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Standard Guide for Conducting In-situ Field Bioassays With Caged Bivalves[1]

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1. Scope

1.1 This guide describes procedures for conducting controlled experiments with caged bivalves under field conditions. The purpose of this approach is to facilitate the simultaneous collection of field data to help characterize chemical exposure and associated biological effects in the same organism under environmentally realistic conditions. This approach of characterizing exposure and effects is consistent with the US EPA ecological risk assessment paradigm. Bivalves are useful test organisms for in-situ field bioassays because they (*1*) concentrate and integrate chemicals in their tissues and have a more limited ability to metabolize most chemicals than other species, (*2*) exhibit measurable sublethal effects associated with exposure to those chemicals, (*3*) provide paired tissue chemistry and response data which can be extrapolated to other species and trophic levels, (*4*) provide tissue chemistry data which can be used to estimate chemical exposure from water or sediment, and (*5*) facilitate controlled experimentation in the field with large sample sizes because they are easy to collect, cage, and measure (**1**, **2**)[2]. The experimental control afforded by this approach can be used to place a large number of animals of a known size distribution in specific areas of concern to quantify exposure and effects over space and time within a clearly defined exposure period. Chemical exposure can be estimated by measuring the concentration of chemicals in water, sediment, or bivalve tissues, and effects can be estimated with survival, growth, and other sublethal end points (**3**). Although a number of assessments have been conducted using bivalves to characterize exposure by measuring tissue chemistry or associated biological effects, relatively few assessments have been conducted to characterize both exposure and biological effects simultaneously (**2**, **4**, **5**). This guide is specifically designed to help minimize the variability in tissue chemistry and response measurements by using a practical uniform size range and compartmentalized cages for multiple measurements on the same individuals.

1.2 The test is referred to as a field bioassay because it is conducted in the field and because it includes an element of relative chemical potency to satisfy the bioassay definition. Relative potency is established by comparing tissue concentrations with effects levels for various chemicals with toxicity and bioaccumulation end points (**6**, **7**, **8**, **9**, **10**) even though there may be more uncertainty associated with effects measurements in field studies. Various pathways of exposure can be evaluated because filter-feeding and deposit-feeding are the primary feeding strategies for bivalves. Filter-feeding bivalves may be best suited to evaluate the bioavailability and associated effects of chemicals in the water column (that is, dissolved and suspended particulates); deposit-feeding bivalves may be best suited to evaluate chemicals associated with sediments (**11**, **12**). It may be difficult to demonstrate pathways of exposure under field conditions, particularly since filter-feeding bivalves can ingest suspended sediment and facultative deposit-feeding bivalves can switch between filter- and deposit feeding over relatively small temporal scales. Filter-feeding bivalves caged within 1 m of bottom sediment have also been used effectively in sediment assessments from depths of 10 to 650 m (**5**, **13**, **14**). Caged bivalve studies have also been conducted in the intertidal zone (**15**). The field testing procedures described here are useful for testing most bivalves although modifications may be necessary for a particular species.

1.3 These field testing procedures with caged bivalves are applicable to the environmental evaluation of water and sediment in marine, estuarine, and freshwater environments with almost any combination of chemicals, and methods are being developed to help interpret the environmental significance of accumulated chemicals (**6**, **7**, **9**, **16**, **17**). These procedures could be regarded as a guide to an exposure system to assess chemical bioavailability and toxicity under natural, site- specific conditions, where any clinical measurements are possible.

1.4 Tissue chemistry results from short- and long-term exposures can be reported in terms of concentrations of chemicals in bivalve tissues (for example, µg/g), amount (that is, weight or mass) of chemical per animal (for example, µg/animal), rate of uptake, or bioaccumulation factor (BAF, the ratio between the concentration of a chemical in bivalve tissues and the concentration in the external environment, including

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

water, sediment, and food). Tissue chemistry results can only be used to calculate a BAF because caged bivalves in the field are exposed to multiple sources of chemicals and can accumulate chemicals from water, sediment, and food. Toxicity results can be reported in terms of survival (**3**, **18**), growth rate (**3**, **18**), or reproductive effects (**19**, **20**) after a defined exposure period.

1.5 Other modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of standardized tests. Comparisons of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting field bioassays with bivalves.

1.6 This guide is arranged as follows:

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 may involve hazardous materials, operations, and equipment – particularly during field operations in turbulent waters or extreme weather conditions. This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory requirements prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D3976 Practice for Preparation of Sediment Samples for Chemical Analysis

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

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

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1342 Practice for Preservation by Freezing, Freeze-Drying, and Low Temperature Maintenance of Bacteria, Fungi, Protista, Viruses, Genetic Elements, and Animal and Plant Tissues (Withdrawn 2011)[4]

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

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

E1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates

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

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

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

[3] 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.

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

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 a test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test. "Should" is used to state that a specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is 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 other terms used in this guide, refer to Terminology D1129, Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to IEEE/SI 10.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *bioaccumulation factor (BAF)*—the ratio of tissue chemical residue to chemical concentration in the external environment. BAF is measured at steady state in situations where organisms are exposed from multiple sources (that is, water, sediment, food), unless noted otherwise.

