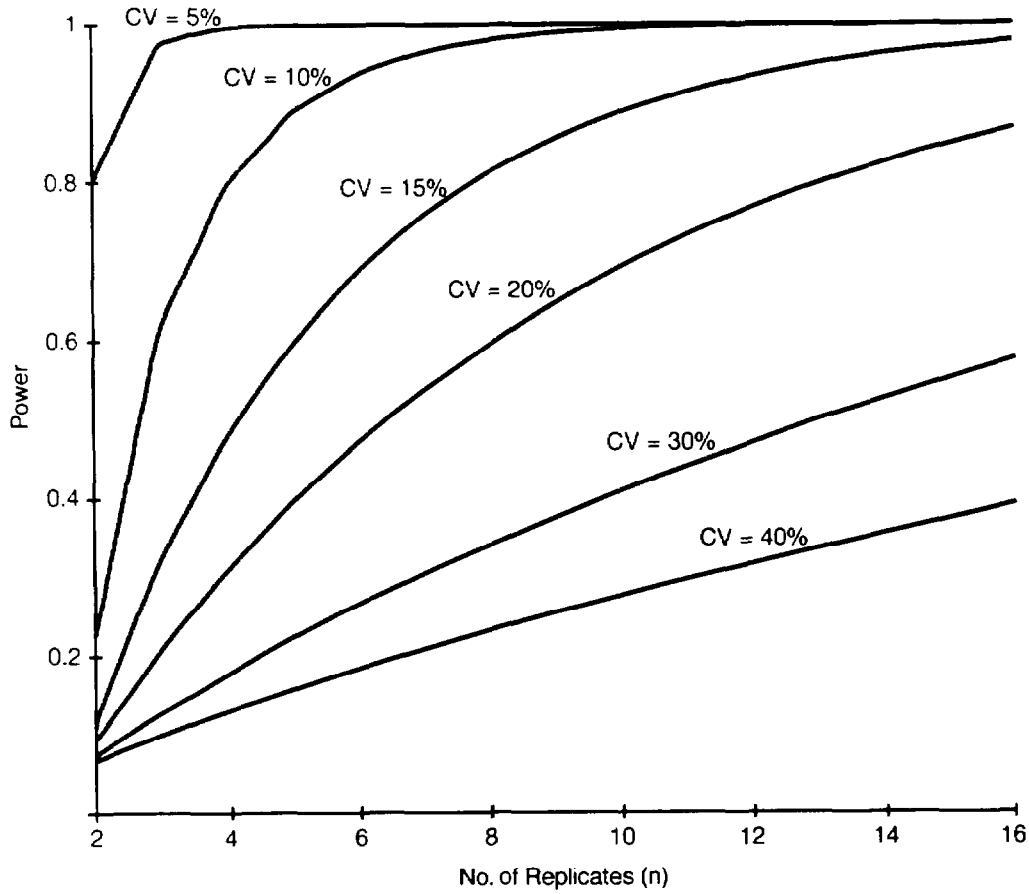


Figure 14.5 Effect of CV and number of replicates on the power to detect a 20% decrease in treatment response relative to the control mean (alpha = 0.05 (one-tailed)).

$$s^2 = \frac{\Sigma x^2 - (\Sigma x)^2 / n}{n-1}$$

14.2.4.3 ***Standard Error of the Mean.*** The standard error of the mean (SE, or $s/\sqrt{n}$) estimates variation among sample means rather than among individual values. The SE is an estimate of the SD among means that would be obtained from several samples of n observations each. Most of the statistical tests in this manual compare means with other means (e.g., dredged sediment mean with reference mean) or with a fixed standard (e.g., FDA action level; Lee et al., 1994). Therefore, the "natural" or "random" variation of sample means (estimated by SE), rather than the variation among individual observations (estimated by s), is required for the tests.

14.2.4.4 ***Tests of Assumptions.*** In general, parametric statistical analyses such as t-tests and analysis of variance are appropriate only if (1) there are independent, replicate experimental units for each treatment, (2) the observations within each treatment follow a normal distribution, and (3) variances for both treatments are equal or similar. The first assumption is an essential component of experimental design. The second and third assumptions can be tested using the data obtained from the experiment. Therefore, before conducting statistical analyses, tests for normality and equality of variances should be performed.

14.2.4.4.1 Outliers (extreme values) and systematic departures from a normal distribution (e.g., a log-normal distribution) are the most common causes of departures from normality or equality of variances. An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, or by analysis of residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should only be discarded with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported. An appropriate transformation, such as the arc sine square root transformation, will normalize many distributions (USEPA, 1985). Problems with outliers can usually be solved only by using nonpara-

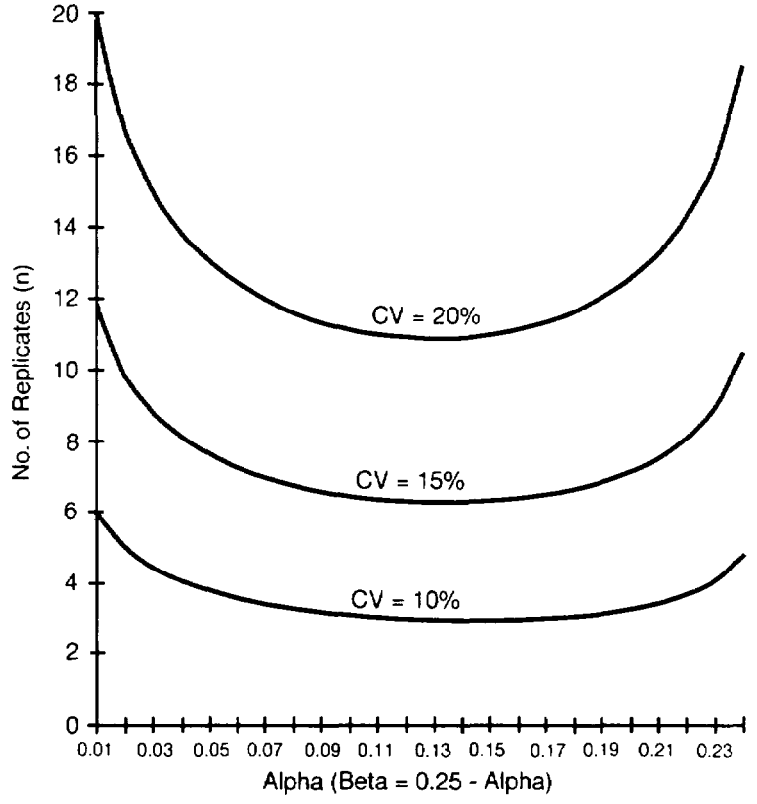


Figure 14.6 Effect of alpha and beta on the number of replicates at various CVs (assuming combined alpha + beta = 0.25).

metric tests, but careful laboratory practices can reduce the frequency of outliers.

14.2.4.4.2 ***Tests for Normality.*** The most commonly used test for normality for small sample sizes (N<50) is the Shapiro-Wilk's Test. This test determines if residuals are normally distributed. Residuals are the differences between individual observations and the treatment mean. Residuals, rather than raw observations, are tested because subtracting the treatment mean removes any differences among treatments. This scales the observations so that the mean of residuals for each treatment and overall treatments is zero. The Shapiro-Wilk's Test provides a test statistic W, which is compared to values of W expected from a normal distribution. W will generally vary between 0.3 and 1.0, with lower values indicating greater departure from normality. Because normality is desired, one looks for a high value of W with an associated probability greater than the pre-specified α level.

14.2.4.4.3 Table 14.1 provides α levels to determine whether departures from normality are significant. Normality should be rejected when the probability associated with W (or other normality test statistic) is less than α for the appropriate total number of replicates (N) and design. A balanced design means that all treatments have an equal number (n) of replicate exposure chambers. A design is considered unbalanced when the treatment with the largest number of replicates (n_{max}) has at least twice as many replicates as the treatment with the fewest replicates (n_{min}). Note that higher α levels are used when the number of replicates is small, or when the design is unbalanced, because these are the cases in which departures from normality have the greatest effects on t-tests and other parametric comparisons. If data fail the test for normality, even after transformation, nonparametric tests should be used for additional analyses.

14.2.4.4.4 Tables of quantiles of W can be found in Shapiro and Wilk (1965), Gill (1978), Conover (1980), USEPA (1989b) and other statistical texts. These references also provide methods of calculating W, although the calculations can be tedious. For that reason, commonly available computer programs or statistical packages are preferred for the calculation of W.

14.2.4.4.5 ***Tests for Homogeneity of Variances.*** There are a number of tests for equality of variances. Some of these tests are sensitive to departures from normality, which is why a test for normality should be performed first. Bartlett's Test or other tests such as Levene's Test or Cochran's Test (Winer, 1971; Snedecor and Cochran, 1989) all have similar power for small, equal sample sizes (n=5) (Conover et al., 1981), and any one of these tests is adequate for the analyses in this section. Many software packages for t-tests and analysis of variance

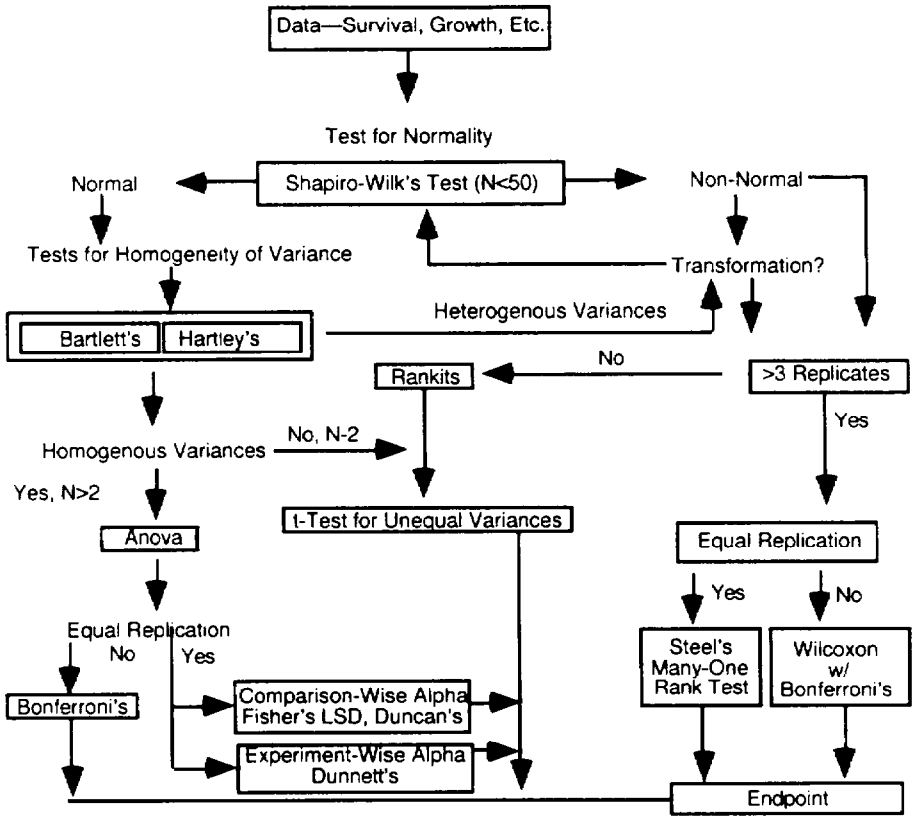


Figure 14.7 Decision tree for analysis of survival and growth data subjected to hypothesis testing.

(ANOVA) provide at least one of the tests. Bartlett's Test is recommended for routine evaluation of homogeneity of variances (USEPA, 1985; USEPA, 1994b; USEPA,1994c).

14.2.4.4.6 If no tests for equality of variances are included in the available statistical software, Hartley's F_{max} can easily be calculated:

$$F_{max} = (\text{ larger of } s_1^2 , s_2^2) / (\text{ smaller of } s_1^2 , s_2^2)$$

Table 14.1 Suggested α Levels to Use for Tests of Assumptions

Test	Number of Observations[1]	α When Design Is Balanced	α When Design Is Unbalanced[2]
Normality	N = 2 to 9	0.10	0.25
	N = 10 to 19	0.05	0.10
	N = 20 or more	0.01	0.05
Equality of variances	n = 2 to 9	0.10	0.25
	n = 10 or more	0.05	0.10

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

[2] $n_{max} \geq 2\, n_{min}$

When F_{max} is large, the hypothesis of equal variances is more likely to be rejected. F_{max} is a two-tailed test because it does not matter which variance is expected to be larger. Some statistical texts provide critical values of F_{max} (Winer, 1971; Gill, 1978; Rohlf and Sokal, 1981).

14.2.4.4.7 Levels of α for tests of equality of variances are provided in Table 14.1. These levels depend upon number of replicates in a treatment (n) and allotment of replicates among treatments. Relatively high α's (i.e., ≥ 0.10) are recommended because the power of the above tests for equality of variances is rather low (about 0.3) when n is small. Equality of variances is rejected if the probability associated with the test statistic is less than the appropriate α.

14.2.4.5 ***Transformations of the Data.*** When the assumptions of normality or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than by a nonparametric technique. The first step in these analyses is to transform the responses, expressed as the proportion surviving, by the arc sine-square root transformation. The arc sine-square root transformation is commonly used on proportionality data to stabilize the variance and satisfy the normality requirement. If the data do not meet the

assumption of normality and there are four or more replicates per group, then the nonparametric test, Wilcoxon Rank Sum Test, can be used to analyze the data. If the data meet the assumption of normality, Bartlett's Test or Hartley's F test for equality of variances is used to test the homogeneity of variance assumption. Failure of the homogeneity of variance assumption leads to the use of a modified t test, and the degrees of freedom for the test are adjusted.

14.2.4.5.1 The arc sine-square root transformation consists of determining the angle (in radians) represented by a sine value. In this transformation, the proportion surviving is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. When the proportion surviving is 0 or 1, a special modification of the transformation should be used (Bartlett, 1937). An example of the arc sine-square root transformation and modification are provided below.

1. Calculate the response proportion (RP) for each replicate within a group, where

   RP = (number of surviving organisms)/(number exposed)

2. Transform each RP to arc sine, as follows:

   a. For RPs greater than zero or less than one:

      Angle (in radians) = *arc sine* $\sqrt{(RP)}$

   b. Modification of the arc sine when RP = 0.

      Angle (in radians) = *arc sine* $\sqrt{\frac{1}{4n}}$

      where n = number of animals/treatment rep.

   c. Modification of the arc sine when RP = 1.0.

      Angle = 1.5708 radians-(radians for RP = 0)

14.2.4.6 ***Two Sample Comparisons (N=2).*** The true population mean (μ) and standard deviation (σ) are known only after sampling the entire population. In most cases samples are taken randomly from the population, and the s calculated from those samples is only an estimate of σ. Student's *t*-values account for this uncertainty. The degrees of freedom for the test, which are defined as the sample size minus one (n-1), should be used to obtain the correct t-value. Student *t*-values decrease with increasing sample size because larger samples provide a more precise estimate of μ and σ.

14.2.4.6.1 When using a t table, it is crucial to determine whether the table is based on one-tailed probabilities or two-tailed probabilities. In formulating a statistical hypothesis, the alternative hypothesis can be one-sided (one-tailed test) or two-sided (two-tailed test). The null hypothesis (H_0) is always that the two values being analyzed are equal. A one-sided alternative hypothesis (H_a) is that there is a specified relationship between the two values (e.g., one value is greater than the other) versus a two-sided alternative hypothesis (H_a) which is that the two values are simply different (i.e., either larger or smaller). A one-tailed test is used when there is an *a priori* reason to test for a specific relationship between two means such as the alternative hypothesis that the treatment mortality or tissue residue is greater than the control mortality or tissue residue. In contrast, the two-tailed test is used when the direction of the difference is not important or cannot be assumed before testing.

14.2.4.6.2 Since control organism mortality or tissue residues and sediment contaminant concentrations are presumed lower than reference or treatment sediment values, conducting one-tailed tests is recommended in most cases. For the same number of replicates, one-tailed tests are more likely to detect statistically significant differences between treatments (e.g., have a greater power). This is a critical consideration when dealing with a small number of replicates (such as 8/treatment). The other alternative for increasing statistical power is to increase the number of replicates, which increases the cost of the test.

14.2.4.6.3 There are cases when a one-tailed test is inappropriate. When no *a priori* assumption can be made as to how the values vary in relationship to one another, a two-tailed test should be used. An example of an alternative two-sided hypothesis is that the reference sediment total organic carbon (TOC) content is different (greater or lesser) from the control sediment TOC. A two-tailed test should also be used when comparing tissue residues among different species exposed to the same sediment and when comparing bioaccumulation factors (BAFs) or biota-sediment-accumulation-factors (BSAFs).

14.2.4.6.4 The t-value for a one-tailed probability may be found in a two-tailed table by looking up t under the column for twice the desired one-tailed probability. For example, the one-tailed t-value for $\alpha = 0.05$ and df = 20 is 1.725, and is found in a two-tailed table using the column for $\alpha = 0.10$.

14.2.4.7 The usual statistical test for comparing two independent samples is the two-sample t-test (Snedecor and Cochran, 1989). The t-statistic for testing the equality of means $\bar{x}_1$ and $\bar{x}_2$ from two independent samples with n_1 and n_2 replicates and unequal variances is

$$t = (\bar{x}_1 - \bar{x}_2) \sqrt{s_1^2 / n_1 + s_2^2 / n_2}$$

where s_1^2 and s_2^2 are the sample variances of the two groups. Although the equation assumes that the variances of the two groups are unequal, it is equally useful for situations in which the variances of the two groups are equal. This statistic is compared with the Student *t*

distribution with degrees of freedom (df) given by Satterthwaite's (1946) approximation:

$$df = \frac{(s_1^2 / n_1 + s_2^2 / n_2)^2}{(s_1^2 / n_1)^2 / (n_1 - 1) + s_2^2 / n_2)^2 / (n_2 - 1)}$$

This formula can result in fractional degrees of freedom, in which case one should round the degree of freedom down to the nearest integer in order to use a t table. Using this approach, the degrees of freedom for this test will be less than the degrees of freedom for a t-test assuming equal variances. If there are unequal numbers of replicates in the treatments, the t-test with Bonferroni's adjustment can be used for data analysis (USEPA, 1993b; USEPA, 1993c). When variances are equal, an *F* test for equality is unnecessary.

14.2.4.8 ***Nonparametric Tests.*** Tests such as the t-test, which analyze the original or transformed data and which rely on the properties of the normal distribution, are referred to as parametric tests. Nonparametric tests, which do not require normally distributed data, analyze the ranks of data and generally compare medians rather than means. The median of a sample is the middle or 50th percentile observation when the data are ranked from smallest to largest. In many cases, nonparametric tests can be performed simply by converting the data to ranks or normalized ranks (rankits) and conducting the usual parametric test procedures on the ranks or rankits.

