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Standard Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates[1]

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1. Scope *

1.1 This guide covers procedures for measuring the bioaccumulation of sediment-associated contaminants by infaunal invertebrates. Marine, estuarine, and freshwater sediments are a major sink for chemicals that sorb preferentially to particles, such as organic compounds with high octanol-water-partitioning coefficients (K_{ow}) (for example, polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT)) and many heavy metals. The accumulation of chemicals into whole or bedded sediments (that is, consolidated rather than suspended sediments) reduces their direct bioavailability to pelagic organisms but increases the exposure of benthic organisms. Feeding of pelagic organisms on benthic prey can reintroduce sediment-associated contaminants into pelagic food webs. The bioaccumulation of sediment-associated contaminants by sediment-dwelling organisms can therefore result in ecological impacts on benthic and pelagic communities and human health from the consumption of contaminated shellfish or pelagic fish.

1.2 Methods of measuring bioaccumulation by infaunal organisms from marine, estuarine, and freshwater sediments will be discussed. The procedures are designed to generate quantitative estimates of steady-state tissue residues because data from bioaccumulation tests are often used in ecological or human health risk assessments. Eighty percent of steady-state is used as the general criterion. Because the results from a single or few species are often extrapolated to other species, the procedures are designed to maximize exposure to sediment-associated contaminants so that residues in untested species are not underestimated systematically. A 28-day exposure with sediment-ingesting invertebrates and no supplemental food is recommended as the standard single sampling procedure. Procedures for long-term and kinetic tests are provided for use when 80 % of steady-state will not be obtained within 28 days or when more precise estimates of steady-state tissue residues are required. The procedures are adaptable to shorter exposures and different feeding types. Exposures shorter than 28 days may be used to identify which compounds are bioavailable (that is, bioaccumulation potential) or for testing species that do not live for 28 days in the sediment (for example, certain *Chironomus*). Non-sediment-ingestors or species requiring supplementary food may be used if the goal is to determine uptake in these particular species because of their importance in ecological or human health risk assessments. However, the results from such species should not be extrapolated to other species.

1.3 Standard test methods are still under development, and much of this guide is based on techniques used in successful studies and expert opinion rather than experimental comparisons of different techniques. Also, relatively few marine/estuarine (for example, *Nereis* and *Macoma*), freshwater (for example, *Diporeia* and *Lumbriculus variegatus*) species, and primarily neutral organic compounds provide a substantial portion of the basis for the guide. Nonetheless, sufficient progress has been made in conducting experiments and understanding the factors regulating sediment bioavailability to establish general guidelines for sediment bioaccumulation tests.

1.4 This guide is arranged as follows:

1.5 Field-collected sediments may contain toxic materials,

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***A Summary of Changes section appears at the end of this standard.**

including pathogens, and should be treated with caution to minimize exposure to workers. Worker safety must also be considered when using laboratory-dosed sediments containing toxic compounds.

1.6 This guide may involve the use of non-indigenous test species. The accidental establishment of non-indigenous species has resulted in substantial harm to both estuarine and freshwater ecosystems. Adequate precautions must therefore be taken against the accidental release of any non-indigenous test species or associated flora or fauna.

1.7 The values stated in SI units are to be regarded as the standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]

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

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[4]

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

E 943 Terminology Relating to Biological Effects and Environmental Fate[3]

E 1022 Practice for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs[3]

E 1367 Guide for Conducting 10-Day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods[3]

E 1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates[5]

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing[3]

E 1525 Guide for Designing Biological Tests with Sediments[3]

E 1706 Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates[3]

2.2 *Federal Document:*

CFR, Title 21, Food and Drugs, Chapter I Food and Drug Administration, Department of Health and Human Services, Part 177, Indirect Food Additives: Polymers[6]

CFR, Title 49, Transportation Chapter 1 Research and Special Programs Administration, Department of Transportation Parts 100–177, Subchapter A—Hazardous Materials Transportation, Oil Transportation and Pipeline Safety, Subchapter B—Oil Transportation and Subchapter C—Hazardous Materials Regulation[6]

[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 11.05.

[4] Discontinued 1997; Replaced by IEEE/ASTM SI-10.

[5] Discontinued 1995; Replaced by E 1706.

[6] Available from Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

3. Terminology

3.1 *Definitions:*

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

3.1.2 For definitions of terms used in this guide, refer to Guide E 729 and Terminologies D 1129 and E 943. For an explanation of units and symbols, refer to Practice E 380.

3.2 *Descriptions of Terms Specific to This Standard:*

3.2.1 *alpha*—see *Type I error*.

3.2.2 *apparent steady-state*—see *steady-state*.

3.2.3 *bedded sediment*—see *whole sediment*.

3.2.4 *beta*—see *Type II error*.

3.2.5 *bioaccumulation*—the net accumulation of a substance by an organism as a result of uptake from all environmental sources.

3.2.6 *bioaccumulation factor (BAF)*—the ratio of tissue residue to sediment contaminant concentration at steady-state.

3.2.7 *bioaccumulation potential*—a qualitative assessment of whether a contaminant in a particular sediment is bioavailable.

3.2.8 *bioconcentration*—the net assimilation of a substance by an aquatic organism as a result of uptake directly from aqueous solution.

3.2.9 *bioconcentration factor (BCF)*—the ratio of tissue residue to water contaminant concentration at steady-state.

3.2.10 *biota-sediment accumulation factor (BSAF)*— the ratio of lipid-normalized tissue residue to organic carbon-normalized sediment contaminant concentration at steady state, with units of g-carbon/g-lipid.

3.2.11 *block*—a group of homogeneous experimental units.

3.2.12 *coefficient of variation (CV)*—a standardized variance term; the standard deviation (SD) divided by the mean and expressed as a percent.

3.2.13 *comparison-wise error*—a Type I error applied to the single comparison of two means. Contrast with *experiment-wise error*.

3.2.14 *compositing*—the combining of separate tissue or sediment samples into a single sample.

3.2.15 *control sediment*—sediment containing no or very low levels of contaminants. Control sediments should ideally contain only unavoidable "global" levels of contaminants. Contrast with *reference sediment*.

3.2.16 *degradation*—metabolic breakdown of the contaminant by a test species.

3.2.17 *depuration*—loss of a substance from an organism as a result of any active (for example, metabolic breakdown) or passive process when the organism is placed into an uncontaminated environment. Contrast with *elimination.*

3.2.18 *dichlorodiphenyltrichloroethane (DDT)*— a common environmental contaminant. Metabolites include dichlorodiphenyldichloroethane (DDD) and dichlorodiphenylethylene (DDE).

3.2.19 *redox potential (Eh)*—a measure of the oxidation state of a sediment.

3.2.20 *elimination*—a general term for the loss of a substance from an organism that occurs by any active or passive means. The term is applicable in either a contaminated environment (for example, occurring simultaneously with uptake) or a clean environment. Contrast with *depuration*.

3.2.21 *equilibrium partitioning bioaccumulation model*—a bioaccumulation model based on equilibrium partitioning of a neutral organic among organism lipids and sediment carbon.

3.2.22 *experiment-wise error*—a Type I error (alpha) chosen such that the probability of making any Type I error in a series of tests is alpha. Contrast with *comparison-wise error*.

3.2.23 *experimental error*—variation among experimental units given the same treatment.

3.2.24 *experimental unit*—an organism or organisms to which one trial of a single treatment is applied.

3.2.25 *fines*—the silt-clay fraction of a sediment.

3.2.26 *gut purging*—voiding of sediment contained in the gut.

3.2.27 *hydrophobic contaminants*—low-contaminant water solubility with a high K_{ow} and usually a strong tendency to bioaccumulate.

3.2.28 *interstitial water*—water within a wet sediment that surrounds the sediment particles.

3.2.29 *kinetic bioaccumulation model*—any model that uses uptake or elimination rates, or both, to predict tissue residues.

3.2.30 *long-term uptake tests*—bioaccumulation tests with an exposure period greater than 28 days.

3.2.31 *metabolism*—see *degradation*.

3.2.32 *minimum detectable difference*—the smallest (absolute) difference between two means that is distinguishable statistically.

3.2.33 *multiple comparisons*—the statistical comparison of several treatments simultaneously, such as with Analysis of Variance (ANOVA).

3.2.34 *no further degradation*—an approach by which a tissue concentration is deemed acceptable if it is not greater than the tissue concentration at a reference site.

3.2.35 *pairwise comparisons*—the statistical comparison of two treatments. Contrast with *multiple comparisons*.

3.2.36 *power*—the probability of detecting a difference between the treatment and control means when a true difference exists.

3.2.37 *pseudoreplication*—the incorrect assignment of replicates, often due to a biased assignment of replicates.

3.2.38 *reference sediment*—a sediment similar to the test sediment in physical and chemical characteristics and not contaminated by the particular contaminant source under study (for example, dredge material, discharge, and non-point runoff). A reference sediment should ideally contain only background levels of contaminants characteristic of the region. Contrast with *control sediment*.

3.2.39 *replication*—the assignment of a treatment to more than one experimental unit.

3.2.40 *sampling unit*—the fraction of the experimental unit that is to be used to measure the treatment effect.

3.2.41 *standard reference sediment*—a standardized sediment and contaminant used to estimate the variability due to variation in the test organisms.

3.2.42 *steady-state*—a "constant" tissue residue resulting from the balance of the flux of compound into and out of the organism, determined operationally by no statistical difference in three consecutive sampling periods.

3.2.43 *total carbon (TC)*—this value includes organic and inorganic carbon.

3.2.44 *test sediment*—the sediment or dredge material of concern.

3.2.45 *test treatment*—treatment that is compared to the control or reference treatment. It may consist of either a test sediment (compared to a reference or control sediment) or a reference sediment (compared to the control sediment).

3.2.46 *thermodynamic partitioning bioaccumulation model*—see *equilibrium partitioning bioaccumulation model.*

3.2.47 *tissue residues*—the contaminant concentration in the tissues.

3.2.48 *toxicokinetic bioaccumulation model*—a bioaccumulation model based on the feeding and ventilatory fluxes of the organism.

3.2.49 *treatment*—the procedure (type of sediment) whose effect is to be measured.

3.2.50 *Type I error*—the rate at which Ho is rejected falsely.

3.2.51 *Type II error*—the rate at which Ho is accepted falsely.

3.2.52 *whole sediment*—consolidated or bedded sediment (that is, not suspended). Also referred to as *bedded sediment.*

3.3 *Symbols* :

Ha—alternate hypothesis.

Ho—null hypothesis.

k_1—uptake rate coefficient from the aqueous phase, in units of g-water $\times$ g-tissue $^{-1} \times$ time^{-1}. Contrast with k_s.

k_2—elimination rate constant, in units of time^{-1}.

K_{oc}—organic carbon-water partitioning coefficient.

K_{ow}—octanol-water partitioning coefficient.

k_s—sediment uptake rate coefficient from the sediment phase, in units of g-sediment $\times$ g-tissue $^{-1} \times$ time^{-1}. Contrast with k_1.

4. Summary of Guide

4.1 This guide provides method descriptions for determining the bioaccumulation of sediment-associated contaminants by infaunal invertebrates. The procedures focus on estimating steady-state tissue residues in sediment-ingesting organisms in a 28-day exposure. Alternative methods for estimating steady-state tissue residues from long-term or kinetic exposures are included, as are procedures for non-steady exposures. Sediments tested may be either collected from the field or spiked

with known compounds. Criteria for the selection of test organisms is provided, and several species are recommended. Recommendations are provided concerning procedures to meet differing study objectives in sediment evaluations. These recommendations address the following: sediment physical and chemical measurements; test organism selection, collection, and maintenance; construction and maintenance of exposure apparatus; sampling methods and test durations; models that may be used to predict bioaccumulation; and statistical design of tests and analysis of test data.

5. Significance and Use

5.1 Sediment exposure evaluations are a critical component for both ecological and human health risk assessments. Credible, cost-effective methods are required to determine the rate and extent of bioaccumulation given the potential importance of bioaccumulation by benthic organisms. Standardized test methods to assess the bioavailability of sediment-associated contaminants are required to assist in the development of sediment quality criteria **(1, 2)**[7] and to assess the potential impacts of disposal of dredge materials **(3)**.

5.2 The extent to which sediment-associated contaminants are biologically available and bioaccumulated is important in order to assess their direct effects on sediment-dwelling organisms and assess their transport to higher trophic levels. Controlled studies are required to determine the potential for bioaccumulation that can be interpreted and modeled for predicting the impact of accumulated chemicals. The data collected by these methods should be correlated with the current understanding of toxicity or human health risks to complete the hazard interpretation for contaminated sediments.

6. Interference

6.1 State-of-the-art sediment quality evaluations are still in their infancy, due largely to methodological difficulties and the complex nature of sediments. The reader is cautioned that the area of sediment bioavailability is highly dynamic. Recommended methods and this guide will be updated routinely to reflect progress in our understanding of sediments and methods of studying them. The following factors should be considered when determining the bioaccumulation of chemicals from whole sediments.

6.1.1 Maintaining the integrity of a sediment environment during its removal, transport, and testing in the laboratory is extremely difficult. The sediment environment is composed of a myriad of microenvironments, redox gradients, and other interacting physicochemical and biological processes. Many of these characteristics influence chemical sorption and speciation, microbial degradation, and the bioavailability of sediment-associated contaminants. Any disruption of this environment complicates interpretations of treatment effects, causative factors, and in situ comparisons.

6.1.1.1 Chemical solubility, partitioning coefficients, and other physical and chemical characteristics will differ for sediments tested at temperatures other than those of their collection.

6.1.2 Changes in the ratios between sediment and overlying water may influence the partitioning and accumulation behavior of compounds.

6.1.3 Interactions may occur among chemicals that may be present in the sediment.

6.1.4 The use of laboratory-spiked sediment may not be representative of contaminants associated with sediments in the field.

6.1.5 An acceptable quality of overlying water should be maintained.

6.1.6 Addition of food to the test chambers may obscure the accumulation of contaminants associated with sediment and may affect water quality.

6.1.7 Resuspension of sediment during the test may alter chemical partitioning and bioavailability.

6.1.8 The natural geochemical properties of test sediment collected from the field may not be within the tolerance limits of the test organisms.

6.1.9 Field-collected sediments may contain endemic organisms including (*1*) predators, (*2*) the same species or a species that is related closely to the species being tested, or (*3*) microorganisms (for example, bacteria and molds) and algae that may grow in or on the sediment and test chamber surfaces.

6.1.9.1 Field-collected sediments may contain concentrations of chemicals concentrations that can elicit toxicity responses or can be detected by the organisms. These concentrations may be sufficient to cause the organism to escape from the sediment. This will result in reduced exposure and accumulation.

6.1.10 The longer the study, the more likely the data will approach steady-state for slowly bioaccumulating compounds. However, long-term tests require greater resources and increase the analytical requirements and likelihood of problems involving the maintenance of the organisms and temporal changes in sediment contaminant concentrations.

6.1.10.1 With longer exposures, there is a greater probability of the test organism reproducing. Spawning can affect lipid content drastically and possibly chemical concentrations **(4)**. Additionally, it is prudent to add extra test organisms for studies of extended duration because many species die after spawning.

6.1.10.2 In addition to spawning, the difficulty of maintaining organism health increases with prolonged exposure, including the possibilities of weight loss due to nutritional insufficiency and disease.

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

6.2 *Static Tests*—Static tests (without the renewal of overlying water) might not be applicable to materials that are highly volatile or are rapidly transformed biologically or chemically. Furthermore, the overlying water quality may change considerably. The procedures can usually be applied to materials that have a high oxygen demand because the experimental chambers are usually aerated. Materials dissolved in interstitial waters might be removed from solution in substantial quantities by absorption to sediment particles and to the test chamber during the test. The dynamics of chemical partitioning between

[7] The boldface numbers in parentheses refer to the list of references at the end of this standard.

solid and dissolved phases at the start of the test should be considered, especially in relation to assumptions of chemical equilibrium.

6.3 *Flow-Through Tests*—The equipment and facilities required to conduct flow-though tests (with the renewal of overlying water) make them inherently more expensive than static tests. Water quality, temperature, or salinity are more difficult to control and may require continuous monitoring equipment. Large volumes of waste water can be produced by flow-though tests. This waste may need to be monitored and treated to remove contaminants or to ensure that nonindigenous species are not released.

7. Apparatus

7.1 *Facilities*—The facility should include separate constant temperature areas for culturing and testing organisms. The exposure system consists of replicate test chambers, any aquaria or tanks that hold the test chambers, the water delivery system, and any pollution abatement system. The test facility should be well ventilated and free of fumes.

7.1.1 Enclosures may be needed to ventilate the test chambers. To reduce the possible contamination by test materials and other substances, acclimation and culture tanks should be in a separate area from that where the tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned.

7.1.2 *Lighting*—Lighting conditions should meet the requirements of the study and test organisms. This may generally be accomplished by means of cool-white fluorescent lights at an intensity of about 100 to 1000 lx. Other sources (incandescent, fluorescent/incandescent, and augmented photosynthetically active radiation) may be required for special purposes. Ultraviolet (UV) radiation, especially UV-B, is generally missing from artificially supplied spectra. Although UV-B radiation can enhance the toxicity of certain chemicals (phototoxicity), this should not be a major limitation with bioaccumulation tests with infaunal species.

7.1.2.1 A timing device should be used to provide a light-:darkness cycle if a photoperiod other than continuous light is used. Practice E 1022 recommends 16 h day, 8 h night as a convenient light/dark cycle. Schedules of 12/12 or 14/10 h day/night are also acceptable and may be useful for delaying the maturation and spawning of some species. The experimental design should consider the specific requirements of the organisms.

7.1.2.2 A 15 to 30-min transition period **(5, 6)** when the lights go on may be desirable to reduce the potential stress from instantaneous illumination; a transition period when the lights go off may also be desirable.

7.1.3 *Temperature*—Test chambers may be placed in a temperature-controlled recirculating water bath or a constant-temperature area to control the temperature. A temperature corresponding to the average spring-summer temperature of the study site should simulate the biologically most active season.

7.2 *Construction Materials*—Materials used to construct the exposure system should not induce any reaction by the organisms or affect the contaminant concentration or bioavailability. Borosilicate glass and soft glass (soda-lime and window) have proved generally nonreactive to metals and organics and are the preferred materials where their fragility is not a major limitation. Most rigid plastics (polyolefins, engineering resins, and fluoropolymers) are acceptable after conditioning, such as soaking in deionized water for several days. Some plastics, generally flexible types that contain mobile plasticizers (phthalate esters), need to be tested for toxicity and should not be used if phthalate ester accumulation is studied. Concrete and rigid plastics may be used for holding, acclimation, and culture tanks and in the water-supply system, but they should be soaked, preferably in flowing water, for several days before use **(7)**. Stainless steel should not be used in direct contact with seawater because the alloy components of many stainless steels may react with saltwater. Cast-iron pipe should probably not be used in freshwater supply systems because colloidal iron will be added to the overlying water and strainers will be needed to remove rust particles. Choose another material if contaminant sorption to the internal surfaces of containers is a problem.

7.2.1 Any sealant used to construct the chambers must be nontoxic, such as a clear, nontoxic silicone-rubber that meets FDA Regulation 21 CFR 177.2600, Office of Federal Register. Such materials are usually specified for aquarium use and do not contain fungicides (for example, arsenic compounds). Exposed sealant at joints should be minimized to minimize contaminant sorption. Place the sealant used for mechanical reinforcement on the outside of the joint. Product literature on the material is helpful for determining the compatibility of a particular sealant to a contaminant. All new test chambers constructed should be soaked for at least 48 h in the overlying water used in the sediment bioaccumulation tests to leach potentially toxic compounds.

7.3 *Water Delivery System*—Adequate amounts of overlying water are required to ensure that the oxygen concentration is not depressed, metabolites do not accumulate, and the organism's behavior is not impaired. The system should deliver water independently to each replicate treatment. Flow-through delivery systems that meet these criteria can be one of several designs (for example, Fig. 1). Various metering systems using different combinations of siphons, pumps, solenoids, valves, etc. have been used successfully to control the water flow rates. If a contaminant is added to the water supply, several dilution systems designs are currently available **(8-10)**.

7.3.1 The metering system should be calibrated before the test by determining the water flow rate through each test chamber. The metering system operation should be checked daily during the test. Flow rates through any two test chambers should not differ by more than 10 % at any particular time during the test.

7.4 *Test Chambers*—Test chamber designs should consider the conditions required to maintain an adequate environment for the test organisms. The designs should also consider the contaminant behavior, construction cost, maintenance, and ease of operation. The following recommendations are based on the standard 28-day exposure duration (see 12.2). Specialized exposure chambers are described in Annex A6.

7.4.1 The test chamber can consist of glass boxes, beakers, aquaria, or other containers of appropriate material. Beakers are an inexpensive exposure chamber for single or a few

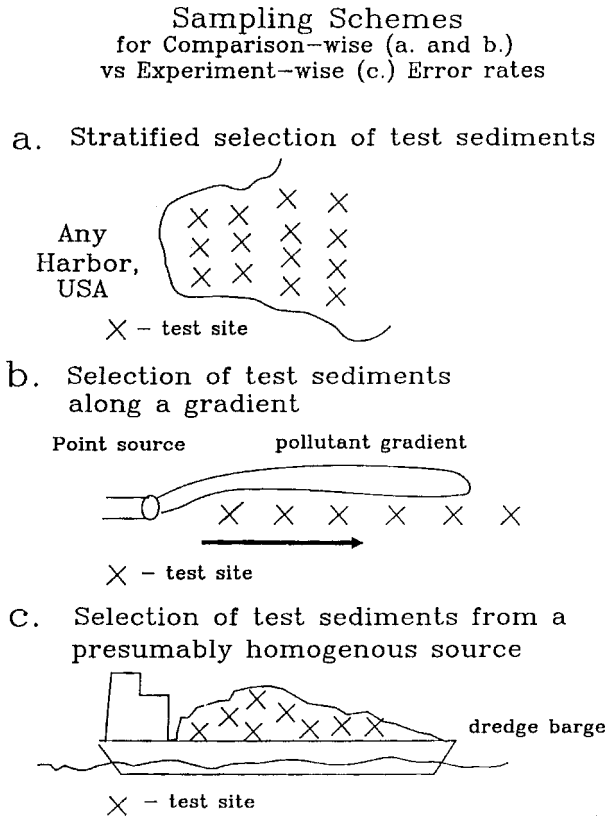


FIG. 1 Representative Sampling Schemes for Comparison-Wise Versus Experiment-Wise Error Rates

individuals for many species. However, an aquarium filled with sufficient sediment may be a more practical exposure chamber if large tissue masses composed of a composite of many individuals are required for analysis. The diameter of the exposure chamber and the sediment depth should be sufficient to allow the organism to bury and construct normal tubes and burrows. The opening of the exposure chamber should be large enough to allow the periodic addition of feeding sediment, if required (see 10.1).

7.5 *Exposure Systems*:

7.5.1 *Static Exposure*—In static exposure systems, test organisms are exposed to sediment without flow-through overlying water, although the overlying water many be exchanged on a periodic basis. The test chambers may be individual aquaria or beakers (for example, Ref **(11)**). A common design for bioaccumulation tests is sets of beakers submerged in aquaria in which overlying water is aerated and replaced with newly prepared water on a regular schedule (for example, Ref **(12)**). A more recent design places the experimental beakers in a water bath for temperature control and permits water renewal to each beaker independently **(10)**. This improves the independence of each beaker as an experimental unit while maintaining the water quality.

7.5.1.1 The beakers or aquaria in a static system should be covered to reduce evaporation and aerated gently to maintain dissolved oxygen levels at 40 to 100 % of oxygen saturation (Guide E 729).

7.5.2 *Flow-Through Exposure Systems*— Chambers may be sets of beakers maintained in aquaria or entire aquaria for flow-through systems. Flow-through systems have the advantages of removing waste products and maintaining oxygen.

7.5.2.1 Water flowing through one container must not flow into another container to prevent cross contamination. Water exiting the system should be passed through a charcoal filter or other appropriate sorptive material. Resuspended sediment should be trapped and retained as waste. Examples of flow-through tests can be found in Guide E 1383 and Refs **(13-15)**.

7.5.3 *Multiple Species Exposures*—If several species are being tested, it is possible to place multiple species within each exposure chamber, which may reduce space requirements. However, mixing multiple species tests has the potential for both negative and positive interactions among species that can alter behavior and could have unknown and varying effects on contaminant accumulation. Multiple species tested in the same exposure chamber can be partitioned with screens to minimize species interactions (for example, Ref **(15)**).

7.5.3.1 Regardless of the specific design, the same numerical ratio of one species to another should be placed in replicate chambers at test initiation. A paired-comparison approach (15.4) should be used when comparing the tissue residues of species kept in the same chambers because the two species are not independent.

7.6 *Cleaning*—To remove organics and metal contamination, the equipment and test chambers are washed initially with a non-phosphate detergent and then rinsed consecutively with distilled water, a water-miscible organic solvent, 5 to 10 % hydrochloric or nitric acid, and finally deionized-distilled water **(16-18)**. Glassware for metal analyses should be stored wrapped in polytetrafluoroethylene (PTFE) sheets or plastic wrap, whereas glassware for organic analyses should be stored wrapped in PTFE or aluminum foil.

8. Safety Precautions

8.1 Personnel involved in bioaccumulation testing need to be protected from exposure to toxic chemicals. Exposure to pathogens must also be considered, especially when working with sediment collected near sewage discharges. The manner of personnel protection must be determined before the start of work, keeping in mind that exposure can occur from breathing vapors, physical contact with the skin, or ingestion. The particular type of protection required depends on the materials involved and is beyond the scope of this guide. Consult Refs **(19-23)** to determine safety approaches. The Integrated Risk Information System (IRIS) is available to local, state, and federal public health officials through the Public Health Network (PHN) of the Public Health Foundation at (202) 898-5600 or through Dialcom, Inc. at (202) 488-0550.

8.2 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, which must 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 for the state in which you are operating. Refer to Ref **(24)** for citations of the Federal requirements.

9. Overlying Water

9.1 *Requirements*—Used both for holding organisms and in

bioaccumulation tests, overlying water should be available in adequate supply and uniform quality. The acceptability of the water for test organisms is determined by satisfactory survival and growth without signs of disease or apparent stress.

9.2 *Freshwater*:

9.2.1 *Source*—Natural overlying water should be uncontaminated and of constant quality to ensure that test organisms are not stressed during holding, acclimation, and testing (see Guide E 1383 for additional details). Water quality should meet the following specifications as established in Guide E 729:

Particulate matter	<5 mg/L
Total organic carbon (TOC)	<5 mg/L
Chemical oxygen demand (COD)	<5 mg/L
Residual chlorine	<11 µg/L

9.3 *Seawater*:

9.3.1 *Source*—Seawater should be uncontaminated and of constant quality (See Guide E 1367 for additional details). If a constant source of seawater is unavailable, collected seawater should be stored in covered containers in the dark at 4°C. Artificial sea water may be used if natural water is not readily available, although it should be demonstrated that the growth and behavior of the test species is not altered by using artificial salts. Prepare artificial water with deionized water or distilled and charcoal-filtered water.

9.3.2 *Salinity*—Practice E 1022 recommends that the overlying water salinity for marine systems should vary less than 2 g/kg or 20 % of the average, whichever is higher. Where the salinity varies (as in water drawn from estuaries with seasonally high river contributions), high-salinity water should be stored in sufficient quantity to supply the test system during the expected period of low salinity.

9.3.3 *pH*—Seawater is well buffered, but metabolites and waste materials (that is, ammonia) can build up in static systems, raising the pH value. Maintain the pH between 6.5 and 8.0 (Practice E 1022). Aeration will help maintain the pH, as will the periodic replacement of water.

9.4 *Filtration*—Because phytoplankton and suspended material are a sink for contaminants and a food for facultative filter-feeders, it is important to filter the water to remove suspended particles (>5 µm) for testing.