3.2.2 *bioassay*—an experiment that includes both an estimate of toxicity and an estimate of relative potency.

3.2.3 *bioavailability*—the fraction of the total chemical concentration in water, on sediment particles, and on food that is available for bioaccumulation.

3.2.4 *biomonitoring*—use of living organisms as "sensors" in water or sediment quality surveillance to detect changes in an effluent or water body or to indicate whether aquatic life may be endangered.

3.2.5 *chemical concentration*—the ratio of the weight or volume of chemicals to the weight or volume of a test sample.

3.2.6 *chemical content*—mass of chemical per whole animal (for example, µg/animal) can be used to normalize the expression of chemical uptake per unit time by eliminating the effects of growth on changing tissues masses.

3.2.7 *chemical fingerprinting*—the use of specific patterns in the ratios of chemicals accumulated in bivalve tissues to identify chemical sources; for example, the ratio of PAH alkylated homologs to parent compounds.

3.2.8 *compartmentalized cage*—a rigid or flexible mesh cage with individual compartments for holding bivalves in a controlled position so that multiple measurements can be made on the same individual organism. The compartmentalized cage helps maximize water flow around individual test organisms and provides even exposure to the test environment.

3.2.9 *growth dilution*—a process whereby the rate of accumulation is exceeded by the rate of tissue growth so that when the concentration is expressed on mass of chemical per mass of tissue over time, it appears as though depuration or elimination is occurring because the concentration (µg/g) is decreasing.

3.2.10 *reference station*—a station similar to the test station(s) in physical and chemical characteristics and with relatively little to no contamination by the particular chemical(s) under study. A reference station should ideally contain only background concentrations of chemicals characteristic of the region.

3.2.11 *scope for growth*—an integrated physiological measure of the energy status of an organism at a particular time, based on the concept that energy in excess of that required for normal maintenance will be available for the growth and reproduction of the organism.

3.2.12 *shell length*—the distance from the tip of the umbo to the distal valve edge.

3.2.13 *site*—a geographical area within a somewhat defined boundary that is being studied. The size of a site is dependent on the extent of suspected perturbation, generally on the order of 0.1 to 50–km^2. Part of the vagueness in size is due to variability in spatial scale and inadequate results from preliminary reconnaissance survey that clearly define the boundary of suspected stressors.

3.2.14 *steady state*—the state in which fluxes of material moving bidirectionally across a membrane or boundary between compartments or phases have reached a balance. An equilibrium between the phases is not necessarily achieved.

3.2.15 *station*—a specific sampling location or area within a site. The size of a station can vary from a single point with one cage to an area of approximately 10 by 10 m including several cages. Vagueness in size is due to variability in spatial scale and experimental design. Several stations in a small geographic area could comprise a site.

3.2.16 *tissue loss magnification*—the process whereby the tissue mass is lost during the exposure period and the chemical mass remains constant over time, so that when the concentration is expressed on mass of chemical per mass of tissue over time, it appears as though bioaccumulation is occurring because the concentration (µg/g) is increasing.

3.2.17 *uptake*—acquisition of a substance from the environment by an organism as a result of any active or passive process.

3.2.18 *whole-animal wet-weight*—the wet weight (g) of the entire bivalve, including water trapped between the valves.

4. Summary of Guide

4.1 This guide describes procedures for exposing marine, estuarine, and freshwater bivalves to chemicals in water, sediment, and food in the field under natural in-situ field conditions. The purpose of this guide is to provide a standard approach for in-situ testing with bivalves. Because of its application to a wide variety of species, many of which have a range of tolerance limits for water quality conditions, it is outside the scope of this guide to provide the tolerance limits for all water quality conditions for all species that can be used for in-situ testing. Tolerance limits are provided for selected species as examples and points of reference. (6.4)

4.2 The approach can be used to characterize exposure and effects over space and time. The primary measurement end points are bioaccumulation of chemicals in bivalve tissues to assess biological availability or bioaccumulation potential, and sublethal effects, like growth, to assess adverse biological effects. The bioavailability of chemical(s) in water, sediment, and food and associated biological effects are determined by the relative differences in these exposure and effects end points among stations over time.

4.3 In practice, the two most commonly measured effects end points are survival and growth. Survival is the easiest effects end point to measure and provides an estimate of toxicity in short- or long-term exposures. The survival end point may be insensitive for some chemicals but can provide important corroborative effects information. Sublethal end-points like growth are generally more sensitive. Growth can be esitmated from changes in whole-animal wet-weight, shell length, tissue weight, or shell weight, with baseline tissue and shell weights for the entire test population estimated from a subsample of that population. Reproduction is another sensitive effects end point, but is more difficult to measure.

4.4 Bioaccumulation and growth are compared among test stations for ranking purposes, among reference and treatment stations, or among stations for temporal and spatial variability as well as short- and long-term trends. It is also possible to use the data to construct dose-response relationships (**6**, **7**) and to identify sources of point and non-point discharges by comparing bioaccumulation and biological effects at various distances away from suspected sources of contamination in a gradient approach (**21**).