14.2.4.8.1 Nonparametric tests are useful because of their generality but have less statistical power than corresponding parametric tests when the parametric test assumptions are met. If parametric tests are not appropriate for comparisons because the normality assumption is not met, data should be converted to normalized ranks (rankits). Rankits are simply the z-scores expected for the rank in a normal distribution. Thus, using rankits imposes a normal distribution over all the data, although not necessarily within each treatment. Rankits can be obtained by ranking the data, then converting the ranks to rankits using the following formula:

$$rankit = z_{[(rank - 0.375) / (N + 0.25)]}$$

where z is the normal deviate and N is the total number of observations. Alternatively, rankits may be obtained from standard statistical tables such as Rohlf and Sokal (1981).

14.2.4.8.2 If normalized ranks are calculated, the ranks should be converted to rankits using the formula above. In comparisons involving only two treatments (N=2), there is no need to test assumptions on the rankits or ranks; simply proceed with a one-tailed t-test for unequal variances using the rankits or ranks.

14.2.4.9 Analysis of Variance (N>2). Some experiments are set up to compare more than one treatment with a control while others may also be interested in comparing the treatments with one another. The basic design of these experiments is the same as for experiments evaluating pairwise comparisons. After the applicable comparisons are determined, the data must to be tested for normality to determine if parametric statistics are appropriate and whether the variances of the treatments are equal. If normality of the data and equal variances are established, then an analysis of variance (ANOVA) may be performed to address the hypothesis that all the treatments including the control are equal. If normality or equality of variance are not established, then transformations of the data may be appropriate or nonparametric statistics can be used to test for equal means. Tests for normality of the data should be performed on the treatment residuals. A residual is defined as the observed value minus the treatment mean, that is, $r_{ik} = o_{ik}$ - (k^{th} treatment mean). Pooling residuals provides an adequate sample size to test the data for normality.

14.2.4.9.1 The variances of the treatments should also be tested for equality. Currently there is no easy way to test for equality of the treatment means using analysis of variance if the variances are not equal. In a toxicity test with several treatments, one treatment may have 100% mortality in all of its replicates, or the control treatment may have 100% survival in all of its replicates. These responses result in 0 variance for a treatment that results in a rejection of equality of variance in these cases. No transformation will change this outcome. In this case, the replicate responses for the treatment with 0 variance should be removed before testing for equality of variances. Only those treatments that do not have 0 replicate variance should be used in the ANOVA to get an estimate of the within treatment variance. After a variance estimate is obtained, the means of the treatments with 0 variance may be tested against the other treatment means using the appropriate mean comparison. Equality of variances among the treatments can be evaluated with the Hartley F_{max} test or Bartlett's test. The option of using nonparametric statistics on the entire set of data ia also an alternative.

14.2.4.9.2 If the data are not normally distributed or the variances among treatments are not homogeneous, even after data transformation, nonparametric analyses are appropriate. If there are four or more replicates per treatment and the number of replicates per treatment is equal, the data can be analyzed with Steel's Many-One Rank test. Unequal replication among treatments requires data analysis with the Wilcoxon Rank Sum test with Bonferroni's adjustment. Steel's Many-One Rank test is a nonparametric test for comparing treatments with a control. This test is an alternative to the Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's test requires equal variances across treatments and the control but is thought to be fairly insensitive to deviations from this condition (USEPA, 1993a). Wilcoxon's Rank Sum Test is a nonparametric test to be used as an alternative to the Steel's test when the number of replicates are not the same within each treatment. A Bonferroni's adjustment of the pairwise error rate for

comparison of each treatment versus the control is used to set an upper bound of alpha on the overall error rate. This is in contrast to the Steel's test with a fixed overall error rate for alpha. Thus, Steel's test is a more powerful test (USEPA, 1993a).

14.2.4.9.3 Different mean comparison tests are used depending on whether an α percent comparison-wise error rate or an α percent experiment-wise error rate is desired. The choice of a comparison-wise or experiment-wise error rate depends on whether a decision is based on a pairwise comparison (comparison-wise) or from a set of comparisons (experiment-wise). For example, a comparison-wise error rate would be used for deciding which stations along a gradient were acceptable or not acceptable, relative to a control or reference sediment. Each individual comparison is performed independently at a smaller α (than used in an experiment-wise comparison) such that the probability of making a Type I error in the entire series of comparisons is not greater than the chosen experiment-wise α level of the test. This results in a more conservative test when comparing any particular sample to the control or reference. However, if several samples were taken from the same area and the decision to accept or reject the area was based upon all comparisons with a reference then an experiment-wise error rate should be used. When an experiment-wise error rate is used, the power to detect real differences between any two means decreases as a function of the number of treatment means being compared to the control treatment.

14.2.4.9.4 The recommended procedure for pairwise comparisons that have a comparison-wise α error rate and equal replication is to do an ANOVA followed by a one-sided Fisher's Least Significant Difference (LSD) test (Steel and Torrie, 1980). A Duncan's mean comparison test should give results similar to the LSD. If the treatments do not contain equal numbers of replicates, the appropriate analysis is the t-test with Bonferroni's adjustment. For comparisons that maintain an experiment-wise α error rate Dunnett's test is recommended for comparisons with the control.

14.2.4.9.5 Dunnett's test has an overall error rate of α, which accounts for the multiple comparisons with the control. Dunnett's procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an ANOVA. Dunnett's procedure can only be used when the same number of replicate test chambers have been used at each treatment and the control.

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

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

where $\bar{Y}_1$ = mean for each treatment

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for treatment "i"

To quantify the sensitivity of the Dunnett's test, the minimum significant difference (MSD=MDD) may be calculated with the following formula:

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

where d = Critical value for the Dunnett's Procedure

S_w = The square root of the within mean square

n = The number of replicates per treatment, assuming an equal number of replicates at all treatment concentrations

n_1 = Number of replicates in the control

14.2.5 Methods for Calculating LC50s, EC50s, and ICps

14.2.5.1 Figure 14.8 outlines a decision tree for analysis of point estimate data. USEPA manuals (USEPA, 1985; USEPA, 1989b; USEPA, 1993b; USEPA, 1993c) discuss in detail the mechanics of calculating LC50 (or EC50) or ICp values using the most current methods.

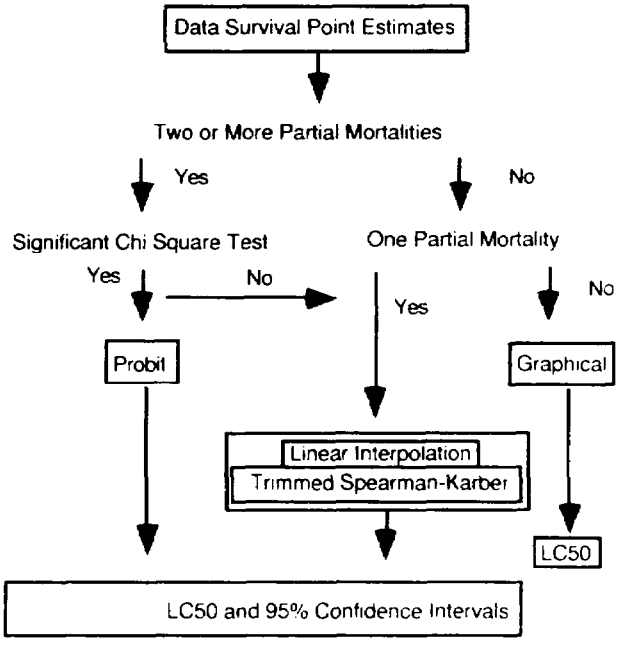


Figure 14.8 Decision tree for analysis of point estimate data.

The most commonly used methods are the Graphical, Probit, Trimmed Spearman-Karber and the Linear Interpolation Methods. In general, results from these methods should yield similar estimates. Each method is outlined below and recommendations are presented for the use of each method.

14.2.5.2 Data for at least five test concentrations and the control should be available to calculate an LC50 although each method can be used with fewer concentrations. Survival in the lowest concentration must be at least 50% and an LC50 should not be calculated unless at least 50% of the organisms die in at least one of the serial dilutions. When less than 50% mortality occurs in the highest test concentration, the LC50 is expressed as greater than the highest test concentration.

14.2.5.3 Due to the intensive nature of the calculations for the estimated LC50 and associated 95% confidence interval using most of the following methods, it is recommended that the data be analyzed with the aid of computer software. Computer programs to estimate the LC50 or ICp values and associated 95% confidence intervals with the methods discussed below (except for the Graphical Method) were developed by USEPA and can be obtained by sending a diskette with a written request to USEPA, Environmental Monitoring Systems Laboratory (EMSL), 26 W. Martin Luther King Drive, Cincinnati, OH 45268 or call 513/569-7076.

14.2.5.4 ***Graphical Method.*** This procedure estimates an LC50 (or EC50) by linearly interpolating between points of a plot of observed percentage mortality versus the base 10 logarithm ($\log_{10}$) of treatment concentration. The only requirement for its use is that treatment mortalities bracket 50%.

14.2.5.4.1 For an analysis using the Graphical Method the data should first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps: Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k treatments. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then

$$p_i^s = (p_i^a - p_0^a)/(1-p_0^a)$$

where p_i^s = the smoothed observed proportion mortality for concentration i.

Adjust the smoothed observed proportion mortality in each treatment for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^a - p_0^a)/(1-p_0^a)$$

where p_i^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for concentration i.

14.2.5.5 ***The Probit Method.*** This method is a parametric statistical procedure for estimating the LC50 (or EC50) and the associated 95% confidence interval (Finney, 1971). The analysis consists of transforming the observed proportion mortalities with a Probit transformation, and transforming the treatment concentrations to $\log_{10}$. Given the assumption of normality for the $\log_{10}$ of the tolerances, the relationship between the transformed variables mentioned above is about linear. This relationship allows estimation of linear regression parameters, using an iterative approach. A Probit is the same as a z-score: for example, the Probit corresponding to 70% mortality is $z_{.70}$ or =.52. The LC50 is calculated from the regression and is the concentration associated with 50% mortality or z=0. To obtain a reasonably precise estimate of the LC50 with the Probit Method, the observed proportion mortalities must bracket 0.5 and the $\log_{10}$ of the tolerance should be normally distributed. To calculate the LC50 estimate and associated 95% confidence interval, two or more of the observed proportion mortalities must be between zero and one. The original percentage mortalities should be corrected for control mortality using Abbott's formula (Section 14.2.5.4.1; Finney, 1971) before the Probit transformation is applied to the data.

14.2.5.5.1 A goodness-of-fit procedure with the chi-square statistic is used to determine if the data fit the Probit model. If many data sets are to be compared to one another, the Probit Method is not recommended because it may not be appropriate for many of the data sets. This method also is only appropriate for percent mortality data sets and should not be used for estimating endpoints that are a function of the control response, such as inhibition of growth. Most computer programs that generate Probit estimates also generate confidence interval estimates for the LC50. These confidence interval estimates on the LC50 may not be correct if replicate mortalities are pooled to obtain a mean treatment response (USEPA-USCOE, 1994). This can be avoided by entering the Probit-transformed replicate responses and doing a least squares regression on the transformed data.

14.2.5.6 ***The Trimmed Spearman-Karber Method.*** The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber, nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton et al., 1977). This procedure estimates the trimmed mean of the distribu-

tion of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution. Use of the Trimmed Spearman-Karber Method is only appropriate when the requirements for the Probit Method are not met (USEPA, 1993b; USEPA, 1993c). This method is only appropriate for lethality data sets.

14.2.5.6.1 To calculate the LC50 estimate with the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5. To calculate a confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

14.2.5.6.2 Smooth the observed proportion mortalities as described for the Probit Method. Adjust the smoothed observed proportion mortality in each concentration for mortality in the control group using Abbott's formula (see Probit Method Section 14.2.5.4.1). Calculate the amount of trim to use in the estimation of the LC50 as follows:

$$\text{Trim} = \max(p_1^a,\ 1 - p_k^a)$$

where p_1^a = the smoothed, adjusted proportion mortality for the lowest treatment concentration, exclusive of the control.

p_k^a = the smoothed, adjusted proportion mortality for the highest treatment concentration.

k = the number of treatment concentrations, exclusive of the control.

14.2.5.7 ***Linear Interpolation Method.*** This method calculates a toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the endpoint of interest and is reported as an ICp value (IC = Inhibition Concentration; where p = the percent effect). The procedure was designed for general applicability in the analysis of data from chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

14.2.5.7.1 As described in USEPA (1993b; 1993c), the Linear Interpolation Method of calculating an ICp assumes that the responses (1) are monotonically nonincreasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically nonincreasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. No assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

14.2.5.7.2 ***The Linear Interpolation Method*** assumes a linear response from one concentration to the next. Thus, the IC is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

14.2.5.7.3 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means. Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. Unusual patterns in the deviations from monotonicity may require an additional step of smoothing. Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

14.2.5.7.4 To obtain the ICp estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [M_1(1 - p/100) - M_J]\ \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

where C_J = tested concentration whose observed mean response is greater than M_1(1 - p/100).

C_{J+1} = tested concentration whose observed mean response is less than M_1(1 - p/100).

M_c = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

IC*p* = estimated concentration at which there is a percent reduction from the smoothed mean control response.

14.2.5.7.5 Standard statistical methods for calculating confidence intervals are not applicable for the ICp. The bootstrap method, as proposed by Efron (1982), is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ij} is randomly resampled with replacement to produce a new set of data Y_{ij}^*, that is statistically equivalent to the original data, but which produces a new and slightly different estimate of the IC*p* (IC*p**). This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associated ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 times, the empirical 2.5% and the 97.5% confidence limits are about the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988). The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

14.2.6 ***Analysis of Bioaccumulation Data.*** In some cases, body burdens will not approach steady-state body burdens in a 28-d test (Lee et al., 1994). Organic compounds exhibiting these kinetics will probably have a log Kow >5, be metabolically refractory (e.g., highly chlorinated PCBs, dioxins), or have low depuration rates. Additionally, tissue residues of several heavy metals may gradually increase over time so that 28 d is inadequate to approach steady-state. Depending on the goals of the study and the adaptability of the test species to long-term testing, it may be necessary to conduct an exposure longer than 28 d (or a kinetic study) to obtain a sufficiently accurate estimate of steady-state tissue residues of these compounds.

14.2.6.1 ***Biotic Sampling.*** In the long-term studies, the exposure should continue until steady-state body burdens are attained. ASTM (1988b) recommends a minimum of five sampling periods (plus t_0) when conducting water exposures to generate bioconcentration factors (BCFs). Sampling in a geometric progression is also recommended with sampling times reasonably close to S/16, S/8, S/4, S/2, and S, where S is the time to steady-state. This sampling design assumes a fairly accurate estimate of time to steady-state, which is often not the case with sediment exposures.

14.2.6.1.1 To document steady-state from sediment exposures, placing a greater number of samples at and beyond the predicted time to steady-state is recommended. With a contaminant expected to reach steady-state within 28 to 50 d, samples should be taken at Day 0, 7, 14, 21, 28, 42, 56, and 70. If the time to steady-state is much greater than 42 d, then additional sampling periods at two week intervals should be added (e.g., Day 84). Slight deviations from this schedule (e.g., Day 45 versus Day 42) are not critical, though for comparative purposes, samples should be taken at t_{28}. An estimate of time to steady-state may be obtained from the literature or estimated from structure-activity relationships, though these values should be considered the minimum times to steady-state.

14.2.6.1.2 This schedule increases the likelihood of statistically documenting that steady-state has been obtained although it does not document the initial uptake phase as well. If an accurate estimate of the sediment uptake rate coefficient (Ks) is required, additional sampling periods are necessary during the initial uptake phase (e.g., Day 0, 2, 4, 7, 10, 14).

14.2.6.2 ***Abiotic Samples.*** The bioavailable fraction of the contaminants as well as the nutritional quality of the sediment are more prone to depletion in extended tests than during the 28-d exposures. To statistically document whether such depletions have occurred, replicate sediment samples should be collected for physical and chemical analysis from each sediment type at the beginning and the end of the exposure. Archiving sediment samples from every biological sampling period also is recommended.

14.2.6.3 ***Short-Term Uptake Tests.*** Compounds may attain steady-state in the oligochaete, *Lumbriculus variegatus,* in less than 28 d (Kukkonen and Landrum, 1993). However, before a shorter test is used, it must be ascertained that the analytes of interest do indeed achieve steady-state in *L. variegatus* in <28 d. Biotic and abiotic samples should be taken at Day 0 and 10 following the same procedure used for the 28-d tests. If time-series biotic samples are desired, sample on Day 0, 1, 3, 5, 7, and 10.

14.2.6.4 ***Estimating Steady-State.*** In tests where steady-state cannot be documented, it may be possible to estimate steady-state concentrations. Several methods have been published that can be used to predict

steady-state contaminant levels from uptake and depuration kinetics (Spacie and Hamelink, 1982; Davies and Dobbs, 1984). All of these methods were derived from fish exposures and most use a linear uptake, first-order depuration model that can be modified for uptake of contaminants from sediment. To avoid confusing uptake from water versus sediment, Ks, the sediment uptake rate coefficient, is used instead of K1. The Ks coefficient has also been referred to as the uptake clearance rate (Landrum et al., 1989). Following the recommendation of Stehly et al. (1990), the gram sediment and gram tissue units are retained in the formulation:

$$Ct(t) = Ks \times Cs / K2 \times (1-e^{-K2 \times t})$$

where Ct = contaminant concentration in tissue at time t

Cs = contaminant concentration in sediment

Ks = uptake rate coefficient in tissue (g sed g^{-1} day^{-1})

K2 = depuration constant (day^{-1})

t = time (days)

As time approaches infinity, the maximum or equilibrium contaminant concentration within the organism (Ct_{max}) becomes

$$Ct_{max} = Cs \times Ks / K2$$

Correspondingly, the bioaccumulation factor (BAF) for a compound may be estimated from

$$BAF = Ks / K2$$

14.2.6.4.1 This model assumes that the sediment concentration and the kinetic coefficients are invariant. Depletion of the sediment concentrations in the vicinity of the organism would invalidate the model. Further, the rate coefficients are conditional on the environment and health of the test organisms. Thus, changes in environmental conditions such as temperature or changes in physiology such as reproduction will also invalidate the model. Despite these potential limitation, the model can provide estimates of steady-state tissue residues.

14.2.6.4.2 The kinetic approach requires an estimate of Ks and K2, which are determined from the changes in tissue residues during the uptake phase and depuration phase, respectively. The uptake experiment should be short enough that an estimate of Ks is made during the linear portion of the uptake phase to avoid an unrealistically low uptake rate due to depuration. The depuration phase should be of sufficient duration to smooth out any loss from a rapidly depurated compartment such as loss from the voiding of feces. Unless there is reason to suspect that the route of exposure will affect the depuration rate, it is acceptable to use a K2 derived from a water exposure. For further discussion of this method for bioconcentration studies in fish, see Davies and Dobbs (1984), Spacie and Hamelink (1982), and ASTM (1988b). For application of this procedure for sediment, see Lee et al. (1994). Recent studies of the accumulation of sediment-associated contaminants by benthos suggest that the kinetics for freshly dosed sediments may require a more complex formulation to estimate the uptake clearance constant than that presented above (Landrum, 1989).