9.5 *Dissolved Gases*—Constant water quality should be maintained in the overlying water of the holding aquaria, keeping the dissolved oxygen above 2.5 mg/L (Guide E 729) and unionized ammonia concentrations <20 µg/L (Practice E 1022). The flow rate of water into the holding aquaria or the aeration rate, or both, should be increased to maintain suitable water quality. Alternatively, the biomass in each holding aquarium can be reduced. Flowing water with a minimum flow rate of 1 L/h/g wet tissue is recommended as a means of maintaining water quality. However, additional flow may be necessary to account for the biological oxygen demand of the sediment.

9.6 *Aeration*—Aeration is usually required in static systems to maintain the oxygen concentration. The air should be filtered (0.22-µm bacterial filter or other suitable system) and free of fumes, oil, and water. The volume should be sufficient to turn the water over but not enough to resuspend sediment. Position the air stone or pipette sufficiently far above the surface to avoid resuspension. Check the bubbler frequently, and remove any salt crystals or encrustations forming at the orifices. If air is provided from a compressed air tank, specify that the composition includes about 0.3 to 1.0 % CO_2 to help control the pH.

9.7 *Tissue Load*—For a flow-through system, Practice E 1022 recommends not more than one filter-feeding bivalve (40 to 60 mm from umbo to edge of distal valve) per litre per hour. This would be equivalent to a minimum flow of 1 L/h/g wet tissue for an oyster. However, this requirement is based on feeding and does not account for the sediment oxygen demand. In addition to the flow rate per gram tissue, flow-through systems should be designed to achieve five turnovers per day (Practice E 1022).

9.7.1 In static systems, the water volume to loading ratio should be sufficient to maintain the oxygen levels at ≥2.5 mg/L of saturation. A gentle aeration helps maintain the oxygen level as does changing the water two or three times per week.

9.7.2 It is important to take into account the total sediment oxygen demand when determining the oxygen demand for the system. In most cases, the sediment microbial demand will be several fold greater than the oxygen used by the test species. The total oxygen demand of sediments ranges from <1 to over 100 mL $O_2/m^2/h$ (for example, Refs **(25-27)**). In general, the total oxygen demand will increase with temperature and organically rich sediments. To maintain appropriate water quality, either increased flow or aeration can account for this increased demand and flow, and aeration should be the same among treatments.

9.8 *Temperature*—The temperature should not vary by more than 1°C in a 12-h period (Practice E 1022) and 3°C over a short period. A storage tank within the laboratory will help ameliorate natural fluctuations in temperature in flow-through systems.

9.9 *Background Contamination*—Regardless of whether flow-through or static systems are used, the water should be analyzed for background levels of contaminants, especially if it is collected from an urbanized area. If a contaminant is detected in the water, its potential uptake can be estimated by multiplying the water concentration by the bioconcentration factor (BCF) for that compound. A different water supply should be used if the calculated tissue residue is greater than that acceptable for a control organism (see Table 1). BCF values and methods for estimating BCFs can be found in Ref **(28)**.

10. Sediment

10.1 *Sediment Amounts*—Sediment serves as the habitat and source of food and contaminants for the test organisms. Adequate amounts of sediment are required to ensure that supplies of food and contaminants are not depleted substantially and that the organism's feeding behavior is not impaired. Deposit-feeding organisms may reingest the same particles if insufficient sediment is added. Alternatively, there may be a reduction in the appropriately sized particles if the fecal pellets are resistant to breakdown, especially for the more selective deposit-feeders. Both of these processes could reduce the mass of bioavailable chemical. Although both reingestion and pelletization of sediments occurs in the field (see Ref **(29)**), the rates may be exaggerated in laboratory systems.

TABLE 1 Representative Control Organism Tissue Residues

Organics[A] (ppb wet weight)	Various East Coast Sites[B]	Puget Sound[C]	Yaquina Bay, OR[D]
CB	<1.0–70		
B(ibk)F		<10	
BaP	0.3–6.0[E]	2.3–<10[E]	1.9
DDT	<0.08–3.8	<1.0–<5.0	3.9
HCB	0.02–0.17	<130	
Naph	<1.0–9.1	<0.05	
PAH	0.02–7.2	<2–17[E]	
PCB	10–70	<2.0–10	
Pesticides	<0.03–0.6		
Metals[A] (ppm wet weight)	**Various East Coast Sites[B]**	**Puget Sound[C]**	**Yaquina Bay, OR[D]**
Ag	0.2–2.6		
As	1.5–3.9		
Cd	<0.06–4.0		<0.005
Cr	0.26–2.5		
Cu	0.1–7.2		<1.5
Hg	<0.05–1.2	1.0	
Ni	<0.4–7.0		
Pb	<0.6–2.6		
Zn	2.4–30		<2.0

[A] CB = chlorinated benzenes, B(ibk)F = benzo(i,b,k)fluoranthene, BaP = benzo(a)pyrene, HCB = hexachlorobenzene, Naph = naphthalene, PAH = polycyclicaromatic hydrocarbons, and PCB = polychlorinated biphenyls.
[B] See Ref. **(30)**.
[C] See Ref. **(31)**.
[D] Unpublished data.
[E] See Refs **(30, 31)**.

10.1.1 The initial amount of sediment placed in each exposure chamber will depend on test species requirements. If sediment is added periodically to the test chambers during the bioaccumulation test, the amount of sediment added initially needs to be deep enough to allow normal burying and feeding and should equal or exceed the consumption requirements for the exposure period. As selective deposit-feeders ingest the fine grain fraction of a sediment selectively, it is important to obtain an accurate estimate of the sediment processing rates of the size fraction ingested by that species. Compilations of sediment processing rates (for example, Ref **(29)**) can be used to estimate these requirements.

10.1.1.1 Assuming periodic sediment additions to the exposure chambers (see Section 13), at least 50 g of wet sediment for each 1 g of wet flesh tissue (excluding shell) should be added initially for surface deposit-feeding bivalves and many larger marine deposit-feeders. For funnel-feeders such as arenicolid worms, at least 200 g of wet sediment to each 1 g of wet flesh tissue may be required for construction of a normal feeding burrow. The initial depth for the deposit-feeding clam *Macoma* should be at least 2 cm and preferably 3 to 5 cm, whereas a large lugworm may require 5 to 10 cm of sediment.

10.1.1.2 For *Lumbriculus variegatus*, the tissue loading rate has been demonstrated to influence the bioaccumulation of contaminants **(32)**. The loading is thus suggested to be no less than 50 g organic carbon in the sediment per gram dry weight of worms. This will provide sufficient food and contaminant for a 28-day test without the depletion of resources.

10.1.2 If periodic sediment additions are not made, the initial amount added should exceed the total amount processed over the duration of the experiment by at least two-fold and preferably five-fold. Thus, for the organism with a 2 g/g-tissue/day sediment processing rate, approximately 250 to 300 g of sediment should be added per gram of tissue. However, an organism can deplete the food or contaminants within its specific feeding zone in a laboratory, especially by surface deposit-feeders, regardless of the amount of sediment added.

10.2 *Sediment Characterization*—All sediments should be characterized for contaminant concentration(s), TOC, percent sand, silt, clay (particle size distribution), and moisture content. Other analyses on sediment might include the following: pH, total volatile solids, biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh or pE, total inorganic carbon, oil and grease, and interstitial water analysis. Acid volatile sulfides (AVSs) may prove helpful when determining the bioavailable fraction of certain metals **(33)**.

10.3 *Control and Reference Sediments*—The difference between control and reference sediments is critical to interpretation of the results.

10.3.1 A control sediment contains no or very low concentrations of the contaminant(s) being tested. The comparison of a test sediment to a control is a measure of the extent of bioaccumulation from the test sediment. Comparisons of control organisms at the beginning and end of an exposure period provides information on whether contamination from the water or exposure system has occurred. Grain size, TOC, and other key physicochemical characteristics of the control sediment should resemble closely those of the test sediment to the extent possible.

10.3.2 In comparison, a reference is sediment collected in the same region as the site of concern and may contain low to moderate levels of contaminants. Reference sediment may be used as an indicator of localized sediment conditions exclusive of the specific contaminant studied. The reference sediment should resemble the test material closely in grain size, TOC, and other physicochemical characteristics.

10.3.3 Bioaccumulation in a test sediment can be compared to that in a reference sediment to determine whether significantly more accumulation is occurring than at some locally designated site. This approach is used for assessing dredge materials **(3)**.

10.3.4 The use of a reference site is appropriate when a "no further degradation" approach is used to determine the suitability of an industrial or municipal discharge or a disposal operation. The reference sediment should not contain high contaminant levels. If contaminant concentrations are too high, the tissue residues in organisms exposed to reference sediment may not differ significantly from those in the test sediment, even though the organisms exposed to the test sediments accumulated an unacceptable tissue residue.

10.3.5 *Criteria for Control and Reference Sediments*—There are no simple criteria available for judging the acceptability of a sediment as a control or reference sediment. Ideally, the concentration of every anthropogenic contaminant (for example, PCBs and DDT) in a control sediment should be significantly indistinguishable from zero, and the concentrations of naturally occurring compounds (for example, metals) should be within natural levels. It will often be difficult to meet these criteria in practice. Sediment with contaminant concentrations similar to the concentrations given in Table 2 represent

TABLE 2 Representative Control Sediment Concentrations

Compound	Southern California[A]	Puget Sound[B]	Oregon[C]	Fresh Water[D]
BaP[E]	...	7–30	10–66	<10
BF[F]	...	7–80	26.2	25
DDT	(15–150)*	0.03–0.6	<6.0	...
NAPH[G]	...	3–30[H]	37[I]	16
PAH[J]	...	2–60	<0.01	...
PCB	(<5.0–18)*	<0.02–1.0	<2.0	27
Ag	0.06–2.0	1.2	0.55[K]	...
As	3–15	3–15	...	<47
Cd	0.001–2	3.1–18.3	0.47	0.32
Cu	6.5–40	20.9	19.3	23.5
Cr	2.8–30	10–50	6.3	10.4
Hg	<1.0	0.02–0.12	...	0.06
Ni	<20.0	13.0	14.5	21.2
Pb	<10.0	8.0	5.5	<32
Zn	<70.0	...	26.3	45

[A] Organics (ppb dry weight), metals (ppm dry weight), * not considered control values, Southern California **(35-37)**.
[B] Puget Sound, WA **(31)**.
[C] Yaquina and Alsea Bays, Newport and Waldport, OR (unpublished data).
[D] An undisturbed agricultural soil collected from Florissant, MO **(38)**.
[E] Benzo(a)pyrene.
[F] Benzo(i,b,k)-fluoranthene.
[G] Napthalene.
[H] See Ref **(36)**.
[I] Schults, unpublished data, U.S. EPA, Newport, OR.
[J] Polyaromatic hydrocarbons.
[K] See Ref **(39)**.

adequate control values for the measured compounds. Alternatively, the concentrations at a putative control site can be compared to the sediment concentrations (normalized by the silt-clay fraction) given in Ref **(34)**. This document presents raw data for both organics and metals for approximately 200 near-coastal sites throughout the United States, with the concentrations for the highest and lowest ten stations. Sediment concentrations falling within or near the ten lowest station values are acceptable as controls. Neither sediment concentrations substantially above those in Table 2 nor the normalized values of the ten lowest stations in Ref **(34)** should be considered control values, except those of sediments containing naturally high levels of certain metals.

10.3.5.1 The acceptability of a reference sediment depends partly on the local background contaminant levels and how the reference sediment will be used. However, the appropriateness of a proposed reference site should be examined carefully if the silt-clay normalized concentrations fall in the upper half of the concentrations presented in Ref **(34)**.

10.3.6 *Standard Reference Sediments*—Variation in organism behavior and physiology can affect contaminant uptake substantially. For example, uptake in a test species could vary seasonally in response to changes in the lipid content or temperature or vary non-seasonally in response to the organism health or site of collection. The extent of this variation should be assessed especially if the results will be compared from tests conducted at different seasons or from tests using organisms collected at different sites.

10.3.6.1 The test variation can be assessed by using a standard reference sediment, which is a well-characterized sediment containing known and constant contaminant (organic and metal) concentrations. An experimental treatment that uses a standard reference sediment is a positive control and may be conducted in addition to the normal (negative) control. Differences found among studies in tissue residues of organisms exposed to standard reference sediments primarily measure the inherent variation associated with a test species but may also reflect the variation associated with other test parameters (for example, overlying water, nutritional quality of the sediment, and analytical variability). Using a standard reference sediment would also help standardize the results from different laboratories or different species.

10.3.6.2 Although positive controls have been suggested for sediment toxicity tests (for example, Ref **(40)**), they have not been used adequately in sediment bioaccumulation tests. Part of the problem is the absence of a standard sediment suitable for bioaccumulation tests. An interim solution is for each laboratory to make its own in the absence of such a national standard.

10.3.6.3 A laboratory-dosed sediment is recommended for use as a standard because of potential spatial and temporal variations in the chemical concentrations of field sediments. Dosing methods are discussed in Guide E 1391. Sediment used for the standard reference can be collected at the site at which the test organisms are collected or are known to exist in nature for laboratory-cultured organisms. If that is impractical, the physical characteristics (for example, grain size and TOC) should match those at the collection or natural habitation site closely. Indigenous organisms will have to be removed before use of the sediment. The undosed sediment can be stored for long periods, by either freezing or drying for the purpose of providing a constant exposure regime. Before either of these storage techniques are used, toxicity tests should be conducted on previously frozen or dried uncontaminated sediment to ensure that the technique does not affect the test species adversely. The sediment would be dosed in a standard manner, and the holding time between dosing and the initiation of organism exposure should be held constant.

10.3.6.4 The standard reference sediment will ideally be dosed with a suite of compounds ranging in chemical properties. Alternatively, a single organic or a single metal could be chosen as a representative compound(s). A specific PCB congener, not an Aroclor, is a good candidate for the organic compounds because of the wealth of information on PCBs, their high bioaccumulation potential, and their resistance to metabolism. A good choice for this congener 2, 2′, 4, 4′, 5, 5′ hexachlorobiphenyl (IUPAC No. 153), which is the most frequently occurring PCB congener in environmental samples **(41)** and is bioaccumulated by marine worms and clams readily **(15, 42, 43)**. It would be useful to include compounds from a second class of chemicals, such as a polycyclic aromatic hydrocarbon (PAH) congener, since PAH congeners exhibit behavior substantially different from the PCB congeners of similar octanol-water partition coefficients **(44, 45)**. Cadmium is suggested as a general reference metal. The bioaccumulation of sediment-associated cadmium has been studied in a number of organisms **(46)** and has been suggested as the reference toxicant for *Neanthes* growth tests **40)**. However, because toxic compounds may alter the behavior of organisms, changes in behavior can alter the bioaccumulation. Thus, metals such as zinc that are much less toxic than cadmium and have been well studied may be better for reference tests.

10.4 *Field-Collected Test Sediment*—Bioaccumulation tests use sediments collected in the field and brought back to the laboratory or manipulated experimentally in the laboratory. The handling can result in both cases in the loss of fine sediments, interstitial water, and water-soluble compounds; oxidation of compounds; or contamination by metals and organic compounds. This disruption can change physicochemical properties such as grain size distributions, chemical concentrations, sorption equilibria, speciation, and complexation, thereby affecting chemical bioavailability **(16, 47, 48)**. Although some changes are unavoidable, they can be minimized with appropriate techniques. The specific techniques used will depend on the goal of the experiment and chemicals of concern. In particular, techniques suited optimally to study metals may not be suitable for organic compounds (see Guide E 1391 and Ref **(16)**). The sediment manipulation methods presented in Guide E 1391 and Classification D 4387 should be followed when possible.

10.4.1 The depths from which sediments are collected can affect bioaccumulation test results; a consistent depth should therefore be used in all collections. Sediments are spatially and temporally variable. 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 (Guide E 1391). 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,

10.4.2 Marine intertidal sediments may be hand collected using shovels, scoops, spatulas, or coring tubes. To maintain the sample layers intact, deposit the sediment sample into an appropriate container or, plug the top and bottom of the tube if a corer is used. Core samples may be sectioned later at specific depth-intervals for analytical and bioaccumulation tests **(16, 34, 48)**.

10.4.3 Box corers and benthic grabs are used commonly to collect subtidal and fresh water sediments. The sampler choice will vary according to the firmness of substrate, volume of sediment needed, and type of ship available. Box corers are the preferred collection device because they disturb sediment layers the least and retain fine particles. Although more disruptive to sediment layers, a Smith-McIntyre or modified Van Veen grab is acceptable. Compared to the box corer, these grabs operate in sandier bottoms, are easier to handle, require fewer personnel, and operate in heavier seas **(16, 34, 48)**. Scrape surficial sediment from the grab or box corer samples and store immediately in appropriate containers (Guide E 1391). Flocculent material should be considered to be part of the sample **(17)**.

10.4.3.1 The original sediment layering needs to be preserved if depth profiles are of interest. Take core samples from the center of the grab sample once on shipboard, and section them vertically at specific depth intervals **(16)**. To minimize oxidation and changes in other chemical properties, place plastic or PTFE bags or containers of appropriate composition and diameter over the ends of core tubes, and extrude the samples to specified depths.

10.4.4 Construct all collecting equipment with appropriate materials and clean equipment to reduce the possibility of contamination. (See 7.2 for general contaminant-materials interactions.)

10.4.5 The collecting apparatus should be cleaned thoroughly before use (see 7.6). Rinsing grabs or corers with site water between stations should suffice in most studies, although it may be necessary to use a brush or a detergent to remove highly cohesive sediments. When it is critical to remove all contaminants, grabs or corers should be rinsed with an organic solvent, for example, methanol, ethanol, acetone, or methylene chloride **(17)**, followed by a water rinse. Hexane might also be used as a solvent for removing non-ionic organic compounds. However, acetone is preferable if only one organic solvent is used to clean equipment.

10.4.6 Specifics of the field sampling design, such as the number of sites and number of samples per site, depend on the goals of the study and type of spatial resolution required. Guidance for designing field sampling programs can be found in Refs **(17, 51, 52)**.

10.5 *Field Measurements*—Upon collection, immediately determine sediment characteristics such as temperature, pH, Eh, and salinity **(16, 48, 50)**. Important information recorded with each sample should include the site (the name and location in appropriate coordinate units) and should include additional information such as the replicate number, depth, sampler description, numbers and kinds of subsamples, sediment characteristics, temperature, salinity, pH, Eh, penetration depth, sieve size, date and time, weather conditions, names of chief scientist and team members, and comments **(17)**.

10.6 *Field Storage and Transport*—Physical, chemical, and biological changes in sediment samples can occur rapidly, resulting in altered sediment quality or bioavailability during the transport of sediment. Temperature, pH, and dissolved oxygen are often the rate-controlling factors for these changes **(47)**.

10.6.1 Store the sediment sample in a bag or jar immediately after collection to diminish these effects. PTFE containers or brown borosilicate glass jars with PTFE-lined lids are recommended for both metal and organic samples, but regular glass jars with PTFE-lined lids are acceptable **(17)**. Containers need to be cleaned completely and stored in a covered container to avoid contamination. Cleaning protocols used for the exposure systems or sampling equipment also apply to storage containers (7.6).

10.6.1.1 Fill jars and bags completely with sediment to eliminate airspace and retard the oxidation of metals, but retain as much of the interstitial water as possible **(3, 17)**. Refrigerate sample containers in insulated cartons or ice chests immediately after collection. A temperature near 4°C can be maintained with frozen, jelled refrigerant packs or ice. Ensure that the samples are protected from the refrigerant to prevent cross contamination and freezing of the sample.

10.6.2 Shipping containers should be durable and leak-proof or lined with two heavy-duty plastic bags. Add adequate absorbent material to soak up any spills. Pack the samples tightly, using dividers between glass containers, and fill all empty spaces with packing material. Mark the containers with "This End Up" and "Fragile" labels. Ship the samples by overnight or 24-h carrier to the laboratory after the completion of sampling. Refrigerate the samples at 4°C upon arrival. Guidance for shipping hazardous materials can be found in CFR 49, Parts 100–177 (Office of Federal Register).

10.7 *Laboratory Sediment Storage*—Keep the time between sediment collection and use in bioassays to a minimum. Store the collected sediments in air-tight containers in the dark at 4°C **(16, 17, 53)** with the possible exception of sediment stored for use as a standard reference sediment (see 10.3.6). The sediment for metals should be stored in the absence of air to minimize the oxidation of reduced forms. Nitrogen can be used to fill the headspace in the container. Glass containers are recommended for sediments polluted with either metals or organic compounds, although high-density polyethylene and PTFE containers are also acceptable. Remove large organisms and extraneous material, such as bivalves or twigs, from the sediment before storing.

10.7.1 Since the chemicals of concern and influencing sediment characteristics are not always known, it is desirable to hold the sediments after collection in the dark at 4°C. Traditional convention has held that sediment tests should be started as soon as possible following collection from the field, although actual recommended storage times range from two weeks (Guide E 1391) to less than eight weeks **(231)**. Discrepancies in recommended storage times reflected a lack of data concerning the effects of long-term storage on the physical, chemical, and toxicological characteristics of the sediment; however, numerous studies have recently been conducted to address issues related to sediment storage (**213-219**). The conclusions and recommendations offered by these studies vary substantially and appear to depend primarily upon the type or class of chemical(s) present. Considered collectively, these studies suggest that the recommended guidance that sediments be tested sometime between the time of collection and eight weeks storage is appropriate. Additional guidance is provided below and in Guide E 1391 and Test Method E 1706.

10.7.2 Extended storage of sediments that contain high concentrations of labile chemicals, for example, ammonia, volatile organic compounds, may lead to a loss of these chemicals and a corresponding reduction in toxicity or bioavailability. Under these circumstances, the sediment should be tested as soon as possible after collection, but not later than within two weeks **(218)**. Sediments that exhibit low-level to moderate toxicity or contamination can exhibit considerable temporal variability in toxicity or contamination although the direction of change often is unpredictable **(214, 215, 217)**. For these types of sediments, the recommended storage time of less than eight weeks may be most appropriate. In some situations, a minimum storage period for low-to-moderately contaminated sediments may help reduce variability. For example, **(215)** observed high variability in survival during early testing periods, for example, less than two weeks, in sediments with low toxicity. DeFoe and Ankley **(215)** hypothesized that this variability partially reflected the presence of indigenous predators that remained alive during this relatively short storage period; thus, if predatory species are known to exist, and the sediment does not contain labile chemicals, it may be desirable to store the sediment for a short period before testing, for example, two weeks, to reduce potential for interferences from indigenous organisms. Sediments that contain comparatively stable compounds, for example, high molecular weight compounds, such as PCBs, or which exhibit a moderate-to-high level of toxicity, typically do not vary appreciably in toxicity in relation to storage duration **(215, 217)**. For these sediments, long-term storage, for example, greater than eight weeks, can be undertaken.

10.8 *Sediment Preparation and Homogenization*—Before using a field sediment, remove any extraneous materials (for example, macroalgae, twigs, rocks, and large organisms). Disturb the sediment as little as possible during this process. This can be accomplished by gently spreading the material out in a glass pan and removing large objects with forceps. However, keep contact with air to a minimum and use plastic tools if metals are the primary focus.

10.8.1 While seiving is not recommended, it may be necessary to sieve field sediments to remove predatory organisms or large amounts of extraneous materials. This could be accomplished by sieving the sediments through a 1 to 2-mm mesh sieve. The sieve size should be as large as is reasonable to minimize sediment disturbance. Using as small a volume of water as possible, sieve the sediment over a large container (for example, a garbage pail) to allow for the retention of sediment fines. After letting the suspended fines settle for 6 to 24 h, siphon off or decant the overlying water carefully, and mix the settled fine particles back into the sediment. The characteristics of the sediment should be determined before and after sieving (see 10.2 of Test Method E 1706).

10.8.2 After settling or storing the sediments, mix them well immediately before taking aliquots for chemical analysis, spiking, or bioaccumulation tests. This helps ensure homogeneity and mix any separated interstitial water back into the sediment. If grab samples were divided into several containers, mix the respective sediment samples together before sampling or using them in biological tests. Large sediment masses can be mixed manually in an appropriately cleaned glass tray or plastic tub or rotated in jars on a rolling mill. Homogenize control and reference sediments in the same manner as test sediments.

10.8.2.1 Inspect the sediment visually to judge the extent of homogeneity. Excess water on the surface of the sediment can indicate the separation of solid and liquid components. If a quantitative measure of homogeneity is required, take replicate subsamples (see 12.3) from the sediment batch and analyze for TOC, chemical concentrations, and particle size.

10.8.2.2 Some changes in the sediment are anticipated with mixing. Prolonged stirring can abrade flocs and change the sediment's physicochemical properties, such as dissolved organic matter (DOM) **(49)**.

10.9 *Sediment Spiking*—The addition or spiking of chemicals to sediments is a frequent sediment manipulation. Other

manipulations include the addition of inert substances to produce a less polluted sediment and alteration of the sediment characteristics, for example, organic content or particle size. Sediment manipulation techniques have not been standardized, so exercise caution when comparing results from different techniques until standard methods are developed or techniques are intercalibrated. Prepare and manipulate control sediments in the same manner as test sediments because manipulations can alter sediment properties (see Guide E 1391 and Test Method E 1706 for additional details on spiking sediment.) Limited studies have been conducting comparing appropriate methods for spiking chemicals in sediment. Additional research is needed before more definitive recommendations for spiking of sediment can be outlined in this standard. The guidance provided in the following sections has been developed from a variety of sources. Spiking procedures that have been developed using one sediment or test organism may not be applicable to other sediments or test organisms. See USEPA (1997) and Guide E1391 for additional detail regarding sediment spiking techniques.

10.9.1 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, for example, identifying standardized and representative sources of organic carbon. (see Test Method E 1706). Mixing time **(220)** and aging **(221, 222)** of spiked sediment can affect bioavailability of chemicals in sediment. Many studies with spiked sediment often are 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 (see 10.9.3.3). 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. See **(223),** Guide E 1391, and Test Method E 1706 for additional detail regarding sediment spiking.

10.9.2 The test material(s) should be at least reagent grade, unless a test using a formulated 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: the identity and concentration of major ingredients and impurities; water solubility in test water; log K_{ow}, BCE (from other test species), persistence, hydrolysis, and photolysis rates of the test substance; estimated toxicity to the test organism and to humans; 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, recommended handling and disposal procedures. Addition of test material(s) to sediment may be accomplished using various methods, such as a rolling mill, feed mixer, or hand mixing (see Guide E 1391; **(223)**). 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 (see Guide E 1391). Duration of contact between the chemical and sediment can affect partitioning and bioavailability **(222)**. Care should be taken to ensure that the chemical is distributed thoroughly and evenly in the sediment. Analyses of sediment subsamples is advisable to determine the degree of mixing homogeneity **(224)**. Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments **(225)**.

10.9.2.1 Organic compounds have been added as follows: directly in a dry (crystalline) form; coated on the inside walls of the container **(224)**; or, coated onto silica sand (for example, 5 % w/w of sediment) which is added to the sediment **(194)**. In Techniques 2 and 3, the chemical is dissolved in solvent, placed in a glass spiking container (with or without sand), then the solvent is evaporated slowly. The advantage of these three approaches is that no solvent is introduced to the sediment, only the chemical being spiked. When testing spiked sediments, procedural blanks (sediments that have been handled in the same way, including solvent addition and evaporation, but contain no added chemical) should be tested in addition to regular negative controls.

10.9.2.2 Organic solvents such as triethylene glycol, methanol, ethanol, or acetone may be used, but they might affect TOC levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microorganisms (guide E1391). Acetone is highly volatile and might leave the system more readily than triethylene glycol, methanol, or ethanol. A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form, or toxicity of the test material.

