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

This standard is issued under the fixed designation E1688; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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 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 containing organic or metal contaminates 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:

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[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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

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Additional Techniques to Correct for Gut Sediment Annex A7
Guidance For Conducting Sediment Bioaccumulation Tests with the Oligochaete *Lumbriculus Variegatus* Annex A8
References

1.5 Field-collected sediments may contain toxic materials, 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 standard. No other units of measurement are included in this 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 8.

1.9 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D4387 Guide for Selecting Grab Sampling Devices for Collecting Benthic Macroinvertebrates (Withdrawn 2003)[3]

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1022 Guide for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks

E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes

E1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates

E1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates (Withdrawn 1995)[3]

E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

E1525 Guide for Designing Biological Tests with Sediments

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

SI10-02 IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

2.2 *Federal Documents:*[4]

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

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

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 E729 and Terminologies D1129 and E943. For an explanation of units and symbols, refer to SI10-02 IEEE/ASTM SI 10 .

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *acid volatile sulfide (AVS)*—sedimentary reduced sulfide phase associated with metal partitioning.

3.2.2 *alpha*—see *Type I error*.

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

3.2.4 *bedded sediment*—see *whole sediment*.

3.2.5 *beta*—see *Type II error*.

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

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

[4] Available from U.S. Government Publishing Office, 732 N. Capitol St., NW, Washington, DC 20401-0001, http://www.gpo.gov.

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

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

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

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

3.2.11 *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.

3.2.12 *black carbon (BC)*—type of environmental carbon formed during the incomplete oxidation of organic substances (for example, fossil fuels, biomass). May consist of phases such as soot, charcoal, tar, and coal. Certain forms have high affinity for hydrophibic contaminants and can reduce the bioavailability of some contaminants.

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

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

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

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

3.2.17 *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*.

3.2.18 *degradation*—biochemical breakdown of the contaminant by a test species.

3.2.19 *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.*

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

3.2.21 *dissolved organic carbon (DOC)*—type of organic carbon soluble in aqueous solutions. Particulate and dissolved organic carbon are the primary organic carbon components in aquatic systems.

3.2.22 *Eh (redox potential)*—a measure of the oxidation state of a sediment relative to the hydrogen half-cell reaction.

3.2.23 *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*.

3.2.24 *equilibrium partitioning (EqP) bioaccumulation model*—a bioaccumulation model based on equilibrium partitioning of a neutral organic between organism lipids and sediment carbon.

3.2.25 *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*.

3.2.26 *experimental error*—variation among replicate experimental units.

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

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

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

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

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

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

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

3.2.34 *metabolism*—see *degradation*.

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

3.2.36 *multiple comparisons*—the statistical comparison of several treatments simultaneously.

3.2.37 *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.

3.2.38 *organic carbon (OC)*—type of environmental carbon resulting from the diagenesis of organic substances (for example, plant and animal matter).

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

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

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

3.2.42 *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*.

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

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

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

3.2.46 *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.

3.2.47 *total carbon (TC)*—this value includes organic, black, and inorganic carbon.

3.2.48 *total organic carbon (TOC)*—includes organic carbon resulting from the diagenesis of organic substances (for example, plant and animal matter) as well as black carbon resulting from incomplete oxidation of organic substances (for example, fossil fuels, biomass).

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

3.2.50 *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).

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

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

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

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

3.2.55 *Type I error*—chance of rejecting the null hypothesis when it should be accepted.

3.2.56 *Type II error*—the chance of accepting the null hypothesis when it should be rejected.

3.2.57 *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 × g-tissue^{-1} × time^{-1}. Contrast with k_s.
k_2	=	elimination rate constant, in units of time^{-1}.
K_{bc}	=	black carbon-water partitioning coefficient.
K_l –lipid	=	water partitioning coefficient.
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 × g-tissue^{-1} × 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 are 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 guidelines (**1, 2, 3**)[5] and to assess the potential impacts of disposal of dredge materials (**4**).

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 augment 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 subject 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 myriad 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

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

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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. Geochemical phases in the sediment, such as total organic carbon (TOC), black carbon (BC), acid volatile sulfides (AVS), and grain size influence contaminant bioavailability and bioaccumulation.