14.2.6.4.3 This model predicts that equilibrium would be reached only as time becomes infinite. Therefore, for practical reasons, apparent steady-state is defined here as 95% of the equilibrium tissue residue. The time to reach steady-state can be estimated by

$$S = \ln[1 / (1.00-0.95)] / K2 = 3.0 / K2$$

where S = time to apparent steady-state (days)

Thus, the key information is the depuration rate of the compound of interest in the test species or phylogenetically related species. Unfortunately, little of this data has been generated for benthic invertebrates. When no depuration rates are available, the depuration rate constant for organic compounds can then be estimated from the relationship between Kow and k2 for fish species (Spacie and Hamelink, 1982):

$$K2 = \text{antilog}[1.47-0.414 \times \log(Kow)]$$

The relationship between S and k2 and between k2 and Kow is summarized in Table 14.2. Estimated time (days) to reach 95% of contaminant steady-state tissue residue (S) and depuration rate constants (k2) are calculated from octanol-water partition coefficients using a linear uptake, first-order depuration model (Spacie and Hamelink, 1982). The k2 values are the amount depurated (decimal fraction of tissue residue lost per day). Table 14.2 may be used to make a rough estimate of the exposure time to reach steady-state tissue residues if a depuration rate constant for the compound of interest from a phylogenetically similar species is available. If no depuration rate is available, then the table may be used

Table 14.2 Estimated Time to Obtain 95 Percent of Steady-State Tissue Residue

Log Kow	K2	S (days)
1	0.114	0.2
2	0.44	0.5
3	0.17	1.4
4	0.0065	3.5
5	0.0025	9.2
6	0.00097	24
7	0.00037	61
8	0.00014	160
9	0.00006	410

for estimating the S of organic compounds from the Kow value. However, as these data were developed from fish bioconcentration data, its applicability to the kinetics of uptake from sediment-associated contaminants is unknown. The portion of organics readily available for uptake may be small in comparison to the total sediment organic concentration (Landrum, 1989). Therefore S values generated by this model should be considered as minimum time periods.

14.2.6.4.4 Using a linear uptake, first-order depuration model to estimate exposure time to reach steady-state body burden for metals is problematical for a number of reasons. The kinetics of uptake may be dependent upon a small fraction of the total sediment metal load that is bioavailable (Luoma and Bryan, 1982). Depuration rates may be more difficult to determine, as metals bound to proteins may have very low exchange rates (Bryan, 1976). High exposure concentrations of some metals can lead to the induction of metal binding proteins, like metallothionein, which detoxify metals. These metal-protein complexes within the organism have extremely low exchange rates with the environment (Bryan, 1976). Thus, the induction of metal binding proteins may result in decreased depuration rate constants in organisms exposed to the most polluted sediments. Additionally, structure-activity relationships that exist for organic contaminants (e.g., relationship between Kow and BCFs) are not well developed for metals.

14.3 Data Interpretation

14.3.1 Sediments spiked with known concentrations of contaminants can be used to establish cause and effect relationships between chemicals and biological responses. Results of toxicity tests with test materials spiked into sediments at different concentrations may be reported in terms of an LC50 (median lethal concentration), an EC50 (median effect concentration), an IC50 (inhibition concentration), or as an NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration; Section 3). Consistent spiking procedures should be followed in order to make interlaboratory comparisons (Section 8.3).

14.3.2 Evaluating effect concentrations for chemicals in sediment requires knowledge of factors controlling the bioavailability. Similar concentrations of a chemical in units of mass of chemical per mass of sediment dry weight often exhibit a range in toxicity in different sediments (Di Toro et al., 1991; USEPA, 1992c). Effect concentrations of chemicals in sediment have been correlated to interstitial water concentrations, and effect concentrations in interstitial water are often similar to effect concentrations in water-only exposures. The bioavailability of nonionic organic compounds are often inversely correlated with the organic carbon concentration of the sediment. Whatever the route of exposure, the correlations of effect concentrations to interstitial water concentrations indicate predicted or measured concentrations in interstitial water can be useful for quantifying the exposure concentration to an organism. Therefore, information on partitioning of chemicals between solid and liquid phases of sediment may be useful for establishing effect concentrations.

14.3.3 Toxic units can be used to help interpret the response of organisms to multiple contaminants in sediment. A toxic unit is the concentration of a chemical divided by an effect concentration. For example, a toxic unit of exposure can be calculated by dividing the measured concentration of a chemical in pore water by the water-only LC50 for the same chemical (Ankley et al., 1991a). Toxicity expressed as toxic units may be summed and this may provide information on the toxicity of chemical mixtures (Ankley et al., 1991a).

14.3.4 Field surveys can be designed to provide either a qualitative reconnaissance of the distribution of sediment contamination or a quantitative statistical comparison of contamination among sites (Burton and Ingersoll, 1994). Surveys of sediment toxicity are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlation can be improved and costs reduced if subsamples are taken simultaneously for sediment toxicity or bioaccumulation tests, chemical analyses, and benthic community structure.

14.3.5 Descriptive methods such as toxicity tests with field-collected sediment should not be used alone to evaluate sediment contamination. An integration of several methods using the weight of evidence is needed to assess the effects of contaminants associated with sediment. Hazard evaluations integrating data from laboratory exposures, chemical analyses, and benthic community assessments provide strong complementary evidence of the degree of pollution-induced degradation in aquatic communities (Chapman et al., 1992; Burton, 1991).

14.3.6 Toxicity Identification Evaluation (TIE) procedures can be used to help provide insights as to specific contaminants responsible for toxicity in sediment (USEPA, 1991a; Ankley and Thomas, 1992). For example, the toxicity of contaminants such as metals, ammonia, hydrogen sulfide, and nonionic organic compounds can be identified using TIE procedures.

14.3.7 ***Interpretation of Comparisons of Tissue Residues.*** If the mean control tissue residues at Day 28 are not significantly greater than the Day 0 tissue residues, it can be concluded that there is no significant contamination from the exposure system or from the control sediment. If there is significant uptake, the exposure system or control sediment should be reevaluated as to suitability. Even if there is a significant uptake in the controls, it is still possible to compare the controls and treatments as long as the contaminant concentrations in the test tissue residues are substantially higher. However, if control values are high, the data should be discarded and the experiment conducted again after determining the source of contamination.

14.3.7.1 Comparisons of the 28-d control (or reference) tissue residues and 28-d treatment tissue residues determines whether there was statistically significant bioaccumulation due to exposure to test sediments. Comparisons between control and reference tissue residues at Day 28 determine whether there was a statistically significant bioaccumulation due to exposure to the reference sediment. If no significant difference is detected when treatment tissue residues are compared to a set criterion value (e.g., FDA Action Limit) with a one-tailed test, the residues must be considered equivalent to the value even though numerically the mean treatment tissue residue may be smaller.

14.3.7.2 ***BAFs and BSAFs.*** Statistical comparisons between ratios such as BAFs or BSAFs are difficult due to computation of error terms. Since all variables used to compute BAFs and BSAFs have errors associated with them, it is necessary to estimate the variance as a function of these errors. This can be accomplished using approximation techniques such as the propagation of error (Beers, 1957) or a Taylor series expansion method (Mood et al., 1974). BAFs and BSAFs can then be compared using these estimates of the variance. See Lee et al. (1994) provide examples of this approach.

14.3.7.3 ***Comparing Tissue Residues of Different Compounds.*** In some cases, it is of interest to compare the tissue residues of different compounds. For example, Rubinstein et al. (1987) compared the uptake of thirteen different PCB congeners to test for differences in bioavailability. Because the values for the different compounds are derived from the same tissue samples, they are not independent and tend to be correlated, so standard t-tests and ANOVAs are inappropriate. A repeated measures technique (repeated testing of the same experimental unit) should be used where the experimental unit (individual) is considered as a random factor and the different compounds as a second factor. See Rubinstein et al. (1987) and Lake et al. (1990) for an example of the application of repeated measures to bioaccumulation data.

14.4 Reporting

14.4.1 The record of the results of an acceptable sediment test should include the following information either directly or by referencing available documents:

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

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

14.4.1.3 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.

14.4.1.4 Source and characteristics of overlying water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

14.4.1.5 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 or life stage, means and ranges of weight or length, observed diseases or unusual appearance, treatments, holding procedures.

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

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

14.4.1.8 Methods used for physical and chemical characterization of sediment.

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

14.4.1.10 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.

14.4.1.11 Methods used for statistical analyses of data.

14.4.1.12 Summary of general observations on other effects or symptoms.

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

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

Section 15
Precision and Accuracy

15.1 Determining Precision and Accuracy

15.1.1 Precision is a term that describes the degree to which data generated from replicate measurements differ and reflects the closeness of agreement between randomly selected test results. Accuracy is the difference between the value of the measured data and the true value and is the closeness of agreement between an observed value and an accepted reference value. Quantitative determination of precision and accuracy in sediment testing of aquatic organisms is difficult or may be impossible in some cases, as compared to analytical (chemical) determinations. This is due, in part, to the many unknown variables that affect organism response. Determining the accuracy of a sediment test using field samples is not possible since the true values are not known. Since there is no acceptable reference material suitable for determining the accuracy of sediment tests, the accuracy of the test methods has not been determined (Section 15.2).

15.1.2 Sediment tests exhibit variability due to several factors (Section 9). Test variability can be described in terms of two types of precision either single laboratory (intralaboratory or repeatability; Section 15.5.1) precision or multi-laboratory (interlaboratory or reproducibility; Section 15.5.2) precision. Intralaboratory precision reflects the ability of trained laboratory personnel to obtain consistent results repeatedly when performing the same test on the same organism using the same toxicant. Interlaboratory precision (also referred to as round-robin or ring tests) is a measure of how reproducible a method is when conducted by a large number of laboratories using the same method, organism, and samples. Generally, intralaboratory results are less variable than interlaboratory results (USEPA, 1991b; USEPA, 1993a; USEPA, 1994b; USEPA, 1994c; Hall et al., 1989; Grothe and Kimerle, 1985).

15.1.3 A measure of precision can be calculated using the mean and relative standard deviation (percent coefficient of variation, or CV% = standard deviation/mean x 100) of the calculated endpoints from the replicated endpoints of a test. However, precision reported as the CV should not be the only approach used for evaluating precision of tests and should not be used for the NOEC effect levels derived from statistical analyses of hypothesis testing. The CVs may be very high when testing extremely toxic samples. For example, if there are multiple replicates with no survival and one with low survival, the CV may exceed 100%, yet the range of response is actually quite consistent. Therefore, additional estimates of precision should be used, such as range of responses, and minimum detectable differences (MDD) compared to control survival or growth. Several factors can affect the precision of the test, including test organism age, condition, sensitivity, handling and feeding of the test organisms, overlying water quality, and the experience of the investigators in conducting tests. For these reasons, it is recommended that trained laboratory personnel conduct the tests in accordance with the procedures outlined in Section 9. Quality assurance practices should include (1) single laboratory precision determinations using reference toxicants for each of the test organisms that are used to determine the ability of the laboratory personnel to obtain precise results. These determinations should be made before conducting a sediment test and should be routinely performed as long as whole sediment tests are being conducted; (2) control charts (Section 15.3) should be prepared for each reference toxicant and test organism to determine if the test results are within prescribed limits; and (3) tests must meet the minimum criteria of test acceptability specific for each test organism (Tables 11.3, 12.3, 13.4; USEPA, 1991b).

15.1.4 Intralaboratory precision data are routinely calculated for test organisms using water-only 96-h exposures to a reference toxicant, such as KCl. Intralaboratory precision data should be tracked using a control chart. Each laboratory's reference toxicant data will reflect conditions unique to that facility, including dilution water, culturing, and other variables (Section 9). However, each laboratory's reference toxicant CVs should reflect good repeatability.

15.1.5 To date, interlaboratory precision (round-robin) tests have been completed with both *Hyalella azteca* and *Chironomus tentans* using 4-d water-only and 10-d whole sediment tests. The results of these round-robin studies are described below.

15.2 Accuracy

15.2.1 The relative accuracy of toxicity tests cannot be determined since there is no acceptable reference material. The relative accuracy of the reference toxicity

tests can only be evaluated by comparing test responses to control charts.

15.3 Replication and Test Sensitivity

15.3.1 The sensitivity of sediment tests will depend in part on the number of replicates per concentration, the probability levels (alpha and beta) selected, and the type of statistical analysis. For a given level of variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

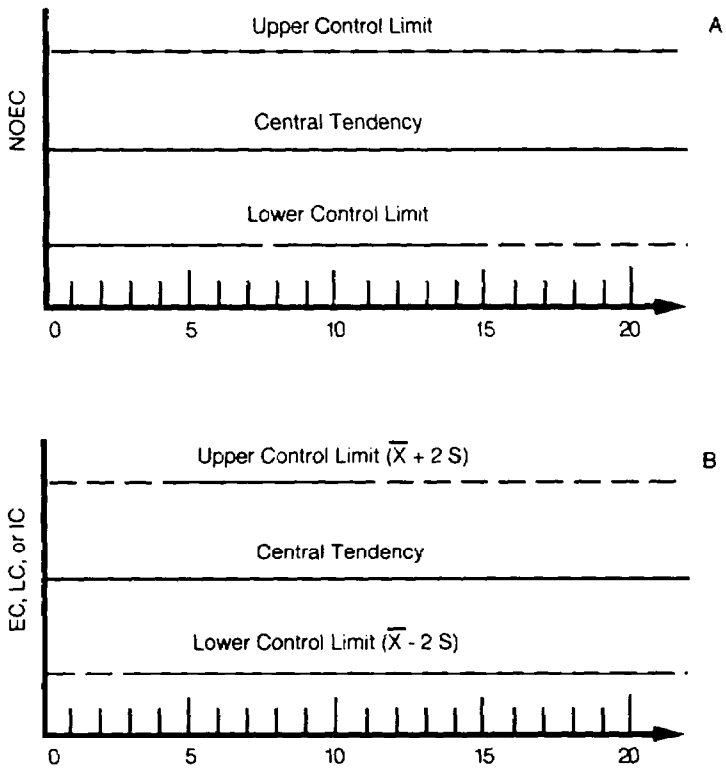
15.4 Demonstrating Acceptable Laboratory Performance

15.4.1 It is the responsibility of a laboratory to demonstrate its ability to obtain precise results with reference toxicants before it performs sediment tests (Section 9.16). Intralaboratory precision, expressed as a coefficient of variation (CV), of the range for each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (e.g., the same test duration, type of water, age of test organisms, feeding), and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical (Section 9.14, Table 9.1, 9.2).

15.4.2 The quality of test organisms obtained from an outside source must be verified by conducting a reference-toxicity test concurrently with the sediment test. The supplier should provide data with the shipment describing the history of the sensitivity of organisms from the same source culture. If the supplier has not conducted five reference toxicity tests with the test organism, it is the responsibility of the testing laboratory to conduct five reference toxicity tests before starting a sediment test (Section 9.14.1).

15.4.3 Before conducting tests with contaminated sediment, the laboratory should demonstrate its ability to conduct tests by conducting five exposures in control sediment as outlined in Table 11.1, 12.1, or 13.1. It is recommended that these five exposures with control sediment be conducted concurrently with the five reference toxicity tests described in Section 15.4.2.

15.4.4 A control chart should be prepared for each combination of reference toxicant and test organism. Each control chart should include the most current data. Endpoints from five tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the values (X_i) from successive tests with a given reference toxicant (Figure 15.1), and the endpoints (LC50, NOEC, ICp) are examined to determine if



Toxicity Test with Reference Toxicants

$$\bar{x} = \frac{\sum_{i=1} x_i}{n}$$

$$s = \sqrt{\frac{\sum_{i=1}^{n} x_i^2 - \frac{(\sum_{i=1}^{n} x_i)^2}{n}}{n-1}}$$

where x_i = Successive toxicity values of toxicity tests.

n = Number of tests.

$\bar{x}$ = Mean toxicity value.

S = Standard deviation.

Figure 15.1 Control (cusum) charts: (A) hypothesis testing and (B) point estimates (LC, EC, or IC).

they are within prescribed limits. Control charts as described in USEPA (1993a) and USEPA (1993b) are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits (±2 SD) are recalculated with each successive test result. After two years of data collection, or a minimum of 20 data points, the control (cusum) chart should be maintained using only the 20 most recent data points.

15.4.5 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified using

control charts. With an alpha of 0.05, one in 20 tests would be expected to fall outside of the control limits by chance alone. During a 30 d period, if two reference toxicity tests out of a total of the previous 20 fall outside the control limits, the sediment toxicity tests conducted during the time in which the second reference toxicity test failed are suspect and should be considered as provisional and subject to careful review.

15.4.5.1 A sediment test may be acceptable if specified conditions of a reference toxicity test fall outside the expected ranges (Section 9). Specifically, a sediment test should not automatically be judged unacceptable if the LC50 for a given reference toxicity test falls outside the expected range or if mortality in the control of the reference toxicity test exceeds 10%. All the performance criteria outlined in Tables 11.3, 12.3, and 13.4 must be considered when determining the acceptability of a sediment test. The acceptability of the sediment test would depend on the experience and judgment of the investigator and the regulatory authority.

15.4.6 If the value from a given test with the reference toxicant falls more than two standard deviations (SD) outside the expected range, the sensitivity of the organisms and the overall credibility of the test system are suspect (USEPA, 1993a). In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

15.4.7 Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ±2 SD, by definition, will be exceeded 5% of the time, regardless of how well a laboratory performs. Highly proficient laboratories that develop a very narrow control limit may be unfairly penalized if a test that falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not an outlier is to be rejected. This determination may be made by the regulatory authority evaluating the data.

15.4.8 The recommended reference toxicity test consists of a control and five or more concentrations in which the endpoint is an estimate of the toxicant concentration that is lethal to 50% of the test organisms in the time period prescribed by the test. The LC50 is determined by an appropriate procedure, such as the Trimmed Spearman-Karber Method, or Probit Method, Graphical Method, or the Linear Interpolation Method (Section 14).

15.4.9 The point estimation analysis methods recommended in this manual have been chosen primarily because they are well-tested, well-documented, and are applicable to most types of test data. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis of toxicity data.

15.5 Precision of Sediment Toxicity Test Methods

15.5.1 Intralaboratory Precision

15.5.1.1 Intralaboratory precision of the *Hyalella azteca* and *Chironomus tentans* 10-d tests (as described in Tables 11.1 and 12.1) was evaluated at ERL-Duluth using one control sediment sample in June 1993. In this study, five individuals simultaneously conducted the 10-d whole sediment toxicity tests as described in Tables 11.1 and 12.1 with the exception of the feeding rate of 1.0 mL rather than 1.5 mL for *C. tentans*. The results of the study are presented in Table 15.1. The mean survival for *H. azteca* was 90.4% with a CV of 7.2% and the mean survival for *C. tentans* was 93.0% with a CV of 5.7%. All of the individuals met the survival performance criteria of 80% for *H. azteca* (Table 11.3) or 70% for *C. tentans* (Table 12.3).