10.9.2.3 Sufficient time should be allowed after spiking for the spiked chemical to equilibrate with sediment components. For organic compounds, it is recommended that the sediment be aged at least one month before starting a test. Two months or more may be necessary for chemicals with a high log K_{ow}, for example, greater than six **(226)**. For metals, shorter aging times (one to two weeks) may be sufficient. Periodic monitoring of chemical concentrations in pore water during sediment aging is recommended highly as a means to assess the equilibration of the spiked sediments. Monitoring of pore water during spiked sediment testing also is recommended.

10.9.3 Direct addition of a solvent (other than water) to the sediment should be avoided, if possible. Addition of organic solvents may influence dramatically the concentration of dissolved organic carbon in pore water. If an organic solvent is to be used, the solvent should be at a concentration that does not affect the test organism. 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 Guide E 729).

11. Test Organisms

11.1 *Indigenous Versus Surrogate Species*—Species selection can include either or both indigenous or surrogate test species. The indigenous species have the advantage of being the same as those that will be effected in the field. However, because of natural fluctuations **(54)**, contaminant events **(55)**, or succession during recolonization **(56)**, the species selected for testing may not be closely related phylogenetically or ecologically to the species at the impacted site.

11.1.1 Many common indigenous species do not meet the criteria for use as a bioaccumulation test species, negating any advantage of using a native species. Even when an indigenous species is acceptable, established surrogate test species offer several advantages. There is considerable information on the maintenance and biology of the recommended test species. Furthermore, an available accumulation database for standard test species will permit comparisons of bioaccumulation under different environmental conditions.

11.1.2 Surrogate species are recommended for routine monitoring of sediments. Local species that meet the various criteria discussed as follows can be tested along with the recommended bioaccumulation species. The local species could be substituted in future tests if they prove acceptable and the results intercalibrate with those from the standard species. Local species that do not meet the criteria but are of special concern (for example, lobster) can be tested in addition to surrogate species but should not be substituted for them.

11.2 *Selection Criteria*—The choice of test species can greatly influence the success, ecological significance, and interpretability of a bioaccumulation test. No one species is best suited for all conditions given the potential range in environmental characteristics. However, two characteristics, sediment ingestion and contaminant resistance, are required of bioaccumulation test species, as well as a number of other desirable characteristics. These characteristics are summarized as follows, and in Table 3.

11.2.1 First, test species must ingest sediment because sediment ingestion is the major uptake route for higher K_{ow} compounds for some species **(45, 57-59)**. Many benthic invertebrates can vary their feeding mode, and this requirement does not preclude the use of facultative filter-feeders (for example, *Macoma*) as long as the primary exposure route during the experiment is whole sediment (that is, no resuspended particles or phytoplankton). Obligate filter feeders and obligate predators should not be used as bioaccumulation test species since the sediment ingestion route may be avoided.

11.2.2 The second attribute for bioaccumulation test species is contaminant resistence to survive the exposure with a minimum level of mortality. This requirement precludes the species used routinely in sediment toxicity testing (for example, *Rhepoxynius* and *Hyalella*), at least for more highly polluted sediments.

11.2.3 Environmentally collected sediments display a wide range of toxicities. Organisms that are very pollutant tolerant may thus be required to produce an acceptable test. In general, mortality greater than 10 % is not acceptable for a bioaccumulation test. However, the response of the organism can be altered if significant mortality occurs. Organisms exposed to high concentrations can exhibit accumulation kinetics different from those at lower doses. These alterations can result in either enhanced **(60, 61)** or reduced **(32)** bioaccumulation. The reduced accumulation is often observed with overt avoidance of sediment.

11.3 *Desirable Criteria*—In addition to the required criteria, there are a number of desirable characteristics that either make the tests easier to perform and the interpretation more straight forward or allow the results to be applied to a wider range of habitats.

TABLE 3 Test Species Characteristics[A]

Species	Feeding Type	Biomass,	Salinity Tolerance, %	Pollution Tolerance	Culture Potential	Commercial Availability	Information on Bioaccumulation, and Toxicity
Marine							
Abarenicola Sp.	FUN[B]	+ +	>15	+	−	−	+
Artenicola Sp.	FUN	+ +	>15	+	−	+	+
Callianassa Sp.	SSDF[C]	+ +	>10	−?	−	+	−
Capetella Sp.	SSDF	−	>10	+ +	+	+	+ +
**Macoma balthica*	SDF[D]	+	>10	+	−	−	+ +
**Macoma nasuta*	SDF	+ +	>10	+	−	−	+ +
Nephtys incisa	SSDF	+	>25	+	−	−	+
Neanthes arenaceodentata	SDF/O[E]	+ ?	>28	+	+ +	+	+ +
**Nereis virens*	SDF/O	+ +	>10	+ +	−	+	+ +
**Nereis diversicolor*	SDF/O	+ +	>10	+ +	−	+	+ +
Nucula Sp.	SSDF	+	?	+	−	−	+
Palaemonetes pugio	SDF	+ ?	>10	−	+	+	+ +
**Yoldia limatula*	SSDF	+	>25	+	−	−	+
Freshwater							
Chironomus riparius	FF[F]/SDF?	+	<5	−	+ +	+	+ +
Chironomus tentans	FF/SDF?	+	<5	−	+ +	+	+ +
**Diporeia* Sp.	SSDF	−	≤20[G]	+	−	−	+ +
Hexagenia Sp.	Col[H]	+	?	−	+	+	+ +
Hyalella azteca	SSDF	−	≤15	+	+ +	+	+ +
Oligochaetes (aquatic)	SSDF	−	?	+ +	+	−	+
**Lumbriculus varigatus*	SSDF	−	?	+ +	+ +	+	+ +
Oligochaetes (earthworms)	SSDF	+ +	?	?	+	+	−

[A] + + = very good, + = good, − = poor or insufficient, and * = recommended species.
[B] FUN = funnel feeder.
[C] SSDF = subsurface deposit-feeder.
[D] SDF = surface deposit-feeder.
[E] O = omnivore.
[F] FF = filter feeder.
[G] Tolerance to 28 h.
[H] Col = collects surface particles.

11.3.1 The ease of obtaining test species in sufficient numbers at the correct season is of concern when planning repeated tests. Collection ease is determined by a species' abundance, habitat (intertidal versus subtidal versus offshore), robustness to collection techniques, depth in the sediment, and seasonality. The time required to collect sufficient numbers of healthy individuals for testing can be substantial. In general, it is prudent to collect twice the number required, especially with organisms that are susceptible to damage during collection or transport. Alternatively, test organisms may be purchased from biological supply houses or local collectors. Local bait suppliers may sell marine species such as *Nereis* and *Callianassa* and freshwater species such as *Hexagenia*. The health, age, and contaminant history of these organisms must be considered, as they may be variable from supply houses (see 11.6.1 and Table 1).

11.3.2 Culturing of test organisms may be cost effective if a large number of bioaccumulation tests will be conducted over an extended time period. Culturing will provide a ready supply of organisms of known history. A few sediment-ingesting marine polychaetes (for example, *Capitella capitata* and *Neanthes arenaceodentata*) can be cultured with relatively simple equipment **(62-65)**, as can *Palaemonetes* **(66, 67)**. For freshwater, *Lumbriculus variegatus* can be cultured readily in large numbers for bioaccumulation tests (see Ref **(68)** and Annex A8). Although these organisms are generally suitable test species, most of the species are small. Groups of organisms are thus required to attain sufficient biomass for analysis. Cultures of bivalves, larger polychaetes, and most crustaceans are impractical at this time except for experimental studies.

11.3.3 Regardless of how the test species are obtained, they should be amenable to laboratory conditions and not require elaborate holding facilities. Fortunately, most contaminant-resistant species are relatively hardy and adaptable to laboratory conditions. Most of the bioaccumulation test species listed in Table 3 are reasonably easy to maintain and do not require flowing water.

11.3.4 Whether field-collected or laboratory-cultured specimens are used, gravid individuals or individuals that are likely to become gravid during a test should be avoided if possible. The reduction in tissue lipids often occurs with spawning **(69, 70)** and can result in a corresponding reduction in contaminant accumulation. Spawning may also result in unacceptable mortality. Certain species, such as *Macomanasuta* in Oregon, have a reasonably well-defined spawning cycle and size at reproductive maturity, making it possible to minimize the collection of reproductive individuals. Other species, such as *Neanthes virens*, change appearance when reproductively mature. In extended tests, it may be impossible to avoid gravid individuals completely, although occurrence of the reproductive state should be noted. For *Lumbriculus variegatus*, most reproduction is through budding, so reproduction may not impact the contaminant concentration or lipid content to the extent observed for sexual reproduction.

11.3.5 A very important characteristic is organism size. Test species need to be small enough to be maintained easily, yet large enough to supply sufficient biomass either as individuals or groups of individuals for chemical analysis. The amount of biomass required depends on the analytical procedures used and the types of analyses required (for example, metals, organics, and lipids). At least 1 g of wet tissue is generally required, and up to 5 g tissue will commonly be required. The species should ideally be large enough to allow chemical analysis on individuals. Depending on the techniques, it may be impossible to conduct both metals and organic analyses on an individual, even when using large species. Twice as many exposure chambers are thus required if both contaminant types are measured. An alternative approach to obtaining sufficient biomass is to composite individuals (see Annex A1). When compositing individuals, it is simpler to handle and count a few larger individuals (for example, *Nereis*) than dozens or even hundreds of smaller specimens (for example, *Capitella* and *Lumbriculus variegatus*).

11.3.6 The more tolerant a species to sediment, temperature, and water quality variations, the more types of sediments that can be tested. Using a few widely adaptable species allows direct comparison of sediment bioavailability from a variety of environments or biogeographic regions. Also, collecting and maintaining a few adaptable species is simpler than developing techniques for a larger number of those less adaptable. The approximate environmental conditions of potential bioaccumulation species are given in Table 3. The ranges for environmental conditions are estimates in which the organisms could be used in a bioaccumulation test and are not the physiological limits. The ranges are based on the general literature and discussions with other researchers rather than extensive experimentation. A preliminary survival test is advisable before initiating a large bioaccumulation test to test for both potential physiological limits and toxic responses.

11.3.7 It is important to choose species with high bioaccumulation potential. Unfortunately, insufficient numbers of multi-species tests have been conducted to compare adequately the bioaccumulation potential of a range of species over a range of compounds. In general, tissue residues will be higher in species with higher lipid contents, varying as much as ten-fold among species (for example, Ref **(15)**. Organisms with a minimal biotransformation capability are desirable for those contaminants that are metabolized readily. For example, to study PAHs, at least one test species should have minimal biotransformation capability, such as a bivalve in a marine environment **(71)** or for freshwater *Diporiea spp*. (formerly *Pontoporeia hoyi* **(72)**) or *Lumbriculus variegatus* **(73, 74)**.

11.3.8 Infaunal species are preferable to epibenthic deposit-feeders because the latter are exposed only intermittently to interstitial water. Because interstitial water may be the major uptake route, both for compounds with a K_{ow} below approximately 5 **(75)** and for metals **(46)**, uptake by an epibenthic deposit-feeder may be underestimated.

11.3.9 Compatibility with other species or with the same species is important if multiple species or multiple individuals of the same species are exposed in the same chamber. Several of the nereid worms are aggressive to members of the same sex **(40, 76)**. Some nereids also prey on smaller species, and *Palaemonetes* may crop the siphons of bivalves.

11.4 *Recommended Species*—An evaluation of the suitability of potential test species is summarized in Table 3. This

evaluation is not based on extensive comparative studies and should be considered a guide rather than a definitive characterization of the species.

11.4.1 *Marine Species*—Five recommended bioaccumulation test species and another eight "secondary" taxa are identified in Table 3. The recommended species meet all or nearly all of the desired criteria and are well established as bioaccumulation test species. The recommended species are the polychaetes *Nereis diversicolor* and *Neanthes (Nereis) virens* and the bivalves *Macoma nasuta, Macoma balthica,* and *Yoldia limatula*. These species have been used in a substantial number of experimental bioaccumulation studies and in regulatory monitoring. They should serve as suitable test species within their tolerance levels. Using at least one of these species in all tests is recommended, at least until the suitability of other species has been demonstrated locally.

11.4.1.1 The secondary marine bioaccumulation species meet the required characteristics but are deficient in one or more of the important desired characteristics. Insufficient information often exists for making a final evaluation. However, some of these secondary taxa offer potential advantages, such as large size (arenicolid worms), additional phylogenetic groups (that is, crustaceans), adaptability to culturing (for example, *Neanthes arenaceodentata*), and high-pollution tolerance (*Capitella* spp.). The importance of these various advantages depend on the site-specific situation (for example, the level of toxicity of sediment).

11.4.2 *Freshwater Species*—Table 3 recommends two primary bioaccumulation organisms, *Diporeia* spp. (see the annex on *Diporeia* in Guide E 1383) and *Lumbriculus variegatus. Diporeia* spp. are easy to handle and have high lipid content and thus a high bioaccumulation potential. *Diporeia* are exposed to contaminants by means of all appropriate routes including porewater and sediment ingestion. *Diporeia* do not biotransform PAHs and are relatively insensitive to contaminants and sediment characteristics. *Diporeia* can tolerate relatively high salinity, 20 parts per thousand; and they can thus be used for both freshwater and estuarine exposure conditions **(77)**. A large database on contaminant bioaccumulation and toxicokinetics is available for *Diporeia*. However, procedures for culturing *Diporeia* in the laboratory have not been developed, and groups of organisms are required to attain sufficient mass for analysis.

11.4.2.1 Oligochaetes are infaunal benthic organisms that meet many of the test criteria previously listed. Oligochaetes are exposed to contaminants by means of all appropriate exposure routes, including pore water and ingestion of sediment particles. Various oligochaete species have been used in toxicity and bioaccumulation evaluations **(68, 78-82)**, and field populations have been used as indicators of pollution of aquatic sediments **(83-90)**. Specifically, *Lumbriculus variegatus* are handled and cultured easily and are tolerant of varying sediment physical and chemical characteristics (See Annex A8). For analysis, groups of organisms are generally required to attain sufficient mass. *L. variegatus* does not biotransform PAHs **(74)**. They do not need to be fed during long-term bioaccumulation exposures **(68)**. Verifications of bioaccumulation between laboratory studies and field conditions have been performed **(89)**.

11.4.2.2 The secondary freshwater species meet many of the important criteria for bioaccumulation but are deficient in one or more aspects. Freshwater clams provide an adequate tissue mass, are easily handled, and can be used in long-term exposures. However, few freshwater species are available for testing. The exposure of clams is uncertain because of valve closure. Furthermore, clams are filter feeders and may accumulate lower concentrations of contaminants compared to detritivores **(43)**. Chironomids can be cultured readily, are easy to handle, and reflect appropriate exposure routes. However, their rapid life-cycle makes it difficult to perform long-term exposures with hydrophobic compounds, and chironomids can biotransform organic compounds such as benzo[a]pyrene readily **(74)**. Larval mayflies reflect appropriate exposure routes, have adequate tissue mass for residue analysis, and can be used in long-term tests. Mayflies cannot be cultured continuously in the laboratory and consequently are not always available for testing. They are also sensitive to sediment-associated contaminants. The background concentrations of contaminants and health of field-collected nymphs of mayflies may be uncertain. *Hyalella azteca* can be cultured in the laboratory, are easy to handle, can tolerate 15 % salinity, and reflect appropriate exposure routes. However, their size may be insufficient for residue analysis, and *H. azteca* are sensitive to contaminants in sediment. Because of exposure routes, sensitivity, and short life spans, these secondary freshwater species are useful as bioaccumulation test species only under special conditions.

11.4.3 *Multiple Species Tests*—Species and larger phylogenetic groups vary in their tendencies to bioaccumulate contaminants in response to both their modes of exposure and their metabolic characteristics. The extent of these interspecific variations are not well understood, and both the magnitude and direction of species differences can vary with contaminant (for example, metals versus organics) and perhaps with sediment type. The use of two or more species from different major taxa thus increases the probability of assessing the maximum field tissue residues accurately.

11.4.3.1 The actual number of species and taxa used depends on the goals and scale of the project and the range of contaminants in the sediment. In general, a single species should be adequate for a general area survey or for assessing a small discharge or volume of dredge material. The data from a single species test should not be interpreted as the likely maximum for all contaminants. Multiple species, at least two, from different major taxa are recommended to assess a moderate- to large-sized discharge or dredging operation.

11.4.3.2 A polychaete and a bivalve are recommended for marine tests. It is especially important to include a bivalve if PAH contamination is of concern since bivalves have a reduced capability to metabolize PAHs compared to amphipods or polychaetes **(71)**. The addition of an arthropod species or additional polychaete or bivalve species may be justified when assessing a large discharge or dredging operation, especially if there is a wide range of contaminants.

11.4.3.3 Only tests with *L. variegatus* are currently standardized for testing for freshwater bioaccumulation (Annex A8). However, other test species such as *Diporeia* or *Chironomus* may be useful for particular applications.

11.5 *Age*—The organisms should be as uniform as possible in age and size class. The age or size class chosen should not be overly sensitive to contaminants; nor should organisms that are reproductively ripe or recently spawned be used. For bioconcentration tests Practice E 1022 stipulates that the length (umbo to distal valve) of the largest clam should be no greater than 1.5 times larger than the smallest clam.

11.6 *Test Organism Acceptability*—The specimens selected for a test should be able to tolerate the physico-chemical conditions (for example, TOC content and interstitial salinity) of the test substrate and should not show signs of disease or stress from capture or handling. Field-collected specimens should be collected from the same site and preferably at the same time. It is important to identify the test species correctly, and voucher specimens should be kept from each collection.

11.6.1 High-contaminant background levels in the test specimens may confound the results, making it difficult to detect differences between treatments. Tissue residues in the test organisms should therefore be no greater than those expected in organisms living in control sediment. Approximate background tissue concentrations for test species are given in Table 1. These concentrations are from organisms collected from sites that appeared to meet the criteria for a control site. The Practice E 1022 criterion of the background tissue residue not exceeding 10 % of the expected steady-state can be applied for compounds not listed in Table 1. First-order estimates of steady-state tissue residues can be obtained from data on other species or from the thermodynamic-based bioaccumulation model for neutral organics (see Annex A1).

11.6.2 *Culture Acceptability*—Organisms obtained from cultures should meet performance-based acceptance criteria, such as those described, before use in bioaccumulation testing. Laboratories should examine culture organisms routinely for concentrations of contaminants before testing. Cultured organisms should be tested periodically in water only, 96-h toxicity tests, to assess culture sensitivity (see Test Method E 1706). Laboratories should monitor and record the frequency of population doubling, particularly for *L. variegatus*, under the culture conditions as criteria for population health. The food and paper towels substrates used to culture organisms should be analyzed for compounds to be evaluated in the bioaccumulation test before the test start. The following water quality characteristics of cultures should be measured and recorded at least quarterly and the day before the start of the test: pH, hardness, alkalinity, and ammonia. Dissolved oxygen should be measured weekly. Temperature should be recorded daily. Physiological measurement such as lipid content might provide useful information regarding the health of the cultures.

11.7 *Source of Test Organisms*:

11.7.1 *Field Collection*—The logistics of collecting intertidal marine specimens are usually much simpler than those of collecting deeper water specimens; intertidal collection is recommended when possible. Infaunal organisms can be collected by turning the sediment over with a shovel and picking out larger species (for example, clams) or by sieving the sediment in the field gently. For most marine bioaccumulation test species, a sieve size of 4 to 6 mm will collect adequate numbers while minimizing damage and sorting time. In freshwater systems, the screen size will generally have to be a finer mesh, with a 0.5 to 1.0-mm opening. Even smaller mesh sizes will occasionally be required, depending on the organisms to be collected. The collection equipment should not have been used in contaminated sites or should have been cleaned adequately.

11.7.1.1 Freshwater and subtidal marine organisms can be collected by grabs, dredges, or suction samplers **(48, 91)**. Dredges sample a larger area than grabs and are usually more proficient at collecting shallow-buried organisms, although there is a possibility of damaging some organisms. Grabs are recommended for collecting more deeply buried species. Suction lifts are also useful for collecting larger, deeply buried bivalves, although they require the use of SCUBA divers and a greater likelihood of damage exists. Holding collected organisms in the laboratory before use can help eliminate damaged organisms. Electro-shocking, chemical poisons, and other harsh collection methods are not recommended.

11.7.1.2 Remove the organisms from the collection device as soon as possible, and submerge them in ambient water or sediment contained in ice chests or uncontaminated plastic buckets. Avoid overcrowding the animals in collection containers. Discard organisms with signs of disease or obvious defects (for example, bivalves with cracked shells).

11.7.1.3 State or local authorities may require collection permits or ban collection from specified areas. The collection of regulated species may require a local license, be limited to a season, and preclude certain collection techniques. Additional permits or precautions may be required when importing non-indigenous species. Check with state authorities concerning local regulations before collecting or importing specimens.

11.7.1.4 *Transport*—Practice E 1022 recommends not more than a 3°C change in water temperature within a 12-h period and an oxygen concentration of between 60 and 100 % of saturation. Simple precautions should meet these requirements if the time between collection and return to the laboratory is short (less than 1 to 2 h) and the ambient temperature is not extreme. If possible, collection buckets or ice-chests should be kept out of direct sunlight and should not be left in closed vehicles. Water in the containers should be changed periodically while collecting and immediately before returning. Use an aerator to maintain oxygen concentrations if the time before returning to the laboratory is several hours or the air temperature is high.

(*1*) Successful long-distance transport of organisms, whether in a vehicle or through the mail, requires packaging that retains moisture and maintains an adequate supply of oxygen. For many species, this can be accomplished by placing the animals in a minimum amount of water (a few millilitres) in a sealed container filled with air. Alternatively, marine test animals may be placed between wet nylon or seagrass (for example, *Zostera*) and surrounded by layers of wet paper towels, all contained in polyethylene bags. Wet sediments of low organic content (for example, ashed sediment and beach

sand) can also be used to retain moisture and are not as prone to anoxia as natural sediment. Regardless of the moisture-retaining agent, the container should have a large air space to maintain aerobic conditions. Air trapped within a plastic bag has the added advantage of preventing the animals from being crushed. Containers with organisms should be placed in ice chests or insulated shipping containers with packets of jelled refrigerant placed at or taped to the inside of the top of the container. Jelled refrigerants are preferred over ice to avoid melted water, and a layer of insulating material should be placed between the refrigerant and animals. Add sufficient refrigerants to maintain the water temperature in the containers at or a few degrees below the water temperature at the collection site, taking care not to cold shock the organisms. Insulating material should fill all extra space in the shipping container, protecting and securing the containers in the carton. Pack the shipment containers to obtain a low center of gravity, and label them plainly to keep the package upright. Every effort should be made to provide overnight or 24-h delivery. If the organisms are transported by vehicle, monitor the temperature periodically and drain any melt water, and replace the ice as required.

11.7.2 *Culturing Test Organisms*—A successful culture of an appropriate test species has the advantage of providing a ready supply of specimens with a known history. Only a few marine sediment-ingesting organisms can be cultured currently. Polychaetes can be cultured with relatively simple equipment (see Annex A6), but the majority of recommended marine test species are not cultured routinely. Culturing procedures for *Lumbriculus variegatus* are given in Annex A8. Culturing conditions for *Chironomus*, *Hexagenia*, and *Hyalella* are outlined in Guide E 1383 Annex . Culturing conditions for these organisms are not provided in this guide since these species can be used for bioaccumulation tests only under special conditions. Other freshwater organisms for use will need to be field collected, for example, *Diporeia*.

11.7.3 *Purchasing Test Organisms*—Some test organisms can be purchased from biological supply houses, local collectors, universities, or bait shops. There are several companies that specialize in supplying bioassay organisms, although most do not presently supply appropriate benthic bioaccumulation organisms on a routine basis. Check with a supplier even if bioaccumulation test species are not carried currently because the availability of particular species may change or the supplier may be able to fill special orders.

11.7.3.1 Maintain purchased organisms in the laboratory for at least one week to acclimate them to the local conditions and to monitor their health. Before beginning the bioaccumulation tests, analyze the purchased organisms for background contaminant levels to determine whether they meet the criteria for control organisms.

11.8 *Preexperimental Conditions*—Most bioaccumulation test species are adaptable to laboratory conditions, so elaborate procedures are not usually required for maintenance of the adults. Additional information on the maintenance of marine benthic invertebrates can be found in Refs **(65, 92-94)**. Permits may be required from state or local authorities when maintaining a nonindigenous species. This may require fail-safe precautions against the accidental release of such organisms into the local environment (that is, double containment, diked water drains, siphon breaks, etc.). Equipment, water, wastes, and dead animals may require sterilization before disposal.

11.8.1 *Sediment Quality for Holding Organisms*—Maintain animals in a sufficient amount of sediment to allow them to burrow naturally. This sediment should be analyzed for contaminant concentrations, which should not exceed the level acceptable for a control sediment (Table 2). Periodically add fresh sediment of the same type to maintain an adequate food supply (that is, detritus and associated microbes). For large marine deposit-feeders (for example, *Macoma*) add approximately 2 mm of fresh control sediment to the sediment surface one to three times per week. This sediment replenishment should be sufficient if the organisms are not overcrowded. Remove the organisms and replace the sediment if the sediments become heavily loaded with fecal material. The addition of other types of food is not recommended except in special cases of long-term maintenance. These foods include detritus (for example, decaying seaweeds), cultured phytoplankton and zooplankton, micro-encapsulated diets, formulated feeds such as fish flakes, or small bits of tissue for omnivors **(95)**. Check the background contaminant levels of all foods.

11.8.2 *Handling of Test Organisms*—Field-collected and shipped organisms should be held in the laboratory for at least four days before starting an exposure, and purchased organisms should be held for at least one week. The longer holding time for purchased organisms is necessary because of the greater uncertainty of the organisms' health prior to control by the laboratory performing the test. Discard any organism if injured or behaving abnormally. Field-collected animals should generally not be held longer than two weeks before testing. If longer maintenance periods are needed, the investigators should have experience with the species and should monitor for any signs of stress (for example, a reduced sediment processing rate and unusual tube construction). A flow-through system for delivering overlying water is advised if long-term maintenance is planned.

11.8.2.1 To prevent the spread of diseases, organisms collected more than one week apart should be maintained in separate aquaria, each with an independent water supply. The organisms should be checked daily, and any diseased, dying, and dead organisms should be removed promptly. Black spots on the surface of the sediment can mark the location of dead organisms. Should a question arise concerning the health of the animals, a behavioral test such as time to rebury or analysis of lipid content is recommended.

11.8.2.2 If the holding and experimental conditions are different, acclimate the test organisms gradually to the experimental conditions. This transition may be accomplished using serial water dilutions until the proper temperature, salinity, and pH are reached. Acclimation for temperature should proceed no faster than 3°C in 72 h (Practice E 1022). Maintain the animals at the test temperature and salinity for at least two days before the commencement of an experiment. No more than 3 % mortality is permitted within 48 h before the test (Practice E 1022).

12. Experimental Design

12.1 *Statistical Considerations*—The experimental objectives are to quantify the contaminant bioaccumulation by organisms exposed to sediments or dredge materials and determine whether this accumulation is statistically greater than that 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 there is no contamination from the experimental setup, and some designs will also require a reference sediment. Uptake from the control sediment or reference sediment (when appropriate) is used to provide baseline values to compare with accumulation from the test sediment. The reference sediment thus functions as the "control" during comparisons with test sediment but also functions as a test treatment during comparisons with the control sediment. The combined descriptor control-reference will be used when referring to the sediment used as the "control" since the statistical term "control" could be confused with the control sediment.

12.1.1 *Experimental Unit*—The organism(s) to which a single application of treatment is applied is the experimental unit. This will be either a single organism or group of organisms exposed to an aliquot of a particular type of sediment. The specific type of sediment constitutes the treatment. If a clam is placed in a beaker containing sediment, the clam is the experimental unit and the beaker is the exposure chamber. If several worms have to be composited to supply sufficient biomass for chemical analysis, the group of worms would constitute the experimental unit, and the beaker or aquarium containing them would constitute the exposure chamber. The important concept is that the treatment (sediment) is applied to the experimental unit as a discrete unit. Experimental units must be independent, for example, there is no flow of water between replicates and they do not differ systematically.