5. Significance and Use

5.1 The ecological importance of bivalves, their wide geographic distribution, ease of handling in the laboratory and the field, and their ability to filter and ingest large volumes of water and sediment particles make them appropriate species for conducting field bioassays to assess bioaccumulation potential and associated biological effects. The test procedures in this guide are intended to provide guidance for conducting controlled experiments with caged bivalves under "natural," site-specific conditions. It is important to acknowledge that a number of "natural" factors can affect bivalve growth and the accumulation of chemicals in their tissues (Section 6, Interferences). This field bioassay can also be conducted in conjunction with laboratory bioassays to help answer questions raised in the field exposures. The field exposures can also be used to validate the results of laboratory bioassays.

5.2 The ultimate resources of concern are communities. However, it is often difficult or impossible to adequately assess the ecological fitness or condition of the community or identify and test the most sensitive species. Bivalves are recommended as a surrogate test species for other species and communities for the following reasons: (*1*) They readily accumulate many chemicals and show sublethal effects associated with exposure to those chemicals (**2**); (*2*) they accumulate many chemicals through multiple pathways of exposure, including water, sediment, and food (**22**, **23**, **24**, **25**, **26**, **27**), and (*3*) caged bivalves have been shown to represent effects on the benthos more accurately than traditional laboratory tests (**28**, **29**). Although bivalve species might be considered insensitive because of their wide use as indicators of chemical bioavailability, it has been suggested that sensitivity is related to the type of test, end points being measured, and duration of exposure (**2**). In short-term toxicity assessments in which survival is the end point, bivalves may appear to be more tolerant to and less affected by chemicals because of their ability to close their valves for short periods and avoid exposure (**30**, **31**, **32**, **33**). However, studies comparing the mortality end point in bivalves and other test species have found bivalves to be equally (**34**, **35**) or more sensitive (**36**, **37**) than the other species (Table 1). When the bivalve growth end point was compared to the mortality end point in other test species, the bivalve growth end point was more sensitive (**18**, **28**, **29**, **38**, **39**).

5.2.1 Chronic tests designed to monitor sublethal end points, such as growth, are recommended because bivalves generally show increasing sensitivity with increasing exposure period. Sublethal end points measured in bivalves that have demonstrated high levels of sensitivity include growth (**3**, **18**), reproduction (**19**), DNA damage (**40**, **41**), metallothioneins and other biochemical markers (**42**, **43**, **44**).

TABLE 1 Relative Sensitivity of Bivalves Compared to Other Test Species

Bivalve Species	Species Compared	Exposure	End Point	Sensitivity
Anodonta grandis (**35**) (giant floater; currently *Pyganodon grandis*)	daphnia, fathead minnow, rainbow trout	municipal effluent	LC-50	equal
Anodonata imbecilis (**36**) (paper pondshell; currently *Utterbackia imbecilis*)	daphnia	pulp and paper mill effluent	10-d vs 7-d mortality	more
Anodonata imbecilis (**34**) (paper pondshell; currently *Utterbackia imbecilis*)	daphnia, midge, fathead minnow	metals	7-d mortality	equal
Musculium transversum (**37**) (fingernail clam)	17 diferent species	ammonia	20-d mortality	more sensitive than 16 species
Mercenaria mercenaria (**28**, **29**) (hard clam)	2 amphipods, microtox	sediment	7-d growth, 10-d mortality	more
	Caged *Mercenaria* more sensitive than lab *Mercenaria* (**28**, **29**)			
Mullinia lateralis (**38**) (dwarf surf clam)	amphipod	sediment	7-d growth, 10-d mortality	more
Mytilus galloprovincialis (**18**) (Mediterranean mussel)	amphipod	in-situ water column	84-d growth, 10-d mortality	more, [tissue TBT]

5.2.2 There are many field monitoring programs in the US which use bivalves, including the NOAA Status and Trends Program (**45**), the California Mussel Watch (**46**), and the California Toxics Monitoring Program, a freshwater monitoring program (**47**). Similar field-monitoring programs exist in other countries. Numerous laboratory studies throughout the world have examined bioaccumulation and biological effects in bivalves. The existing databases which have compiled bioaccumulation and effects in bivalves and other species (**8, 9**) make it possible to use tissue residues associated with effects in bivalves as surrogates to estimate effects in both water column and benthic organisms in many freshwater, estuarine, and marine environments.

5.3 Bivalves are an abundant component of many soft bottom marine, estuarine, and freshwater environments. Intertidal marine bivalves make up a significant portion of many habitats and provide habitats for many additional species. It is important to monitor freshwater bivalves for the following reasons: they are among the first taxa to disappear from benthic communities impacted by chemicals; they have been shown to be more sensitive than several other major taxa in laboartory tests.(**48**) The threatened and endangered status of many freshwater bivalve species also make them an important group to monitor.

5.4 If practical, the species to be used in a field bioassay should be one that is endemic to the area under investigation. In many cases, the specific area under investigation may not support bivalves due to a variety of factors including high concentrations of chemicals, competition or predation, or lack of suitable habitat or substrate. Under these conditions, it may be desirable to use a species that would normally be found in the environment if all conditions were favorable; however, it may be necessary to use a surrogate species, that is, a species that can tolerate the environmental conditions but is not normally found in the area, if native species are unavailable in the test area.