15.5.2 Interlaboratory Precision

15.5.2.1 Interlaboratory precision using reference toxicity tests and 10-d whole sediment toxicity tests using the methods described in this manual (Tables 9.1, 9.2, 11.1, and 12.1) were conducted by federal government laboratories, contract laboratories, and academic laboratories that had demonstrated experience in sediment toxicity testing (Table 15.2). The only exception to the methods outlined in Table 9.1 and 9.2 was that 80% rather than the current recommendation of 90% survival was used to judge the acceptability of the reference toxicity tests. The round robin study was conducted in two phases for each test organism. The experimental design for the round robin study required each laboratory to conduct 96-h water-only reference toxicity tests in Phase 1 and 10-d whole sediment tests in Phase 2 with *Hyalella azteca* or *Chironomus tentans* over a period of six months. Criteria for selection of participants in the

Table 15.1 Intralaboratory Precision for Survival of *Hyalella azteca* and *Chironomus tentans* in 10-d Whole-Sediment Toxicity Tests, June 1993[1]

	Percent Survival	
Individual	*H. azteca*	*C. tentans*
A	85	85
B	93	93
C	90	93
D	84	94
E	100	100
N	5	5
Mean	90.4	93.0
CV	7.2%	5.7%

[1] Test sample was from a control sediment (T.J. Norberg-King, USEPA, Duluth, MN, personal communication.) The test was conducted at the same time by five individuals at ERL-Duluth. The source of overlying water was from Lake Superior.

round-robin study were that the laboratories had (1) existing cultures of the test organisms, (2) experience conducting tests with the organisms, and (3) would participate voluntarily. The test methods for the reference toxicity tests and the whole sediment toxicity tests were similar among laboratories. Standard operating procedures detailing the test methods were provided to all participants. Culture methods were not specified and were not identical across laboratories.

15.5.2.2 In Phase 1, water-only reference toxicity (KCl) tests were conducted with *H. azteca* for 96-h and LC50s were calculated. In these tests, *H. azteca* were placed in reconstituted hard water in 250-mL beakers containing a small piece of plastic mesh substrate. Ten organisms were randomly added to each of four replicates at five concentrations of KCl and a control. The organisms were fed 0.5 mL of a 1800 mg/L stock solution of YCT on Day 0 and Day 2. Mortality was monitored at 24 h intervals and the test was ended at 96 h (Table 9.2). In Phase 2, the variability of the 10-d whole sediment test procedure for *H. azteca* was evaluated using an automated water renewal exposure system (Table 11.1 and Section B.3). This system consisted of eight replicate 300-mL beakers per treatment with each containing 10 organisms each. Each beaker contained a 100-mL aliquot of sediment and the overlying water was replaced twice a day (Table 11.1). The test sediments that were previously tested at ERL-Duluth to ascertain their toxicity included a control sediment (RR 3), a moderately contaminated sediment (RR 2), and a heavily contaminated test sediment (RR 1). Sediments RR 2 and RR 3 were contaminated primarily with copper. An additional sediment heavily contaminated with polycyclic aromatic hydrocarbons (RR 4) was tested by five laboratories. At the end of a test, the sediment from each replicate was sieved and surviving organisms were counted.

15.5.2.3 Ten laboratories participated in the *H. azteca* reference toxicity test (Table 15.2). The results from the tests with KCl are summarized in Table 15.3. The test performance criteria of ≥80% control survival was met by 90% of the laboratories resulting in a mean control survival of 98.8% (CV = 2.1%). The mean LC50 was 305 mg/L (CV = 14.2%) and the LC50s ranged from 232 to 372 mg/L KCl.

15.5.2.4 In the 10-d whole sediment tests with *H. azteca*, nine laboratories tested the three sediments described above and five laboratories tested a fourth sediment from a heavily contaminated site (Table 15.4). All laboratories completed the tests; however, laboratory C had 75% survival, which was below the acceptable test criteria for survival (Table 11.3). For these tests, the CV was calculated using the mean percent survival for

Table 15.2 Participants in Round Robin Studies[1]

	Chironomus tentans			*Hyalella azteca*	
Laboratory	96 h KCl Test Dec 92	96 h KCl Test May 93	10-d Sediment Test May 93	96 h KCl Test Oct 92	10-d Sediment Test Mar 93
Lab A	Y	N	N	Y	N
Lab B	Y	Y	Y	Y	Y
Lab C	Y	N	Y	Y	Y
Lab D	Y	Y	Y	N	N
Lab E	Y	Y	Y	Y	Y
Lab F	Y	Y	Y	Y	Y
Lab G	Y	Y	Y	Y	Y
Lab H	Y	N	N	Y	N
Lab I	Y	Y	Y	—[2]	Y
Lab J	Y	Y	Y	Y	Y
Lab K	_[3]	—[3]	—[3]	Y	Y
Lab L	_[4]	—[4]	—[4]	Y	Y
N	9	7	8	10	9

[1] Y = Laboratory participated in testing sediment samples.
[2] Test in January 1993.
[3] Participated using *C. riparius* only.
[4] Did not intend to participate with *C. tentans*.

Table 15.3 Interlaboratory Precision for *Hyalella azteca* 96-h LC50s from Water-Only Static Acute Toxicity Tests Using a Reference Toxicant (KCl) (October 1992)

Laboratory	KCl LC50 (mg/L)	Confidence Intervals Lower	Confidence Intervals Upper	Percent Control Survival
Lab A	372	352	395	100
Lab B	321	294	350	98
Lab C	232	205	262	100
Lab D	—[1]	—[1]	—[1]	—[1]
Lab E	325	282	374	100
Lab F	276	240	316	98
Lab G	297	267	331	73
Lab H	336	317	356	100
Lab I	142[2]	101	200	93
Lab J	337	286	398	100
Lab L	250	222	282	100
N	10			10
Mean	289.0[3]			96.2%
CV	23.0%[3]			8.3%
N	9			9
Mean	305.0[4]			98.8
CV	14.2%[4]			2.1%

[1] Laboratory did not participate in *H. azteca* test in October.
[2] Results are from a retest in January using three concentrations only; results excluded from analysis.
[3] Mean 1 and CV 1 include all data points.
[4] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥80%.

the eight laboratories that met the performance criteria for the test. The CV for the control sediment (RR 3) was 5.8% with a mean survival of 94.5% with survival ranging from 86% to 100%. For sediments RR 2 and RR 4, the mean survival was 3.3% and 4.3%, respectively (Table 15.4). For RR 2, survival ranged from 0% to 24% (CV = 253%) and for RR 4 the survival ranged from 0% to 11% (CV = 114%). Survival in the moderately contaminated sediment (RR 1) was 54.2% with survival ranging from 23% to 76% (CV = 38.9%). When the RR 1 data for each laboratory were compared to the control for that laboratory, the range for the minimum detectable difference between the test sediments and the control sediment ranged from 5 to 24% with a mean of 11% (SD = 6).

15.5.2.5 The Phase 1 *C. tentans* reference toxicity test was conducted with KCl on two occasions (Tables 15.5 and 15.6). Both tests were conducted in 20 mL of test solution in 30-mL beakers using 10 replicates per treatment with 1 organism per beaker. Animals were fed 0.25 mL of a 4 g/L solution of Tetrafin® on Day 0 and Day 2 (Table 9.1). For the first reference toxicity test comparison, 10 laboratories participated, and eight laboratories met the survival criteria of the round robin, which was 80% survival (Table 15.5). The mean LC50 for the eight laboratories that met the survival criterion was 4.25 g/L (CV of 51.8%). The LC50s ranged from 1.25 to 6.83 g/L. Length and instar were determined for a subset of organisms at the start of the tests for some of the laboratories. When length was correlated with the LC50, the larger animals were less sensitive than the smaller animals. The effect level was significantly correlated (r^2 = 0.78) with the organism size, which ranged from 1.56 mm to 10.87 mm (ages of animals ranged from 7- to 13-d post-deposition). The majority of these animals were the third instar, with the smallest animals in their first instar and the largest animals a mix of third and fourth instar (Table 15.5) as determined by head capsule width.

15.5.2.6 For the second Phase 1 KCl reference toxicity tests with *C. tentans,* seven laboratories participated (Table 15.6). The test conditions were identical to those in the previous reference toxicity test except that a minimum size was specified rather than using initial age of the animals. Each laboratory was instructed to start the test when larvae were at least 0.4 to 0.6 mm long. Therefore, a more consistent size of test organisms was used in this test. Six out of the seven laboratories met the ≥80% control survival criterion with a mean LC50 of 5.37 g/L (CV = 19.6%). The LC50s ranged from 3.61 to 6.65 g/L.

15.5.2.7 In the 10-d whole sediment test with *C. tentans* eight laboratories participated. The same three sediments used in the *H. azteca* whole sediment test were used for this test (Table 15.7). All test conditions were those as described in Table 12.1 with the exception of the feeding rate of 1.0 mL rather than 1.5 mL for *C. tentans.* Three laboratories did not meet the control criteria for acceptable tests of ≥70% survival in the control (RR 3) sediment; Table 12.3). For the five laboratories that successfully completed the tests, the mean survival in the control sediment (RR 3) was 92.0% (CV of 8.3%) and survival ranged from 81.2% to 98.8%. For

Table 15.4 Interlaboratory Precision for Survival of *Hyalella azteca* in 10-d Whole Sediment Toxicity Tests Using Four Sediments (March 1993)

	Mean Percent Survival (SD) in Sediment Samples							
Laboratory	RR 1		RR 2		RR 3 (Control)		RR 4	
Lab A		—[1]		—[1]	—[1]		—[1]	
Lab B	76.2	(20.7)	2.5	(7.1)	97.5	(4.6)	11.2	(13.6)
Lab C	57.52[2]	(14.9)	1.2[2]	(0)	75.0[2]	(17.7)	1.2[2]	(0)
Lab D	—[1]		—[1]		—[1]		—[1]	
Lab E	46.2	(17.7)	0	(0)	97.5	(7.1)	—	
Lab F	72.5	(12.8)	23.7	(18.5)	98.7	(3.5)	0	(0)
Lab G	50.0	(28.3)	0	(0)	100	(0)	3.3	(5.2)
Lab H	—[1]		—[1]		—[1]		—[1]	
Lab I	73.7	(32.0)	0	(0)	86.2	(10.6)	—	
Lab J	65.0	(9.3)	0	(0)	96.2	(5.2)	2.5	(7.1)
Lab K	22.5	(18.3)	0	(0)	95.0	(5.3)	—	
Lab L	27.5	(16.7)	0	(0)	86.2	(18.5)	—	
N	9		9		9		5	
Mean 1[3]	54.6		3.0		93.0		3.6	
CV 1	36.2%		256%		9.0%		121%	
N	8		8		8		4	
Mean 2[4]	54.2		3.3		94.5		4.3	
CV 2	38.9%		253%		5.8%		114%	

[1] Laboratory did not participate in *H. azteca* test in March.
[2] Survival in control sediment (RR 3) below minimum acceptable level.
[3] Mean 1 and CV 1 include all data points.
[4] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥80%.

Table 15.5 Interlaboratory Precision for *Chironomus tentans* 96-h LC50s from Water-only Static Acute Toxicity Tests Using a Reference Toxicant (KCl) (December 1992)

Laboratory	KCl LC50 (g/L)	Confidence Interval Lower	Confidence Interval Upper	Control Survival (%)	Mean Length (mm)	Instar at Start of Test	Age at Start of Test (day)
Lab A	6.19	5.37	7.13	75[1]	10.87	3,4	10
Lab B	6.83	6.38	7.31	100	10.43	3	13
Lab C	5.00	4.16	6.01	100	5.78	3	11
Lab D	3.17	2.29	4.40	100	5.86	3	11
Lab E	2.00[2]	—[2]	—	80	6.07	3	11
Lab F	1.25	—[3]	—	80	1.56	1	12
Lab G	6.28	5.26	7.50	95	7.84	3	11
Lab H	2.89	2.39	3.50	95	6.07	3	7
Lab I	6.66	6.01	7.24	100	—[4]	—[4]	10
Lab J	1.77	0.59	5.26	65[1]	4.42	2,3	7
N	10			10	8		10
Mean 1[5]	4.20			89.0	6.6		10.3
CV 1	52.7%			14.5%	46.6%		17.9%
N	8			8	7		8
Mean 2[6]	4.25			93.8	6.2		10.75
CV 2	51.8%			9.3%	39.5%		15.2%

[1] Control survival below minimum acceptable level.
[2] Unable to calculate LC50 with trimmed Spearman Karber; no confidence interval could be calculated.
[3] Confidence intervals cannot be calculated as no partial mortalities occurred.
[4] No animals were measured.
[5] Mean 1 and CV 1 include all data points.
[6] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥80%.

Table 15.6 Interlaboratory Precision for *Chironomus tentans* 96-h LC50s from Water-only Static Acute Toxicity Tests Using a Reference Toxicant (KCl) (May 1993)

Laboratory	KCl LC50 (g/L)	Confidence Interval Lower	Confidence Interval Upper	Control Survival (%)	Age at Start of Test (day)
Lab A	—[1]	—	—	—	—
Lab B	6.65	—[2]	—	90	12
Lab C	—[1]	—	—	—	—
Lab D	5.30	4.33	6.50	55[3]	10
Lab E	5.11	4.18	6.24	100	11
Lab F	3.61	2.95	4.42	90	10
Lab G	5.36	4.43	6.49	93	12
Lab H	—[1]	—	—	—	
Lab I	5.30	4.33	6.52	95	10-11
Lab J	6.20	4.80	7.89	100	13
n	7			7	7
Mean 1[4]	5.36			89	11.1
CV 1	17.9%			17.5%	9.46%
n	6			6	6
Mean 2[5]	5.37			94.7	11.2
CV 2	19.6%			4.8%	9.13%

[1] Did not participate in reference toxicity test in April.
[2] Confidence intervals cannot be calculated as no partial mortalities occurred.
[3] Control survival below minimum acceptable level.
[4] Mean 1 and CV 1 include all data points.
[5] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥70%.

the RR 2 sediment sample, the mean survival among the five laboratories was 3.0% (CV = 181%) and for the RR 1 sediment sample, the mean survival was 86.8% (CV = 13.5%). A significant effect on survival was not evident for the RR 1 sample, but growth was affected (Table 15.8). When the RR 1 data for each laboratory were compared to the control for that laboratory, the minimum detectable difference for survival among laboratories ranged from 2.3 to 12.1 with a mean of 8% (SD = 4).

15.5.2.8 For *C. tentans,* growth is a sensitive indicator of sediment toxicity (Ankley et al., 1993) and growth was also measured in the round-robin comparison (Table 15.8). Using the data from five laboratories with acceptable control survival in the control sediment (RR 3), the mean weight of *C. tentans* for the control sediment (RR 3) was 1.254 mg (CV = 26.6%). The *C. tentans* in the moderately contaminated sediment (RR 1) had a mean weight of 0.546 mg (CV = 31.9%). No growth measurements were obtained for *C. tentans* in sediment RR 2 because of the high mortality. The mean minimum detectable difference for growth among laboratories meeting the survival performance criteria was 11% (SD = 5) and the MDD ranged from 4.8 to 23.6% when the RR 1 data were compared to the RR 3 data.

15.5.2.9 These tests exhibited similar or better precision than many chemical analyses and effluent toxicity test methods (USEPA, 1991b; USEPA, 1993a). The success rate for test initiation and completion of the EPA's round-robin evaluations is a good indication that a well equipped and trained staff will be able to successfully conduct these tests. This is an important consideration for any test performed routinely in any regulatory program.

Table 15.7 Interlaboratory Precision for Survival of *Chironomus tentans* in 10-d Whole-Sediment Toxicity Tests Using Three Sediments (May 1993)

	Mean Percent Survival (SD) in Sediment Samples					
Laboratory	RR 1		RR 2		RR 3 (Control)	
Lab A	—[1]		—[1]		—[1]	
Lab B	67.5	(14.9)	2.5	(7.1)	98.8	(3.5)
Lab C	15.0[2]	(12.0)	0[2]	(0)	62.5[2]	(26.0)
Lab D	60.0[2]	(20.0)	0[2]	(0)	66.3[2]	(27.7)
Lab E	85.0	(11.9)	0	(0)	93.8	(9.2)
Lab F	87.5[2]	(12.5)	0[2]	(0)	43.8[2]	(30.2)
Lab G	90.0	(13.1)	12.5	(3.5)	87.5	(10.3)
Lab H	—[1]		—[1]		—[1]	
Lab I	97.5	(4.6)	0	(0)	98.8	(3.5)
Lab J	93.8	(11.8)	0	(0)	81.2	(8.3)
N	8		8		8	
Mean 1[3]	74.5		1.88		79.1	
CV 1	36.7%		233%		25.1%	
N	5		5		5	
Mean 2[4]	86.8		3.0		92.0	
CV 2	13 5%		181%		8.3%	

[1] Did not participate in *C. tentans* test in May.
[2] Survival in control sediment (RR 3) below minimum acceptable level.
[3] Mean 1 and CV 1 include all data points.
[4] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥70%.

Table 15.8 Interlaboratory Precision for Growth of *Chironomus tentans* in 10-d Whole-Sediment Toxicity Tests Using Three Sediments (May 1993)

	Growth—Dry Weight in mg (SD) in Sediment Samples					
Laboratory	RR 1		RR 2		RR 3 (Control)	
Lab A	—[1]		—[1]		—[1]	
Lab B	0.370	(0.090)	0	(0)	1.300	(0.060)
Lab C	0.883[2]	(0.890)	0	(0)	0.504	(0.212)
Lab D	0.215[2]	(0.052)	0	(0)	1.070	(0.107)
Lab E	0.657	(0.198)	0	(0)	0.778	(0.169)
Lab F	0.210[2]	(0.120)	0	(0)	0.610	(0.390)
Lab G	0.718	(0.114)	0	(0)	1.710	(0.250)
Lab H	—[1]		—[1]		—[1]	
Lab I	0.639	(0.149)	0	(0)	1.300	(0.006)
Lab J	0.347	(0.050)	0	(0)	1.180	(0.123)
n	8		8		8	
Mean 1[3]	0.505		—		1.056	
CV 1	49.9%		—		38.3%	
n	5		5		5	
Mean 2[4]	0.546		—		1.254	
CV 2	31.9%		—		26.6%	

[1] Did not participate in testing in May.
[2] Survival in control sediment (RR 3) below minimum acceptable level.
[3] Mean 1 and CV 1 include all data points.
[4] Mean 2 and CV 2 exclude data points for all sediment samples from laboratories that did not meet minimum control survival of ≥70%.

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Appendix A
Summary USEPA Workshop on Development of Standard Sediment Test Methods

A.1 The USEPA Office of Water, Office of Science and Technology, and Office of Research and Development held a workshop September 16-18, 1992, in Washington, DC, to provide an opportunity for experts in the field of sediment toxicology and staff from USEPA regional and Headquarters program offices to discuss the development of standard freshwater and marine sediment testing procedures (USEPA, 1992a). As part of USEPA's Contaminated Sediment Strategy, the Agency's program offices agreed to develop and use consistent tests for the assessment of sediment contamination. USEPA sponsored research to address uncertainties associated with the use of sediment tests discussed at the workshop. The results of discussions held at the workshop were used to identify research issues and develop manuals for conducting sediment toxicity and bioaccumulation tests. The following test organisms were selected for sediment test method development in 1993: (1) Freshwater toxicity tests: *Hyalella azteca* and *Chironomus tentans*, (2) Freshwater bioaccumulation tests: *Lumbriculus variegatus*, (3) Marine toxicity tests: *Ampelisca abdita, Rhepoxynius abronius, Eohaustorius estuarius*, and *Leptocheirus plumulosus*, (4) Marine bioaccumulation tests: *Macoma nasuta* and *Nereis* spp.