12.1.2 *Replication*—Replication is the assignment of a treatment to more than one experimental unit, which in the bioaccumulation experiment is the organism (or composite of organisms) to which a single treatment (for example, test or control/reference sediment) is applied. 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 (see 12.1.4).

12.1.2.1 *Minimum Detectable Difference*— The smaller the minimum detectable difference between treatments, the greater the number of replicates required for a given significance level, power, and extent of variance. Although there is no consensus concerning what constitutes an acceptable minimum difference, it is suggested that the bioaccumulation experiment be designed to detect a two-fold difference between tissue residues in the test and control sediments or the test and reference sediments. A two-fold difference should provide a sufficiently precise result to address the ecological and human health concerns in most cases.

12.1.2.2 *Minimum Number of Replicates*—The risk of a Type II error must be selected when determining the minimum number of replicates. Because a Type II could have serious environmental or health consequences, it is advisable to assign the same risk of 5 % to both Type I and Type II errors providing a power of 95 %. Practice E 1022 recommends at least four replicates to determine bioconcentration factors but does not specify which power is used for this estimation. Because of the likelihood of a greater variation in sediment exposures compared to water exposures and the choice of a power of 95 %, eight replicates is recommended as the default number of replicates for bioaccumulation tests. Fewer replicates can be used in some cases, for example, when variability is low, the difference between the control or reference and the test is large, or less power is required. It is prudent to include an extra replicate or two for each treatment in case of mortality or the loss of samples during chemical analysis.

12.1.3 *Randomization*—Randomization is the unbiased assignment of treatments to the experimental units (that is, organisms or composites of organisms) ensuring that no treatment is favored and that observations are independent. It is often performed by using tables of random numbers. For these experiments, it is important to assign the organisms to the control and test treatments, to allocate the sediment between replicates, and to locate the exposure units in a non-biased manner.

12.1.4 *Pseudoreplication*—The appropriate assignment of treatments to experimental units is critical in order to prevent a common error in design and analysis recently termed "pseudoreplication" **(96)**. 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 **(96)**.

12.1.4.1 The simplest form of pseudoreplication is treating subsamples of the experimental unit as true replicates. For example, two aquaria are prepared, one with control sediment and the other with test sediment, and five organisms are placed in each aquarium. Even if each organism is analyzed individually, the five organisms replicate only the biological response and do not replicate the treatment (that is, the sediment type). The experimental unit in this case is the five organisms, and each organism is a subsample.

12.1.4.2 A less obvious form of pseudoreplication is the physical segregation of replicates by treatment, potentially resulting in a systematic error. For example, if all of the control experimental units are placed in one area of a room and all of the test experimental units are in another, spatial effects (for example, different lighting and temperature) could bias the results for one set of treatments. Random physical intermixing of the experimental units is necessary to prevent this type of pseudoreplication.

12.1.4.3 A more common form of segregating replicates is the use of separate aquaria for each treatment. For example, segregation would occur if all of the control experimental chambers (for example, beakers) are placed in one aquarium and all of the test experimental chambers are in another aquarium. Any effects due to temperatures or different lighting conditions could bias the results for one of the treatments. Replicate aquaria are necessary in this case.

12.1.4.4 Randomized spatial interspersion does not necessarily preclude pseudoreplication. If the replicates are physically interdependent, spurious effects can bias one treatment over another. This can occur if all of the aquaria replicates of the control are serviced by the same water supply system while all of the treatment aquaria replicates are serviced by another water supply system. Any differences between supply systems may potentially bias one set of aquaria over another. Thus, the replicates are not independent, even if the aquaria replicates are interspersed physically. To prevent pseudoreplication, each experimental unit should have its own water or air supply, all branching off a common supply, and there should be no flow of water from one exposure system to another.

12.1.5 *Preventing or Reducing Pseudoreplication*—Pseudoreplication can be prevented or reduced by identifying the experimental unit properly, providing replicate experimental units for each treatment, and applying the treatments to each experimental unit in a manner that includes interspersion and independence.

12.1.5.1 The simplest design that prevents pseudoreplication is the completely randomized design. Treatments are assigned randomly to the experimental units independent of location in this design, and each experimental unit is maintained in a separate exposure chamber with a separate water and air supply.

12.1.5.2 A randomized block design is also appropriate. A block is a set of relatively homogeneous units to which treatments are to be applied, such as an aquarium (block) containing several beakers (experimental units). In the randomized block design, all of the treatments are assigned randomly to each block, and there are multiple blocks. For example, if there are two treatments and one wishes to contain the experiment in only two aquaria with eight beakers per aquarium, each aquarium (block) is randomly assigned four beakers with control sediment and four beakers with test sediment. One drawback of this design, however, is that since both the test and control organisms are in one aquarium, the potential exists for the cross contamination of controls by test sediment. This is especially likely with organisms that eject sediment into the water, such as *Macoma* during the production of pseudofeces. If this design is used, the aquaria or control exposure chambers, or both, need to be monitored to ensure that cross-contamination does not occur.

12.1.5.3 Preventing pseudoreplication completely may be difficult or impossible given resource constraints. For example, one common experimental design segregates the experimental treatments in separate aquaria. In this case, the beakers containing the test sediment are placed in aquaria separate from the beakers containing the control sediment. Such a design prevents the problem of cross-contamination between the test and control sediment and does not require a separate aquarium for each beaker. However, because the beakers are segregated by treatment type, their distribution is not random. The experimental unit may be defined in such cases as the replicated unit (organism(s) in the beaker with each beaker as a replicate), but with the stated assumption that there is no effect due to the physical segregation (aquaria effect in this example). Using replicate aquaria for each treatment type is recommended with this design to enable the comparison of results between aquaria within a given treatment using a nested ANOVA. The data from one or more aquaria may be considered invalid if aquaria effects are apparent. The organism(s) within each beaker may be considered the experimental unit and each beaker a replicate if no significant aquaria effects are detected. The analysis is then performed as if the beakers were not segregated into aquaria.

12.1.6 *Compositing Samples*—Compositing consists of combining samples (for example, organisms and sediment) and chemically analyzing the mix rather than the individual samples **(97)**. The chemical analysis of the mix provides an estimate of the average concentration of the individual samples comprising the composite. Compositing will be used in bioaccumulation experiments primarily when the biomass of an individual organism is insufficient for chemical analysis. Several individuals can be composited into a single experimental unit with sufficient biomass and the analysis performed on the composite. Compositing is also used when the cost of analysis is high.

12.1.6.1 Individuals must be assigned randomly to the various treatments for the tissue composite to be unbiased. Each organism, group of organisms, or sediment sample added to the composite must be of equal size (that is, wet weight), and the composite must be homogenized completely 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 replicate 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.

12.1.6.2 If composites are made of individuals or samples varying in size or quality (for example, a disproportionate number of gravid females in one composite), 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. The variance of the composites can exceed the population variance in extreme cases **(98)**. It is therefore important to keep the individuals or sediment samples comprising the composite equivalent in size and quality. If sample sizes vary, consult the tables in Ref **(99)** to determine whether replicate composite variances will be higher than individual sample variances, which would make compositing inappropriate.

12.1.6.3 It is not advisable to composite samples if either an estimate of the population variance is required or information concerning the range in concentrations obtained for individuals is needed. Compositing also requires more individuals (assuming that individuals can be analyzed), so it is not advised when space or cost keeps the number of individuals at a minimum. When there is extra sediment or tissue, archive individual samples in case a measure of the population variance or the concentration in a particular exposure chamber is desired latter.

12.2 *Test Duration*—Ideally, the duration of a bioaccumulation test should be sufficient for the organisms to reach steady-state tissue residues, where steady-state is defined operationally as the absence of any significant difference (ANOVA, alpha = 0.05) among tissue residues taken at three consecutive sampling intervals (Practice E 1022). The time to reach or approach steady-state varies drastically among different compounds, but the tests should generally be designed to generate environmentally relevant data on high K_{ow} organic compounds (for example, PCBs and DDT) and heavy metals. A 28-day exposure is considered the standard duration because the 28-day exposure will result in tissue residues generally within 80 % of the steady-state tissue residues for most cases. A 28-day exposure provides inherently better estimates of steady-state than a 10-day bioaccumulation test (Table 4), which has been used previously in the evaluation of dredge materials **(100)**. Because of the recognized limitations of the 10-day exposures, updated procedures for evaluating dredge materials require a 28-day exposure if organic compounds are present **(3)**. Additionally, a 28-day duration test is the recommended standard length for conducting bioconcentrations tests (Practice E 1022). See Annex A3 for details comparing the adequacy of 10 and 28-day bioaccumulation tests. When steady-state is not approached within 28 days, tissue residues of organic compounds usually appear to be within two- to four-fold of steady-state concentrations (Table 4), which is considered acceptable for the ASTM bioconcentration test

TABLE 4 Percent of Steady-State Tissue Residues of Neutral Organics and Metals Obtained After 10 and 28-Day Exposures to Whole Sediment

Organic Compound	Steady-State[A] Tissue Residue, % 10-Day	28-Day	Species	Estimate by	Reference
PCBs					
Aroclor 1242	18	87	*Nereis virens*	G	**(101)**
Aroclor 1242	29	82	*Cerastodema edule*	G	**(101)**
Aroclor 1254	12	82	*Macoma balthica*	G	**(101)**
Aroclor 1254	25	56	*Nereis virens*	K	**(102)**
Aroclor 1254	27	100	*Cerastodema edule*	G	**(101**
Aroclor 1260	27	100	*Cerastodema edule*	G	**(101)**
Aroclor 1260	53	100	*Macoma balthica*	G	**(101)**
Hexachlorobiphenyl	88	100	*Hexagenia limbata*	K	**(103)**
Hexachlorobiphenyl	17	41	*Pontoporeia hoyi*	K	**(104**
Total PCBs	21	54	*Nereis virens*	G	**(105)**
Total PCBs	48	80	*Macoma nasuta*	G	**(105)**
Total PCBs	23	71	*Macoma nasuta*	G	**(106)**
PAHs					
Benzo(a)pyrene	32	66	*Pontoporeia hoyi*	K	**(103)**
Benzo(a)pyrene	43	75	*Macoma inquinata*	G	**(107)**
Benzo(a)pyrene	96	100	*Hexagenia limbata*	K	**(103)**
Benzo(bk)fluor	71	100	*Macoma nasuta*	G	**(108)**
Chrysene	43	87	*Macoma inquinata*	G	**(107)**
Fluoranthene	100	100	*Macoma nasuta*	G	**(108)**
Phenanthrene	67	95	*Pontoporeia hoyi*	K	**(103)**
PAHs					
Phenanthrene	100	100	*Hexagenia limbata*	K	**(103)**
Phenanthrene	100	100	*Macoma inquinata*	G	**(107)**
Phenanthrene	100	100	*Macoma nasuta*	G	**(108)**
Pryene	84	97	*Macoma nasuta*	G	**(108)**
PCDD/PCDF					
2,3,7,8-TCDD	6	22	*Nereis virens*	G	**(105)**
2,3,7,8-TCDD	63	80	*Macoma nasuta*	G	**(105)**
2,3,7,8-TCDF	43	62	*Nereis virens*	G	**(105)**
2,3,7,8-TCDF	92	100	*Macoma nasuta*	G	**(105)**
Miscellaneous					
DDE	21	48	*Pontoporeia hoyi*	K	**(104)**
Dieldrin	27	65	*Macoma nasuta*	G	**(109)**
4,4′DDT	17	10	*Macoma nasuta*	G	**(109)**
4,4′DDD	31	60	*Macoma nasuta*	G	**(109)**
2,4′DDD	31	56	*Macoma nasuta*	G	**(109)**
4,4′DDE	20	50	*Macoma nasuta*	G	**(109)**
Hexachlorobenzene	35	70	*Macoma nasuta*	K	**(59)**
Hexachlorobenzene	36	98	*Macoma nasuta*	G	**(106)**
Metals					
Americium	36	47	*Nereis diversicolor*	G	**(110)**
Americium	50	95	*Venerupis decussata*	G	**(111)**
Americium	32	67	*Hermione hystrix*	G	**(111)**
Cadmium	17	50	*Callinassa australiensis*	G	**(47)**
Copper	75	100	*Macoma nasuta*	G	**(108)**
Iron	11	59	*Nereis diversicolor*	G	**(112)**
Lead	81	100	*Macoma nasuta*	G	**(108)**
Plutonium	43	83	*Nereis diversicolor*	G	**(110)**

[A] All steady-state values are estimates since steady state was not documented rigorously (see 12.2) in any of these studies. K = steady-state tissue residue estimated from the kinetic uptake model. G = steady-state tissue residue estimated by visual inspection of graphs of tissue residue versus time.

(Practice E 1022). However, if steady-state cannot be documented from the experimental results, the tissue residue is only an estimate of steady-state and can be a substantial underestimate of the true value for some compounds. A longer-term bioaccumulation test (>28 days) or an approach that uses a kinetic uptake model should be considered for cases in which more accurate estimates of the steady-state tissue residues are needed. If long-term bioaccumulation tests are considered, the design should address their inherent problems of changing sediment contaminant concentrations and characteristics, as well as possible changes in the physiology of the test organism (for example, the loss of tissue lipids). An exposure duration of 10 to 14 days may be sufficient to achieve steady state for many compounds in sediment bioaccumulation tests with the oligochaete *Lumbriculus variegatus* (see A8.5.2).

12.3 *Biotic Sampling Schedule*—Biological samples are used to determine the amount of chemicals accumulated from the test sediment and to compare these values statistically to the amount of chemicals accumulated from control and reference sediments. Bioassay organisms should be analyzed for chemical and lipid content immediately before the start of the experiment (t_0 samples) to set the baseline conditions for these comparisons. Eight replicates is assumed to be the number required to achieve sufficient statistical power. Therefore, eight replicate organisms or composites (that is, experimental units) should be analyzed at t_0 (see 12.1.2.2). The replicates sampled at t_0 should be chosen randomly from the same set of organisms used in the various sediment treatments. The same compositing scheme should be used for all sampling periods throughout the experiment if compositing of individuals is necessary to obtain sufficient biomass. Eight replicate organisms or composites should be taken from each of the treatments and analyzed for chemicals and lipids at the end of the 28-day test period (t_{28}). The simplest design for comparing test and control sediments results in 24 tissue samples (8 controls at t_0, 8 controls at t_{28}, and 8 test samples at t_{28}). It is recommended that one or two extra replicates be included in each treatment in case a sample is lost. Additionally, several extra individuals or composites should be taken at the initiation of the experiment. These extra samples should be frozen until the tissue residue data have been analyzed and interpreted. This experimental design is considered the minimum data set needed to document bioaccumulation, but it does not supply sufficient data to document that steady state has been attained.

12.3.1 Time-series samples may be taken during the 28-day exposure to document uptake kinetics and steady state. This type of information can be very helpful, even if it is necessary to limit the analytical load by taking only a single sample or, preferably, a single composite at each sampling period. However, if the data will be compared statistically to determine whether steady state has been attained, replicates are required at each sampling period. The sampling interval for these samples should approach a geometric progression with sampling periods of no greater than one week (for example, day 0, 2, 4, 7, 14, 21, and 28). A sample at 10 days is recommended if there are previous 10-day exposure data.

12.4 *Abiotic Sampling*—The physical and chemical properties of each test, control, and reference sediment should have been characterized immediately after collection (see 10.2). Depending on the length of storage, it may be necessary to remeasure these physical and chemical characteristics, with the possible exception of grain size distribution, immediately before the start of the bioaccumulation test (that is, t_0). Additionally, if these t_0 samples will be compared statistically to samples taken at the end of the test period (t_{28}), eight replicate samples are suggested (see 12.4.1).

12.4.1 At the end of the bioaccumulation test (t_{28}), take sediment samples from each exposure chamber for measurements of contaminant concentrations, TOC, and moisture content. It is usually not necessary to remeasure grain size. These analyses should preferably be conducted on the sediment from each beaker or aquarium (that is, experimental unit). Measurements on individual experimental units may help explain any unexpected variation among the replicates. If eight replicates are used per treatment, this would result in a total of 32 sediment samples (8 controls and 8 tests at t_0, 8 controls, and 8 tests samples at t_{28}).

12.4.2 An alternative sampling scheme could be used to reduce analytical loads. This would be conducted by making a composite sample for each experimental unit composed of equal aliquots of sediment from each beaker or aquarium within the treatment. Additionally, a sediment sample from each beaker or aquarium should be taken and archived. If the tissue residue data are more variable than expected, or if there are "unusual" data points, these individual sediment samples should be analyzed. Additionally, individual sediment samples should be analyzed if the differences in contaminant concentrations in the t_0 and t_{28} sediment samples are greater than would be expected from analytical variation alone.

12.5 *Long-Term Uptake Tests*—In some cases, body burdens will not approach steady-state body burdens in a 28-day test (see Table 4). Organic compounds exhibiting these kinetics will probably have a log $K_{ow} > 5$, be metabolically refractory (for example, highly chlorinated PCBs and dioxins), or have low depuration rates. Additionally, tissue residues of several heavy metals may increase gradually over time so that 28 days 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 days (or a kinetic study) to obtain a sufficiently accurate estimate of the steady-state tissue residues of these compounds.

12.5.1 *Biotic Sampling*—In long-term studies, the exposure should continue until steady-state body burdens are attained (see 12.2). Practice E 1022 recommends a minimum of five sampling periods (plus t_0) when conducting water exposures to generate BCFs. For bioconcentration tests, Practice E 1022 recommends sampling in a geometric progression with sampling times reasonably close to $S/16$, $S/8$, $S/4$, $S/2$, and S, where S = the time to steady-state. This sampling design presupposes a fairly accurate estimate of time to steady-state, which is often not the case with sediment exposures.

12.5.1.1 Placing a greater number of samples at and beyond the predicted time to steady-state is recommended to document steady-state from sediment exposures. With a contaminant expected to reach steady-state within 28 to 50 days, samples should be taken at days 0, 7, 14, 21, 28, 42, 56, and 70. If the

time to steady-state is much greater than 42 days, additional sampling periods at two-week intervals should be added (for example, Day 84). Slight deviations from this schedule (for example, Day 45 versus Day 42) are not critical, although samples should be taken at t_{28} for comparative purposes. An estimate of time to steady-state may be obtained from the literature or approximated from structure-activity relationships (Annex A5), although these values should be considered the minimum times to steady-state.

12.5.1.2 Compared to the ASTM bioconcentration sampling schedule, this schedule increases the likelihood of documenting statistically that steady-state has been obtained, although it does not document the initial uptake phase as well. Add sampling periods during the initial uptake phase (for example, Days 0, 2, 4, 7, 10, and 14) if accurate estimates of the sediment uptake rate coefficient (K_s) are required.

12.5.1.3 The loss of replicates due to mortality and spawning can be a problem with long-term exposures. However, increasing the total number of replicates by an additional 10 to 20 % should suffice in most cases. If not needed, archive these extra individuals at the end of the test as replacement samples in case of analytical failures, or analyze them to increase the statistical power of the final sampling period.

12.5.1.4 Avoidance of the sediment can occur, particularly at high doses. The exposure will decrease if this occurs, and the expected kinetics and overall steady state will be altered.

12.5.2 *Abiotic Samples*—The bioavailable fraction of the contaminants as well as the nutritional quality of the sediment are more prone to depletion in these extended tests than in 28-day exposures. To document statistically whether such depletions have occurred, eight replicate sediment samples are ideally required for physical and chemical analysis from each sediment type at the beginning and end of the exposure. Additionally, archiving sediment samples from every biological sampling period is recommended.

12.5.2.1 To minimize the depletion of sediment contaminants or nutrients, sediment can be completely replaced stored sediment or freshly dosed sediment on a regular basis (for example, monthly). Sediment must be renewed carefully to avoid damaging the test organisms, especially polychaete worms. Another way of minimizing the depletion of contaminants is by periodically adding fresh sediment (see 13.3.1). However, over a long experiment the exposure container may be filled entirely, necessitating replacement of the sediment anyway. Replenishment sediment should be sampled and characterized completely for the recommended characteristics (see 10.2). Test organisms should not be given a supplemental food source (for example, fish flakes) since this will reduce exposure to ingested sediment and may result in an underestimation of the sediment bioavailability and steady-state tissue residues **(113)**.

12.6 *Estimating Steady State*—It may be possible to estimate steady-state levels in tests in which steady-state cannot be documented (see 12.2). Several methods have been published that can be used to predict steady-state contaminant levels from uptake and depuration kinetics **(114–115)**. All of these methods were derived from fish exposures, and most use a linear uptake, first-order depuration model that can be modified for contaminant uptake from sediment. To avoid confusing uptake from water versus sediment, k_s, the sediment uptake rate coefficient, is used instead of k_1**(116)**. The k_s coefficient has also been referred to as the uptake clearance rate **(45)**. Following the recommendation of Stehly, et al **(117)**, the gram sediment and gram tissue units are retained in the following formulation:

$$C_t(t) = k_s \times C_s/k_2 \times (1 - e^{-k_2 \times t}) \quad (1)$$

where:

C_t = contaminant concentration in tissue at time t,
C_s = contaminant concentration in sediment,
k_s = uptake rate coefficient in tissue, g sed g^{-1} day^{-1},
k_2 = depuration constant, day^{-1}, and
t = time, days.

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

$$Ct_{max} = C_s \times k_s/k_2 \quad (2)$$

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

$$BAF = k_s/k_2 \quad (3)$$

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

12.6.1 The kinetic approach requires an estimate of k_s and k_2, 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 k_s is made during the linear portion of the uptake phase to prevent 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. It is acceptable to use a k_2 derived from a water exposure unless there is reason to suspect that the route of exposure will affect the depuration rate. The durations of the uptake and depuration experiments will vary with animal species, compound, contaminant concentration, analytical detection limits, and test sediment. As a result, no specific guidelines will be presented here. For a discussion of this method for bioconcentration studies in fish, see Practice E 1022 and Refs **(114, 115)**. Its application to sediment is discussed in Ref **(116)**. 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 previously presented **(44)**.

13. Procedure

13.1 *Preexperimental Preparations*—Coordinate the collection and acclimation of the test organisms with collection of the sediments so that the experiment can begin with a minimum of delay. The glassware, water delivery system, and any stored water, as well as sampling containers, labels, and related

materials, should be ready. Beakers and other containers should be pre-labeled. A detailed work schedule, showing daily tasks and the individuals responsible for accomplishing them, should be prepared before the sediment arrives. A prearranged numbering scheme should be agreed upon by the analytical chemists. It is critical to keep the analytical chemists well informed of the sampling schedule so they can prepare for the sample load. Arrange with maintenance personnel to look for power failures, pump leaks, breakage of aquaria, inadvertent switching on of lights at night, and other accidents. Provide telephone numbers for key personnel responsible for maintenance of the experiment in a prominent location (for example, on the door of the laboratory). Any safety warnings should also be posted at entry points.

13.2 *Experiment Initiation*—Weigh all individual organisms or composites of organisms, while taking care to minimize the exposure of soft-bodied organisms to air. In cases in which it is not possible to pre-weigh soft-bodied aquatic organisms without damage, an average population weight should be determined by random sampling of the population of organisms that will be used in the test. Maintain the air temperature of the room near the experimental water temperature to prevent temperature shock. Large bivalves should be measured (anterior to posterior valve), weighed, and marked individually with a random number. Discard any organisms not meeting the criteria for size or condition. Maintain a few extra individuals for potential replacements (see 13.3). Also, choose randomly an appropriate number of specimens for wet-to-dry weight conversions and for lipid analysis.

13.2.1 Distribute measured aliquots of homogenized sediment to each exposure chamber. Weighing the sediment aliquot is preferable, but the sediment volume can be used to estimate the mass for a particular sediment type. During the process of measuring aliquots of sediment, re-stir the source periodically to prevent separation of the fines and interstitial water. If beakers are used as exposure chambers, tap the beaker gently to consolidate the sediment and eliminate air bubbles. To prevent the loss of surficial fines when filling the beakers, place a plastic film over the sediment surface, slowly fill the beaker with water, and then withdraw the film using forceps. Place the water-filled beakers into filled aquaria carefully, and allow any suspended fines to settle.

13.2.2 If aquaria or other large containers are used as the exposure chambers, stir the sediment gently after adding the sediment and to remove bubbles. As with the beakers, a plastic film can be placed over the sediment surface when filling the aquarium with water. Position any aerating device so that the induced turbulence does not resuspend sediment. Allow the sediment to consolidate and the suspended particles to settle before adding organisms. Settling times will vary with the grain size of the test sediment, but it is often convenient to add the sediment to the test chamber the day before the test is initiated, which allows the sediment to settle overnight.

13.2.3 *Adding Organisms*—Place animals on the surface of the sediment, and allow them to bury. Mobile organisms, such as the polychaetes or oligochaetes, should be observed for a sufficient period to ensure that they bury in the correct chamber and do not swim into another chamber. For mobile animals, it may be necessary to place screens on the tops of beakers to keep them from swimming out. It is also important to ensure that sediment samples are not toxic to the test organisms (see 13.5) or that the organisms do not exhibit significant sediment avoidance to ensure appropriate exposure (see Table A8.5).

13.3 *Experiment Maintenance*—Replace animals whose behavior is abnormal (failure to bury in the sediment, etc.) within the first 24 h if possible. Observe the chambers daily, and note any signs of abnormal activity (for example, reduced production of fecal pellets and avoidance of the sediment). Remove beakers with dead organisms. It is especially important to check for dead organisms in a static system. (Note that the previous recommendations may not be practical for the smaller test organisms.) Record the temperature and other water-quality characteristics on a weekly basis. Replenish the water in water renewal experiments according to a preplanned schedule, and dispose of drained water in accordance with the applicable rules for hazardous waste.

13.3.1 *Sediment Renewal*—It is recommended for some test organisms (for example, *Macoma*) that periodic additions of small amounts of the appropriate sediment type be made to each exposure chamber. Because the bioavailable fraction may constitute a small portion of the total sediment chemical (see Ref **(44)**), sediment-ingesting organisms may deplete the available fraction, especially if they have a restrictive feeding zone. Accordingly, depletion of the bioavailable fraction may be the reason that tissue residues of 35 of 37 compounds declined between Day 39 and Day 79 in Oliver's study **(118)** of uptake by oligochaetes. Also, without organic input from settling phytoplankton and with low light levels inhibiting benthic microalgae, it is possible that the nutrient quality of the sediment could decline over the course of a long-term experiment. Periodic sediment renewal should reduce these potential laboratory artifacts and help maintain a more constant chemical concentration and food supply. The amount of sediment added daily should equal or exceed the daily sediment processing rate of the organism. Sediment-ingesting clams such as *Macoma* require about 1 g of wet sediment per gram of wet tissue mass per day, and arenicolid worms (2 to 6 g wet weight) require about 10 g of sediment per day. It is sufficient to add the sediment two or three times per week (for example, about 3.5 g twice per week for a 1-g *Macoma*).

13.3.1.1 Periodically replacing all of the sediment in the chambers is recommended for long-term exposures (>28 days). Replacement of the sediment reduces the possibility of depletion of the bioavailable fraction of the chemicals or food and prevents excessive pelletization of the sediment. Additionally, the periodic addition of surface sediments will overfill most chambers within a few weeks. Replacement on a monthly schedule should suffice, and it coordinates with the long-term sampling schedule. All of the sediment should be collected at the same time and the renewal sediment stored until needed if a field sediment is tested. It may be preferable to dose new sediment for replacement if a dosed sediment is tested. All added or replacement sediments need to be analyzed for physical and chemical characteristics (see 10.2).