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 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 (**5**). 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. Toxic compounds that occur naturally, 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 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. Guide E1022 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.

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7.1.2.2 A15 to 30-min transition period (**6, 7**) 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 (**8**). 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 (See Test Method E1706 for examples). 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 (**9-11**).

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 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 (**12**)). 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 (**13**)). A more recent design places the experimental beakers in a water bath for temperature control and permits water renewal to each beaker independently (**11**). 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 E729).

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 E1383 and Refs (**14-16**).

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

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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 (**16**)).

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 (**17-19**). 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 (**20-24**) 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 (**25**) 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 E1383 for additional details). Water quality should meet the following specifications as established in Guide E729:

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 Test Method E1367 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*—Guide E1022 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 (Guide E1022). 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 E729) and unionized ammonia concentrations <20 µg/L (Guide E1022). 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, Guide E1022 recommends not more than one filter-feeding bivalve (40 to 60 mm from umbo to edge of distal valve) per liter per hour. This would be equivalent to a minimum flow of 1 L/h/g wet tissue

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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 (Guide E1022).

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 (**26-28**)). 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 (Guide E1022) 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 (**29**).

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. 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 (**32**)), the rates may be exaggerated in laboratory systems.

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 (**32**)) 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 (**33**). 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 concentrations, the organic

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carbon (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 (**34**). Black carbon (BC) may be a useful sediment variable for determining the bioavailability (and bioaccumulation) of many organic contaminants including polycyclic aromatic hydrocarbons (**35**).

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, BC, 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 (**4**).

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 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 (**36**). 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 10 stations. Sediment concentrations falling within or near the 10 lowest station values are acceptable as controls. Neither sediment concentrations substantially above those in Table 2 nor the normalized values of the 10 lowest stations in (**36**) should be considered control values, except those of sediments containing naturally high levels of certain metals.

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 (**37-39**).
[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 (**40**).
[E] Benzo(a)pyrene.
[F] Benzo(i,b,k)-fluoranthene.
[G] Napthalene.
[H] See Ref (**38**).
[I] Schults, unpublished data, U.S. EPA, Newport, OR.
[J] Polyaromatic hydrocarbons.
[K] See Ref (**41**).

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 (**36**).

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 provide a measure of the

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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 (**42**)), 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 E1391. 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 (**43**) and is bioaccumulated by marine worms and clams readily (**16, 44, 45**). 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 (**46, 47**). Cadmium is suggested as a general reference metal. The bioaccumulation of sediment-associated cadmium has been studied in a number of organisms (**48**) and has been suggested as the reference toxicant for *Neanthes* growth tests (**42**). However, because toxic compounds may alter the behavior of organisms, changes in behavior can alter 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 (**17, 49, 50**). 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 E1391 and Ref (**17**)). The sediment manipulation methods presented in Guide E1391 and Guide D4387 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 E1391). 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 (**17, 36, 50**).

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 (**17, 36, 50**). Scrape surficial sediment from the grab or box corer samples and store immediately in appropriate containers (Guide E1391). Flocculent material should be considered to be part of the sample (**18**).

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 (**17**). To minimize oxidation and changes in other chemical properties, place plastic or PTFE bags or containers of appropriate composition

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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 (**18**), 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 (**18, 51, 52**).

10.5 *Field Measurements*—Upon collection, immediately determine sediment characteristics such as temperature, pH, Eh, and salinity (**17, 50, 53**). 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 (**18**).

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 (**49**).

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 (**18**). 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 (**4, 18**). 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, Title 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 (**17, 18, 54**) 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 E1391) to less than eight weeks (**55**). 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 (**56-62**). 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 E1391 and Test Method E1706.

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 (**61**). 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 (**57, 58, 60**). 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, (**58**) high variability was observed in survival during early testing periods, for example, less than two weeks, in sediments with low toxicity. DeFoe and Ankley (**58**) hypothesized that this variability partially reflected the presence of indigenous predators that remained alive during this relatively short storage

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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 (**58, 60**). 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 sieving 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, carefully siphon off or decant the overlying water 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 E1706).