A.2 If funds are available in future years, additional work will be started on developing chronic toxicity tests, toxicity identification evaluation (TIE), and test development for other organisms. USEPA plans to develop two other methods documents: (1) one on sediment spiking and (2) one on sediment collection, handling, and storage. Parts of the document on collection, handling, and storage methods are already under development for a Quality Assurance and Quality Control guidance document that will supplement both the Inland and Ocean Testing Manuals for disposal of dredged material (USEPA-USCOE, 1991; 1994).

A.3 Before the workshop on July 13, 1992, a questionnaire was sent out to freshwater workshop invitees and other selected researchers. Information was requested on culture methods for and testing of *H. azteca, C. tentans*, and *L. variegatus* and any additional organisms used for sediment tests. The discussion topics for culturing and testing of the three species were ranked in order of importance for developing standard methods based on the similarity of the issues across all tests. The following section summarize results of the questionnaire for each test organism.

1. Development of a Standard Testing Method for *Hyalella azteca*. Twenty-one responses to the survey were received and eighteen laboratories reported information on *H. azteca* (USEPA, 1992a and Table A.1). The summary of the survey responses follow. The most common response is underlined and when no item is underlined it indicates no single most common response. Published procedures for conducting sediment toxicity tests with *H. azteca* are also listed in Table A.2.

Table A.1 List of Laboratories Responding to the Survey

Laboratory	*Hyalella azteca*	*Chironomus tentans*	*Lumbriculus variegatus*
Dept. of Fish. & Oceans, Canada	x		
Environ. Canada, Burlington, ON	x	x	
EPA-Duluth, Duluth, MN	x	x	x
EPA Region 1, Lexington, MA	x	x	
EPA Region 8, Denver, CO	x		
EPA-Newtown, Cincinnati, OH	x	x	
EVS Consultants, Vancouver, BC	x	x	
MD Dept. Environ., Baltimore, MD	x		
Miami Univ., Oxford, OH	x		
Mich. State Univ., E. Lansing, MI	x	x	x
NBS-Athens, Athens, GA	x	x	
NBS-Columbia, Columbia, MO	x	x	x
NOAA-Ann Arbor, Ann Arbor, MI	x	x	x
Old Dominion, Norfolk, VA	x		
State of WA, Manchester, WA	x		
Univ. of MS, University, MS	x		
Univ. of WI-Superior, Superior, WI	x	x	x
Wright State Univ., Dayton, OH	x	x	

Table A.2 Summary of Testing Procedures Used to Evaluate the Toxicity of Whole Sediments with *Hyalella azteca*

Condition	Citation [1]	[2]	[3]	[4]	[5]	[6]
Temperature (°C)	20	20	22	20-25	20-23	20-22
Light intensity (foot candles)	NR	25-50	NR	50-100	25-50	NR
Photoperiod	NR	16-8	16-8	16-8	16-8	16-8
Test chamber (mL)	1000	1000	300	300	30-300	2500
Sediment volume (mL)	200	200	100	40-50	5-100	~150
Overlying water volume (mL)	800	100	175	160-200	20-150	~1350
Renewal rate of overlying water (additions/day)	0	1-4	1-4	variable	0-2	0
Age of organisms (days)	juvenile	juvenile	7-14	juvenile	7-14	0-7
Size of organisms (mm)	NR	1-2	NR	NR	1-2	NR
Number of organisms/ chamber	15	20	10	10	3-10	20
Number of replicate chambers/treatment	NR	4	3	4	5-10	2
Food	RC	RC	YCT	RC	YCT, mL	TM
Aeration	Yes	None	None	DO <3	None	Yes
Overlying water	Natural	Natural	Natural	Natural	Reconst.	Natural
Test duration (d)	10	10-28	10	7	10	28
Endpoints	S	S,G,M	S	S	S	S,G
Test acceptability (survival %)	NR	80	80	80	80	NR

Citations

[1] Nebeker et al. (1984a)
[2] Ingersoll and Nelson (1990)
[3] Ankley et al. (1993a)
[4] Burton et al. (1989)
[5] Winger and Lasier (1993)
[6] Borgmann and Munawar (1989)

Conditions

Food: YCT = yeast-cerophyll-trout chow, RC = Rabbit chow, TM = Tetramin®, ML = maple leaves.
Endpoints: S = survival, G = growth (length or weight), M = maturation, NR = Not reported

A. Survey Summary of Culture Methods for *H. azteca*

Flow:	Static vs. renewal
Temperature:	19 to 25°C (23°C)
Light:	16L:8D photoperiod; about 500 to 1000 lux
Chamber:	1 L to 80 L
Age of organisms:	Known age vs. mixed age
Frequency restart:	Monthly, every two months
Water Quality:	Natural vs. reconstituted
Source of Strains:	ERL-Duluth, ERL-Corvallis, Burlington, Michigan State (most cultured in moderately-hard or hard water)
Aeration:	Moderate
Feeding:	Leaves, Tetramin®, rabbit chow, diatoms, yeast, wheat grass, Chlorella, alfalfa, Nutrafin®, YCT, paper towels, Selenastrum, Ankistrodesmus, brine shrimp, aquatic plants, sediment. Feed 2 to 3 times/week typical.
Substrate:	Leaves, nylon mesh, cotton gauze, 3-M web plastic, paper towels
Reference toxicants:	Cd, Cu, KCl, Zn, NaCl, Cr (water-only exposures)

B. Survey Summary of Testing Procedures for *H. azteca*

Flow:	Static vs. renewal
Aeration:	None or moderate
Temperature:	20 to 25°C (20°C)
Light:	16L:8D photoperiod; about 250 to 1000 lux
Chamber:	30 mL to 1 L (250 to 300 mL)
Sediment ratio:	1:1 to *1:4* ratio sediment:water
Age of organisms:	Known age (0 to 7 d, 7 to 14 d) vs. mixed age (size about 7 to 14 d) (sieved)
Number of organisms:	5 to 20/chamber (10/chamber)
Number replicates:	2 to 10/treatment (3 to 5/treatment)
Duration:	2- to 28-d (10-d)
Feeding:	None, Rabbit Chow, YCT, maple leaves, Tetramin®
Endpoints:	Survival, length, weight, sexual maturation (males), young production, bioaccumulation
Acceptability:	Survival (80%), length, weight

2. Development of a Standard Testing Method for *Chironomus tentans*. Twenty-one responses to the survey were received and twelve laboratories reported information on *C. tentans* (USEPA, 1992a and Table A.1). The summary of the survey responses follow. The most common response is underlined and when no item is underlined it indicates no single most common response. Published procedures for conducting sediment toxicity tests with *C. tentans* are also listed in Table A.3.

Table A.3 Summary of Testing Procedures Used to Evaluate the Toxicity of Whole Sediments with *Chironomus tentans*

Condition	Citation [1]	[2]	[3]	[4]	[5]
Temperature (°C)	20	22	22	23	22
Light intensity (foot candles)	NR	~100	NR	NR	NR
Photoperiod	NR	16-8	16-8	NR	NR
Test chamber (mL)	1000	3000	300	50	2000
Sediment volume (mL)	200	~250	100	~7.5	1500
Overlying water volume (mL)	800	2000	175	47	~200
Renewal rate of overlying water (additions/day)	0	0-5	1-4	0	0
Age of organisms (instar)	2nd	2nd	2nd	2nd	2nd
Size of organisms	NR	0.15 mg	NR	0.5 g	6-8 mm
Number of organisms/ chamber	15	25	10	1	20
Number of replicate chambers/treatment	NR	2	NR	15	NR
Food	TM,CP	TM	TF	TF	None
Aeration	Yes	None	None	Yes	Yes
Overlying water	Natural	Natural	Natural	Natural	Natural
Test duration (d)	10	14	10	10	17
Endpoints	S,G	S,G	S,G	G	S,G
Test acceptability (survival %)	NR	NR	70	NR	NR

Citations:
[1] Nebeker et al. (1984a)
[2] Adams et al. (1985)
[3] Ankley et al. (1993a)
[4] Giesy et al. (1988)
[5] Wentsel et al. (1977)

Conditions:
Food: CP = cerophyll, RC = Rabbit chow, TM = Tetramin®, TF = Tetrafin®
Endpoints: S = survival, G = growth (length or weight), M = maturation, NR = not reported

A. Survey Summary of Culture Methods for *C. tentans*

Flow:	Static vs. renewal
Temperature:	19 to 25°C (23°C)
Light:	16L:8D photoperiod; about 500 to 1300 lux
Chamber:	1 L to 80 L
Age of organisms:	Known age vs. mixed age
Frequency restart:	2x/week to every 6 months
Age restart:	Egg masses to ≤24-h old larvae
Water Quality:	Natural vs. reconstituted
Aeration:	Moderate
Feeding:	Tetramin®, Nutrafin®, YCT and algae, alfalfa and Tetrafin®, feed daily to 3x/week
Substrate:	Paper towels (bleached or unbleached); sand
Reference toxicants:	Cu, NaCl, Cd, KCl (water-only exposures)

B. Survey Summary of Testing Procedures for *C. tentans*

Flow:	Static vs. renewal
Aeration:	None or moderate
Temperature:	20 to 25°C (23°C)
Light:	16L:8D photoperiod; about 250 to 1300 lux
Chamber:	50 mL to 2 L
Sediment ratio:	1:1 to 1:4 ratio sediment:water
Age of organisms:	Known age (0 to 16 d; 10 to 14 d)
Number of organisms:	10 to 80/chamber (10 to 15/chamber)
Number replicates:	2-15 (3 to 4)
Duration:	2 to 14-d (10-d)
Feeding:	Trout chow, Tetrafin®, YCT
Endpoints:	Survival, weight
Acceptability:	Survival (70%), weight (dry weight)

3. Development of a Standard Testing Procedure for *Lumbriculus variegatus*. Twenty-one responses to the survey were received and five laboratories reported information on *L. variegatus* (USEPA, 1992a and Table A.1). The summary of the survey responses follow. The most common response is underlined and when no item is underlined it indicates no single most common response. Published procedures for conducting sediment bioaccumulation tests with *L. variegatus* are also listed in Table A.4.

Table A.4 Summary of Testing Procedures Used to Conduct Whole Sediment Bioaccumulation Tests with *Lumbriculus variegatus*

Condition	Citation [1]	[2]	[3]	[4]
Temperature (°C)	20	23	23	20
Light intensity (foot candles)	NR	NR	25-50	NR
Photoperiod	NR	Various	16-8	NR
Test chamber (L)	3-5	0.15-0.6	4	3-3.8
Sediment volume (L)	1.5-2	30-180g	1	0.3-0.35
Overlying water volume (L)	1.5-3	0.1-0.45	3	2.7-3
Renewal rate of overlying water (additions/day)	2-6	0.5-1	1	0
Age of organisms	Adult	Adult	Adult	Adult
Loading (g/chamber)	1	1:50*	1	0.1-0.39/L
Number of replicate chambers/treatment	NR	3-4	3-5	3
Food	None	None	None	Yes
Aeration	None	Yes	Yes	Yes
Overlying water	Natural/ Reconst.	Natural	Natural	Natural
Test duration (d)	10-60	10-60	56	28-44
Test acceptability	NR	Biomass lipid	Biomass	NR

* = 50 g dry weight organism:sediment organic carbon
NR = not reported

Citations:
[1] Phipps et al. (1993)
[2] Kukkonen and Landrum (1993)
[3] E L. Brunson, NBS, Columbia, MO, unpublished data
[4] Schuytema et al. (1988)

A. Survey Summary of Culture Methods for *L. variegatus*

Flow:	Static vs. renewal
Temperature:	22 to 24°C
Light:	16L:8D photoperiod; illuminance unspecified
Chamber:	1 L to 80 L
Age of organisms:	Mixed-age adults
Frequency restart:	Monthly, every two months
Water Quality:	Natural vs. reconstituted
Aeration:	Moderate
Feeding:	Frozen silver cup trout chow, salmon starter, sediment, Tetramin®, yeast, wheat grass. Chlorella, alfalfa, Nutrafin®, YCT, paper towels. Feed 2 to 3 times/week typical.
Substrate:	Paper towels, sediment
Reference toxicants:	No reference toxicants specified

B. Survey Summary of Testing Procedures for *L. variegatus*

Flow:	Static vs. renewal
Aeration:	None or moderate
Temperature:	10 to 23°C
Light:	16L:8D photoperiod; illuminance unspecified
Chamber:	1 to 6 L
Sediment Ratio:	1:1 to 1:4 ratio sediment: water (sediment volumes should be adequate to allow feeding and burrowing)
Age of organisms:	Adults, 3.8 cm.
Number of organisms:	Adequate number to provide tissue mass for analysis of residue of concern
Number replicates:	4 to 5/treatment
Duration:	10 to 28 d
Endpoints:	Bioaccumulation
Feeding:	None
Acceptability:	Adequate tissue mass for residue analysis

A.4 Workgroup participants arrived at a consensus on several culturing and testing procedures. Where it was not possible to make a decision because of lack of information, the group identified research items that need further consideration before a specific decisions could be made.

A.5 In developing guidance for culturing test organisms to be included in the methods manual for sediment tests, it was generally agreed that no one method had to be used to culture organisms. The success of the tests should rely on the health of the culture from which the organisms were taken for testing. That is, having healthy organisms of known quality and age for testing was deemed to be the key consideration relative to culture procedures. Therefore, performance-based criteria were selected as the preferred approach laboratories should use to evaluate cultures rather than using control-based criteria. Performance criteria were chosen to allow each laboratory to optimize culture techniques and meet quality control monitoring requirements.

A.6 The selection of test organisms for standardization were based on (1) current and historical acceptance, (2) logistical considerations, and (3) availability of testing methods. Major differences between freshwater and marine tests were discussed: (1) freshwater organisms are cultured in the laboratory and marine organisms are collected from the field, (2) freshwater organisms are smaller and younger, (3) freshwater organisms are generally epibenthic and marine test organisms are generally infaunal, (4) freshwater test conditions are sensitive to organic carbon, sediment oxygen demand, ammonia, and the buffering capacity of the overlying water. The overlying water is more stable in saltwater tests compared to freshwater tests.

A.7 Performance-based culturing and testing criteria were outlined for freshwater species at the workshop (Table 11.3, 12.3, 13.4). Consensus was reached on criteria that *must* be met and criteria that *should* be considered. Factors that *must* be met include reference toxicants with short-term water only exposures, survival of control organisms, age or size of organisms at the beginning or end of tests, and consistent water quality. Factors that *should* be considered include parental survival, food quality, frequency of restarting cultures, and time to emergence (midges). In addition, a performance-based criterion for culture of *L. variegatus* to monitor is population doubling time (number of organisms).

A.8 The following topics were discussed related to the use of *Hyalella azteca* and *Chironomus* spp. in sediment toxicity tests: (1) Age of organisms used to start a test: *Hyalella azteca*: Organisms of age 0- to 14-d old are typically used to start a test. It may be best to test organisms 7- to 14-d old, since organisms 0- to 7-d old are difficult to recover from sediment. *Chironomus tentans:* 9- to 11-d old organisms should be tested. *Chironomus riparius:* 6- to 8-d old organisms should be tested; (2) Length of test: The length of the test agreed upon was 10 d with survival as the endpoint. Additional research is ongoing to evaluate growth as an endpoint; (3) Feeding: A minimal amount of food is required to consistently achieve adequate control survival and growth. Additional research is ongoing to evaluate the influence of feeding on sediment toxicity; (4) Water renewal: Limited renewal of overlying water was recommended (about 1 to 2 volume/d): (5) Sediment volumes: Sediment volume up to in the 200 mL have been routinely tested, but smaller volumes would be acceptable; (6) Grain size: There does not seem to be a substantial effect of sediment grain size in the 10-d exposures with *H. azteca*. Additional research is ongoing to evaluate the influence of grain size on the response of amphipods and midges; (7) Strains of organisms: Different strains of *H. azteca* have been used for testing. Reference toxicant comparisons of the strains are needed.

A.9 The following topics were discussed related to the use of *L. variegatus* sediment bioaccumulation tests: (1) Age of test organisms: Adults should be tested; (2) Length of test: 28 d. Additional research is ongoing to evaluate duration of the exposure; (3) Feeding: No feeding is required during the test; (4) Water renewal: Limited renewal of overlying water was recommended (about 1 to 2 volume/d); (5) Sediment volumes: Sediment volume up to in the 200 mL have been routinely tested, but smaller volumes would be acceptable; (6) Grain size: There does not seem to be a substantial effect of sediment grain size in 10-d exposures with *L. variegatus;* (7) Additional discussion topics: Standard lipid content should be addressed in the document, sediment avoidance may be important, and rigorous techniques have been developed to purge the gut in clean water. Research is needed to see if purging is necessary.

Appendix B
Exposure Systems

B.1 Renewal of overlying water is recommended during sediment tests (Section 11.3, 12.3, 13.3). The overlying water can be replaced manually (e.g., siphoning) or automatically. Automated systems require more equipment and initially take more time to build, but manual addition of water takes more time during a test. In addition, automated systems generally result in less suspension of sediment compared to manual renewal of water.

B.2 At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%. Mount and Brungs (1967) diluters have been modified for sediment testing, and other diluter systems have also been used (Maki, 1977; Ingersoll and Nelson, 1990; Benoit et al., 1993; Zumwalt et al., 1994). The water-delivery system should be calibrated before a test is started to verify that the system is functioning properly. Renewal of overlying water is started on Day -1 before the addition of test organisms or food on Day 0. Water-delivery systems are described by Benoit et al. (1993) in Section B.3 and by Zumwalt et al. (1994) in Section B.4. A 60-mL syringe with a mesh screen over the end can be used to manually remove and replace overlying water (J. Lazorchak, USEPA, Cincinnati, OH, personal communication).

B.3 Benoit et al. (1993) describe a sediment testing intermittent-renewal (STIR) system (stationary or portable) for invertebrate toxicity testing with sediment. Either stationary or portable systems enable the maintenance of acceptable water quality (e.g., dissolved oxygen) by automatically renewing overlying water in sediment tests at rates ranging from 1 to 21 volume renewals/d. The STIR system not only reduces the labor associated with renewal of overlying water but also affords a gentle exchange of water that results in virtually no sediment suspension. Both gravity-operated systems can be installed in a compact vented enclosure. The STIR system has been used for conducting 10-d whole-sediment tests with *Chironomus tentans, Hyalella azteca* and *Lumbriculus variegatus.*

B.3.1 STIR systems described in Benoit et al. (1982) can be modified to conduct sediment tests and at the same time maintain their original capacity to deliver varying concentrations of toxicants for water-only toxicity tests. A STIR system (stationary or portable) solely for sediment toxicity tests was designed, which offers a simple, inexpensive approach for the automated renewal of variable amounts of overlying water (Figures B.1 and B.2). This system is described below. The system can be built as a two-unit system (Section B.3.2) or with more exposure treatments (Section B.3.4). All exposure systems consist of exposure holding tanks, head tanks, head tank support stands, and a water bath (Section B.3.2 and B.3.3). The automated delivery system includes design descriptions for a support stand, water renewal supply, and water delivery apparatus (Section B.3.4).