13.3.2 Test organisms should not be fed a supplemental source of food in either 28-day or long-term experiments.

Studies on long-term maintenance (>28 days) of deposit-feeding bivalves (for example, Ref **(119)**), polychaetes (for example, Ref **(42)**), and crustaceans (for example, Ref **(44)**) have shown that an artificial food was not necessary. By ingesting added food, the organisms presumably ingest less sediment, resulting in less uptake of the sediment-associated contaminants. Supplemental food may also enhance the rate of loss by passing uncontaminated material though the intestinal track.

13.4 *Contaminant Samples*—Samples of sediment, water, and biota should be taken for chemical analysis before, during, and after testing (see 12.3-12.5). The sampling techniques and apparatus will vary with the nature of the sediment, species of test organism, and compound(s) of interest. Consistency in sampling for any given characteristic is essential since the manner in which the samples are taken may affect the analysis.

13.4.1 *Overlying Water*—Although no contaminants are intentionally added to overlying water in sediment bioaccumulation tests, contaminants may be introduced from the water supply system, leached from the sediment, or present on resuspended particulates. The activities of some species (for example, *Yoldia*) can resuspend considerable amounts of fine-grain material directly into the water column. Depending on the design of the exposure system, this bioturbation may lead to cross-contamination between treatments. This potential uptake from the water needs to be quantified to differentiate it from uptake from the whole sediment and to check for possible cross-contamination among treatments.

13.4.1.1 At a minimum, overlying water should be sampled for contaminants from each treatment at the beginning, middle, and end of the test period (that is, T_0, T_{14}, and T_{28}). A sample from each aquarium should be analyzed if statistical comparisons are planned, although it would be acceptable to composite water samples from aquaria of the same treatment in many cases. If samples are composited, individual samples from each aquarium should be archived in case a more detailed analysis is required. Samples should also be taken during periods of high turbidity or other unusual water quality.

13.4.1.2 Overlying water should be sampled at mid-depth from each exposure unit. Overlying water should be sampled from mid-depth of the entire container. Care should be taken to avoid disturbing the flocculent material at the sediment-water interface. Sampling apparatus (pipettes and sample vials) should be made of materials that do not absorb or leach contaminants appreciably. Rinse the sampling apparatus after each use to guard against cross-contamination. Sample volumes will depend on the analytical technique used but may range from about 1 to 100 mL.

13.4.2 *Sediment and Interstitial Water*— Sample all test, control, and reference sediments before the addition of organisms (t_0 sample) and at the end of the exposure (typically t_{28}). These sediment samples should be analyzed for chemical concentrations, TOC, and moisture content. It is adequate to conduct the grain size analysis only on the initial sample in most cases.

13.4.2.1 One procedure for sampling sediment for organic compounds from exposure chambers is as follows:

(1) Remove overlying water from the exposure chamber by siphoning or decanting, taking care not to disturb the surface floc. Use PTFE or glass tubing for siphoning or decanting if metals are also to be analyzed. Depending on the procedure, interstitial water samples may be taken at this stage (see Guide E 1391 and Test Method E 1706 for guidance on sampling interstitial water).

(2) Remove the test organism(s) from the sediment. Larger bivalves can be removed directly with forceps (use PTFE forceps if metals are a concern). Spread the sediment out on a tray to remove small bivalves, polychaetes, and oligochaetes. Do not use any water to remove the sediment from the exposure chambers.

(3) Homogenize the test sediment from each exposure chamber by stirring with a PTFE-coated spoon or glass rod. Take a sediment sample from each exposure chamber, place it in a labeled sample glass or plastic (for metals) vial, and freeze it leaving a head space above the sample. These individual samples will be either analyzed or archived if composites are analyzed.

(4) If composites are going to be taken, the compositing strategy will depend on how the exposure chambers were allocated among aquaria. Composite all of the beakers within an aquarium if only one treatment type is placed in each aquarium. If the exposure chambers are allocated randomly among aquaria, combine all of the sediment from each treatment (that is, sediment type) regardless of the aquarium. In both cases, homogenize the sediment, take replicate samples from each composite, and freeze until analyzed.

13.4.2.2 Extra sediment samples should be taken from individual exposure chambers (and from any composites) and frozen in case there is an analytical failure or greater statistical power is required.

13.4.2.3 Reduced metal forms will be oxidized because the removal of organisms exposes the sediment to air. The organisms should be removed from the sediment sample in a glove bag under a controlled atmosphere if metal speciation will be studied. Interstitial water should be collected at the same time as the sediment samples. Interstitial water may be collected by a variety of methods, including centrifugation, sediment squeezing, and dialysis membranes (see Guide E 1391).

13.4.3 *Tissue Samples*—Test organisms need to be removed carefully from the sediment by gentle seiving or other mechanical means, and all adhering particles need to be removed. A gentle rinse with clean water will help remove particles from benthos. Use seawater or freshwater, as appropriate. Organisms should generally be placed in clean water for 24 h to purge their gut contents before chemical analysis.

13.4.3.1 *Gut Purging*—When a whole-body tissue analysis is conducted on a deposit-feeder, any contaminants associated with the mineral particles and detritus in the gut are included. Depending on the mass of sediment and the associated contaminant concentration, the gut sediment can increase the apparent whole-body tissue residue measurably. Allowing the organism to purge its gut contents (that is, defecate) in a clean environment can reduce or eliminate this positive bias. Contaminants can depurate or be metabolized during purging, resulting in an underestimation of the bioaccumulation. The type and extent of the error will depend on many factors,

including the nature of the contaminant, feeding behavior of the organism, and ability of the organism to metabolize and eliminate the contaminant. Factors influencing the errors associated with purging are summarized in Table 5. Purging in either uncontaminated water or sediment have both been used. However, purging in clean sediment can enhance the depuration of compounds from the organism and add uncontaminated sediment to the organism weight, which will result in concentration dilution **(32, 120)**. While complete evacuation of the organism's gut contents may not occur in water-only purging, the error of dilution by the addition of uncontaminated gut contents will probably be greater than the contribution from incomplete gut clearance.

13.4.3.2 After collection, rinse the organisms with clean water, blot them dry, and then weigh them. Measure the shell length of bivalves. Organisms should be analyzed immediately or frozen in baked-out aluminum foil or glass vials. Use non-contaminating plastic (linear or high-density polyethylene or equivalent for metals). The entire soft-tissue of each individual or composite of individuals from an experimental unit should be prepared for analysis. In many cases, the tissue from each experimental unit will be homogenized first, and then subsamples will be taken for organic, metal, and lipid analyses and archiving. The type of homogenization technique will depend on the size and tissue consistency of the organism, contaminant of interest, and analytical procedures used for the contaminant analysis.

13.4.4 *Standard 24-h Purge*—Organic compounds with high K_{ow} values (for example, PCBs, DDT, and BaP) are usually the greatest environmental concern in terms of bioaccumulation. Most of these compounds are depurated slowly, so a relatively small amount should be lost during purging. A 24-h gut purging is therefore recommended as the standard procedure for sediments known or suspected to contain more than trace amounts of these contaminants. A 24-h depuration period is sufficient for most organisms to defecate the majority of their gut contents without introducing substantial errors from contaminant depuration or metabolism. If the rate of compound depuration is unknown, a time series of samples can be taken to determine the elimination rate coefficient and estimate the extent of loss during the 24-h purge.

13.4.4.1 Many deposit feeders require the ingestion of sediment to void their gut contents completely, and organisms have been placed in control sediment to ensure complete purging. A clean control sediment should be used if purging is performed in sediment. Reference sediment may contain substantial contaminant concentrations and should not be used. The experimental conditions (for example, temperature and salinity) should be maintained. An estimate of the gut content mass must be made to correct for dilution by the uncontaminated material. If metals are to be measured, the uncontaminated sediment may contain as high a mineral metals concentration as the test sediment. The organisms in the control and reference sediment(s) should undergo the same purging treatment as individuals exposed to the test sediment. Organisms from different treatments should be kept in separate containers to prevent any possibility of cross-contamination. Observations should be made on whether feces were produced during the purging period and on the general health of the organisms.

TABLE 5 Errors Associated With Gut Sediment/Purging

(*1*)	Gut sediment introduces greatest error:
	(*a*) In organisms that ingest high-organic particles selectively.
	(*b*) In organisms with a large gut capacity.
	(*c*) During the early stages of uptake when tissue residues are low.
	(*d*) For compounds not extensively bioaccumulated, especially high K_{oc} compounds with steric hindrance to uptake.
(2)	Purging introduces greatest error:
	(*a*) For rapidly depurated/metabolized compounds.
	(*b*) Purging in councontaminated sediment can introduce a dilution error.

13.4.4.2 Whether to purge organisms in water only or an uncontaminated sediment depends on the expected extent of the bias. Oliver **(118)** indicated that no bias existed for chlorinated hydrocarbons and that purging did not have to be performed. For the oligochaete, *Lumbriculus variegatus*, purging in water only results in rapid gut content elimination, and back extrapolation along the elimination curve suggests a contribution of approximately 10 % of the total concentration to be attributed to gut contents for both pyrene and benzo(a)pyrene **(32, 120)**. The depuration rate will be much greater if the purging is performed in clean sediment; it is thus critical to correct for depuration losses. Furthermore, a negative bias due to dilution by uncontaminated sediment exists and reduces the expected concentration by an additional 10 to 15 % when the elimination curve is back extrapolated **(32, 120)**. This is almost exactly the fractional mass of material eliminated by the oligochaetes. Purging the organisms in sediment will thus require a correction for dilution (Annex A7). The decision to purge needs to consider the potential bias from remaining gut contents if the purge is water only, the potential bias from depuration and metabolism during the purging period, and the potential bias from errors in estimating the dilution mass if purging in uncontaminated sediment.

13.4.4.3 *When Not to Purge*—Gut purging may introduce an error in some situations that is greater than that associated with retaining gut sediment. If the purpose of the study is to compare laboratory and field organisms, it is often impractical to purge field-collected organisms. Therefore, to ensure that the laboratory and field results are directly comparable, laboratory organisms should not be purged. If the purpose of the study is to determine contaminant trophic transport, do not purge because predators usually ingest the entire prey item. If the compounds of concern are lower molecular weight PAHs, purging is not recommended since these compounds may be depurated and metabolized rapidly (see Table 6), so that a 24-h purge can result in a greater error than leaving the gut sediment.

13.5 *Acceptable Levels of Mortality*— According to Practice E 1022 guidelines for bioconcentration tests, a test is unacceptable if "more than 10 % of the organisms in any treatment died or showed signs of disease, stress, or other adverse effects." This criterion is applicable to studies of dosed sediments in which it is possible to adjust contaminant concentrations. Repeat any 28-day spiking experiment at a lower contaminant concentration if 10 % or more of the organisms in any treatment die or show overt signs of stress. Signs of stress

TABLE 6 Depuration Loss Of Contaminants During 24 and 72-h Gut Purges[A]

Compound	Organism	%Lost, h		Reference
		24	72	
PCB	*Crangon septemsapinosa*	3	8	**(102)**
HCB	*Macoma nasuta*	4	12	**(58)**
BaP	*Pontoporeia hoyi*	4 ± 3	12 ± 8	**(44)**
Phe	*Pontoporeia hoyi*	11 ± 7	33 ± 19	**(44)**
BaP	*Hexagenia limbata*	14–26	43–99	**(103)**
Phe	*Hexagenia limbata*	77–100		**(103)**
HCBP	*Hexagenia limbata*	12–41	36–99	**(103)**

[A] PCB = Aroclor 1254, HCB = hexachlorobenzene, BaP = benzo(a)pyrene, Phe = phenanthrene, and HCBP = hexachlorobiphenyl.

include avoidance of the sediment, non-burial, casting off of siphons, abnormal tube construction, and reduced ventilation or sediment processing rates.

13.5.1 Many of the field sediments or dredge materials of environmental concern will have moderate to high toxicity, in contrast to most experimental studies of bioavailability. It may be impossible or difficult to meet the 10 % mortality criterion with these sediments. However, this may not represent a serious problem because the purpose of evaluating these sediments is to determine the extent of bioaccumulation resulting from a particular sediment. The mortality in the laboratory would presumably mimic the response in the field and so represent the actual effect of the sediment. However, when sediments produce toxicity, the bioaccumulation response may be lower than if toxicity did not occur. Altered behaviors due to stress and avoidance of sediment have both been observed when sediment-associated contaminants produced a toxic response. These altered behaviors have been associated with altered exposure and accumulation.

13.5.1.1 Because tests conducted on field and dosed sediments have different purposes, it may not be necessary to reject the tests when mortality in the test sediment is greater than 10 %. The determining factors in deciding to accept a test treatment with high mortality is whether there are adequate replicates to obtain sufficient statistical power and consideration of the potential for altered exposure. The test should be considered invalid if overt sediment avoidance is observed. The experiment should be repeated if the statistical power is insufficient. Also, mortality or stress at greater than 10 % in the control or reference sediment could indicate that the organisms were stressed initially, the system was contaminated, or the control or reference sediment was unacceptable. The cause of the problem should be determined and the experiment repeated. Consider using a more contaminant-resistant species in any future tests if the mortality in the test sediment exceeds 25 %.

13.5.1.2 High mortality in field sediments may be a moot problem because a sediment sufficiently toxic to kill a substantial proportion of the recommended test species would presumably be unacceptable based on toxicity. Even in cases in which a sediment is rejected on the basis of toxicity, a bioaccumulation test conducted on the diluted sediment may help identify the compounds responsible for the toxicity. However, diluting a sediment with uncontaminated sediment may alter the contaminant bioavailability.

14. Analytical Methodology

14.1 *Contaminant Analysis*—Explanation of specific techniques used to analyze sediment, water, and tissues for contaminants is a complex subject beyond the scope of this guide. Discussions of analytical techniques can be found in Refs **(21, 121-126)**. However, it is possible to offer several guidelines. First, analytical techniques are media dependent. Time should thus be allocated for modifying procedures for the various media and any special conditions (for example, high TOC sediment and low tissue biomass). Second, a harsh extraction technique should not be used when analyzing sediments for metals since such a technique can extract biologically unavailable metals from the mineral matrix. A discussion of various metal extraction techniques is found in Refs **(127, 128)**. Third, the PCB analysis should be at the level of identifying and reporting specific congeners rather than Aroclor equivalents to the extent possible. In particular, the more toxic planar congeners need to be identified. A thorough review of PCB congeners, including which to analyze, can be found in McFarland and Clarke **(41)**.

14.1.1 The required or desired detection limits will have a major effect on the choice of analytical techniques and on the ability to interpret the data. The detection limits and analytical procedures will be specified by the pertinent regulation in some cases, while the decision will be determined by the researcher in other cases. If no detection limits are specified, the minimum requirements of the analytical techniques should meet the requirements of the U.S. Environmental Protection Agency's (EPA's) Contract Laboratory Program **(21, 126)**. The quantification limits from these documents are summarized in Table 7. These limits cover organics in water and sediment and metals in water. Although tissues are not addressed, it should be possible to obtain the same quantification limits as with the sediments.

14.1.2 Control samples or samples from relatively clean areas contain low contaminant concentrations and may require lower detection limits to achieve satisfactory results. The

TABLE 7 U.S. EPA Contract Laboratory Program Quantification Limits for Water and Sediment With Estimates for Tissue Matrices

Organics	Water, µg/L	Sediment, µg/kg[A]	Tissue, µg/kg[B]
Volatiles	5–10	0.5–10	0.5–10
Semivolatiles	10–50	330–1600	330–1600
Pesticides/ PCBs	0.05–1	8–160	8–160
For individual contaminants, refer to CLP Statement of Work			
Metals	**Water, µg/L**		
Antimony	20–300		
Arsenic	5–100		
Cadmium	0.5–10		
Copper	5–100		
Lead	5–100		
Mercury	0.2–20		
Nickel	5–100		
Silver	1-25		
Zinc	0.2–4		
Metals not listed, refer to CLP Statement of Work			

[A] µg/kg wet weight.
[B] µg/kg wet weight basis. Values for tissues were estimated from the sediment values on the premise that tissue and sediment contaminant concentrations are of a similar magnitude and are analyzed by similar techniques.

methods developed for measuring contaminants in samples collected from the Puget Sound Dredge Disposal Analysis study (PSDDA) control sites **(18)** are suggested in such cases. The PSDDA values include tissues as well as water and sediment and are summarized in Table 8.

14.1.3 A complete quality assurance/quality control QA/QC plan is a central part of any analytical procedure. Information on analytical QA/QC procedures is available from several sources **(21, 126, 129)**. An important part of any QA/QC program is the use of reference samples and standards. Reference samples and standards are available from the U.S. EPA in Cincinnati, OH; Las Vegas, NV; and Research Triangle Park, NC, as well as the National Institute of Standards and Technology (Office of Standard Reference Materials, Room B311, Chemistry Building, NIST, Gaithersburg, MD 20899).

14.2 *Lipid Analysis*—A number of studies have demonstrated that lipids are the major storage site for organic contaminants in a variety of organisms **(130-132)**. Bioaccumulated concentrations for nonpolar organics should be normalized to the tissue lipid concentration because of the importance of lipids. Lipid concentration is one of the factors required in deriving the Biota-sediment accumulation factor (BSAF) (see Annex A1). However, the difficulty in using this approach is that each lipid method generates different lipid concentrations. (See Kates **(133)** for a discussion of lipid methodology.) The differences in lipid concentration translate directly to a similar variation in the lipid-normalized contaminant concentrations or BSAFs.

14.2.1 To allow lipid-normalized tissue residues or BSAFs to be compared, it is necessary to either promulgate a standard lipid technique or intercalibrate the various techniques. Standardization on 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 **(134)** is recommended as a temporary "intercalibration standard."

14.2.1.1 The potential advantages of Bligh-Dyer include its ability to extract neutral lipids not extracted by many other solvent systems and the use of Bligh-Dyer (or the same solvent system) in numerous biological and toxicological studies (for example, Refs **(44, 130, 131)**). 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 **(135, 136)**.

14.2.1.2 A potential disadvantage of the Bligh-Dyer is that, by extracting many of lipids not extracted by other techniques, the method may extract lipids that are not important to the storage of neutral organic contaminants. Solvents used in the Bligh-Dyer method are not commonly used in analytical methodologies used to quantify nonpolar organic contaminants, and as a result it may be necessary to quantify lipids on a subsample of the tissue used to quantify the tissue residues.

14.2.1.3 Compare the chosen lipid method with Bligh-Dyer for each tissue type if the Bligh-Dyer method is not the primary lipid method used. 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.

14.3 *Sample Storage*—For organics, the U.S. EPA Contract Laboratory Program **(21)** requires that the samples be protected from light and refrigerated at 4°C (±2°C) from the time of receipt until they are extracted and analyzed. Water samples shall be extracted within five days of receipt of the sample. Sediment samples shall be extracted within ten days of receipt of the sample, and the extraction of water samples shall be started within five days of receipt of the sample if continuous extraction procedures are used.

14.3.1 For inorganics, the U.S. EPA Contract Laboratory Program **(126)** requires that soil and sediment samples be maintained at 4°C (±2°C) until analyzed. Samples for mercury shall be analyzed within 26 days of receipt of the sample. Samples for metals shall be analyzed within 180 days of receipt of the sample.

14.3.2 If other priorities interfere with the requirements set by the Contract Laboratory Program, it is suggested that in those cases the samples either be frozen (−20°C) in airtight containers or dried, depending on the type of sample and analyses required. Purging the container with nitrogen before sealing will delay the degradation of some contaminants as well as lipids. Sample containers should be as full as practical to prevent moisture loss from the sample. Sediment samples so preserved are stable for at least six months, if not longer **(123)**. Tissue and water samples are expected to be at least as stable as sediments.

14.4 *Reporting of Results*—Investigators have reported results on either a dry or wet basis, usually without a conversion factor between the two and sometimes without any indication of which was used. This makes it difficult, or impossible, to compare the results from different studies. A dry-weight basis is generally preferred for both sediment and tissue contaminant concentrations. However, certain analytical techniques use wet

TABLE 8 PSDDA Low Limits of Detection for Water, Sediment, and Tissue Matrices

Organics		Sediment, µg/kg[A]	Tissue, µg/kg[B]
Volatiles		10–20	5–10
Semivolatiles		1–50	10–20
Pesticides/PCBs		0.1–15	0.1–20
Metals	**Water, µg/L[C]**	**Sediment, mg/kg[D]**	**Tissue, mg/kg[E]**
Antimony	3	0.1	0.02
Arsenic	1	0.1	0.02
Cadmium	0.1	0.1	0.01
Copper	1	0.1	0.01
Lead	1	0.1	0.03
Mercury	0.2	0.01	0.01
Nickel	1	0.1	0.02
Silver	0.2	0.1	0.01
Zinc	1	0.2	0.20

[A] µg/kg dry weight, ppb.
[B] µg/kg wet weight, ppb.
[C] µg/L, ppb.
[D] mg/kg dry weight, ppm.
[E] mg/kg wet weight, ppm.

tissue or wet sediment, necessitating the calculation of wet-weight concentrations. The wet-to-dry weight ratios should be reported for each tissue and sediment type to allow comparisons among studies. As previously mentioned, lipid values should be reported in "Bligh-Dyer equivalents," along with any conversion factor(s) between lipid methods.

15. Data Interpretation

15.1 *Objective*—The main objective of statistical testing is to determine whether the mean tissue residues in animals exposed to the test sediment are significantly greater than those in the control or reference sediments, or greater than a specified criterion value such as a Food and Drug Administration (FDA) action limit. Additional statistical tests comparing the means of other tissue residues (for example, control versus reference) or sediment characteristics will also be conducted, but the same principles and methods apply. A summary of the standard statistical tests and their interpretation are presented in Table 9 and Table 10.

15.2 *Requirements for Statistical Testing*—To perform statistical testing, replicate samples must have been taken to provide an estimate of variability. Non-replicated samples (that is, a concentration from a single composite sample) cannot be compared using these methods. The concentration of each chemical in a tissue or sediment sample is considered statistically independent in these tests and is compared separately. Comparisons of tissue residues of different chemicals within the same organisms require the use of "repeated measures."

15.2.1 The standard deviations (SDs) or standard errors (SEs) and number of replicates (*n*) should always be reported in addition to the mean values. When composited values are used, report the number of organisms per composite (if the composite comprises the experimental unit) or the number of experimental units per composite, as well as the number of replicate composites sampled.

15.2.1.1 It is necessary to decide whether the comparisons between means are to be multiple or pairwise before conducting any statistical analyses. Pairwise comparisons include comparisons of a test and control/reference mean for tissue concentrations, sediment characteristics, etc. Pairwise comparisons may also include comparison of the control with the reference mean and comparisons of a mean and a specified criterion value such as comparison of a test tissue residue with a constant such as an FDA action limit. Multiple comparisons involve comparisons of more than two means simultaneously. Multiple comparisons are used in cases such as determining whether three or more test tissue concentrations are equal or whether all of the TOC values for the sediments (test(s), control, and reference) are equal.

15.2.2 After the applicable comparisons are determined, the data need to be tested for normality to determine the whether parametric statistics are appropriate and the variances of the means to be compared are homogeneous. If normality and homogeneity of variances are established, *t*-tests can be performed in the case of pairwise comparisons or ANOVA in the case of multiple comparisons. Transformations of the data or nonparametric statistics may be used if normality or homogeneity of variance are not established.

15.3 *Tests for Normality and Homogeneity of Variances*—The data need to be checked for both normality and homogeneity of variances before conducting parametric statistics. The

TABLE 9 Summary of Statistical Analyses

Pairwise Comparisons Hypothesis	Test(s)[A]	Comments
Normality	Chi-square or Kolmogorov-Smirnov	try transformations if not normal
Equality of variances	*F*-test	try transformations if not equal
Equality of means	*t*-test	one-tailed with a priori knowledge, otherwise two-tailed
Equality of a means	modified *t*-test	if variances are not equal
Equality of a mean and a constant	*t*-test	one-tailed with a priori knowledge, otherwise two-tailed
Equality of means	nonparametric tests	if normality is not established
Multiple Comparisons		
Normality	Chi-square or Kolmogorov-Smirnov	try transformations if not normal
Equality of variances	Bartlett's test	try transformations if not equal
Equality of means	ANOVA	if normality is established
Equality of means	nonparametric tests	if normality is not established

[A] More than one test can often be used for the same hypothesis. Each test will have different assumptions. Choose the test with the assumptions most closely matching your specific conditions and requirements.

TABLE 10 Examples of Analyses and Interpretation of Results[A]

Hypothesis		Interpretation of Test Rejection of Null Hypothesis
	Physical Characteristics	
Ho: $TOC_c = TOC_i$	two-tailed *t*-test	TOC not equal between control and test sediment *i*
Ha: $TOC_c = \backslash TOC_i$		
Ho: $TOC_c = TOC_r$	two-tailed *t*-test	TOC not equal between control and reference
Ha: $TOC_c = \backslash TOC_r$		
Ho: $TOC_c = TOC_r = TOC_1 = ... = TOC_n$	ANOVA	TOC of one or more sediment differs
Ha: $TOC_c = \backslash TOC_r = \backslash TOC_1 = ... = \backslash TOC_n$		
	Adequacy of Control	
Ho: $Ct_c = Ct_u$	one-tailed *t*-test	exposure system contaminated
Ha: $Ct_c > Ct_u$		
	Treatment Differences	
Ho: $Ct_i = Ct_c$	one-tailed *t*-test	significant uptake from test sediment *i* above control
Ha: $Ct_i > Ct_c$		
Ho: $Ct_i = Ct_r$	one-tailed *t*-test	significant uptake from test sediment *i* above reference
Ha: $Ct_i > Ct_r$		
Ho: $Ct_r = Ct_c$	one-tailed *t*-test	significant uptake from reference sediment above control
Ha: $Ct_r > Ct_c$		
Ho: $Ct_c = Ct_r = Ct_1 = ... = Ct_n$	ANOVA	uptake from one or more sediment differs
Ha: $Ct_c = \backslash Ct_r = \backslash Ct_1 = ... = \backslash Ct_n$		
Ho: $Ct_1 = Ct_2 = ... = Ct_n$	ANOVA	uptake from one or more test sediment differs
Ha: $Ct_1 = \backslash Ct_2 = ... = \backslash Ct_n$		
	Long-Term Exposures	
Ho: $Ct(j)_i = Ct(j-1)_i = Ct(j-2)_i$	ANOVA	Ct_i has not reached steady state
Ha: $Ct(j)_i = \backslash Ct(j-1)_i = \backslash Ct(j-2)_i$		

[A] Ho = null hypothesis, Ha = alternative hypothesis, *Ct* = concentration of contaminant in tissue at Day 28. Subscripts: *c* = control organisms or sediment, i = 1, 2, ..., *n* test organisms or sediment, j = last sampling period, n = total number of test treatments, r = reference organisms or sediments, and u = unexposed organisms.

data for each chemical or sediment characteristic are tested separately. Tests used commonly for testing normality are the Kolmogorov-Smirnov one-sample test and the chi-square test **(137)**. However, these tests are not very powerful, especially if the sample sizes are small (such as eight replicates). More powerful, but less common, tests of normality such as Shapiro-Wilk and K^2 tests **(138)** can be used for small sample sizes.

15.3.1 If the data are not distributed normally, they can often be transformed to achieve normality. The logarithmic and arcsine are two commonly used transformations. It may be necessary to apply different transformations to different chemical or sediment characteristics. See Ref **(137)** for a more extensive discussion on transformations.

15.3.2 The variances of the samples to be compared should be tested for homogeneity. This is performed using an *F*-test when comparing two variances or Bartlett's test when comparing more than two variances. A *t*-test or ANOVA is appropriate if the variances are considered homogeneous. The data can be transformed in an attempt to achieve homogeneity if the variances are heterogeneous. A modified *t*-test for comparisons of two means or approximate tests for multiple comparisons can be performed under conditions of variance heterogeneity. See Ref **(137)** for a more extensive discussion on appropriate tests when different treatments have unequal variances.