5.5 Bivalves generally utilize one of two primary modes of feeding: filter-feeding or deposit feeding. However, all known deposit-feeding bivalves are facultative in that they can either deposit- or filter-feed. Filter-feeders assimilate dissolved organics as well as suspended particulate matter, including plankton and suspended sediments, from the water column and have the potential for exposure to chemicals associated with this ingested material. Facultative deposit-feeding bivalves can be exposed to chemicals associated with sediments as they ingest sediments. They also ingest particulate material from the water column as they filter feed. As such, bivalves are capable of integrating exposure to chemicals dissolved in water and sorbed on sediment particles on the bottom or in suspension. It should be acknowledged that bivalves transplanted in the overlying water above sediment or transplanted directly on or in sediment may not exclusively accumulate or be affected by chemicals in a particular medium. That is, bivalves in or on sediment may still filter and accumulate chemicals from overlying water. Conversely, bivalves transplanted in the water column may filter suspended sediment and accumulate chemicals from that sediment. Bivalves can also assimilate chemicals as they ventilate overlying water.

5.6 Field bioassays are conducted to obtain information concerning the bioavailability of chemicals in the water column or bedded sediments and subsequent biological effects on bivalves after short- and long-term exposure to water and sediment under site-specific conditions. These bioassays do not necessarily provide information about whether delayed effects will occur, although a post-exposure observation period could provide such information. Sublethal post-exposure observations may include gonad development, spawning success, gamete survival, and development. The decision to conduct post-exposure studies in the field or in the laboratory depends on the observations being made, test conditions required, and experimental logistics.

5.7 The in-situ exposures described in this guide could be followed by laboratory measurements, such as scope for growth (**2**), filtration rate (**49**), byssal thread production (**50, 51, 52**), and biomarkers (**53, 54**).

5.8 The bivalve field bioassay can be used to determine the spatial or temporal trends of chemical bioavailability in water and sediment and effects due to exposure to those chemicals. Spatial comparisons of parameters of concern can be made by distributing the caged bivalves along physical and chemical gradients at scales commensurate with the desired level of discrimination. For example, station locations might be distributed along a known physical or chemical gradient in relation to the boundary of a disposal site (**55, 56, 57, 58, 59**), sewage outfall (**60**), or effluent pipe or at stations identified as containing elevated concentrations of chemicals in water or sediment as identified in a reconnaissance survey (**3, 61, 62**). This can be accomplished by placing caged bivalves along horizontal transects or at different depths in the water column. Temporal comparisons can be made by conducting before-and-after studies. For example, the effectiveness of dredge activities, effluent diffuser construction, effluent reduction, or remedial action can be determined by conducting field bioassays before the action, during the action, and after the action.

5.9 The relative bioavailability of chemicals from the various pathways of exposure (that is, aqueous phase, suspended particulate matter, sediment) and subsequent effects can be determined by simultaneously deploying bivalves with different feeding strategies and making supplementary measurements. A combination of filtration and the use of sediment traps followed by chemical analysis of the various environmental compartments can be used to identify the relative contribution of the aqueous phase, suspended particulate matter, and sediment. Lipid bags or semi-permeable membrane devices (SPMDs), which predominantly collect the dissolved fraction of chemicals, could also be used (**63, 64, 65, 66, 67**). The bioaccumulation of chemicals and effects among different bivalve species deployed side-by-side can be compared and used to help explain the spatial variability of chemical contamination, particularly if the different species are placed in different locations (that is, in the water column, on top of the sediments, within the sediments). This field assessment approach could be supplemented with laboratory studies designed to answer specific questions regarding dissolved versus particulate pathways of exposure.

5.10 Results of bivalve field bioassays might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E1023) or when deriving water or sediment quality guidelines for aquatic organisms (**15, 68**). They might also be useful for establishing tissue residue criteria. Bivalve field bioassays can be useful in making decisions regarding the extent of remedial action needed for contaminated sites. They also provide a convenient method for manipulative field experiments, hypothesis testing, and monitoring specific sites before, during, and after cleanup operations (**61, 62**).

6. Interferences

6.1 As with all bioassay procedures, there are limitations to the methods described in this guide. However, these limitations should not be considered as a reason for not using the methods described in this guide.

6.2 Results of bivalve field bioassays will depend, in part, on natural factors, including temperature, food supply, other physical and chemical properties of the test environment, selection of adequate reference areas, species selected, condition of the test organisms, exposure technique, and handling of the bivalves prior to deployment. Taking bivalves out of their habitat and weighing and measuring them may be stressful to the bivalves. The degree of handling, holding time, and differences between water and sediment conditions at the collection site versus the transplant site may also be stressful. Careful handling and appropriate acclimation can minimize these stresses.