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 flocculants and change the sediment's physicochemical properties, such as dissolved organic matter (DOM) (**63**).

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 E1391 and Test Method E1706 for additional details on spiking sediment.) Limited studies have been conducted 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 (**64**) 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 E1706). Mixing time (**65**) and aging (**66, 67**) 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 . 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 (**64**), Guide E1391, and Test Method E1706 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 E1391; (**64**)). 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 E1391). Duration of contact between the chemical and sediment can affect partitioning and bioavailability (**67**). Care should be taken to ensure that the chemical is distributed thoroughly and evenly in the sediment. Analyses of sediment

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subsamples is advisable to determine the degree of mixing homogeneity (**68**). Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments (**69**).

10.9.2.1 Organic compounds have been added as follows: *(1)* directly in a dry (crystalline) form; *(2)* coated on the inside walls of the container (**68**) ; or, *(3)* coated onto silica sand (for example, 5 % w/w of sediment) which is added to the sediment (**70**). 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 and BC 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 (**71**). 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 E729).

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 affected in the field. However, because of natural fluctuations (**72**), contaminant events (**73**), or succession during recolonization (**74**), 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 (**47, 75-77**). 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 resistance 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 (**78,79**) or reduced (**33**) 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.

11.3.1 The ease of obtaining test species in sufficient numbers at the correct season is of concern when planning repeated

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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	+	–	–	+
Arenicola sp.	FUN	+ +	>15	+	–	+	+
Callianassa Sp.	SSDF[C]	+ +	>10	–?	–	+	–
Capitella 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	+ +	–	+	+ +
**Hediste 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.

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 (**80-83**), as can *Palaemonetes*(**84, 85**). For freshwater, *Lumbriculus variegatus* can be cultured readily in large numbers for bioaccumulation tests (see Ref (**86**) 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 (**87, 88**) 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 *Nereis 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. However, selection of smaller individuals of *L. variegatus* for use in the tests may be desirable because reproduction by larger individuals can result in cessation of sediment ingestion (**89**), which could cause additional variability in bioaccumulation.

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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 experiment 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 10-fold among species (for example, (**16**). 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 (**90**) or for freshwater *Diporiea spp*. (formerly *Pontoporeia hoyi* (**91**)) or *Lumbriculus variegatus*(**92, 93**).

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 log_{10} K_{ow} below approximately 5 (**94**) and for metals (**48**), 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 (**42, 95**). 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 *Hediste diversicolor* and *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 E1383) 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 (**96**). 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 (**86, 97-101**), and field populations have been used as indicators of pollution of aquatic sediments (**102-109**). Specifically, *L. 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

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attain sufficient mass. *L. variegatus* does not biotransform PAHs (**93**). They do not need to be fed during long-term bioaccumulation exposures (**86**). Verifications of bioaccumulation between laboratory studies and field conditions have been performed (**108**).

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 (**45**). 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 (**93**). 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 is 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 (**90**). 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 Guide E1022 stipulates that the length (umbo to distal valve) of the largest clam should be no greater than 1.5 times larger than that of 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, total organic carbon (TOC) content and interstitial water 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 Guide E1022 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 E1706). 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

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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 gently sieving the sediment in the field. 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 (**50**, **110**). 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*—Guide E1022 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 milliliters) 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 E1383, 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

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benthic invertebrates can be found in Refs (**83, 111-113**). 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 omnivores (**114**). 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 (Guide E1022). 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 (Guide E1022).

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 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

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reference sediments. A two-fold difference should provide a sufficient signal for 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 error 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 %. Guide E1022 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" (**115**). 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 (**115**).

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

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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 or sediment) and chemically analyzing the mix rather than the individual samples (**116**). 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 (**117**). 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 (**118**) 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 later.

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 (Guide E1022). 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 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 (**119**). 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 (**4**). Additionally, a 28-day duration test is the recommended standard length for conducting bioconcentration tests (Guide E1022). 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 (Guide E1022). 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 A8.4.2).

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 organisms sampled*

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