15.3.3 An alternative approach is to use non-parametric tests which do not require that normality be assumed or that data be transformed. Non-parametric tests for comparisons of two means, such as the Mann-Whitney test and the Tukey's Quick test may be used. Another test for paired data, the Wilcoxon Rank Sum (also known as, Wilcoxon paired-sample test) procedure has been recommended **(139-141)**. Non-parametric tests are more robust than parametric tests, since they make no assumptions as to distribution. If the distribution is non-normal, these methods are preferred. If the assumptions concerning normality can be met, then parametric tests will be more powerful in determining significant differences and therefore should be used. See Refs **(139** and **142)** for discussion of non-parametric statistics.

15.4 *Pairwise Comparisons*—Pairwise comparisons are performed using Student's *t*-test, using a pooled variance estimate when variances are homogeneous. A modified *t*-test can be used under conditions of variance heterogeneity (see Ref **(113)**). Before analysis, it must be established whether the *t*-test performed will be a one-tailed or two-tailed test and whether the Type I error rate should be a comparison-wise or experiment-wise error rate.

15.4.1 *One-Tailed Versus Two-Tailed Tests*— In formulating a statistical hypothesis, the alternative hypothesis can be one-sided (one-tailed test) or two-sided (two-tailed test). The null hypothesis (Ho) is always whether two values are equal. A one-sided alternative hypothesis (Ha) is that there is a specified relationship between the two values (for example, one value is greater than the other) versus a two-sided alternative hypothesis (Ha), which is that the two values are different. 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 test tissue residue is greater than the control 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. An example of an alternative two-sided hypothesis is that the reference sediment TOC is different (greater or lesser) from the control sediment TOC.

15.4.1.1 Conducting one-tailed tests is recommended in most cases because control tissue residues and sediment contaminant concentrations are presumed lower than reference values, which are presumed lower than test values. For the same number of replicates, one-tailed tests are more likely to detect statistically significant differences between treatments (that is, they have a greater power). This is a critical consideration when dealing with a small number of replicates (such as eight per treatment). The other alternative to increasing statistical power is to increase the number of replicates, which increases the cost of the bioassay.

15.4.1.2 There are cases in which a one-tailed test is inappropriate. A two-tailed test should be used when no a priori assumption can be made concerning which treatment is higher than the other. For example, a two-tailed test should be used when comparing TOCs of the test and reference sediments. A two-tailed test should also be used when one regulatory action will be taken when the two treatments are equal and another when they are not equal, regardless of which one was larger or smaller. This would be unusual for tissue residues, but it would apply to other benthic characteristics. For example, a two-tailed test should be used when comparing the benthic biomass at a control and test site because both enhanced and reduced biomass are indicators of organic enrichment **(55)**. A two-tailed test should also be used when comparing tissue residues among different species exposed to the same sediment and when comparing BAFs or BSAFs (see Annex A1).

15.4.2 The non-parametric Wilcoxon Rank Sum procedure has been recommended for paired sample data **(139)**. Non-parametric tests are usually not as powerful as parametric tests. However, Lehman suggests that the power of the Wilcoxon procedure is only about 5 % less than the *t* test under normal distributions, and has equivalent power under other distributions **(139)**. Under skewed distributions, the power of the *t*-test declines while the power of the Wilcoxon Rank Sum remains constant **(143)**. The Wilcoxon test has also been recommended where there are unequal numbers of observations between experimental treatments **(140, 141)**.

15.5 *Comparison-Wise Versus Experiment-Wise Error Rates*—The Type I error rate used in the tests will be chosen either as a comparison-wise or experiment-wise error rate, depending on whether one decision is made for each pairwise comparison or from a set of pairwise comparisons. A comparison-wise Type I error rate of 0.05 should be used for each comparison for cases in which test sediments are chosen in a stratified manner or along a gradient (see examples in Fig. 1(a) and 1(b)) and any decisions will be made on a case by case basis. For example, a comparison-wise error would be used for deciding which specific stations along a gradient were acceptable or not acceptable.

15.5.1 If the test sediments are selected from a supposedly homogeneous source (for example, multiple sediment samples from a dredge barge; see example in Fig. 1(c)), and the decision to accept or reject the sediment will be made from the

results of several pairwise comparisons, an experiment-wise error rate of 0.05 should be used. Each individual comparison is performed at a lower error rate such that the probability of making a Type I error in the entire series of comparisons is not greater than 0.05. This results in a more conservative test when comparing any particular sample to the control or reference. A single sediment sample from the barge that would have been rejected at the 0.05 level may thus not be rejected at the lower experiment-wise error rate, although the probability of rejecting Ho for the entire set of samples is still 0.05. The use of experiment-wise error rates adjusts for the possibility of random differences when multiple samples are taken from a homogeneous source. (For example, if 100 samples were taken, a certain percentage would be greater than the control/reference because of random variation.) The error rate used in each comparison is a function of the number of comparisons to be used in the decision "experiment" and can be computed using the method of Dunn-Sidak **(137)** as follows:

$$\text{alpha} = 1 - (1 - \text{alpha})^{1/k} \quad (4)$$

where:

alpha = Type I error rate used for each pairwise comparison,
alpha = experiment-wise Type I error rate (0.05), and
k = number of comparisons.

When an experiment-wise error is used, the power to detect real differences between any two means decreases as a function of k, the number of comparisons.

15.6 *Multiple Comparisons*—For comparisons involving several means, as in the case of comparing TOC values among all sediment types, an ANOVA is first performed to establish whether any of the means are different. The ANOVA also provides a "best" estimate of the variance (within-treatment error). If there are significant differences, a series of *t*-tests can be performed for any planned (a priori) comparisons (such as between test and control) to distinguish which means are different. Tests such as the T-Method or Tukey Kramer procedure (Dunnett's test) are more appropriate for unplanned (a posteriori) comparisons, such as between two reference tissue residues. See Ref **(137)** for unplanned multiple comparison tests to determine which is most suited for each case.

15.6.1 It is important to note that an ANOVA is inherently for two-tailed comparisons. If the comparisons can be broken down into a series of one-tailed pairwise comparisons, it is therefore preferable to perform the analysis in this manner because of the increase in power. However, if the series of comparisons are two-tailed, an ANOVA can be performed first to determine whether any additional comparisons should be made.

15.7 *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 control sediment. If there is significant uptake, the exposure system or control sediment, or both, should be reevaluated for 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.

15.7.1 Comparisons between the 28-day control or reference, or both, tissue residues and 28-day test tissue residues determines whether statistically significant bioaccumulation exists due to exposure to test sediment. Comparisons between control and reference tissue residues at Day 28 determine whether statistically significant bioaccumulation exists due to exposure to the reference sediment. If no significant difference is detected when test tissue residues are compared with a one-tailed test using a set criterion value (for example, FDA action limit), the residues must be considered equivalent to the value even though the mean tissue residue may be numerically lower.

15.8 *Additional Analyses*:

15.8.1 *Testing BAFs and BSAFs*—Statistical comparisons between ratios such as BAFs or BSAFs are difficult due to the 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 **(144)** or a Taylor series expansion method **(145)**. BAFs and BSAFs can then be compared using these estimates for the variance. See Ref **(12)** for an example of this approach.

15.8.2 *Comparing Tissue Residues of Different Compounds*—It is of interest to compare the tissue residues of different compounds in some cases. For example, Rubinstein, et al **(15)** 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. Rather, a repeated measures technique (repeated testing of the same individual) should be used where the individual (experimental unit) is considered as a random factor and the different compounds are considered as a second factor. See Refs **(15, 43)** for an example of the application of repeated measures to bioaccumulation data.

15.8.3 *Analyses for Alternative Test Designs*—Long-term exposures require a test to show that steady state has been reached. An ANOVA should be performed on the last three sample sets. Practice E 1022 requires that there be no significant difference ($p > 0.05$) between the means of these sample sets. If apparent steady state is reached, the mean of the samples taken during apparent steady state should be used for the steady-state concentration value. For steady state estimates based on uptake and depuration tests, see Refs **(114, 115)** for details on the nonlinear parameter estimation methods required to estimate these rate constants and steady-state concentrations.

16. Keywords

16.1 bioavailability; freshwater invertebrates; marine invertebrates; sediment-associated contaminants; sediment bioaccumulation

ANNEXES

(Mandatory Information)

A1. ADDITIONAL METHODS FOR PREDICTING BIOACCUMULATION

A1.1 *Field Collection of Organisms*—The most direct method of assessing the tissue residues in existing sediments is by measuring the tissue residues in organisms from a potentially contaminated site and comparing these values to the tissue levels for control and reference sites. The field approach is appealing because it avoids laboratory artifacts, as well as additional time, expense, and facilities required for laboratory tests. However, the routine use of field-collected organisms has several limitations.

A1.1.1 The greatest problem is collecting sufficient tissue biomass from selected species for chemical analysis. This problem exists at the most contaminated sites because smaller species dominate both stressed communities and the early stages of recolonization **(55, 56)**. In addition, benthic densities are reduced under severe stress **(55)**. Even when sufficient biomass of a particular species can be collected at a given station, it will often be impossible to collect the same species from either other stations located along a pollution gradient, seasonally within a single station, or at an estuarine dredge site and an open ocean disposal site.

A1.1.1.1 An approach to collecting sufficient biomass is to composite individuals from each species at each site. Although this will increase biomass, the species composited at one station may not be present at another station. Additionally, the presence of a given species at a station may vary seasonally. These compounding factors will make it unclear whether the patterns in tissue residues are due to site differences (for example, physicochemical differences and bioavailability) or to interspecific differences in bioaccumulation (for example, population differences and metabolism). For example, if the PAH tissue residues in a bivalve composite are compared to an amphipod composite from a different site, the difference in PAH tissue residues between sites could reflect the greater ability of amphipods to metabolize PAHs **(71)** as opposed to a real difference in sediment bioavailability.

A1.1.2 Unknown exposure histories of field-collected specimens is an additional problem. For example, many benthic species, especially amphipods and some polychaetes, are mobile during some stages of development (for example, Refs **(146-148)**) and may migrate to new sites. Another source of unknown exposure is resuspension. Although contaminant concentrations in sediments are relatively constant, resuspension events can obscure recent or historical sediment-bioaccumulation relationships. In this example, the deposition of resuspended contaminated sediment in an uncontaminated site may form a surface veneer available to surface-deposit-feeders or filter-feeders. A bulk sediment analysis may underestimate the actual exposure if this were the case. Also, field organisms may be exposed to contaminated phytoplankton and contaminants dissolved in the overlying water. If the water column contaminant concentration is important to uptake, relating tissue residues to the field sediment will generate incorrect conclusions regarding sediment bioavailability.

A1.1.3 *Sediment Parameters*—In addition to the organisms, sediment must be collected from the same site and characterized. This characterization needs to be just as extensive as that previously described for sediments used in laboratory experiments (see 10.2).

A1.1.4 With these limitations, field collections are not as well suited as laboratory experiments for routine predictions of the tissue residues resulting from sediments and contaminant discharges or for between-site comparisons. However, field collections are a powerful regulatory tool if used in the context of periodic monitoring of existing sites. When comparing changes at the same stations over time, problems with the comparison of different species are reduced, although there may still be problems with collecting sufficient biomass. Field collections also complement the laboratory studies as a QA check and by providing data on commercially important species difficult to maintain in the laboratory. In some cases, both laboratory and field assessments of tissue residues are justified by the size of a discharge or dredging operation or by a high contaminant concentration. Guidelines on sampling designs for field surveys can be found in Refs **(34, 51, 52)**, while Ref **(48)** contains information on the sampling techniques.

A1.2 *Bioaccumulation Factors (BAFs)*—Several approaches have been developed for predicting benthic tissue residues directly from sediment concentrations, thereby obviating the need for field collections or bioassays. The simplest of these approaches is the BAF, which is

$$\mathrm{BAF} = C_t/C_s \tag{A1.1}$$

where:
C_t = tissue concentration, µg/g, and
C_s = sediment concentration, µg/g.

BAFs are derived empirically from either laboratory bioassays or field-collected organisms. Both tissue and sediment concentrations are preferably given in dry weight units, but the units must be reported in any case. Assuming that the BAFs were constant among species and sediments, multiplying the BAF of a compound times the sediment concentration would predict the steady-state tissue residue. BAFs are analogous to the BCFs, which are used to predict tissue residues from water concentrations:

$$\mathrm{BCF} = C_t/C_w \tag{A1.2}$$

where:
C_w = concentration in water, µg/g.

Although the formulas are analogous, the terms are not interchangeable, and BCFs should be limited to uptake from water.

A1.3 *Biota-Sediment Accumulation Factors (BSAFs)*—Sediment characteristics, such as TOC, have a major influence on the bioavailability of nonpolar contaminants and increase the among-site variation in BAFs. The BAF variability is reduced by normalizing the sediment concentrations to the TOC content **(149)**. Normalizing tissue residues to tissue lipid concentrations reduces the variability in contaminant concentrations among individuals of the same species and between species (for example, Refs **(150, 151)**). These normalizations are combined in a simple thermodynamic-based bioaccumulation model for contaminant uptake from sediment **(15, 152)**. The fundamental assumptions of this thermodynamic model are that the tissue concentration is controlled by the contaminant's physical partitioning between sediment carbon and tissue lipids and that the organism and the environment may approach thermodynamic equilibrium. The method assumes that lipids in different organisms and TOC in different sediments partition contaminants in similar manners. The key value in the model is the BSAF, which predicts the lipid-normalized tissue residue when multiplied by the TOC-normalized sediment contaminant concentration.

NOTE A1.1—Some previous studies such as Refs **(42, 152)** reported Preference Factors, which is the inverse of the BSAF.

A1.3.1 In its simplest form, the model is as follows:

$$C_t/L = \text{BSAF} \times (C_s/\text{TOC}) \quad \text{(A1.3)}$$

or

$$\text{BSAF} = (C_t/L)/(C_s/\text{TOC}) \quad \text{(A1.4)}$$

where:

L = concentration of lipid in organism, g/g dry weight,
TOC = total organic carbon in sediment, g/g dry weight, and
BSAF = biota-sediment accumulation factor, g carbon/g lipid.

A1.3.2 The BSAFs should not vary with sediment type or among species in theory. Based on the relationship between K_{oc} and lipid-normalized BCFs, the maximum BSAF for neutral organic compounds has been calculated at approximately 1.7 **(153)**. Measured BSAFs would be lower than this maximum if metabolism of the compound by the organism is rapid or the organism fails to reach steady-state body burdens due to limited exposure durations or kinetic limitations to accumulation (for example, steric hindrances to uptake and slow desorption from sediment particulates to interstitial water). Measured BSAFs could exceed the calculated thermodynamic maximum if there is active uptake of the contaminant in the gut or if there is an increase in the contaminant's gut fugacity, driving the contaminant from the gut into the body. The contaminant fugacity in the gut could increase as the volume of food decreases during digestion or as a result of a reduction in lipids **(154)**.

A1.3.3 Laboratory and field validation of the thermodynamic partitioning model suggests that BSAF values do not exceed the maximum value for a large number of organic contaminants **(12)**. However, BSAFs for some highly lipophilic PCB congeners can exceed the theoretical maximum of 1.7 by as much as an order-of-magnitude **(15)**. Sediments with the lowest TOCs tend to have the highest BSAF values **(12, 15, 42, 43)**, which is not explained by the present model.

A1.3.4 The BSAFs are also dependent on the accuracy of the lipid measurement. A standard lipid extraction method is needed since total lipids can vary several fold based on the extraction technique used. As discussed in 15.2.1, using the Bligh-Dyer lipid method is recommended as an interim standard method for BSAF determinations. If another lipid extraction technique is used, a conversion factor should be provided to allow the conversion of the lipid values to chloroform-methanol extraction values.

A1.3.5 Although laboratory and field evaluations of the BSAFs have shown that they are not statistically constant in all cases, BSAFs are less variable for predicting sediment uptake than BAFs **(12, 15, 43)**. The BSAFs have great potential as a cost-effective, first-order estimate of tissue residues because of their minimal data requirements. The predicted tissue residues can then be used for determining whether bioaccumulation tests or field surveys are needed.

A1.3.5.1 For these reasons, the data required to calculate BSAFs should be collected and reported in all laboratory tests and field collections. The development of a BSAF database would be extremely useful for determining the limits of applicability of this approach, as well as generating values for specific chemicals.

A1.4 *Toxicokinetic Bioaccumulation Models*—Toxicokinetic bioaccumulation models are an alternative to thermodynamic-based partitioning approaches. Toxicokinetic models assume that contaminant uptake is a function of the feeding behaviors and physiological characteristics of the organism as well as the physicochemical characteristics of the contaminant and sediment. Most of these toxicokinetic models (for example, Ref **(155)**) assume that the tissue residue can be predicted as the sum of the uptake from each individual phase (for example, interstitial water and ingested sediment) minus any loss due to depuration or metabolism.

A1.4.1 In its simplest form, uptake from all phases may be expressed as follows:

$$dC_t/dt = \Sigma\,(F_x \times CP_x \times EP_x) - L \quad \text{(A1.5)}$$

where:

dC_t/dt = change in tissue residue with time,
F_x = flux of Phase x through organism,
CP_x = concentration of contaminant in Phase x,
EP_x = fraction of contaminant extracted from Phase x by the organism,
L = summation of loss of contaminant through metabolism and depuration, and
x = phase (W = water, F = food, and S = sediment).

A1.4.1.1 For example, the uptake from water would be the product of the amount of water ventilated across the gills (*FW*), the contaminant concentration in the water (*CPW*), and the efficiency with which the contaminant is extracted from the water (*EPW*). These kinds of models usually assume that uptake efficiency values do not change as body burdens approach steady-state and that loss (L) can be modeled as a first-order process. Opposed to the thermodynamic model, the

toxicokinetic model assumes the uptake from each route is independent and additive, so that an organism exposed to two uptake phases (for example, interstitial water and sediment) would have a higher steady-state tissue residue than an organism exposed to one phase.

A1.4.2 These models have been used successfully to predict the PCB, methylmercury, and kepone levels in marine and freshwater fish **(155-157)**. This approach has been applied to benthic species only recently and has been used to model the uptake of hexachlorobenzene by a marine clam **(58, 59, 158-160)**. A slightly different toxicokinetic model has been used to predict the uptake of various PAHs by freshwater amphipods **(44, 73, 103)**. Landrum used this model to determine the relative importance of interstitial water versus ingested particulates as an uptake route for these PAHs.

A1.4.3 In contrast to thermodynamic approaches, toxicokinetic models can predict tissue residues under non-steady-state conditions and can account for differences in organism feeding or ventilatory behaviors due to toxic or natural effects (for example, growth-related changes). The models can also predict the time course of uptake and depuration. However, the approach requires relatively sophisticated laboratory experiments to measure the input parameters. This approach is not presently suited for the routine prediction of bioaccumulation because of the extensive data needs and the ongoing process of developing the laboratory methods. The toxicokinetic models are appropriate when detailed analyses of sediment or biological effects on bioaccumulation are required and as a method to test the assumptions of various sediment assessment approaches.

A2. DETERMINING THE NUMBER OF REPLICATES

A2.1 *Number of Replicates*—Adequate replication is essential for determining statistically significant differences between treatments with sufficient power. If there is a question that the eight replicates recommended (see 12.3) will not provide sufficient statistical power, the techniques in this annex can be used to determine the appropriate number. Determining the appropriate number of replicates requires estimates of the variability of each treatment and the minimum detectable difference. The minimum detectable difference is the smallest difference between two means, or between a mean and a constant value, that needs to be distinguishable statistically. The variability is a measure of the within-treatment variation and is expressed as a standard deviation (SD) or coefficient of variation (CV) and can be obtained from previous experiments or the literature. This information is needed because treatments with high variation will require more replication to distinguish differences between treatments than less variable ones. See Table A2.1 for a listing of CVs for tissue residues reported for a variety of contaminants.

TABLE A2.1 Ranges of CVs for Tissue Residues Reported for Benthic Organisms

Contaminant	Organism	CV,%	Reference
Cadmium	*Modiolus demissus*	4–54[A]	**(161)**
	Mytilus edulis	4–61[A]	**(161)**
	Mya arenaria	18–22	**(162)**
	Mulinia lateralis	35–49	**(162)**
	Callianassa australiensis	5–67	**(46)**
Mercury	*Modiolus demissus*	5–34[A]	**(161**
	Mytilus edulis	5–53[A]	**(161)**
Copper	*Neanthes arenaceodentata*	8–60	**(163)**
Zinc	*Nereis diversicolor*	42	**(164)**
	Octolasion tyrtaeum	12–30[A]	**(165)**
	Corbicula fluminea	7–8[A]	**(165)**
Kepone	*Crassostrea virginica*	8–80	**(166)**
PCB	*Octolasion tyrtaeum*	2–23[A]	**(165)**
	Corbicula fluminea	10–74[A]	**(165, 167)**
	Nereis virens	5–40	**(168)**
	Uca spp.	31–75	**(169)**
HCB	*Macoma nasuta*	23–33	**(170)**
BaP	Amphipods	4–22	**(170)**
	Macoma inquinata	4–36	**(107)**
	Abarenicola pacifica	9–24[A]	**(107)**
Napthalene	*Macoma inquinata*	50–100[A]	**(171)**
Phenanthrene	*Macoma inquinata*	17–56	**(107)**
	Abarenicola pacifica	10–31[A]	**(107)**
Chrysene	*Macoma inquinata*	11–46	**(107)**
	Abarenicola pacifica	2–46[A]	**(107)**

[A] Samples were composited resulting in (usually) lower CVs.

A2.2 *Minimum Detectable Difference*—The number of replicates required is related to the minimum detectable difference, and detecting a two-fold increase in tissue concentrations requires many more replicates than detecting a 100-fold increase. No standards exist for an acceptable minimum detectable difference; however, it is recommended that there be sufficient replication to detect, at a minimum, two-fold differences in tissue concentrations between two treatments (12.3.1).

A2.3 Error rates for Type I and Type II errors must additionally be chosen. A Type I error (alpha) is the probability of rejecting the null hypothesis when no true difference exists between treatment means and is usually given a value of 0.05. A Type II error (beta) is the probability of accepting the null hypothesis when a true difference exists between treatment means. As discussed in 12.1.2.2, a beta of 0.05 is recommended. This is equivalent to a power of 0.95, where the power of a test is the probability of rejecting the null hypothesis correctly.

A2.4 One equation that can be used to estimate the number of replicates (n) required to detect a minimum detectable difference between two means (adapted from Ref **(137)**) is as follows:

$$n > 2 \times (s/d)^2 \times (t_{\text{alpha},v} + t_{2\text{beta},v})^2 \qquad \text{(A2.1)}$$

where:

n = sample size for each treatment,
s = standard deviation (often a pooled value of the two sample variances),
d = minimum detectable difference,
v = number of degrees of freedom ($v = 2 \times (n - 1)$ for the comparison of two means; $v = (n - 1)$ for the comparison of a mean and a constant),

alpha = experiment-wise or comparison-wise Type I error (see Section 15). If a two-tailed test is performed, each tail will consist of alpha/2. If a one-tailed test is performed, the single tail is alpha,

beta = Type II error (or 1 − power of test),

$t_{alpha,v}$ = critical value for alpha of Student's *t*-distribution with *v* degrees of freedom. (Use a two-tailed *t*-table for a two-tailed test and a one-tailed table for a one-tailed test),

$t_2$beta,*v* = critical value for 2 × beta of Student's *t*-distribution with *v* degrees of freedom. (Use a two-tailed table. The critical value is beta if a one-tailed table is used. The critical value is the same whether the test is one- or two-tailed.)

For the comparison of one mean and a constant (for example, FDA Action Limit), the formula becomes

$$n > (s/d)^2 \times (t_{alpha,v} + t_2\text{beta},v)^2 \quad \text{(A2.2)}$$

A2.5 An iterative approach is used to calculate *n* since $t_{alpha,}v$ and t_{2beta},v are dependent on *n* through *v*. The values for $t_{alpha,}v$, $t_{2beta,}v$, alpha, beta, and *v* are either set by the investigator or found in tables. Therefore, only the SD and the minimum detectable difference must be estimated. Although a minimum detectable difference (*d*) of 2 is recommended (see 12.3.1), an estimate of the SD will not be available in many cases. However, the ratio of the two (*s/d*) can be described in several ways, providing different approaches to estimating these parameters. Three methods of estimating *s/d* and their advantages and disadvantages are as follows:

A2.5.1 *Method No. 1*:

$$(s/d) = [s/(u_1 - u_2)] \quad \text{(A2.3)}$$

where:

$u_1 - u_2$ = difference between mean u_1 and mean u_2, or mean u_1 and a constant.

A2.5.1.1 *Advantages*— There may be cases in which an absolute difference between two numbers is of interest, as in a comparison of a measured tissue residue and a regulatory action limit.

A2.5.1.2 *Disadvantages*— It requires an estimate of the SD of the sample, a value often difficult to obtain.

A2.5.2 *Method No. 2*:

$$(s/d) = [(CV/100)/m_1] \quad \text{(A2.4)}$$

where:

CV = coefficient of variation, %, and

m_1 = a multiplicative factor of u_1 that is the minimum detectable difference between mean u_1 and mean u_2(or criterion value) (for example, if $m_1 = 5$, the minimum detectable difference between u_1 and u_2 will be five times the value of u_1).

A2.5.2.1 *Advantages*— The CV is often easier to estimate than the SD. The CVs in Table A2.1 can be used as estimates if no other information is available, although it would be prudent to consider these values as the minimum estimates of variation.

A2.5.2.2 *Disadvantages*— The value for m_1 will change whether the comparisons are between control and test values or a test and a criterion value. Control values (tissue residues) will tend to be low compared to test values (tissue residues), while test values may be large and close to a criterion value (for example, FDA action limits).

A2.5.3 *Method No. 3*:

$$(s/d) = [s/(m_2 \times s)] = [1/m_2] \quad \text{(A2.5)}$$

where:

m_2 = multiplicative factor of *s*.

A2.5.3.1 For example, if $m_2 = 2$, the minimum detectable difference is 2 SDs (that is, u_2 will have to be 2 SDs from u_1 to be able to detect a difference).

A2.5.3.2 *Advantages*— No estimates are required of the SD or CV.

A2.5.3.3 *Disadvantages*— The value of m_2 may have to vary whether the comparisons are between control and test values or test and action limits.

A2.5.4 If a comparison between more than two means is anticipated (as in the determination of steady-state conditions), see Ref **(137)** for a modification of this approach or Ref **(98)** for tables of estimates.

A3. ADEQUACY OF 10-DAY AND 28-DAY EXPOSURES

A3.1 Organisms should ideally be exposed to test sediments for a period sufficient to attain steady-state tissue residues. However, cost considerations often prove prohibitive to conducting tests long enough to document that steady-state has been attained. Bioaccumulation tests have historically been conducted for a preset duration as a result. Choosing a single time period is complicated by the multitude of organic contaminants and metals found in most field sediments or dredge materials, with each having differing uptake and elimination kinetics. To date, a ten-day exposure to assess "bioaccumulation potential" has been the most commonly used time period for the testing of marine sediments (primarily dredge materials) **(100)**. Bioaccumulation potential is the potential for any uptake of a contaminant by organisms exposed to a sediment, and the basic premise was that if there was going to be bioaccumulation, it should be possible to detect it within ten days. The original intent of the ten-day test was thus as a qualitative rather than quantitative measure. Since 1977, however, data from ten-day tests have frequently been extended beyond their original intent and used as a quantitative result.