6.3 Condition of the test organisms is critical to the success of the field bioassay. The most important consideration is spawning cycle because of possible interferences on bioaccumulation and growth and with subsequent data interpretation. Generally, chemicals are lost during spawning, resulting in potential underestimation of chemical bioavailability (**69**). Conversely, the energy used for gonad development and spawning can make bivalves more sensitive to chemicals, reduce their growth rates, and overestimate potential toxicity. Tests should be conducted with populations that will most likely not spawn during the exposure period. The spawning cycle of candidate test species should be evaluated prior to developing the study design, and species that do not spawn during the proposed exposure period should be selected.

6.4 Temperature of the test environment could affect both bioaccumulation and biological effects. Water temperatures should be monitored over the course of the study to quantify the exposure conditions and the potential effects of temperature. As a general guide, examples of temperature tolerance for the most commonly used species are provided in Table 2. Temperature conditions during the exposure period can be quantified using in-situ monitoring devices. These devices can be attached to the deployment cages and set to collect temperature data at specified time intervals for the duration of the test.

6.5 Lack of acclimation to deployment water quality conditions could be an interference. If water quality conditions differ at collection and deployment sites, it may be necessary to acclimate the test organisms gradually to the deployment conditions. This transition is particularly important near the bivalve's tolerance limits and may be accomplished using serial water dilutions until the proper water quality conditions (for example, temperature, salinity, and pH) are reached. Acclimation for temperature should proceed no faster than 3°C in 72 h (Guides E1022 and E1688). Once acclimated, bivalves should be maintained under these conditions for a minimum period of time. Holding bivalves for extended periods under laboratory conditions can induce stress because bivalves are particularly sensitive to temperature, nutrition, and water flow. If test specimens are held for an extended period of time in the laboratory, the effect of this holding can be assessed by comparing soft tissue weights, or other indicators of bivalve health, to that of bivalves of the same size group freshly collected from the field. Alternatively, bivalves could be acclimated in the field under conditions similar to the proposed transplant sites.

6.6 Food supply is important because it affects both biological availability and associated biological effects. Food availability may be more difficult to quantify during the test than temperature or other physical factors. Until in-situ monitoring devices for chlorophyll and other nutrient sources are developed, it is suggested that food availability be estimated at least three times during the study (that is, beginning, middle, and end of test). The measurements made (that is, chlorophyll-a, particulate or total organic carbon, and suspended solids) will depend on the feeding strategy of the test species.

6.7 Current speed is important for filter-feeding bivalves because currents regulate the food supply to the test organisms. Currents are also important to facultative deposit-feeding and filter-feeding bivalves in the benthos because flushing may reduce the potential effects of chemicals by dilution with clean water from outside the assessment area. Currents can be quantified during the exposure period with a continuously recording, in-situ current meter or quantified intermittently during the suggested sampling intervals used to measure food availability.

6.8 Salinity is particularly important in estuarine areas, where salinity can range from 0 ppt at the head of a river to 33 ppt at the mouth. Salinity should be evaluated prior to species selection. If there is a wide salinity range, it may be necessary to identify two or more bivalve species for the assessment: one species for the lower end of the salinity range and another for the upper end of the salinity range. It is recommended that both species be deployed in the area where salinity is in the middle, as this provides a means to compare results between species.

6.9 Possible interferences influencing retrieval of test organisms from the field include caged bivalves being washed away during storm events, buried by underwater sediment shifts, theft, vandalism, fouling, disease, and consumption by predators.

6.10 Depending on the environment under assessment, it is possible for the bivalve cages, including the external predator mesh (see 11.3) and the mesh bags, to become fouled with both epiphytic plant and animal growth. Fouling occurs most

TABLE 2 Temperature (°C) and Salinity (Parts per Thousand (ppt)) Tolerance Limits for Selected Bivalve Species (Months when spawning may occur and species distribution are also shown)