B.3.2 Two Unit Portable STIR System Construction (Figure B.1 and B.2)

B.3.2.1 Exposure Holding Tanks (2) (Figure B.3).

1. Outer diameter: 15.8 cm wide x 29.3 cm long x 11.7 cm high

2. Cutting dimensions: (double strength glass, 3 mm)

2 Bottoms:	15.8 cm x 29.3 cm
4 Sides:	11.4 cm x 28.7 cm
4 Ends:	11.4 cm x 15.8 cm

3. Hole: 1.6 cm centered between sides and 7.2 cm from bottom edge of 11.4 cm high end piece.

4. Standpipe Height: 10.3 cm above inside of tank bottom.

B.3.2.2 Head Tanks (2) (4-L capacity; Figure B.3)

1. Outer diameter: 15.8 cm wide x 24 cm long x 14.5 cm high

2. Cutting dimensions: (acrylic plastic, 6 mm)

2 Bottoms:	15.8 cm x 24 cm
4 Sides:	13.9 cm x 22.8 cm
4 Ends:	13.9 cm x 15.8 cm

3. Acrylic plastic sheets should be cut with a smooth cutting fine toothed table saw blade. Dimension cut pieces can most easily be glued together with Weld-On® #16 clear-thickened cement for acrylic

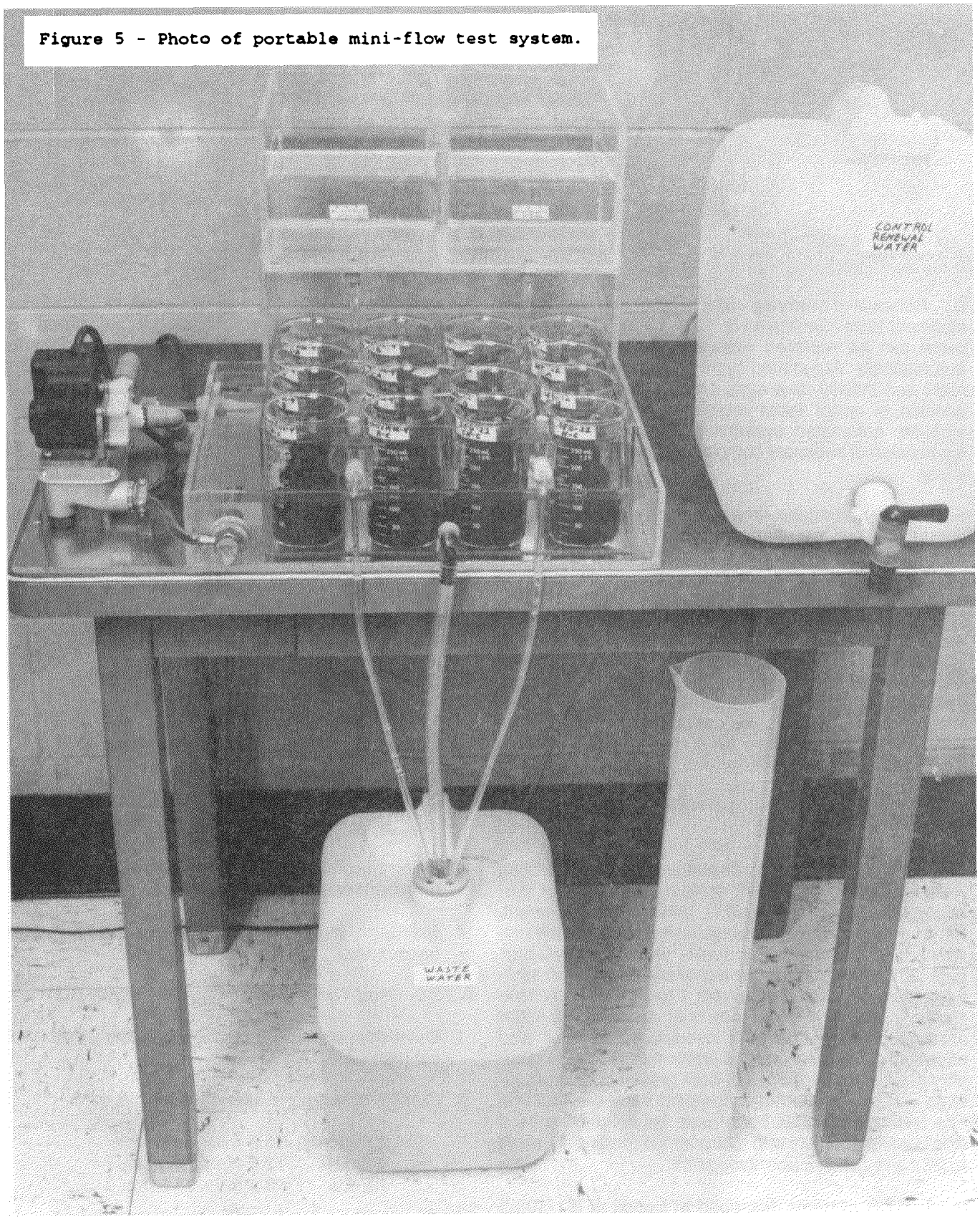


Figure B.1 Portable table top STIR system described in Benoit et al. (1993).

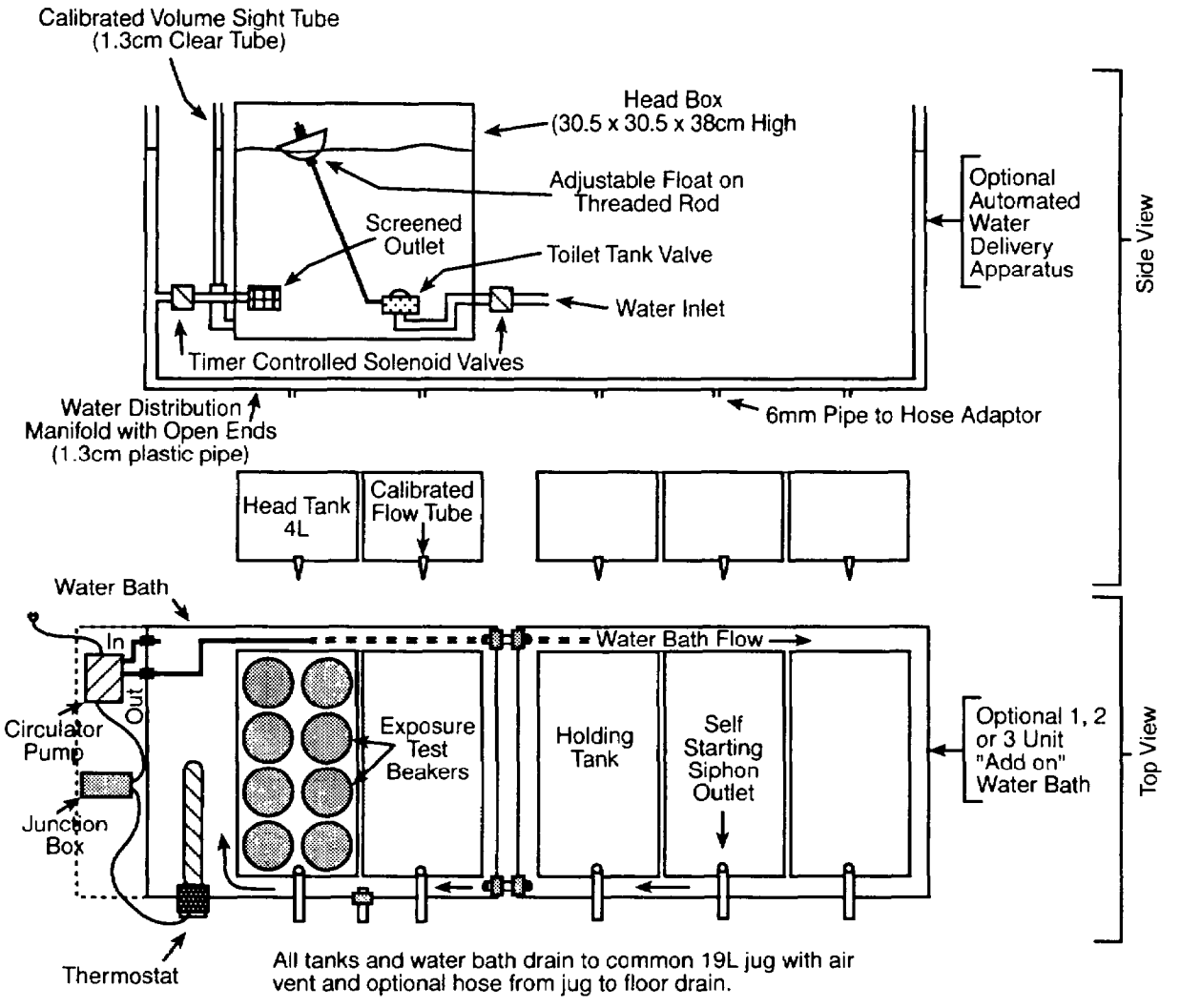
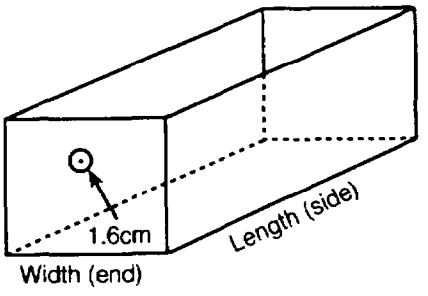
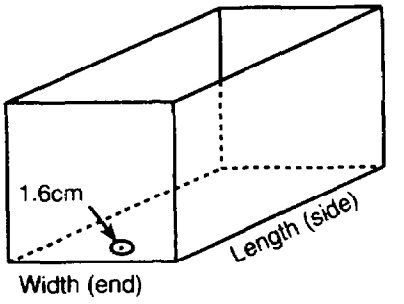


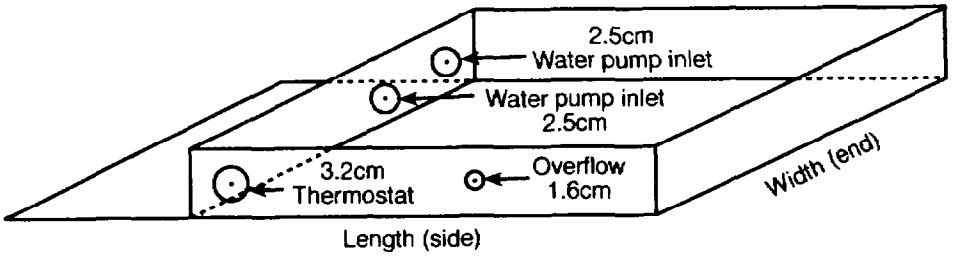
Figure B.2 Portable table top STIR system with several additional options as described in Benoit et al. (1993).



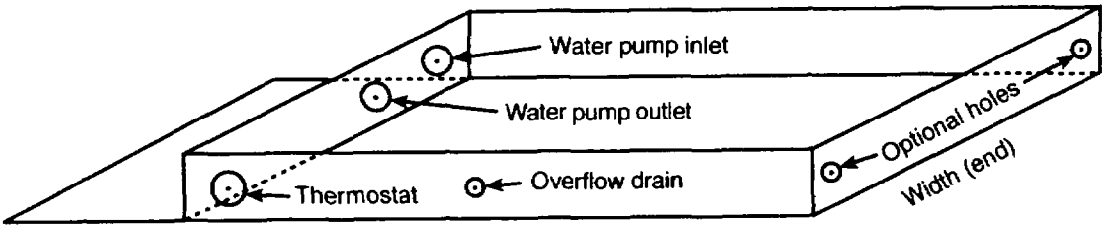
Exposure Holding Tank



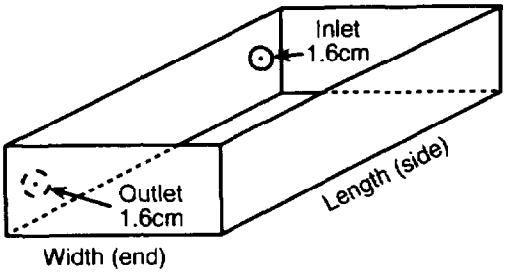
Head Tank



Basic Water Bath



Basic Water Bath with Optional Holes for Water Bath



Add on Water Bath for One Additional Unit

Figure B.3 Tanks for the STIR system in Benoit et al. (1993).

plastic (Industrial Polychemical Service, P.O. Box 471, Gardena, CA, 90247).

4. Hole: 1.6 cm centered between sides and 2 cm from front edge of 24 cm long bottom piece. Holes can most easily be drilled in acrylic plastic by using a wood spade bit and drill press.

5. Flow Tubes: 10-mL pipet tip initially cut off at the 6 mL mark and inserted flush with top of #0 stopper. Top of stopper should be inserted nearly flush with head tank bottom. With 2 L of water in head tank, calibrate flow tube to deliver 32 mL/min.

B.3.2.3 Head Tank Support Stand (1) (Figure B.3)

1. Outer diameter: 16.7 cm wide x 33.7 cm long x 17.8 cm high

2. Cutting dimensions: (acrylic plastic, 6 mm)

1 Bottom:	16.7 cm x 33.7 cm
2 Sides:	17.2 cm x 32.5 cm
2 Ends:	17.2 cm x 16.7 cm

3. Size is such that both head tanks fit into support stand for storage and transport.

B.3.2.4 Water Bath (1) (Figure B.3)

1. Outer diameter: 33 cm wide x 40.6 cm long x 7.4 cm high

2. Cutting dimensions: (acrylic plastic, 6 mm)

1 Bottom:	33 cm x 55.9 cm
2 Ends:	33 cm x 6.8 cm
2 Sides:	39.4 cm x 6.8 cm

3. Holes:

   a. Overflow drain; 1.6 cm centered 2.9 cm from bottom edge of 39.4 cm long side piece and 17.8 cm from right edge.
   b. Thermostat; 3.2 cm centered 2.5 cm from bottom edge of 39.4 cm long side piece and 3.2 cm from left edge.
   c. Water pump outlet; 2.5 cm centered 2.5 cm from bottom edge of 33 cm long end piece and 8.3 cm from back edge.
   d. Water pump inlet; 2.5 cm centered 2.5 cm from bottom edge of 33 cm long end piece and 2.0 cm from back edge.

4. A small 90° elbow made of glass or plastic is attached to the water pump inlet tube and turned downward so the circulator pump will not pick up air at the water surface.

5. The bottom piece for the water bath includes 15.3 cm extension for motor mount and the thermostat electrical junction box.

6. Motor Mount: 5.1 cm wide x 11.4 cm long x 3.8 cm thick mount made from 6 pieces of 6-mm acrylic plastic. Four of these pieces are glued together. The other two pieces are glued together, motor attached to the edge with two screws and the two pieces (with motor attached) are then screwed to the top of the four pieces. The entire unit is then glued to water bath extension after 6-mm PVC piping is attached and secured with stoppers to the inlet and outlet water bath holes.

7. Thermostat Conduit Junction Box: (1.3-cm small left back (SLB)) is attached to the water bath extension by screwing a 1.3-cm PVC plug into junction box and securing this plug with a screw, countersunk up through the bottom and into the PVC plug.

B.3.2.5 Latex Rubber Mold: If you plan to construct a substantial number of exposure test beakers, as described in Benoit et al. (1993), then it would be to your advantage to make a latex rubber mold to give support to the underside of the glass when drilling holes. It significantly reduces the number of broken beakers. Liquid latex, with hardener that can be purchased from the local hardware store is commonly used to coat the handles of tools. The rubber mold is constructed as follows:

1. Mix latex with hardener as per instructions.

2. Fill one exposure test beaker with the mixture.

3. Suspend one 5 cm eye bolt (5 mm diameter) with nut on end so that the eye is protruding just above the top of the mixture.

4. Allow the latex plenty of time to "set up."

5. With proper eye protection and wearing heavy gloves, gently break the beaker with a small hammer and remove all of the glass from the mold.

6. Using a long drill bit for wood, drill an air vent hole through the mold from top through bottom.

7. When using the mold, wet the mold and the beaker with water before inserting. Place the beaker, with pre-marked location of holes, on its side in a 3.5-L stainless steel pan filled with coolant water so that the beaker is just below the surface. The beaker is then held in position with one hand while the other hand operates the drill press. Operator should wear proper eye protection.

8. After the two holes are drilled, the mold can be easily removed, with some effort, by inserting the eye bolt into the handle of a securely attached "C"

clamp and physically pulling the beaker from the mold.

B.3.3 Suggested options for more exposure treatments (examples given are for a three unit treatment system)

B.3.3.1 Exposure Holding Tanks and Head Tanks: Same dimensions as for two unit system except that three (3) of each should be made.

B.3.3.2 Head Tank Support Stand (1) (Figure B.3)

1. Outer diameter: 16.7 cm wide x 49.5 cm long x 17.8 cm high

2. Cutting dimensions:(acrylic plastic, 6 mm)

1 Bottom:	16.7 cm x 49.5 cm
2 Sides:	17.2 cm x 48.3 cm
2 Ends:	17.2 cm x 16.7 cm

3. Size is such that the three head tanks will fit into the support stand for storage and transport.

B.3.3.3 Water Bath (1) (Figure B.3)

1. Outer diameter: 33 cm wide x 56.4 cm long x 7.4 cm high

2. Cutting dimensions:(acrylic plastic, 6 mm)

1 Bottom:	33 cm x 71.7 cm
2 Ends:	33 cm x 6.8 cm
2 Sides:	55 cm x 6.8 cm

3. Holes: All hole sizes and locations are the same as for the two unit system except that overflow drain is located 25.7 cm from right edge of 55-cm side. Also, two optional 1.6-cm holes centered 2.5 cm from bottom edge of 33-cm long end piece and 1.8 cm from corner edges are shown in the drawing for future additions of "add-on" water baths.

4. Motor mount and junction box installation are the same as for two unit system.

B.3.3.4 "Add-On" Water Bath (example given is for one additional unit treatment system; Figure B.3)

1. Outer diameter: 18.5 cm wide x 33 cm long x 8 cm high

2. Cutting dimensions: (acrylic plastic, 6 mm)

1 Bottom:	18.5 cm x 33 cm
2 Ends:	17.3 cm x 7.4 cm
2 Sides:	33 cm x 7.4 cm

3. Holes: Inlet and outlet holes (1.6 cm) are centered 2.5 cm from bottom edge of 33-cm long side piece and 1.8 cm from corner edges.

4. The above holes will match the previously drilled holes in the main water bath. The "add-on" water bath is connected using #2 stoppers and 6.4 cm lengths of clear plastic tubing (1.3 cm diameter). The circulator pump outlet tubing (Tygon®) in the main water bath is extended through the inlet connection as shown in Figure B.2. Circulating water is then forced into the "add-on" bath and flows back to the main water bath by gravity.

5. Note that the walls of the "add-on" bath are 6 mm higher than the main water bath to accommodate the small head of water that builds up.