A3.2 Because of the widespread use of the ten-day exposures, it is worth assessing their utility as both a qualitative measure of bioaccumulation potential and a quantitative

method of generating data for ecological and human health risk assessments. The percent of steady-state tissue residue obtained after ten days for several organic contaminants was used as a simple measure of accuracy (Table 4). To assess bioaccumulation potential adequately, the exposure should result in a sufficient percentage of the steady-state tissue residues to identify which sediments could be an environmental problem. Also, the percentage of the steady-state tissue residue obtained should be relatively consistent for the same contaminant in different species. That is, the exposure should yield a strong and consistent signal. Benthic tissue residues will be used in the quantitative risk assessments to predict the amount of contaminants transported from the sediment to higher trophic levels, including man. A large error at the base of the food-web will result in errors throughout the analysis, especially as some of the errors may be multiplicative. As a preliminary measure, for data to be acceptable for quantitative risk assessment, the resulting tissue residues should be within 80 % of the steady-state tissue concentrations. An accuracy of 80 % for each trophic step results in the prediction of tissue residues being within two-fold of the actual residues for a three-step chain (that is, sediment to benthos to demersal predator to higher predator or man; or $0.8 \times 0.8 \times 0.8 = 0.51$).

A3.2.1 For organic contaminants in these studies, only 33 % of the organisms approached within 80 % of the steady-state level in ten days (Table 4). Ten-day tissue residues averaged 56 % of the estimated steady-state value, and this average included some rapidly accumulated PAHs. Tissue residues of PCBs achieved after ten days averaged only approximately 34 % of the steady-state values and ranged from 100 % to a low of 12 %. In contrast, 28-day tissue residues averaged 82 % of the estimated steady-state value, with 67 % of the tests within 80 % of the steady-state level (Table 4).

A3.3 *Metals*—Ten days is also likely to generate a relatively low percentage of the steady-state tissue residues for metals. For example, mercury levels in fish may not attain steady-state during the lifetime of the organism **(172, 173)**, and the minimum time for lead to attain steady-state in *Mytilus edulis* was greater than 230 days **(174)**. In the few studies in which 10 and 28-day values could be compared (Table 4), only 13 % attained 80 % of the steady-state value in 10-day tests, while 50 % of organisms exposed for 28 days attained this value.

A3.4 Several conclusions are apparent based on this preliminary review. First, a ten-day exposure generates a low percentage of the steady-state tissue residues for PCBs and presumably other high K_{ow} organics and some heavy metals. These compounds are the most likely to represent an ecological and human health risk through bioaccumulation and biomagnification. Second, the percentage of the steady-state tissue residue obtained varies several-fold even within a single compound. Third, the amount accumulated within ten days is such a small percentage of the steady-state concentration that it may be below the detection limits of standard analytical methods or may not be significantly different from the control values. The ten-day exposure can thus result in false negatives concerning the bioaccumulation potential of a sediment. Fourth, the percentage of the steady-state tissue residues accumulated over ten days is inadequate for a quantitative risk assessment. Finally, the ten-day exposure does not generate any additional insights into the bioaccumulation potential of neutral organics that are not generated by use of the BSAFs (see summary in Table A3.1).

A3.5 A 28-day exposure is a practical compromise between cost, data accuracy, and data utility. When 28-day organic and metal contaminant levels were compared to observed or estimated steady-state levels (Table 4), steady-state tissue residues were approached (that is, ≥80 % of steady-state) in 69 % of the tests, and the mean steady-state contaminant tissue level increased to 84 % of the steady-state maximum. An average of 83 % of the PCB steady-state tissue residues was obtained after 28 days. This level of accuracy should be sufficient in nearly all cases to test for bioaccumulation potential with a reasonable level of statistical certainty. The data should be sufficiently accurate for quantitative risk analysis in most cases. In cases in which more accurate estimates are required, either a long-term exposure (12.5) or an alternative approach (Annex A1) can be used.

A3.6 In addition to underestimating tissue residues because of insufficient duration, single-point tests can underestimate maximum tissue residues when a compound reaches a maximum value before the sampling period and then declines. For example, phenanthrene approaches its maximum tissue residue in freshwater amphipods after approximately ten days and then declines **(44)**. A 28-day test would generate a lower value than a 10-day test in this case. The decline is presumably the result of an increase in the metabolic degradation rate of the contaminant and should be most common with the lower

TABLE A3.1 Information Gained and Requirements of Different Approaches to Estimating Benthic Tissue Residues

Method	Bioaccumulation Potential	False Negative Bioaccumulation Potential	Estimates Equilibrium Residue	Additional Requirements
Accumulation factors	yes	no	yes?	sediment concentration, TOC, lipids
10-Day test	yes	yes	no	10 days laboratory time, tissue concentration
28-Day test	yes	no	approximate to yes	18 days additional laboratory time
Kinetic models	yes	no	yes	additional tissue concentration, additional laboratory time? development of techniques
Long-term exposures	yes	no	yes	28 to 70 days additional laboratory time, additional tissue concentration[A]

[A] Bioaccumulation potential = qualitative ability to detect uptake. False negative bioaccumulation potential = amount accumulated is so low that it is concluded incorrectly that no uptake will occur. Estimates equilibrium residue = tissue residue data sufficiently accurate for use in quantitative risk assessments. Experiment techniques = resources devoted to determining the correct uptake and depuration periods for specific compounds and organisms. Laboratory time = laboratory time required for biological exposure. Lipids = tissue samples analyzed for lipid content. Sediment concentration = sediment samples analyzed for contaminants. Tissue concentration = tissue samples analyzed for contaminants. TOC = sediment samples analyzed for TOC.

molecular weight PAHs and other rapidly degraded contaminants. The ability to degrade PAHs varies among taxa **(71)** so different taxa may not show the same pattern. Time series samples should be taken before Day 28 if low-molecular-weight PAHs or other rapidly metabolized compounds are of interest (see 12.3.1).

A3.7 *When Steady-State Is Not Achieved*—If steady-state cannot be documented from the experimental results, the tissue residue is only an estimate of steady-state and can be a substantial underestimate of the true value for some compounds.

A4. ALTERNATIVE TEST DESIGNS

A4.1 *Short-Term Test*—Some compounds (for example, volatiles) may attain steady-state in less than 28 days (see Table 4), so that a 28-day exposure may not be necessary. Generally, 10-day tests should be acceptable with organic compounds that have log K_{ow} s <3 that have been dosed into sediments. Even with these compounds, a 10-day test should be used only after it has been documented to approach steady-state in phylogenetically similar species in less than ten days or documented that the depuration rate (k_2) in phylogenetically similar species is >0.5/day. However, when determining the bioaccumulation of contaminants from field sediments, a 28-day test should be used because nearly all field sediments contain some contaminants with slow uptake kinetics. 10-day test may also be appropriate when the goal of the study is to estimate tissue residues in insect larvae that have larval stages shorter than 28 days (for example, *Chironomus*). Biotic and abiotic samples should be taken at Day 0 and Day 10 following the same protocol as that used for the 28-day tests. Sample on Days 0, 1, 3, 5, 7, and 10 if time-series biotic samples are desired.

A4.2 *Estimating Steady-State from Uptake Rates*—In theory, it is possible to estimate both the uptake clearance, k_s and elimination rate constant, k_2, from the uptake phase alone if the experiment continues past the point at which the tissue residues begin to "bend over," indicating that the elimination is sufficient to slow the net uptake. This approach obviates the need to run a separate elimination experiment, as is required in the kinetic approach (see 12.6). However, since both k_s and k_2 are estimated from the fitting of nonlinear mathematical models, this method can have more variance in parameter estimates than the kinetic approach, that uses independent measures of k_s and k_2. This approach nonetheless has utility when time or analytical support is limited, or if a long-term, time-series uptake test is terminated before steady-state is attained. In this design, the sampling schedule should follow closely that of the uptake phase of the kinetic approach using both uptake and depuration rates. Refer to Refs **(175, 176)** for the specifics of estimating k_s(or k_1) and k_2. If a mathematical model is used for estimating k_s and k_2 simultaneously, caution should be used to ensure that the model will account for complexities that occur with sediment exposures such as changes in the bioavailability of sediment compartments with time **(44)**.

A4.3 *Growth Dilution*—Growth dilution, the dilution of contaminant concentrations in the tissues by the increase in tissue mass, will occur if the test organisms grow during an experiment. Taking an extreme example, if an organism doubled its weight during a depuration study, it would appear that half of the contaminants had been depurated, even if none of the contaminants were excreted from the organism. Without correction for growth, the depuration rate (k_2) calculated from this experiment would be incorrect for an organism growing at a different rate. Many experiments have not taken growth dilution into account, which may contribute to the variation among measured depuration rates (see Ref **(177)**).

A4.3.1 For the larger benthic test species (for example, *Macoma*), growth dilution is usually not a problem in 28-day tests since the growth is relatively slow. However, growth dilution can cause errors in estimating uptake and depuration parameters for the kinetic approach, resulting in errors in predicting steady-state concentrations and time to steady-state.

A4.3.2 If substantial growth occurs during experiments to determine the rate constants, the uptake rate constants will be underestimated and the depuration rate constants will be overestimated. If these erroneous constants are used in the kinetic model ((Eq 1 and 2) of 12.6) under no growth conditions, both steady-state tissue concentrations and time to steady-state will be underestimated. Conversely, an error occurs when correct (that is, derived under no growth) uptake and depuration rate constants are used in this kinetic model when the organisms are growing. Both the steady-state concentrations and time to steady-state will be overestimated in this case because the model does not compensate for growth dilution.

A4.3.3 If possible, experiments should be conducted with organisms that grow very slowly or under environmental conditions that keep growth at a minimum (such as low temperatures). If growth cannot be prevented, growth dilution must be taken into consideration when a kinetic approach is used.

A4.3.4 Assuming that growth dilution is a first-order process and that growth occurs at a constant rate, the kinetic model (12.6) becomes the following:

$$C(t) = k_s \times C_s/(k_2 + k_3) \times [1 - e^{-(k_2 + k_3)\times t}] \qquad \text{(A4.1)}$$

where:

C_t = concentration in the organism at time t,
C_s = concentration in the sediment,
k_s = sediment uptake rate coefficient, g sediment/g tissue × days,
k_2 = depuration rate constant, days^{-1},
k_3 = growth rate constant, days^{-1}, and
t = time, days.

A4.3.4.1 The growth rate constant (k_3) can be measured from the weight change during the exposure experiment or

during a separate growth experiment under similar environmental conditions. (Eq A4.1) assumes that the k_s and k_2 values are constants and were measured under no growth conditions or, if growth occurred, that growth dilution was taken into account. If the depuration rate is measured while the organisms are growing, the rate measured will actually be a function of growth and depuration and can be modeled as $k_2 + k_3$.

A4.3.4.2 Under growth conditions, and using an estimated growth constant (k_3), the maximum tissue residue becomes the following:

$$C_{t_{max}} = k_s \times C_s/(k_2 + k_3) \quad \text{(A4.2)}$$

A4.4 Kinetic coefficients determined for specific experimental designs are conditional on both the environmental and physiological conditions of the test. The coefficients will be altered if the temperature is raised or lowered. Similarly, the coefficients will be altered by changes in the organism's physiology such as changes in reproductive status or lipid content. Generalizing results to conditions different from the test must therefore be made with caution.

A5. CALCULATION OF TIME TO STEADY-STATE

A5.1 Having an estimate of the time to reach steady-state tissue residues is helpful when designing long-term studies and assessing the adequacy of a 28-day test. If no estimate for a contaminant in phylogenetically similar organisms is available, the time required to approach steady-state can be estimated from a linear uptake, first-order depuration model (see 12.6). This model is an approximation for benthic invertebrates since it was developed for fish exposed to dissolved organic contaminants **(114)**. The uptake of organic contaminants from the dissolved phase is modeled as follows:

$$C_t(t) = k_s \times C_w/k_2 \times (1 - e^{-k_2 \times t}) \quad \text{(A5.1)}$$

where:

C_t = contaminant concentration in tissue at time t,
C_w = dissolved contaminant concentration in water,
k_s = sediment uptake rate coefficients, g sediment/g tissue × days,
k_2 = depuration rate constant, days^{-1}, and
t = time, days.

A5.1.1 This model predicts that equilibrium would be reached only as time becomes infinite. For practical reasons, apparent steady-state is therefore defined here as 95 % of the equilibrium tissue residue. The time to reach steady-state can be estimated by the following:

$$S = \ln[1/(1.00 - 0.95)]/k_2 = 3.0/k_2 \quad \text{(A5.2)}$$

where:

S = time to apparent steady-state, days.

A5.1.2 The key information is thus 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 K_{ow} and k_2 for fish species **(114)**:

$$k_2 = \text{antilog}\,[1.47 - 0.414 \times \log(K_{ow})] \quad \text{(A5.3)}$$

A5.1.3 The relationship between S and k_2 (using (Eq A5.2)) and between k_2 and K_{ow} (using (Eq A5.3)) is summarized in Table A5.1. The estimated time (days) to reach 95 % of contaminant steady-state tissue residue (S) and depuration rate constants (k_2) is calculated from octanol-water partition coefficients using a linear uptake, first-order depuration model **(114)**. Values of k_2 are the amount depurated (the decimal fraction of tissue residue lost per day). Table A5.1 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. The table may be used for estimating the S of organic compounds from the K_{ow} value if no depuration rate is available. However, since these data were developed from fish bioconcentration data, their applicability to the kinetics of uptake from sediment-associated contaminants is unknown. The portion of organics readily available for uptake may be small compared to the total sediment organic concentration **(44)**. The S values generated by this model should therefore be considered to be minimum time periods. Also, (Eq A5.2) does not account for growth dilution (see Annex A4). To correct for growth dilution, (Eq A5.3) becomes the following:

TABLE A5.1 Estimated Time to Obtain 95 % of Steady-State Tissue Residue

Log K_{ow}	k_2(days^{-1})	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

$$S = \ln[1/(1.00 - 0.95)]/(k_2 + k_3) = 3.0/(k_2 + k_3) \quad \text{(A5.4)}$$

where:

k_3 = growth rate constant, days^{-1}.

A5.1.4 Using a linear uptake, first-order depuration model to estimate the exposure time to reach steady-state body burden for metals is problematic for a number of reasons. The kinetics of uptake may be dependent on a small fraction of the total sediment metal load that is bioavailable **(178)**. Depuration rates may be more difficult to determine, as metals bound to proteins may have very low exchange rates **(179)**. High exposure concentrations of some metals can lead to the induction of metal binding proteins, such as metallothionein, which detoxify metals. These metal-protein complexes within the organism have extremely low exchange rates with the environment **(179)**. The induction of metal binding proteins may thus result in decreased depuration rate constants in organisms exposed to the most polluted sediments. Additionally,

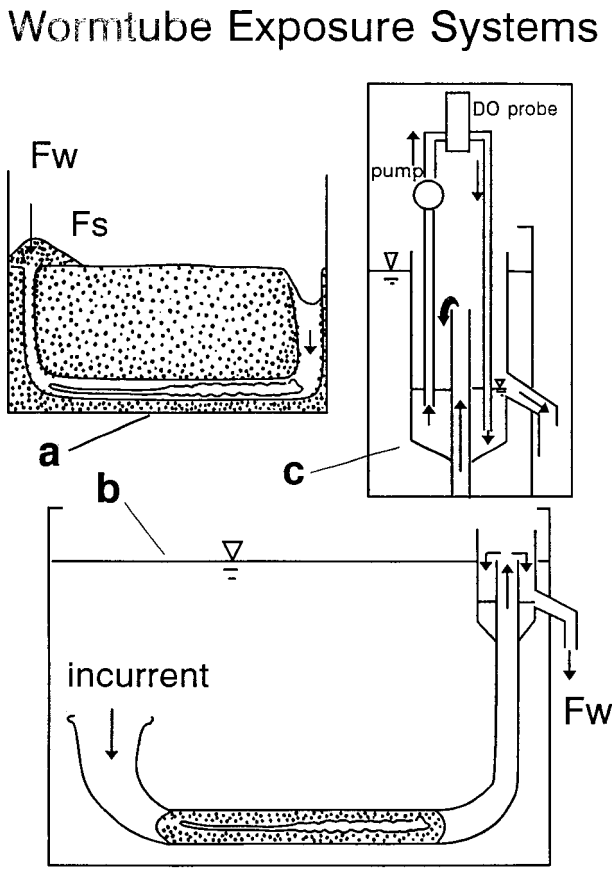
structure-activity relationships that exist for organic contaminants (for example, relationships between K_{ow} and BCFs) are not well developed for metals.

A6. SPECIAL PURPOSE EXPOSURE CHAMBERS

A6.1 *Clambox*—This exposure chamber is designed to separate the inhalant and exhalant siphons of sediment-ingesting clams having independent siphons (see Fig. A6.1). The technique is applicable for *Macoma* spp. and other tellinids, although the two siphons are fused together to form the "neck" in most bivalves. The apparatus allows isolation and collection of the feces from the parent sediment and ventilated (pumped) water from the input supply. This permits a direct measure of short- and long-term ventilation and sediment processing rates (the F_x terms of (Eq A1.5) **(119)**. By analyzing the contaminant content in the feces or ventilated water, the amount of contaminant extracted by the clam (the *EPx* term of (Eq A1.5)) can be estimated. The chamber has been used to determine the efficiency of the uptake of dissolved hexachlorobenzene (HCB) by the gills **(158)**, HCB uptake through the gut from ingested sediment **(159)**, uptake from ventilated interstitial water **(160)**, and passive sorption of HCB to the soft-tissues **(180)**.

A6.2 *Worm Tubes*—These exposure chambers are tubes open on each end, simulating the burrow of sediment-ingesting polychaete such as *Abarenicola pacifica* and *Arenicola marina*. The worms pump water and sediment in one direction through the tubes (Fig. A6.2). As with the clamboxes, the feces can be collected and separated from the parent sediment, allowing measurement of the sediment processing rate and collection of the feces for chemical analysis. These systems have been used to study the effects of crude oil on sediment processing rates **(181)** and on the uptake rate of cadmium as a function of the addition of sewage carbon to sediment **(182)**. Some versions also allow simultaneous measurement of the ventilation rate and oxygen consumption **(183, 184)**.

A6.3 *Sediment Resuspension Systems*—This flow-through device maintains a constant suspended sediment load in the water column automatically, using an electro-optical feedback mechanism **(13)** that employs an airlift dosing system, a transmissometer to measure particle concentration, and a microcomputer that calculates the dose required to achieve a programmed turbidity (see Fig. A6.3) **(185-187)**. The system has been used in several studies on the uptake and effects of contaminants from resuspended sediments using the mussel *Mytilus edulis* and the infaunal polychaete *Nephtys incisa* **(186, 188, 189)**. A guide to conducting sediment resuspension toxicity tests is presented in Guide E 1525. Additional systems for maintaining suspended sediments are given in Refs **(14, 190)**. These chambers should be used when there is concern about bioaccumulation in obligate benthic filter-feeders (for example, *Mercenaria*, *Mya*, and *Mytilus*) or facultative filter-feeders (for example, *Macoma*) by means of resuspended



a. Worm in sediment, 1L glass box
b. Worm in glass tube, 30 L aquarium
c. Expanded view of ventilated water collection and monitoring device

FIG. A6.2 Worm Tube Exposure Chamber

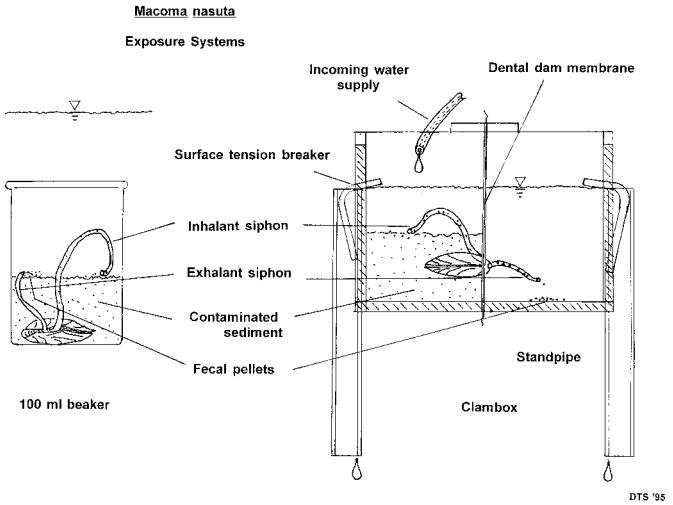


FIG. A6.1 Clam Exposure Chambers

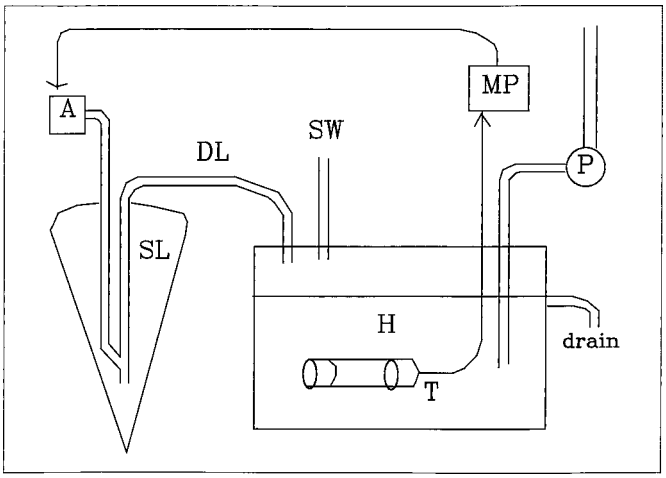


FIG. A6.3 Flow-Through Sediment Resuspension System: A = Air Lift Pump, DL = Slurry Delivery, H = Head Tank, MP = Microprocessor, P = Pump for Dose Delivery to Exposure Chambers, SL = Sediment Slurry, SW = Seawater or Freshwater Inflow, and T = Transmissometer

sediments. This mode of exposure is important in areas in which current or wave action resuspend sediments periodically and in areas with a flocculent surface layer.

A7. ADDITIONAL TECHNIQUES TO CORRECT FOR GUT SEDIMENT

A7.1 *Modifications to 24-h Purge and Dissection*—There are a number of other techniques or modifications to the standard 24-h purge in control sediment (13.4.4) that should be considered in specific cases. When it is unclear whether a species is voiding all of its gut contents within 24 h, a marker "sediment" can be added to the control sediment during the purging. Marker sediments are inert particles of a contrasting color or phosphorescence under UV radiation added to the control sediment. The observation of feces composed of these marker sediments is an indicator that the gut has been voided. Techniques for marking sediments for use as tracers are given in Ref **(191)**. When purging in an uncontaminated sediment, corrections must be made for both the mass dilution based on the mass of the uncontaminated material and for the enhanced elimination that occurs in the presence of ingestable material. In cases in which it is critical to not have any sediment in the gut, such as in certain studies of metals, it may be necessary to purge the organisms in clean water without sediment. It is necessary to determine whether the test species will void its gut satisfactorily in the absence of sediment before using this approach.

A7.1.1 Another approach is removing the gut sediment by dissection. Dissection avoids problems with loss of tissue contaminants during purging, but it requires the use of larger test species (for example, *Abarenicola*). Care has to be taken to minimize the loss of body fluids and to prevent contamination, especially with the metals. General instructions for minimizing contamination are available in Ref **(17)**.

A7.2 *Calculating Contaminant Mass of Gut Sediment*—It is possible to calculate the contaminant mass associated with the gut sediment if both the mass and the contaminant concentration of gut sediment can be estimated. The contaminant concentration of the ingested sediment for selective deposit-feeders may be several fold greater than the concentration of the bulk sediment **(159)**, so the bulk sediment concentration should not be used as an estimate of the gut sediment. Instead, the gut concentrations can be estimated from either the contaminant concentrations of the ingested sediment or the feces. Using the fecal pellet concentrations as the input parameter, the whole body tissue residue (C_{tw}, including both the tissue and gut sediment contaminants) can be expressed as follows:

$$C_{tw} = \frac{(M_s \times \text{CPSf}) + (M_t \times C_t)}{M_s + M_t} \quad \text{(A7.1)}$$

where:

C_{tw} = whole body tissue concentration (tissue and gut sediment), µg/g,
M_s = mass of gut sediment, g,
CPSf = contaminant concentration in feces, µg/g,
M_t = mass of tissue, g, and
C_t = tissue concentration without gut sediment, µg/g.

A7.2.1 Expressed on a tissue residue-only basis (that is, no gut sediment), the formula becomes

$$Ct = \frac{C_{t_w} \times (M_s \times M_t) - (\text{CPSf} \times M_s)}{M_t} \quad \text{(A7.2)}$$

A7.2.2 If the ingested contaminant concentration (CPSi) is

used, the formula is the same except that CPSi is substituted for CPSf. Use of the fecal pellet contaminant concentration underestimates the average gut contaminant content because some of the contaminants are extracted from the sediment before defecation. Ingested sediment conversely overestimates the average gut contaminant content because some of the contaminants have been extracted. These errors are not expected to be large, but both methods could be calculated and the results averaged for the most accurate estimate. Fecal pellets can be collected for chemical analysis by using special exposure chambers such as the clambox with *Macoma* or worm tubes with polychaetes (see Annex A6). A method for estimating ingested dose is given in Ref **(159)**.

A7.3 *Use of Conservative Trace Elements*—Using the concentration of a conservative, non-biologically active element as a means to determine sediment mass in the gut **(192)** is another approach to correcting for gut sediment. Knowing the sediment contaminant concentration, it is theoretically possible to calculate the amount of contaminant associated with the gut sediment. Some of the conservative elements common in minerals but not typically found in more than trace amounts in tissues include silicon, aluminum, and iron **(192)**. The difficulty with this approach is that the elemental content of gut sediment in selective deposit-feeders may differ from that of the bulk sediment, especially if the organism ingests organic rather than mineral particles selectively. Additionally, this method will underestimate the gut contaminant mass unless the CPSi is used rather than the bulk sediment concentration.

A8. BIOACCUMULATION TESTING WITH *LUMBRICULUS VARIEGATUS*

A8.1 General guidance for conducting 28-day bioaccumulation tests with the oligochaete *Lumbriculus variegatus* is described in this Annex. Overlying water is renewed daily, and test organisms are not fed during the bioaccumulation tests. Methods are described for determining the bioaccumulation kinetics of different classes of compounds during 28-day exposures with *L. variegatus*.

A8.1.1 *Lumbriculus variegatus* is one of the best developed organisms for testing bioaccumulation in freshwater systems (Table 3 and Table A8.1). It meets most of the criteria of an ideal test organism listed in the main guide except for size, but sufficient biomass can be obtained for bioaccumulation testing since this species can be cultured in large numbers. *Lumbriculus variegatus* in sediment exposures attains steady state rapidly **(32, 120)** and does not biotransform polycyclic aromatic hydrocarbons **(113)**. Furthermore, *L. variegatus* bioaccumulation has been compared with field populations and was found to yield very similar bioaccumulation **(89, 90,229**).

TABLE A8.1 Summary of Testing Procedures Used to Conduct Whole-Sediment Bioaccumulation Tests with *Lumbriculus variegatus*

Condition	Reference				
	(68)	**(32)**	**(90)**	**(193)**	**(194)**
Temperature, °C	20	23	23	20	23
Light intensity (foot-candles)	NR[A]	NR	25–50	NR	50–100
Photoperiod	NR	various	16–8	NR	16–8
Test chamber, L	3–5	0.15–0.6	4	3–3.8	4–6
Sediment volume, L	1.5–2	30–180 g	1	0.3–0.35	1 L or more
Overlying water volume, L	1.5–3	0.1–0.45	3	2.7–3	1 L or more
Renewal rate of overlying water (additions/day)	2–6	0.5–1	1	0	2
Age of organisms	adult	adult	adult	adult	adult
Loading (g/chamber)	1	1:50[B]	1	0.1–0.39/L	1
Number of replicate chambers/treatment	NR	3–4	3–5	3	5
Food	none	none	none	yes	none
Aeration	none	yes	yes	yes	none
Overlying water	natural/reconstituted	natural	natural	natural	natural/reconstituted
Test duration (day)	10–60	10–60	56	28–44	10–28
Test acceptability	NR	biomass lipid	biomass	NR	biomass

[A] NR = not reported.
[B] 1:50 g dry weight organism:sediment organic carbon.

A8.1.2 *Lumbriculus variegatus* inhabit a variety of sediment types throughout the United States and Europe **(193-198)**. *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.