Species and Reference	Temperature Range	Salinity Range	Spawning	Distribution
Corbicula fluminea (Asian clam) (70)	2–25	0–5	may be continuous, usually twice/year spring/early summer; later summer	All west, gulf, and east coastal United States to DE River; NM; OH & MS River systems
Dreissena polymorpha (Zebra mussel) (71)	<0–35	0–6	May to September	Canada and Northeastern United States; Great Lakes, St. Lawrence River; MS, OH, IL & TN River drainages; NY Canals, Hudson River, Finger Lakes
Elliptio complanata (Eastern Elliptio) (72, 73, 74)	0–30	0–3	most June to July; some May to September	Gulf St. Lawrence to GA; Great Lakes, except Lake Michigan & Lake Erie
Pyganodon (Anodonta) grandis (floater mussel) (72)	0–30	0–3	most April to May; some to late August	Canada Interior & St. Lawrence River drainage; Hudson Bay, MI and MO Rivers drainages; NM, CO, TX, Mex
Rangia cuneata (Atlantic Rangia) (75, 76)	8–32	0<19	VA: early April to summer; FL: July-November; LA: Mar-May and late summer to November; Mexico: February-June and September to November	Gulf of Mexico coast from northwest FL to Campeche, Mexico; along Atlantic coast to NJ
Argopecten irradians (Bay scallop) (76)	>7 >30	>14–28	mid-Atlantic: mid-April through early September; NY: June and July; NC and FL: August and December	Atlantic coast; Cape Cod to Gulf of Mexico
Crassostrea gigas (Pacific oyster) (76)	4–24	25–35	July to August	Pacific coast; Pacific Northwest
Crassostrea virginica (Eastern oyster) (77)	-2–36	5–32	Gulf of Mexico: April-October; Malpeque Bay, PEI: July-August; Bideford River Estuary, PEI: July	Gulf of Mexico to Cape Cod
Macoma balthica (Baltic clam) (78, 79, 80)	-2–23	5–30	June-August (Europe); July-September (United States)	Greenland to France; Baltic and Wadden Seas; UK; N. Canada to Chesapeake; AK to San Francisco Bay
Mercenaria mercenaria (Hard clam) (81)	<0–35	12–35	March-November depending on latitude and temperature. Peaks in July	Atlantic and Gulf coasts; abundant MA to VA
Mya arenaria (Soft-shell clam) (82)	-1.7–32	10–32	June-September; once/year north of Cape Cod, twice/year south of Cape Cod	Atlantic coast from Labrador to SC; less in FL; AK and CA
Mytilus californianus (California mussel) (76)	7–28	25–33	Continuous throughout year; peaks in July and December	AK to southern CA
Mytilus edulis (Blue mussel) (83)	0–27	5–33	differs between populations; some low-level thoughout year; first in early summer, second in the fall	Atlantic coast, from Labrador to Cape Hatteras, NC
Mytilus galloprovincialis (Mediterranean mussel) (84)	8–25	10–33	Similar to *M. edulis*, but several weeks later when temperature is maximum	Mediterranean, Europe, Atlantic France and British Isles, Japan, East China to Korea, Australia, South Africa; southern CA to OR
Mytilus trossulus (Pacific blue mussel) (85)	0–29	4–33	July to September	Baltic Sea; west Coast, Central CA to AK; east Coast, Canadian Maritimes
Ostrea lurida (Olympia oyster) (86)	6–20	NA–25	Spring to fall: peaks in spring in south, mid summer in mid-range and north	Southeast AK to Baja California
Protothaca staminea (Littleneck clam) (87)	0–25	20-32	BC, Canada, January to March; AK, mid-July; southern CA, June	Aleutian Islands, AK to Cape San Lucas, Baja California
Venerupis japonica (Manila clam) (76)	13–21	24–31	Washington: once/year May-September; peaks in June/July	British Columbia to CA

frequently in highly productive embayments or areas with restricted flow, such as marinas. Excessive fouling can reduce or eliminate flow of water through the cage material, resulting in highly stressful conditions to the test bivalves. If such conditions are anticipated, the deployed cages should be examined for fouling at regular intervals during the exposure period. Fouling organisms can be removed from the exterior surfaces of the cages by hand or with a stiff brush. If the cages are heavily fouled and it is difficult to remove the attached biomass with brushing or scraping, the bivalves should be transferred to clean, unfouled cages for the remainder of the exposure period.

6.11 Possible interferences associated with interpretation of tissue chemistry data include the use of inappropriate analytical procedures. It is critical to use the most appropriate method for each chemical analysis. Some commonly used methods, may not always be appropriate. For example, when measuring the suite of PAH-alkylated homologs, it is essential to use sufficient silica gel to clean up excess lipids in the sample. A more specific approach for these analyses developed as part of the Exxon Valdez oil spill assessment program included advanced methods specific to that group of researchers. These methods are recommended for bivalve tissues when source identification through chemical fingerprinting is necessary (88, 89, 90, 91).

6.12 Natural variability in the concentrations of chemicals of concern in water or sediment coupled with intermittent chemical discharges may increase the difficulty in interpreting exposure concentrations in these pathways. However, weekly measurements of chemicals in the water column coupled with measurements of bioaccumulation and growth have proven effective in explaining the environmental significance of these variables (3, 18). In practice, it is usually difficult to sample with that frequency, and water samples are generally taken

only at the beginning and end of the test. Since the variability in sediment chemistry is generally less extreme than in water, collecting sediment samples for chemical analysis at the beginning and end of test may be sufficient to characterize exposure conditions. However, sediments may also be highly variable on a small spatial scale (**92**).

6.13 In assessing effects of effluents with high organic loads, it is possible that the organic enrichment from the effluent will increase bivalve growth rates and make it more difficult to assess the adverse effects of associated chemicals. Differentiating between the positive effects of nutrient enrichment and the adverse effects of toxic chemicals is best accomplished by maximizing the number of stations in the assessment area, deploying caged bivalves at various depths, and maximizing the number of effects end points. The processes involved could be better characterized and understood by using various biomarkers in addition to the bioaccumulation and effects end points (**93**).

7. Hazards

7.1 Water and sediment might be contaminated with unknown concentrations of many potentially toxic materials. Any potentially contaminated water or sediment should be handled in a manner to minimize exposure of personnel to toxic compounds. Therefore, skin contact with all potentially toxic sediments and overlying water should be minimized by such means as wearing appropriate protective gloves, laboratory coats, aprons, and glasses particularly when washing equipment or placing hands into test water, effluents, sediment, or cleaning solutions. Respirators may also be necessary in some hazardous waste sites or during oil spills.