6. "Add-on" water baths tend to run a little warmer (0.2°C) than main water bath test temperatures.

B.3.4 Optional Automated Water Delivery Apparatus For Table Top STIR Systems (examples given are for a three unit treatment system)

B.3.4.1 Support Stand: A stand to support the automated water delivery apparatus, shown in Figure B.2, can be made from bolted slotted angle iron bolted with corner braces. A convenient size to construct is 30 cm wide x 85 cm long x 43 cm high. The head box in Figure B.2 sits on top of the stand, and the water distribution manifold as shown in Figure B.2 is placed directly under the top of the stand with two 1.3 cm conduit hangers. A small portion of each angle iron cross piece is cut away to allow the pipe to be clamped into the conduit hanger. This also keeps the manifold up high enough for sufficient clearance between the head tanks and the 6-mm pipe to hose adapters as shown in Figure B.2.

B.3.4.2 Water Renewal Supply: If tests will be conducted in the local water supply, then the head box water inlet shown in Figure B.2 is simply plumbed into the supply line. However, if the tests are conducted with transported water or with reconstituted water, the head box water inlet can be connected to a Nalgene® drum with flexible Tygon® tubing. With a four volume test beaker water renewal flow rate per day, both 114-L and 208-L Nalgene® drums will hold a 5-d supply for a 3-unit treatment system and a 5-unit treatment system, respectively. If the water supply drum is located below the head box, then an open air water pump such as a March® model MDXT pump (PFC Equipment Corp., Minneapolis, MN 55440) can be used between the drum and head box.

B.3.4.3 Operation of Water Delivery Apparatus: The head box water inlet solenoid valve (Figure B.2) and the open air water pump (if needed) are connected to the same timer control switch. The head box water outlet solenoid valve is connected to another separate timer control switch. With four test beaker renewals/d and a 3-unit treatment system, the head box toilet float valve is pre-adjusted to allow the head box to fill to the 12-L mark on the sight tube (Figure B.2).

B.3.4.3.1 With head box filled, the renewal cycle begins when the first timer opens the head box outlet solenoid valve. The distribution manifold is quickly flooded and the 12 L of renewal water divided equally to each of the three 4-L head tanks. Since the timers have a minimum setting of one hour on-off periods, the first timer is set to shut off the head box outlet solenoid valve one hour after it opens.

B.3.4.3.2 About 30 min later, the second timer is set to open the head box water inlet solenoid valve (and pump if needed). As head box water volume reaches the 12-L mark, the pre-adjusted toilet tank valve stops the water flow. One hour after they come on, the second timer will shut off the solenoid valve inlet and water pump.

B.3.4.3.3 The automated system is then ready for the next renewal cycle that is set to begin 12 h after the first cycle. Head box volume dimensions are such that up to five unit treatment systems can be tested simultaneously as shown in Figure B.2.

B.3.5 A criticism of the system described by Benoit et al. (1993) is that the (up to) 8 beakers placed in each holding tank are not true replicates because of the potential for exchange of water overlying the sediments among the beakers. However, this concern is largely semantic with regard to actual test results. The rationale for this position is described below. The data described below are unpublished data from ERL-Duluth (G.T. Ankley, USEPA, Duluth, MN, personal communication).

B.3.5.1 Beakers within a test tank should contain an aliquot of the *same* homogenized sediment and the same test species. The replication is intended to reflect variability in the biology (e.g., health) of the organism, as well as placement and recovery of the animals from the test sediments (i.e., operator variability). To treat even completely separate tanks containing homogenized sediment from the same source as true replicates (of the sediment "treatment") is inaccurate and is pseudoreplication. Hence, because the same sediment is tested in each beaker in a particular tank, and because the replication is focused on defining variability in the biology of the organism (and the operator), this is essentially a non-issue from a theoretical standpoint.

B.3.5.2 From a practical standpoint, it is important to determine the potential influence of one beaker on another over the course of a test. To determine this, a study was designed (which is not advocated) in which treatments were mixed within a tank. In the first experiment, four beakers of highly metal-contaminated sediment from the Keweenaw Waterway, MI, were placed in the same tank as four beakers containing clean sediment from West Bearskin Lake, MN. This was done in two tanks; in one tank, 10 amphipods *(Hyalella azteca)* were added to each beaker, while in the other tank, 10 midges *(Chironomus tentans)* were placed in each beaker. Controls for the experiment consisted of the West Bearskin sediments assayed in separate "clean" tanks. The four contaminated beakers were placed "upstream" of the four clean beakers to attempt to maximize possible exchange of contaminant. At the end of the test, organism survival (and growth in for *C. tentans)* was measured in two of the beakers from each site and sediment Cu concentrations were determined in the other two beakers from each site. The Keweenaw sediments contained concentrations of Cu in excess of 9,000 µg/g (dry wt), and were toxic to both test species (Table B.1). Conversely, survival of both *C. tentans* and *H. azteca* was high in the West Bearskin sediments from the Keweenaw tank, and was similar to survival in West Bearskin sediments held in separate tanks. Most important, there was no apparent increase in Cu concentrations in the West Bearskin sediments held in the Keweenaw tank (Table B.1).

Table B.1 Sediment Copper Concentrations and Organism Survival and Growth at the End of a 10-d Test with West Bearskin Sediment in an Individual Tank Versus 10-d Cu Concentrations and Organism Survival and Growth in West Bearskin Sediment Tested in the Same Tank as Keweenaw Waterway Sediment[1]

Sediment	Tank	Species	Survival (%)	Dry wt (mg/organism)	Cu (µg/g)
WB[2]	1	Amphipod	90	ND[3]	22.4
WB	2	Amphipod	100	ND	13.8
KW[4]	2	Amphipod	20	ND	9397.0
WB	3	Midge	95	1.34	12.3
WB	4	Midge	100	1.33	15.6
KW	4	Midge	5	ND	9167.0

[1] All values are the mean of duplicate observations (G.T. Ankley, USEPA, Duluth, MN, unpublished data)
[2] West Bearskin
[3] Not determined
[4] Keweenaw Waterway

B.3.5.3 A similar design was used to determine transfer of contaminants among beakers containing sediments spiked with the organochlorine pesticide dieldrin. In this experiment, sediment from Airport Pond, MN, was spiked with dieldrin and placed in the same tank as clean unspiked Airport Pond sediments. Two different concentrations were assayed: (1) in the midge test sediment concentrations were about 150 µg dieldrin/g (dry weight) and (2) in the amphipod test sediments contained in excess of 450 µg dieldrin/g sediment. The control for the experiment again consisted of clean Airport Pond sediment held in a separate tank. The spiked sediments were toxic to both test species, and survival of organisms held in the clean Airport Pond sediments was similar in the two different tanks. However, there was an effect on the growth of *C. tentans* from the clean Airport Pond sediment assayed in the tank containing the spiked sediment. This corresponded to the presence of measurable dieldrin concentrations in unspiked Airport Pond sediments in the tank with the mixed treatments

Table B.2 Sediment Dieldrin Concentrations and Organism Survival and Growth at the End of a 10-d Test with Airport Pond Sediment in an Individual Tank Versus 10-d Dieldrin Concentrations and Organism Survival and Growth in Airport Pond Sediment Tested in the Same Tank as Dieldren-spiked Airport Pond Sediment[1]

Sediment	Tank	Species	Survival (%)	Dry wt (mg/organism)	Dieldrin (µg/g)
AP[2]	1	Amphipod	75	ND[3]	<0.01
AP	2	Amphipod	80	ND	0.07
DAP[4]	2	Amphipod	20	ND	446.4
AP	3	Midge	85	1.71	<0.01
AP	4	Midge	85	0.13	0.04
DAP	4	Midge	0	ND	151.9

[1] All values are the mean of duplicate observations (G.T. Ankley, USEPA, Duluth, MN, unpublished data)
[2] Airport Pond
[3] Not determined
[4] Dieldren-spiked Airport Pond

(Table B.2). The concentrations of dieldrin in the unspiked sediment, although detectable, were on the order of 5,000-fold lower than the spiked sediments, indicating relatively minimal transfer of pesticide.

B.3.5.4 Using a similar design, an investigation was made to evaluate if extremely low dissolved oxygen (DO) concentrations, due to sediment oxygen demand, in four beakers in a test system would result in a decrease in DO in other beakers in the tank. In this experiment, trout chow was added to each of four beakers containing clean Pequaywan Lake sediment, and placed in a test tank with four beakers containing Pequaywan Lake sediment without exogenous organic carbon. Again, the control consisted of Pequaywan Lake sediment held in a separate tank under otherwise identical test conditions. Assays were conducted, without organisms, for 10 d. At this time, DO concentrations were very low in the beakers containing trout chow-amended sediment (ca., 1 mg/L, n = 4). However, overlying water DO concentration in the "untreated" vs. the "treated" beakers in a separate tank, i.e., 6.8 vs. 6.9 mg/L, respectively. This indicates, that from a practical standpoint, even under extreme conditions of mixed treatments (which again, is *not* recommended), interaction between beakers within a tank is minimal.

B.3.5.5 One final observation germane to this issue is worth noting. If indeed beakers of homogenized sediment within a test tank do not serve as suitable replicates, this should be manifested by a lack of variability among beakers with regard to biological assay results. This has not proven to be the case. For example, in a recent amphipod test with a homogenized sediment from the Keweenaw Waterway in which all eight replicates were held in the same tank; mean survival for the test was 76%; however, survival in the various beakers ranged from 30 to 100%, with a standard deviation of 21%. Clearly, if the test system were biased so as to reduce variability (i.e., result in unsuitable replicates due to common overlying water), this type of result would not be expected.

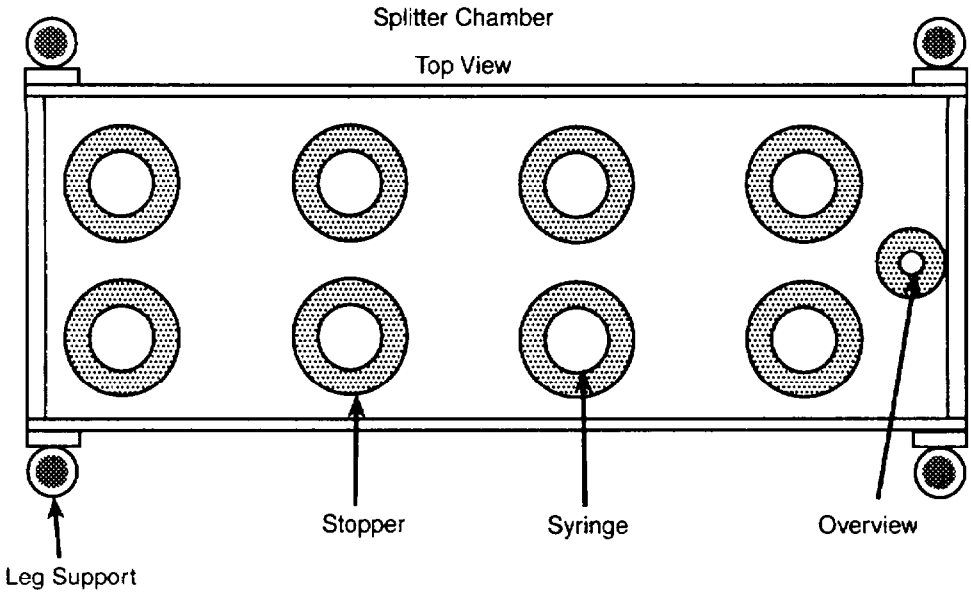
B.3.5.6 In summary, in both a theoretical and practical sense, use of the system described by Benoit et al. (1993) results in valid replicates that enable the evaluation of variability due to factors related to differences in organism biology and operator effects. To achieve this, it is important that treatments not be mixed within a tank; rather, the replicates should be generated from the same sediment sample. Given this, and the fact that it is difficult to document interaction between beakers using even unrealistic (and unrecommended) designs, leads to the conclusion that variability of replicates from the test system can be validly used for hypothesis testing.

B.4 Zumwalt et al. (1994) also describe a water-delivery system that can accurately deliver small volumes of water (50 mL/cycle) to eight 300-mL beakers to conduct sediment tests. The system was designed to be comparable with the system described by Benoit et al. (1993).

B.4.1 Eight 35-mL polypropylene syringes equipped with 18-gauge needles are suspended from a splitting chamber (Figure B.4). The system is suspended above eight beakers and about 1 L of water/cycle is delivered manually or automatically to the splitting chamber. Each syringe fills and empties 50 mL into each beaker and the 600 mL of excess water empties out an overflow in the splitting chamber (Section B.4.3.1). The volume of water delivered per day can be adjusted by changing either the cycling rate or the size of the syringes. The system has been used to renew overlying water in whole-sediment toxicity tests with *H. azteca* and *C. tentans*. Variation in delivery of water among 24 beakers was less than 5%. The system is inexpensive (<$100), easy to build (<8 h), and easy to calibrate (<15 min).

B.4.2 Water Splitting Chamber

B.4.2.1 The glass water-splitting chamber is 14.5 cm wide, 30 cm long, and 6.5 cm high (inner diameter). Eight 3.8-cm holes and one 2.5-cm hole are drilled in a 15.5 cm x 30.5 cm glass bottom before assembly (Figure B.4 and Table B.3). The glass bottom is made from 4.8- (3/16 inch) or 6.4 mm (1/4 inch) plate glass. An easy way to position the 3.8-cm holes is to place the eight 300- mL beakers (2 wide x 4 long) under the bottom plate and mark the center of each beaker. The 2.5-cm hole for overflow is centered at one end of the bottom plate between the last two holes and endplate (Figure B.4). After drilling the holes in the bottom plate, the side (6.5 x 30.5 cm) and end (6.5 x 14.5 cm) plates are cut from 3.2-mm (1/8 inch) double-strength glass and the splitting box is assembled using silicone adhesive. Sharp glass edges should be sanded smooth using a whetstone or a piece of carborundum wheel. After the



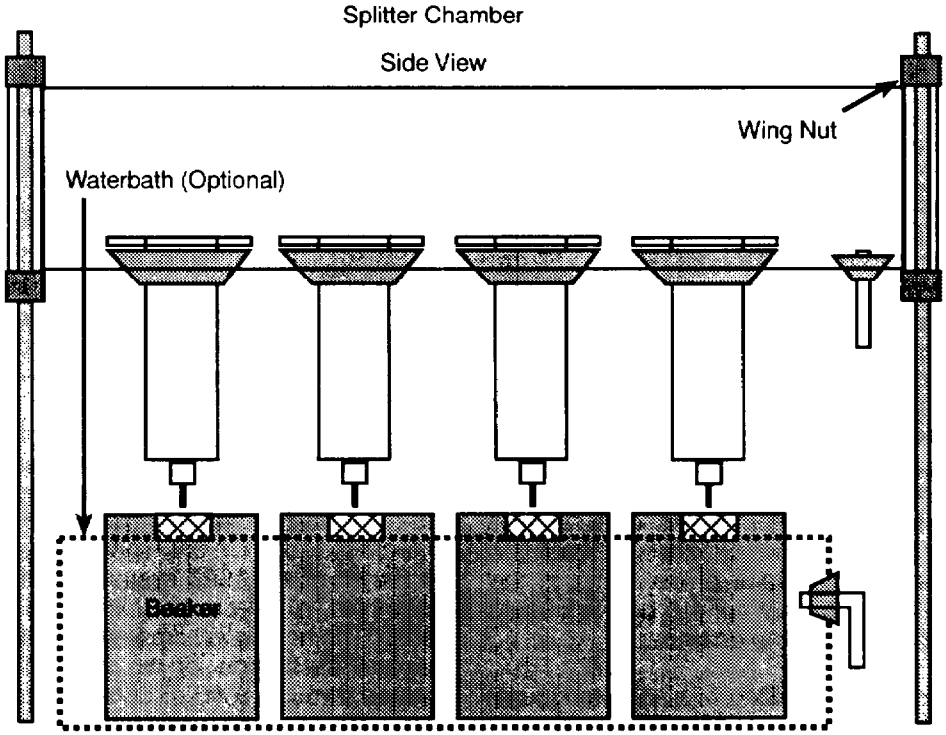


Figure B.4 Water splitting chamber described in Zumwalt et al. (1994).

Table B.3 Materials Needed for Constructing a Zumwalt et al. (1994) Delivery System

Equipment
Drill press
Glass drill bits (2.54 cm [1 inch] and 3.8 cm [1.5 inch])
Cork boring set
Table-top saw equipped with a carborundum wheel
Small level (about 30 cm long)
Supplies
300-mL beakers (lipless, tall form; e.g., Pyrex Model 1040)
Stainless-steel screen (50- x 50-mesh)
9.5 mm (3/8 inch x 16) stainless-steel threaded rod
9.5 mm (3/8 inch x 16) nylon wingnuts
9.5 mm (3/8 inch x 16) nylon nuts
35 mL Mono-ject syringes (Sherwood Medical, St. Louis, MO)
18-gauge Mono-ject stainless-steel hypodermic needles
Silicone stoppers (#0, 5, and 7)
Plate glass (6.4 mm [1/4 inch], 4.8 mm [3/16 inch], 3.2 mm [1/8 inch])
Glass tubing (8 mm outer diameter)
Stainless-steel tubing (12 mm outer diameter)
Silicone adhesive (without fungicide)
5-way stainless-steel gang valves and pasteur pipets (14.5 cm [5.75 inch])

splitting chamber has dried for 24 h, four 12-mm (outer diameter) stainless-steel tubes (7 cm long) are glued to each corner of the splitting chamber (the surface of the steel tubes is scored with rough emery paper to allow better adhesion of the silicone). These tubes are used as sleeves for attaching the legs to the splitting chamber. The legs of the splitting chamber are threaded stainless-steel rods (9.5 mm [3/8 inch] diameter, 36 cm long). The location of the tubes depends on the way that the beakers are to be accessed in the waterbath. If the tubes are placed on the side of the splitting chamber, a 3.2-mm-thick x 2-cm-wide x 7-cm-long spacer is required so beakers and the optional waterbath can be slid out the ends (Figure B.4). If the sleeves and legs are attached to the ends of the splitting chamber, the beakers and waterbath can be removed from the side. The legs are inserted into the 12-mm tubes and secured using nylon nuts or wingnuts. The distance between the tips of the needles to the surface of the water in the 300-mL beakers is about 2 cm. Four 1-L beakers could also be placed under the splitting chamber.

B.4.2.2 A #7 silicone stopper drilled with a 21-mm (outer diameter) core borer is used to hold each 35-mL polypropylene syringe (45 mL total capacity) in place. Glass syringes could be used if adsorption of contaminants on the surface of the syringe is of concern. A dilute soap solution can be used to help slide the syringe into the #7 stopper (until the end of the syringe is flush with the top of stopper). Stoppers and syringes are inserted into 3.8-cm holes and are visually leveled. A #5 silicone stopper drilled with an 8 mm (outer diameter) core borer is placed in the 2.5 cm overflow hole. An 8-mm (outer diameter) glass tube (7.5 cm long) is inserted into the stopper. Only 3 mm of the overflow tube should be left exposed above the stopper. This overflow drain is placed about 3 mm lower than the top of the syringes. A short piece of 6.4-mm (1/4 inch; inner diameter) tubing can be placed on the lower end of drain to collect excess water from the overflow.