A8.1.2.1 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 **(68, 90, 199)**. The lipid content is about 1.0 % (wet weight **(200)**). *Lumbriculus variegatus* reproduce asexually most commonly, although sexual reproduction has been reported **(195)**. Newly hatched worms have not been observed in cultures **(68, 90, 201)**. Cultures consist of adults of various sizes. Populations of laboratory cultures double (number of organisms) every 10 to 14 days at 20°C **(68)**.

A8.1.2.2 *Lumbriculus variegatus* tolerate a wide range of substrates. Ankley, et al **(202)** evaluated the effects of natural sediment physicochemical characteristics on the results of 10-day laboratory toxicity tests with *Hyalella azteca, Chironomus tentans*, and *L. variegatus*. Tests were conducted with and without the addition of exogenous food. The survival of organisms was decreased in tests without added food. The physicochemical sediment characteristics, including grain size and TOC, were not correlated significantly to reproduction or growth of *L. variegatus* in either fed or unfed tests.

A8.1.3 Concentrations of total PCBs in laboratory-exposed *L. variegatus* were similar to concentrations measured in field-collected oligochaetes from the same sites **(89)**. PCB homolog patterns also were similar to between laboratory-exposed and field-collected oligochaetes. The more highly

chlorinated PCBs tend to have greater accumulation 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 sediment concentrations at the same sites.

A8.1.4 Chemical concentrations measured in *L. variegatus* after 28-day exposures to sediment in the laboratory were compared to chemical concentrations in field-collected oligochaetes from 13 pools of the upper Mississippi River where these sediments were collected **(229)**. Chemical concentrations were relatively low in sediment and tissue concentrations from the pools evaluated. Only polycyclic aromatic hydrocarbons (PAHs) and total PCBs were frequently measured above detection limits. A positive correlation was observed between lipid-normalized concentrations of PAHs detected in laboratory-exposed *L. variegatus* and field-collected oligochaetes across all sampling locations. Rank correlations for concentrations of individual compounds between laboratory-exposed and field-collected oligochaetes were strongest for benzo(e)pyrene, perylene, benzo(b,k)fluoranthene, and pyrene (Spearman rank correlations > 0.69). About 90 % of the paired PAH concentrations in laboratory-exposed and field-collected oligochaetes were within a factor of three of one another indicating laboratory results could be extrapolated to the field with reasonable certainty.

A8.2 *Culturing Procedures for Lumbriculus variegatus:*

A8.2.1 The culturing procedures described are based on methods described in Refs **(68, 90, 194, 203)**. The bioaccumulation tests are started with adult organisms.

A8.2.2 *Lumbriculus variegatus* are generally cultured with daily renewal of water (57 to 80-L aquaria containing 45 to 50 L of water). Phipps, et al **(68)** recommend starting a new culture with 500 to 1000 worms.

A8.2.3 Paper towels can be used as a substrate for culturing *L. variegatus* **(68)**. Substrate is prepared by cutting unbleached brown paper towels into strips, either with a paper shredder or with a scissors. Cut toweling is packed loosely into a blender with culture water and blended for a few seconds. Small pieces should be available to the organisms. Blending too long will result in a fine pulp that will not settle in the culture tanks. Blended towels then can be added directly to the culture tanks eliminating any conditioning period for the substrate. The paper towel substrate should be renewed with conditioned towels when thin or bare areas appear in the cultures. The substrate in the chamber will generally last for about two months.

A8.2.4 Oligochaetes probably obtain nourishment from ingesting the organic matter in the substrate **(204)**. *Lumbriculus variegatus* in each culture chamber are fed a 10-mL suspension of 6 g of trout starter three times per week. The particles will disperse on the surface film temporarily, 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 or substrate used to culture oligochaetes should be analyzed for compounds to be evaluated in the bioaccumulation tests. Recent studies in other laboratories, for example, have indicated elevated concentrations of PCBs in substrate or food used in culturing of oligochaetes **(194)**.

A8.2.5 Oligochaetes can be isolated on the day before the start of a test 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 inside diameter (ID); **(68)**). Water can be trickled slowly into the beaker. The oligochaetes will form a mass, and most of the remaining substrate will be flushed from the beaker **(90)**. Organisms can be placed in glass or stainless steel pans on the day the test is started. 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, weighed quickly, and introduced immediately into the appropriate test chamber. Organisms should not be blotted with a paper towel to remove excess water. Brunson et al. **(229)** recommended adding about 1.33× of the target stocking weight. This additional 33 % should account for the excess weight from water in the sample of nonblotted oligochaetes at the start of the test (see A8.4.4.1).

A8.2.6 The culture population generally doubles (the number of organisms) in about 10 to 14 days. See Table A8.2, Section B for additional details on procedures for evaluating the health of the cultures.

A8.3 *Guidance for Conducting a 28-Day Sediment Bioaccumulation Test with Lumbriculus variegatus:*

A8.3.1 Recommended conditions for conducting a 28-day sediment bioaccumulation test with *L. variegatus* are summarized in Table A8.3. A general activity schedule is outlined in Table A8.4. Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers per treatment, and water quality characteristics should be based on the purpose of the test and methods of data analysis. The number of replicates and concentrations tested depends partly on the significance level selected and type of statistical analysis. The sensitivity of a test increases as the number of replicates increases when variability remains constant. Additional research is needed on the standardization of bioaccumulation procedures with sediment; therefore, A8.3.2 describes general guidance for conducting a 28-day sediment bioaccumulation test with *L. variegatus*. Methods outlined in USEPA **(194)** were used for developing this general guidance. Results of tests using procedures different from the procedures described in A8.3.2 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 standard, additional tests are required to determine comparability of results.

TABLE A8.2 Test Acceptability Requirements for a 28-Day Sediment Bioaccumulation Test with *Lumbriculus variegatus*

(*A*)		It is recommended for conducting a 28-day test with *L. variegatus* that the following performance criteria are met:
	(*1*)	Numbers of *L. variegatus* in a 4-day toxicity screening test should not be reduced significantly in the test sediment relative to the control sediment.
	(*2*)	Test organisms should burrow into test sediment. Avoidance of the test sediment by *L. variegatus* may decrease bioaccumulation.
	(*3*)	The hardness, alkalinity, pH, and ammonia of overlying water within a treatment typically should not vary by more than 50 % during the test and dissolved oxygen should be maintained above 2.5 mg/L in the overlying water.
(*B*)		Performance-based criteria for culturing *L. variegatus* include the following:
	(*1*)	It may be desirable for laboratories to perform periodically 96-h water-only reference toxicity tests to assess the sensitivity of culture organisms (see Test Methods E 1706). Data from these reference toxicity tests could be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.
	(*2*)	Laboratories should monitor the frequency with which the population is doubling in the culture (the number of organisms) and record this information using control charts (the doubling rate would need to be estimated on a subset of animals from a mass culture). Records also should be kept on the frequency of restarting cultures. If static cultures are used, it may be desirable to measure water quality more frequently.
	(*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 in the cultures should be measured weekly. Temperatures of the cultures should be recorded daily.
	(*5*)	Laboratories should characterize and monitor the 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*)	Storage of sediment collected from the field should follow guidance outlined in 10.7.
	(*3*)	All test chambers (and compartments) should be identical and should contain the same amount of sediment and overlying water.
	(*4*)	Negative-control sediment or appropriate solvent controls, must be included in a test. The concentration of solvent used must not affect test organisms adversely.
	(*5*)	Culture and test temperatures must be the same. Acclimation of test organisms to the test water is not required.
	(*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 physicochemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

TABLE A8.3 Recommended Test Conditions for Conducting a 28-Day Sediment Bioaccumulation Test with *Lumbriculus variegatus*

Parameter	Conditions
(*1*) Test type	whole-sediment bioaccumulation test with renewal of overlying water
(*2*) Temperature	23°C
(*3*) Light quality	wide-spectrum fluorescent lights
(*4*) Illuminance	about 100 to 1000 lx
(*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/day; continuous or intermittent (for example, one volume addition every 12 h)
(*10*) Age of test organisms	adults
(*11*) Loading of organisms in chamber	Ratio of TOC in sediment to organism dry weight should be no less than about 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.
(*13*) Feeding	none
(*14*) Aeration	None, unless dissolved oxygen in overlying water drops below 2.5 mg/L.
(*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	28 days
(*19*) Endpoint	bioaccumulation
(*20*) Test acceptability	performance-based criteria specifications outlined in Table A8.2

A8.3.2 The recommended 28-day sediment bioaccumulation test with *L. variegatus* can be conducted with adult oligochaetes at 23°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lx. While a specific light regim has been suggested, no specific tests on light requirements for bioaccumulation testing have been performed to date. Test chambers are 4 to 6-L that contain 1 to 2 L of sediment and 1 to 4 L of overlying water. The number of replicates per treatment depends on the objective of the test. Five replicates are recommended for routine testing. To minimize the depletion of sediment contaminants, the ratio of TOC in sediment to dry weight of organisms should be no less than about 50:1 **(32)**. 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 per day of overlying water. Benoit, et al **(205)** and Zumwalt, et al **(10)**, and Brunson et al.**(229)** describe water-renewal systems that can be used with minor modifications 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 at which the sediment is collected. The requirements for test acceptability are outlined in Table A8.2.

A8.3.2.1 If there is concern that the test samples may exhibit overt toxicity, and hence reduced bioaccumulation, a 4-day toxicity screening test can be conducted before starting

TABLE A8.4 General Activity Schedule for Conducting a 28-Day Sediment Bioaccumulation Test with *Lumbriculus variegatus*

(*A*) Conducting a 4-Day Toxicity Screening Test (Conducted Before the 28-Day 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, and ammonia). Measure the weight of a subset of 20 organisms used to start the test. Transfer 10 worms into each test chamber. Observe the behavior of test organisms.
1–2	Measure temperature and dissolved oxygen. Observe the 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 the weight of survivors. Bioaccumulation tests should not be conducted with *L. variegatus* if a test sediment significantly reduces the number of oligochaetes relative to the control sediment or if oligochaetes avoid the sediment.

(*B*) Conducting a 28-Day 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, and ammonia). Sample a subset of worms used to start the test for residue analyses. Transfer appropriate amount of worms (based on weight) into each test chamber. Observe the behavior of test organisms.
1	Measure temperature and dissolved oxygen. Observe the behavior of test organisms.
2–6	Same as Day 1
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 the uptake by collecting the worms with a sieve. Separate any indigenous organisms from *L. variegatus*. Determine the weight of survivors. Eliminate the gut contents of surviving worms in water for 6 to 8 h. Longer purging periods (not to exceed 24 h) may be used if all target analytes have Log K_{ow}>5 (see A8.4.7.3).

a 28-day sediment bioaccumulation test with *L. variegatus* (Table A8.5, **(80)**). The preliminary toxicity screening test is conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lx. The test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten adult oligochaetes per replicate are used to start a test. Four replicates are recommended for routine screening tests. Oligochaetes are not fed during the test. Each chamber receives 2 volume additions per day of overlying water. Benoit, et al **(205)** and Zumwalt, et al **(10)** and Brunson et al. **(229)** describe 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 reduced significantly in the test sediment relative to the control sediment. The test organisms should burrow into test sediment. Avoidance of the

TABLE A8.5 Recommended Test Conditions for Conducting a Preliminary 4-Day Sediment Toxicity Screening Test with *Lumbriculus variegatus*

Parameter	Conditions
(*1*) Test type	4-day whole-sediment toxicity test with renewal of overlying water
(*2*) Temperature	23°C
(*3*) Light quality	wide-spectrum fluorescent lights
(*4*) Illuminance	about 100 to 1000 lx
(*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/day; continuous or intermittent (for example, 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 min
(*13*) Feeding	none
(*14*) Aeration	none, unless dissolved oxygen in overlying water drops below 2.5 mg/L
(*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 days (minimum; up to 10 days)
(*19*) Endpoints	Number or 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 A8.2

test sediment by *L. variegatus* may decrease bioaccumulation.

A8.4 *General Procedures:*

A8.4.1 *Sediment into Test Chambers*—The day before the sediment test is started (Day − 1), each sediment should be mixed thoroughly and added to the test chambers. The sediment should be inspected visually 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 characteristics such as TOC, chemical concentrations, or particle size.

A8.4.1.1 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 the suspension of sediment. This can be accomplished by pouring water along the sides of the chambers gently or by pouring water onto a baffle (for example, a circular piece of PTFE 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).

A8.4.2 *Renewal of Overlying Water*—Renewal of overlying water is recommended during a test. Flow rates through any two test chambers should not differ by more than 10 % at any particular time during the test. Mount and Brungs **(8)** diluters have been modified for sediment testing, and other automated water delivery systems have also been used **(9, 10, 38, 205,230)**. 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 on Day 0.

A8.4.2.1 In water-renewal tests with one to four volume additions of overlying water/day, water quality characteristics generally remain similar to the in-flowing water **(38, 200)**; however, in static tests, water quality may change profoundly during the exposure **(206)**. 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 **(38)**. Additionally, concentrations of metabolic products (for example, ammonia) may also increase during static exposures, and these compounds can be either 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 **(207)** and organic **(208)** 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 the interstitial water.

A8.4.3 *Acclimation*— Test organisms should be cultured and tested at the same temperature. The test organisms should ideally be cultured in the same water that will be used in testing. However, acclimation of test organisms to the test water is not required. If the test organisms are to be acclimated, they could be held for 2 h in a 50 to 50 mixture of culture water to overlying water and then for 2 h in a 25 to 75 mixture of culture water to overlying water, followed by a transfer into 100 % overlying water for 2 h **(38)**.

A8.4.4 *Placing Test Organisms in Test Chambers*—Paragraph A8.2.5 describes a procedure for isolating oligochaetes for starting a test. At the start of the test, a subset of *L. variegatus* should be sampled to determine the starting concentrations of chemicals of concern. Mean group weights should be measured on a subset of at least 100 organisms used to start the test. The ratio of TOC in sediment to dry weight of organisms at the start of the test should be no less than about 50:1.

A8.4.4.1 Oligochaetes added to each replicate should not be blotted to remove excess water. Brunson et al. **(229)** recommend adding about 1.33× of the target stocking weight. This additional 33 % should account for the excess weight from water in the sample of non-blotted oligochaetes at the start of the test.

A8.4.5 *Monitoring a Test*—All chambers should be checked daily and observations made to assess test organism behaviors such as sediment avoidance. However, monitoring effects on the 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.

A8.4.5.1 *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 composite water samples from individual replicates. The pipet should be checked to ensure that no organisms are removed during the sampling of overlying water. The hardness, alkalinity, conductivity, and ammonia of overlying water within a treatment should not vary by more than 50 % during a test.

(1) Dissolved oxygen should be measured daily and should be above 2.5 mg/L (Guide E 729). If a probe is used to measured dissolved oxygen in the overlying water, it should be inspected thoroughly between samples to ensure that the 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 2.5 mg/L, that is, about one bubble/s in the overlying water. Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

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

A8.4.6 *Feeding—Lumbriculus variegatus* should not be fed during a bioaccumulation test.

A8.4.7 *Ending a Test*— Care should be taken to isolate at least the minimum amount of tissue mass from each replicate chamber needed for analytical chemistry.

A8.4.7.1 Sediment at the end of the test can be sieved through a fine-meshed screen sufficiently small to retain the

oligochaetes (for example, U.S. Standard Sieve 40 (425-µm mesh) or 60 (250-µm mesh)). The sieved material should be transferred rapidly to a shallow pan to keep the oligochaetes from moving through the screen. Immobile organisms should be considered dead.

A8.4.7.2 The sediment contribution to the body weight of *Lumbriculus variegatus* is reported to be about 20 % of the wet weight and the contribution to chemical concentrations ranges from 0 to 11 % in two laboratory studies **(32, 120)**. Analyses by Mount et al. **(226)** suggest that under certain conditions substantially larger errors may occur if gut contents are included in samples for tissue analysis. Accordingly, after separating the organisms from the sediment, test animals are held in clean water to allow the worms to purge their guts of sediment. To initiate gut purging, live oligochaetes are transferred from the sieved material to a 1-L beaker containing overlying water only. Oligochaetes should not be placed in clean sediment to eliminate gut contents. Clean sediment can add to the dry weight of the oligochaetes, which would result in a dilution of chemical concentrations on a dry weight basis. Further, purging in clean sediment is thought to accelerate depuration of chemical from tissues **(32)**. The elimination beakers may need to be aerated to maintain dissolved oxygen above 2.5 mg/L.

A8.4.7.3 The previous version of this guide (E 1688–97a) specified a 24-h holding period for gut purging, based on the findings of Call et al. **(199)** who reported that *L. variegatus* clear more than 90 % of their gut contents in 24 h. Kukkonen and Landrum **(120)** reported *L. variegatus* will purge out the intestinal contents in 10 h in water, and more recently, Mount et al. **(226)** found that gut purging of *L. variegatus* was essentially complete in only 6 h. Shorter purging periods may be preferable to reduce depuration of chemical from tissue during holding in clean water, particularly for compounds with log K_{ow} < 5 (see Fig. A8.1). Mount et al. **(226)** estimated that after a 6-h purging period, compounds with log K_{ow} > 3.85 would remain at >90 % of their initial concentrations, but after 24 h, only compounds with log K_{ow} > 5 would be at >90 % of the initial concentration in tissue. For this reason, it is recommended that the purging period last 6 to 8 h. Longer purging periods (not to exceed 24 h) may be used if all target analytes have log K_{ow} > 5.

A8.4.7.4 Field-collected sediments may include indigenous oligochaetes. The behavior and appearance of indigenous oligochaetes is 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 **(68)**. Dwyer, et al **(90)** evaluated the bioaccumulation of chemicals by indigenous oligochaetes that were exposed in the same chamber with introduced *L. variegatus* in a 28-day test. The peak concentrations of select PAHs and DDT were similar in the indigenous oligochaetes and *L. variegatus* exposed in the same chamber for 28 days.

A8.4.8 *Test Data*—Sensitivity of tissue analyses is dependent largely on the mass of tissue available and the sensitivity of the analytical procedure. To obtain meaningful results from bioaccumulation tests, it is essential that desired detection limits be established before testing, and that the test design allow for sufficient tissue mass. Tissue masses required for various analyses at selected lower limits of detection are listed

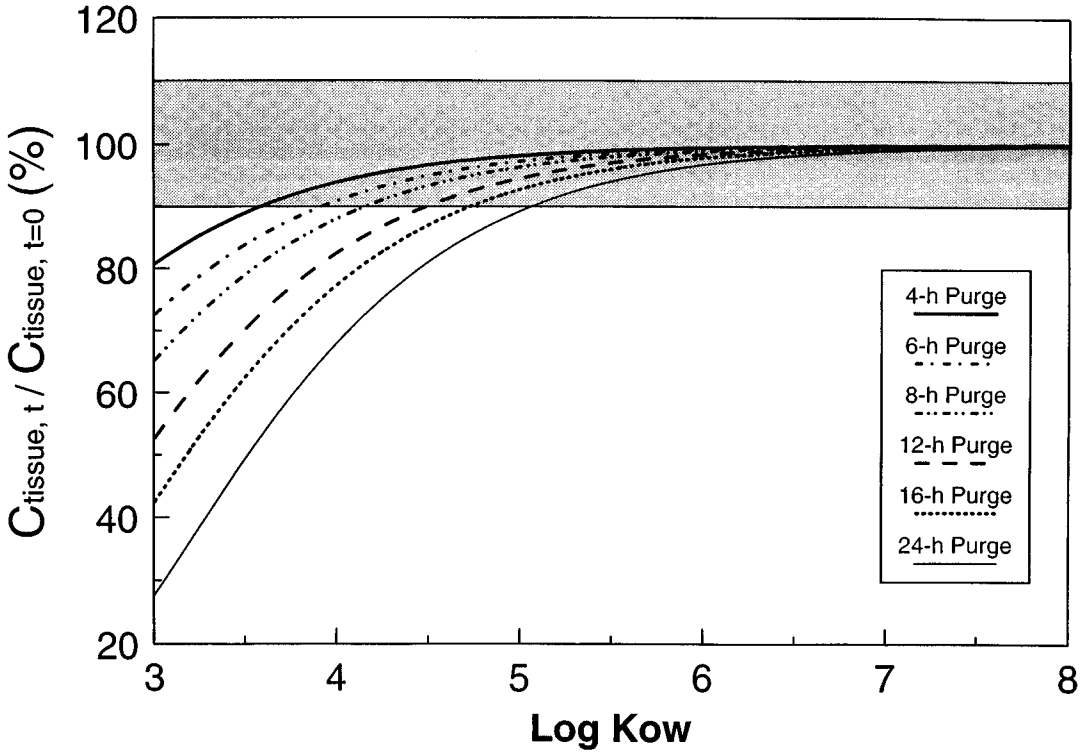


FIG. A8.1 Predicted depuration of nonionic organic chemicals from tissue of *Lumbriculus variegatus* as a function of K_{ow} and duration of depuration, assuming no contribution of sediment in the gut. Shaded area represents ±10 % of tissue concentration at the beginning of the depuration period (Mount et al., (226)).

in Table A8.6. Detection limits for individual PAHs in tissue are listed in Table A8.7. For most chemicals, a minimum mass of 1 g/replicate (wet weight) and preferably 5 g/replicate (wet weight) should be tested. Again, however, to insure results will be meaningful, required masses for analytes of interest to the study should be evaluated specifically before the study is designed.

A8.4.8.1 If an estimate of dry weight is needed, a subsample should be dried to a constant weight at approximately 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 approximately 1 % lipid (dry weight). It may be desirable to determine ash-free dry weight (AFDW) of oligochaetes instead of dry weight. Measurement of AFDW is recommended over dry weight for *C. tentans* due to the contribution of sediment in the gut to the weight of midge (see Test Method E 1706). Additional data are needed to determine the contribution of sediment in the gut of *L. variegatus* to body weight before a definitive recommendation can be made to measure AFDW of oligochaetes routinely.

A8.4.8.2 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 **(136)** describe procedures for measuring lipids in 1 mg of tissue. Different methods of lipid analysis can yield different results **(209)**. The analytical method used for lipid analysis should be calibrated against the chloroform-methanol extraction method described by **(134, 210)**. The dry weight of oligochaetes can be determined on a separate subsample from each replicate.

(1) A number of studies have demonstrated that lipids are the major storage site for organic compounds in a variety of organisms **(130-132)** . It may be desirable to normalize bioaccumulated concentrations of nonpolar organic compounds to the tissue lipid concentration because of the importance of lipids. Lipid concentration is one of the factors required for deriving the BSAF. However, the difficulty with using this approach is that each lipid method generates different lipid concentrations. (See Ref **(133)** for a discussion of lipid methodology.) The differences in lipid concentrations translate directly to a similar variation in the lipid-normalized chemical concentrations or BSAF.

(2) For comparisons of lipid-normalized tissue residues or BSAFs, it is necessary to either promulgate a standard lipid technique or intercalibrate the various techniques. Standardization on a single method is difficult because the lipid methodology is often intimately tied in with the extraction procedure for chemical analysis. As an interim solution, the Bligh-Dyer lipid method **(134)** is recommended as a temporary "intercalibration standard."

(3) The potential advantages of Bligh-Dyer include (*1*) its ability to extract neutral lipids not extracted by many other solvent systems and (*2*) the wide use of this method (or the same solvent system) in biological and toxicological studies (for example, Refs **(44, 130-132)**). 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 **(136, 211)**

(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.

A8.5 *Interpretation of Results:*

A8.5.1 *Test Acceptability*—For the test results to be acceptable, numbers of *L. variegatus* should not be reduced in test sediments relative to the control sediment in a 4-day screening toxicity test, and the organisms should burrow into the test sediment. Avoidance of the test sediment by *L. variegatus* will decrease bioaccumulation.

A8.5.2 *Duration of Exposure*—Because the data from bioaccumulation tests will often be used in ecological or human health risk assessments, the procedures are designed to generate quantitative estimates of steady-state tissue residues. Eighty

TABLE A8.6 Wet Weight (g) Tissue Required for Various Analytes at Selected Lower Limits of Detection (208)

Analyte	Grams of Tissue		
	1.0	5.0	2.0
	Lower Limit of Detection, µg/g		
PCB (total)	0.600	0.300	0.120
PCB 1–3 chlorines	0.025	0.0125	0.005
PCB 4–6 chlorines	0.050	0.025	0.010
PCB 7–8 chlorines	0.075	0.0375	0.015
PCB 9–10 chlorines	0.125	0.0625	0.025
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
α-Chlordane	0.050	0.025	0.010
γ-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
PAH (total)	0.012	0.006	0.002
Dioxins	0.020 (ng/g)	0.010 (ng/g)	0.004 (ng/g)
TCDD	0.005 (ng/g)	0.0025 (ng/g)	0.001 (ng/g)
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

TABLE A8.7 Detection Limits (ng) of Individual PAHS by HPLC-FD (209)

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

percent of steady-state is used as the general criterion. Because the results from a single or few species will often be extrapolated to other species, the procedures are designed to maximize exposure to sediment-associated contaminants so as not to underestimate the residues in untested species systematically.

A8.5.2.1 A kinetic study can be conducted to estimate steady-state concentrations instead of conducting a 28-day bioaccumulation test (for example, sample on Day 1, 3, 7, 14, and 28; **(3, 90, 100, 194)**; Section 16). A kinetic test can be used when 80 % of steady-state will not be obtained within 28 days or when more precise estimates of steady-state tissue residues are required.

A8.5.2.2 Dwyer, et al **(90)** reported DDT to reach 90 % of steady state by Day 14 of a 56-day exposure with *L. variegatus*. However, low molecular weight PAHs (for example, acenaphthylene, fluorene, and phenanthrene) generally peaked at Day 3 and tended to decline to Day 56. In general, concentrations of high molecular weight PAHs (for example, benzo(b)fluoranthene, benzo(e)pyrene, and indeno (1,2,3-c,d)pyrene) either peaked at Day 28 or continued to increase during the 56-day exposure.

A8.5.3 *Isolating Organism at the End of a Test*—Quantitative recovery of *L. variegatus* at the end of a sediment test should not be a problem. Laboratory personnel need to be able to distinguish between native oligochaetes and *L. variegatus*.

A8.5.4 *Influence of Indigenous Organisms*—Field-collected sediments may include indigenous oligochaetes. The presence of a second oligochaete in a laboratory study altered bioaccumulation compared to its absence **(81, 82)**. Phipps, et al **(68)** recommends testing extra chambers without the addition of *L. variegatus* to check for the presence of indigenous oligochaetes in field-collected sediment.

A8.5.5 *Sediment Toxicity in Bioaccumulation Tests*—Toxicity or altered behavior of organisms in a sample may not preclude the use of bioaccumulation data; however, information on the adverse effects of a sample should be included in the report.

A8.5.5.1 *Grain Size—Lumbriculus variegatus* are tolerant of a wide range of substrates. Physicochemical characteristics (for example, grain size) of sediment were not correlated significantly to the growth or reproduction of *L. variegatus* in 10-day toxicity tests in which the organisms were fed **(200)**.

A8.5.5.2 *Sediment Organic Carbon*—Reduced growth of *L. variegatus* may result from exposure to sediments with low organic carbon concentrations **(200)**. For this reason, reduced growth observed in bioaccumulation tests could be caused by either direct toxicity or insufficient nutrition of the sediment. Testing additional replicate chambers with supplemental food could be used to help make this distinction, although the effect of added food on accumulation of chemicals would need to be considered in the test interpretation.

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SUMMARY OF CHANGES

The primary changes from the previous two versions of this guide are summarized in this Section.

E 1688 – 99 and E 1688 – 00:

The following sections were revised in 1999 and 2000 and additional guidance has been provided on:

(*1*) bioaccumulation testing with the oligochaete *Lumbriculus variegatus* (Section Annex A8),;

(*2*) sediment collection (Section 10.4.1);

(3) sediment storage (Section 10.7), and

(4) sediment spiking (Section 10.9; USEPA 2000 **(194)**; Test Method E 1706).