7.2 Water and sediment, particularly in effluent areas, might contain organisms that can be pathogenic to humans. Special precautions when working in these areas might include immunization prior to deployments and the use of bactericidal soaps after working in the water and touching sediments.

7.3 Use of ground fault systems is strongly recommended during measurements at the beginning and end of the tests where electronic equipment such as portable computers are used to record data electronically to help prevent electrical shocks because water is a good conductor of electricity.

8. Experimental Design

8.1 Field bioassays can be designed to provide either a qualitative reconnaissance or a quantitative assessment involving statistical comparisons of measured end points (that is, chemical concentration in tissues and effects end points) among stations. The object of a qualitative reconnaissance survey is to identify sites with the potential for bioaccumulation and associated biological effects. Qualitative surveys are often conducted in areas where little is known about contamination patterns. Quantitative assessments are conducted to test for statistically significant differences among stations.

8.2 Experimental design considerations, such as station location, number of stations per site, number of cages per station, and number of bivalves per cage, should be based on the purpose of the test and the procedure(s) used to analyze the results. Various experimental designs can be applied, with the most common used to:

(1) Compare bivalve tissue chemistry and growth at one or more stations to reference, background, or pre-test conditions.

(2) Compare bivalve tissue chemistry and growth among multiple stations to characterize patterns, trends, or gradients.

8.3 Experimental control of all test variables can be difficult to achieve in field tests that assess or monitor resident populations. The use of in-situ field bioassays allows the investigator to control the following: species; number; and size range of test animals, specific location(s) to be assessed, and exposure duration. Generally, the concentration of chemicals of concern and natural factors, such as temperature, salinity, dissolved oxygen, pH, current speed, and food supply, are not manipulated or controlled as they are in laboratory testing. However, temperature could be increased by heating dissolved oxygen by aeration, current speed by pumping, and food supply by adding nutrients. The intent of field bioassays is to determine chemical bioavailability and subsequent effects under natural, site-specific conditions, which includes intrinsic, site-specific variability. With an adequate number of stations, statistical testing can often identify the importance of these uncontrolled variables with respect to exposure and effects.

8.4 *Measurement End Points*

8.4.1 At a minimum, biological effects should be characterized by measuring survival and growth. Survival may not always be a very sensitive indicator of effects in bivalves (**3**), but it is an important parameter to monitor. Several factors can affect survival, including handling prior to test initiation and physical-chemical factors at the deployment stations. Survival can be easily quantified, although it is possible to have some individuals missing at the end of the test due to shell decomposition. Under some circumstances, more individuals may be present at the end of the test than at the start. This would most likely be due to the settlement of juvenile bivalves during the course of the test. This can easily be accounted for as new recruits should be smaller than the test bivalves. All recruits should be removed prior to determining survival and assessing effects end points. Only effects measurements from surviving bivalves should be used to calculate summary statistics. It is possible for shells to stick together due to mucilaginous material or sediment within the shells, prohibiting a precise determination of death. Thus, all dead bivalves may not be identified until the tissue removal process when the shells are opened to reveal the internal tissues.

8.4.2 Growth is a sensitive sublethal effects end point that is easy to measure and is recommended for all field bioassays. It is generally more sensitive than mortality, and reductions in growth have been related to adverse effects on bivalve populations (**1**). As many growth end points as are practical should be measured for assessing growth in a weight-of-evidence approach. For example, it has been shown that shell growth and tissue growth are decoupled. Measuring only one of these end points could provide misleading results and lead to a spurious interpretation of environmental effects on growth (**94**,**95**). Growth end points include, but are not limited to, whole-animal wet weight, shell length, tissue weight, shell weight

(18). Whole-animal wet-weights and shell lengths are nondestructive measurements and can be made multiple times over the course of the exposure period. At a minimum, whole-animal wet-weights and shell lengths should be measured at the beginning and end of the test. Since tissue weights and shell weights provide a different perspective on animal health and may be related to different stressors, they should also be measured at the beginning and end of the test (18). Because these measurements are destructive, beginning-of-test tissue and shell weights for bivalves to be deployed can be estimated from a subsample of the bivalves distributed to the individual cages. The bivalves used to estimate beginning-of-test tissue and shell weights should be within the same size range as those identified for deployment. Because the initial tissue and shell weights are based on a subsample of the test population, the change in these metrics over the test period is an indirect determination and has some uncertainty. However, tissue and shell weights can provide the most discriminating measurements under certain conditions, particularly when growth rates are low (18).

8.4.3 Although tissue dry weights are less variable than wet weights, drying the tissues has some limitations. *(1)* It is more time consuming to dry all the tissues and make the weight measurements. *(2)* In a combined bioaccumulation and biological effects test, the same wet tissues can be used for chemical analysis and wet-weight measurements (drying tissues may destroy organic chemicals, and limit their ability to predict bioaccumulation potential). *(3)* A wet-weight approach has been used successfully (18), and may provide better correlations with other growth metrics. Nevertheless, if additional testing clearly demonstrates an advantage to measuring dry weights, or if particular studies require more emphasis on the accuracy of tissue weight measurements, it would be relatively simple to alter the procedures accordingly.