B.4.2.3 The splitting chamber is leveled by placing a level on top of the chamber and adjusting the nylon nuts. Eighteen-gauge needles are attached to the syringes. About 6 mm of the needle should remain after the sharp tip has been cut off using a carborundum wheel. Jagged edges left in the bore of the needle can be smoothed using a small sewing needle or stainless-steel wire.

B.4.2.4 When about 1 L of water is delivered to the splitting chamber, the top of each syringe should be quickly covered with water. The overflow tube will quickly drain excess water to a level just below the tops of the syringes. The syringes should empty completely in about 4 min. If water remains in a syringe, the needle should be checked to ensure that it is clean and does not have any jagged edges.

B.4.3 Calibration and Delivery of Water to the Splitting Chamber. Flow adjustments can be made by sliding either the stoppers or syringes up or down to deliver more or less water. A splitting chamber with eight syringes can be calibrated in less than 15 min. Delivery of water to the splitting chamber can be as simple as manually adding about 1 L of water/cycle. Water can be added automatically to the splitting chamber using a single cell or a Mount and Brungs (1967) diluter that delivers about 1 L/cycle on a time delay. About 50 mL will be delivered to each of the 8 beakers/cycle and 600 mL will flow out the overflow. A minimum of about 1 L/cycle should be dumped into the splitting chamber to ensure each syringe fills to the top. If the quantity of water is limited at a laboratory, the excess water that drains through the overflow can be collected and recycled.

B.4.4 Waterbath and Exposure Beakers. The optional waterbath surrounding the beakers is made from 3.2-mm (1/8-inch) double-strength glass and is 15.8 cm wide x 29.5 cm long x 11.7 cm high (Figure B.4 [Figure B.3 in the Benoit et al., 1993 system]). Before the pieces are assembled, a 1.4-cm hole is drilled in one of the end pieces. The hole is 7.2 cm from the bottom and centered between each side of the end piece. A glass tube inserted through a #0 silicone stopper can be used to drain water from the waterbath. A notch is made in each 300-mL beaker by making two cuts with a carborundum wheel 1.9 cm apart to the 275 mL level. The beaker is etched across the bottom of the cuts, gently tapped to remove the cut section, and the notch is covered with 50- x 50-mesh stainless-steel screen using silicone adhesive. The waterbath illustrated in Figure B.4 is optional if the splitting chambers and beakers are placed in a larger waterbath to collect waste water. This smaller waterbath could be used to collect waste water and a surrounding larger waterbath could be used for temperature control.

B.4.4.5 Operation and Maintenance

B.4.4.5.1 Maintenance of the system is minimal. The syringes should be checked daily to make sure that all of the water is emptying with each cycle. As long as the syringe empties completely, the rate of flow out of the syringes is not important because a set volume of water is delivered from each syringe. If the syringe does not empty completely with each cycle, the needle tip should be replaced or cleaned with a thin wire or sewing needle. If the screens on the beakers need to be cleaned, a toothbrush can be used to brush the outside of screens.

B.4.4.5.2 Overlying water can be aerated by suspending pasteur pipets (e.g., Pyrex disposable 14.5-cm [5.75 inch] length) about 3 cm above the sediment surface in the beakers. Five-way stainless steel gang valves are suspended from the splitting chamber using stainless steel hooks. Latex tubing (3.2-mm [1/8 inch] inner diameter) is used to connect valves and pipets. Flow rate of air should be maintained at about 2 to 3 bubbles/s and the pipets can be placed on the outside of the beakers when samples of overlying water are taken during a test.

B.4.4.5.3 The splitting chambers were used to deliver water in a toxicity test with the midge *Chironomus tentans* exposed to metal-contaminated sediments (Zumwalt et al., 1994). Ten third-instar midges were exposed in 300-mL beakers containing 100 mL of sediment and 175 mL of overlying water at 23°C. Midges in each beaker received a daily suspension of 4 mg Tetrafin® flake food and survival and growth were measured after 10 d. Splitting chambers delivered 50 mL/cycle of overlying water to each of the eight replicate beakers/sediment sample. One liter of water was delivered with a single-cell diluter to each splitting chamber 4 times/d. This cycle rate resulted in 1.1 volume additions of overlying water/d to each beaker ([4 cycles/d x 50-mL volume/cycle]/175 mL of overlying water). The variation in delivery of water between 24 beakers was less than 5%.

B.4.4.5.4 Hardness, alkalinity, and conductivity in water overlying the sediments averaged about 20% higher than inflowing water. These water-quality characteristics tended to be more similar to inflowing water at the end of the exposure compared with the beginning of the exposure. The average pH was about 0.3 units lower than inflowing water. Ammonia in overlying water ranged from 0.20 to 0.83 mg/L. The dissolved oxygen content was about 1 mg/L lower than inflowing water at the beginning of the exposure and was about 2 to 3 mg/L lower than inflowing water by the end of the exposure. Survival and growth of midges were reduced with exposure to metal-contaminated sediments. Water delivered at a similar rate to a second set of beakers using a system described by Benoit et al. (1993) resulted in similar overlying water quality and similar toxic effects on midges.

B.4.4.5.5 The system has been used to deliver 33 ‰ salt water to exposure chambers for 10 d. Precipitation of salts on the tips of the needles reduced flow from the syringes. Use of a larger bore needle (16-gauge) reduced clogging problems; however, daily brushing of the needle tips is required. Use of larger bore needles with 300-mL beakers containing 100 mL of sediment and 175 mL of overlying water results in some suspension of sediment in the overlying water. This suspension of sediment can be eliminated if the stream of water from the larger bore needle falls on a baffle (e.g., a piece of glass) at the surface of the water in the beaker.

Appendix C
Food Preparation

C.1 Yeast, Cerophyl®, and Trout Chow (YCT) for feeding the cultures and *Hyalella azteca*. Food should be stored at 4°C and used within two weeks from preparation; however, once prepared, YCT can be frozen until use.

C.1.1 Digested trout chow is prepared as follows:

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

2. Add 5.0 g of trout chow pellets to 1 L of deionized water. Mix well in a blender and pour into a 2-L separatory funnel or similar container. Digest before use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a ventilated area.

3. At the end of digestion period allow material to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (e.g., Nitex® 110 mesh). Combine with equal volumes of the supernatant from Cerophyl® and yeast preparation (below). The supernatant can be used fresh, or it can be frozen until use. Discard the remaining particulate material.

C.1.2 Yeast is prepared as follows:

1. Add 5.0 g of dry yeast, such as Fleishmann's® Yeast, Lake State Kosher Certified Yeast, or equivalent, to 1 L of deionized water.

2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.

3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and Cerophyl® preparations (below). Discard excess material.

C.1.3 Cerophyl® is prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves are available as "Cereal Leaves," from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO, 63178 (800/325-3010); or as Cerophyl®, from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY, 14692-9012 (716/359-2502). Dried, powdered, alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet shops.

2. Add 1 L of deionized water.

3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.

4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

C.1.4 Combined yeast-cerophyl-trout chow (YCT) is mixed as follows:

1. Thoroughly mix equal (e.g., 300 mL) volumes of the three foods as described above.

2. Place aliquots of the mixture in small (50 mL to 100 mL) screw-cap plastic bottles.

3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings and is used for a maximum of two weeks. Do not store YCT frozen over three months.

4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7 to 1.9 g solids/L. Cultures are fed 10 mL/L on Monday and 5 mL/L on Wednesday and Friday (USEPA, 1993).

C.2 Algal Food: Starter cultures of the green algae, *Selenastrum capricornutum* and the diatom *Navicula* (or *Synedra)* are available from the following sources: American Type Culture Collection (Culture No. ATCC 22662), 12301 Parklawn Drive, Rockville, MD 10852, or Culture Collection of Algae, Botany Department, University of Texas, Austin, TX 78712.

C.2.1 Algal Culture Medium for the green algae and diatoms *(Navicula* or *Synedra)* prepared as follows (USEPA, 1993a):

Table C.1 Nutrient Stock Solutions for Maintaining Algal Stock Cultures

Stock solution	Compound	Amount dissolved in 500 mL deionized water
1. Macronutrients		
A.	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4 \cdot 7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. Micronutrients		
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg[5]

[1]$ZnCl_2$—Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to micronutrient stock.

[2]$CoCl_2 \cdot 6H_2O$—Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to micronutrient stock.

[3]$Na_2MoO_4 \cdot 2H_2O$—Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to micronutrient stock.

[4]$CuCl_2 \cdot 2H_2O$—Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to micronutrient stock.

[5]Na_2SeO_4—Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to micronutrient stock.

Table C.2 Final Concentration of Macronutrients and Micronutrients in the Algal Culture Medium

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14
Micronutrient	**Concentration (µg/L)**	**Element**	**Concentration (µg/L)**
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	—	—
Na_2SeO_4	2.39	Se	0.91

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

2. Add 1 mL of each stock solution, in the order listed in Table C.1, to about 900 mL of deionized water. Mix well after the addition of each solution. Dilute to 1 L, mix well. The final concentration of macronutrients and micronutrients in the culture medium is listed in Table C.2.

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

4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels. Unused sterile medium should not be stored more than one week before use, because there may be substantial loss of water by evaporation.

C.2.2 Algal Cultures: Two types of algal cultures are maintained: (1) stock cultures and (2) "food" cultures.

C.2.2.1 Establishing and Maintaining Stock Cultures of Algae:

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

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

3. Culture temperature is not critical. Stock cultures may be maintained at 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of about 4300 lux).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One to 3 mL of 7-d old algal stock culture, containing about 1.5×10^6 cells/mL are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of about 10,000 to 30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.

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

C.2.2.2 Establishing and Maintaining "*S. capricornutum* food" Cultures:

1. "*S. capricornutum* food" cultures are started 7 d before use. About 20 mL of 7-d-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL are added to each liter of fresh algal culture medium (e.g., 3 L of medium in a 4-L bottle or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of about 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are used in 7 to 10 d. A one-month supply of algal food can be grown at one time and stored in the refrigerator.

2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of about 4300 lux).

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

C.2.2.3 Preparing Algal Concentrate of *S. capricornutum* for Use as Food:

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

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

3. Assuming a cell density of about 1.5×10^6 cells/mL in the algal food cultures at 7 d, and 100% recovery in the concentration process, a 3-L culture at 7 to 10 d will provide 4.5×10^9 algal cells.

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

5. Cultures of *Hyalella azteca* are fed 10 mL/L on Monday and 5 mL/L on Wednesday and Friday (ERL- Duluth, 1993).

C.2.2.4 Establishing and Maintaining Stock Cultures of Diatoms *(Navicula* sp. or *Synedra* sp.):

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

2. The stock cultures are used as a source of diatoms to initiate "diatom food" cultures. The volume of stock culture maintained at any one time will depend on the amount of food required for culture. Stock culture volume may be rapidly "scaled up" to several liters using 4-L serum bottles or similar vessels containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained at 23°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of about 1075 lux).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "diatom food" cultures or can be transferred to new medium weekly. One to 3 mL of 7-d old algal stock culture, containing about 1.5 X 10^6 cells/mL are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of about 10,000 to 30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.

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

C.2.2.5 Establishing and Maintaining "Diatom food" Cultures:

1. "Diatom food" cultures are started about 10 d before use. About 20 mL of 7-d-old algal stock culture (described in the previous paragraph) are added to each liter of fresh culture medium (e.g., 3 L of medium in a 4-L bottle). Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are used in 7 to 10 d. A one-month supply of diatom food can be grown at one time and stored in the refrigerator.

2. Food cultures may be maintained at 23°C in environmental chambers, but not with the algae stock cultures. The illumination is continuous "cool-white" fluorescent lighting of about 1075 lux). Higher temperatures can be problematic for diatom cultures.

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) in a moderately aerated separatory funnel, or are manually mixed twice daily. Cultures become very brown before harvesting. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be taken to prevent the culture temperature from rising more than 2 to 3°C.

C.2.2.6 Preparing Concentrate of Diatoms for Use as Food:

1. A diatom concentrate containing 1 X 10^9 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer and used to determine the dilution (or further concentration) required to achieve a final cell count of 1 X 10^9 cells/mL.

3. Algal concentrate can be stored in the refrigerator for one month.

4. Cultures of *Hyalella azteca* are fed 10 mL/L on Monday and 5 mL/L on Wednesday and Friday (ERL- Duluth, 1993).

C.2.2.7 Cell counts:

1. Several types of automatic electronic and optical particle counters are available to rapidly count cell number (cells/mL) and mean cell volume (MCV; μm^3/cell). The Coulter Counter is widely used and is discussed in detail in USEPA (1978). When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a 1% sodium chloride electrolyte solution, such as Coulter ISOTON®, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-µm

diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

A. Mix the algal culture in the flask thoroughly by swirling the contents of the flask about six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.

B. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.

C. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON®).

D. Determine the cell density (and MCV, if desired).

2. Manual microscope counting methods for cell counts are determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA (1992) and USEPA (1973). Whenever feasible, 400 cells per replicate are counted to obtain ±10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

C.3 Tetrafin® food for culturing and testing *C. tentans*. Food should be stored at 4°C and used within two weeks from preparation or can be frozen until use.

1. Blend the Tetrafin® food in deionized water for 1 to 3 min or until very finely ground.

2. Filter slurry through an #110 Nitex screen to remove large particles. Place aliquot of food in 100- to 500-mL screw-top plastic bottles. It is desirable to determine dry weight of solids in each batch of food before use. Food should be held for no longer than two weeks at 4°C. Food can be frozen before use, but it is desirable to use fresh food.

3. Tetrafin® food is added to each culture chamber to provide about 0.04 mg dry solids/mL of culture water. A stock suspension of the solids is prepared in culture water such that a total volume of 5.0 mL of food suspension is added daily to each culture chamber. For example, if a culture chamber volume is 8 L, 300 mg of food would be added daily by adding 5 mL of a 56 g/L stock suspension (USEPA, 1993).

4. In a sediment test, Tetrafin® food (4 g/L) is added at 1.5 mL daily to each test chamber.

Appendix D
Sample Data Sheets

Culture Aquarium	Date of Egg Mass Deposition	Date 4th Instar Larvae Were Weighed	Age of Weighed 4th Instar Larvae	Mean Dry Weight of 4th Instar Larvae (n = 10)	Date of Observed First Emergent Adult	Total Number of Egg Masses Produced	General Comments	Initials of Culturist
A								
B								
C								
D								
E								
F								

Figure D.1 Data sheet for the evaluation of a *Chironomus tentans* culture.

Brood Stock Source_____

Test Type (circle one)[1]: SU SM RU RM FU FM

No. of Animals Tested Per Replicate_____

No. of Replicates_____

Method of LC50 Estimate_____

Reference Toxicant ($CuSO_4$ or KCl)_____

Reference Toxicant Supplier and Lot No.

_____

Reference Toxicant Purity_____

Test Initiation Date_____

Toxicologist_____

Exposure Duration (H)	Number of Mortalities											
	Control		Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5	
	A	B	A	B	A	B	A	B	A	B	A	B
0												
24												
48												
72												
96												

Current Test 96 h LC50 = _____

Number of Reference Toxicant Test Used to Determine Cumulative Mean 96 h LC50_____

Mean 96 h LC50 for All Tests to Date_____

Acceptability of Current Test[2] Yes_____ No_____

[1] SU = Static unmeasured
SM = Static measured
RU = Renewal unmeasured
RM = Renewal measured
FU = Flow-through unmeasured
FM = Flow-through measured

[2] Based on two standard deviations around the cumulative mean 96 h LC50

Figure D.2 Data sheet for performing reference toxicant tests.

Sediment Sample Source_____

Date of Test Initiation_____

Toxicologist Conducting Test _____

Test Day	Test Replicate Sampled	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Hardness (mg/L)	Alkalinity (mg/L)	Specific Conductance (umhos/cm)	Total Ammonia (mg/L)
0								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Figure D.3 **Data sheet for temperature and overlying water chemistry measurements.**

Daily Checklist for Sediment Tests

Study Code_____

Study Name_____

Building_____Diluter_____Waterbath_____

Study Director_____

Lead Technician_____ Month_____

Target temperature _____°C

Acceptable Range _____°C to _____°C

Dissolved Oxygen
Minimum Acceptable Concentration
(40% of Saturation at Target Temp)

= _____mg/L

Day of Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Day of Study																															
Diluter Operation																															
Number of Cycles																															
Time of Day																															
Temperature																															
Air Pressure																															
Aeration																															
Brush Screens																															
Clean Needles																															
Feeding																															
Total Water Quality																															
Partial Water Quality																															
Initials																															

Comments_____

Approved by_____ Date_____

Figure D.4 Data sheet for daily checklist for sediment tests.

Water Quality Data Sheet

Study Code________ Date________ Test Day_____ Study Director____________ Investigator____________

Study Name____

Sample Code	Dissolved Oxygen (mg/L)	Temp. °C	Salinity (ppt)	Conductivity (umhos or uS)	pH	(ml Titrant x mult. factor =)		Alkalinity (ppm $CaCO_3$)	(ml Titrant x mult. factor =)		Hardness (ppm $CaCO_3$) __ml	Turbidity (NTU) __ml	Total Ammonia as NH_3 (mg/L) __ml	ml of ISA	Other ____	____
Volume of Sample =	__ml	__ml	__ml	__ml	__ml		__ml		__ml						__ml	__ml
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____ =						
______						____ x	____	=	____ x	____						
______						____ x	____	=	____ x	____ =						
Meter #																
Initials																
Comments																

Approved by______________________ Date__________

Figure D.5 **Data sheet for water quality parameters.**

Chemistries

Test Type________________ Sample Info________________ Water Type________________

Organism________________ ________________ Experimenter________________

Test Dates________________ ________________ Test System________________

I.D.	Day	-1	0	1	2	3	4	5	6	7	8	9	10	Remarks
	pH													
	DO (mg/L)													
	Temp (°C)													
	Hard/Alk													
	pH													
	DO (mg/L)													
	Temp (°C)													
	Hard/Alk													
	pH													
	DO (mg/L)													
	Temp (°C)													
	Hard/Alk													
	pH													
	DO (mg/L)													
	Temp (°C)													
	Hard/Alk													

Figure D.6 **Chemistry data sheet.**

Study Director

Study Code

Study Name_____

Daily Comment Sheet

Day_____ Date_____-_____-_____ Initials_____

Day_____ Date_____-_____-_____ Initials_____

Day_____ Date_____-_____-_____ Initials_____

Day_____ Date_____-_____-_____ Initials_____

Day_____ Date_____-_____-_____ Initials_____

Figure D.7 Daily comment data sheet.

Weight Data Form

Test Dates			Species				
Test Material			Weighing Date			Food	
Location			Oven Temp (°C)			Age Organisms	
Analyst			Drying Time (h)			Initial No/Rep	
Sample	Replicate	Wt. of (mg) oven dried pan	Wt. of (mg) pan + organism	Total (mg)	Survival (#)	Mean dry (mg) wt./_____	Mean/Sample

Figure D.8 Weight data sheet.

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Center for Environmental Research Information
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