8.5 *Reference Stations*—The use of one or more reference stations may be used for field bioassays with caged bivalves. It is the responsibility of the investigator to determine the need for reference station(s) based on the experimental design. It may be difficult or problematic to identify a true reference in the field because of the variability in field conditions and the influence of natural factors on site-specific conditions. If reference stations are used, the physical and natural factors (that is, temperature, salinity, dissolved oxygen, vegetation, and currents) at the reference station(s) should be as similar as possible to those conditions at the area under investigation. Multiple reference stations may help account for natural differences and variability among uncontaminated areas. It may be more useful to employ a gradient design with decreasing chemical gradients in bivalve tissue chemistry associated with changes in growth rate rather than comparing treatments to reference conditions or upstream versus downstream sites.

8.6 Natural population of bivalves could also be used for comparative purposes, but these comparisons should be made cautiously because there is evidence that caged bivalves can have different growth rates and different rates of accumulation than natural populations under certain conditions (3, 11, 12). It would be useful if growth rates of natural populations and caged bivalves were compared, if practical.

8.7 *Statistical Design*—Field bioassays with caged bivalves can be used to support a variety of statistical designs. The experimental design is a function of the technical and environmental issues to be answered as well as the most appropriate statistical design for analyzing the data. The level of replication is a function of desired power and confidence. The following null hypotheses can be used to determine statistical differences in bivalve bioaccumulation and associated biological effects among stations as well as relationships between tissue chemistry, sediment or water chemistry, if measured, and measured effects:

(1) Null Hypothesis #1—There is no difference in bioaccumulation of chemicals of concern (as determined by tissue burdens) between test and reference station(s),

(2) Null Hypothesis #2—There is no difference in effects between test and reference station(s),

(3) Null Hypothesis #3—There is no relationship between effects end points in bivalves and tissues, water, or sediments containing chemicals of concern among stations.

(4) Null Hypothesis #4—There is no relationship between bioaccumulation and associated biological effects with distance from the suspected chemical source.

The preceding null hypotheses can also be used when it is appropriate to pool the stations to allow comparisons among sites. It may also be appropriate to apply these hypotheses to gradient designs in both horizontal and vertical planes in the water column or in bottom sediments.

8.8 *Replication*—The individual bivalves or the cage may be considered as the experimental unit. It is the investigator's responsibility to define the experimental unit and level of replication, which are appropriate for the study design. Additional guidance on statistical approaches can be found in other ASTM standards (E1847 and E1191). The distance between stations, or cages, is a function of the size of the area under investigation, the expected gradient or change in monitoring parameter(s), and the expected variability in conditions. Typically, stations can be placed 50 to 500 m apart. However, stations can be closer together, or further apart, as determined during development of the study design and hypotheses.

8.8.1 For the exposure assessment, a chemical replicate may be formed by combining the tissues of all living bivalves from one cage (see 9.3). Compositing may be necessary because, in most cases, individual bivalves do not contain sufficient tissue for chemical analysis. The cage can be used as a way to identify the bivalves to be combined for a chemical replicate. The number of chemical replicates prepared for each station depends on the level of replication desired for the bioaccumulation assessment. If statistical comparisons are desired, a minimum of three replicate tissue samples for each station is recommended. The number of bivalves required for each tissue sample is a function of the tissue mass requirements for the chemical analyses being performed and the tissue mass of the individual bivalves. The analytical laboratory performing the chemical analyses should be contacted to identify the amount of tissue required for each analysis. For example, if the analytical laboratory requires a minimum of 50 g wet tissue, and the average individual tissue weight is 0.5 g wet, then a minimum of 100 bivalves will be required for each chemical

composite (that is, 100 bivalves per cage). With larger bivalves there may be sufficient tissue to conduct chemical analyses on individuals, particularly if only a few chemicals are being analyzed. This approach could improve the discriminating power of the assessment.

8.8.2 For the effects assessment, each individual bivalve may be considered a replicate, although concerns about individuals in the same experimental unit have been noted (Guide E1847). The bivalves within a predetermined size range are assigned to cages (see Section 11), and the cages are randomly assigned to stations. Independence among bivalves within each cage is assumed. In addition to the tissue chemistry biomass requirements, the minimum number of bivalves per cage should also consider the following with respect to effects endpoints: (*1*) the expected variance within cages, (*2*) the expected variance between cages, and (*3*) either the maximum acceptable width of the confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing.

8.9 *Statistical Analyses and Data Interpretation*—The calculating procedure(s) and interpretation of results should be appropriate to the experimental design. Procedures used to calculate results of these field bioassays can be divided into two general categories: those that test hypotheses regarding differences among stations, and those that establish relationships along suspected chemical gradients or between bioaccumulation and growth in the test organisms. No procedure should be used without careful consideration of (*1*) the advantages and disadvantages of various alternative procedures, and (*2*) appropriate preliminary tests such as those for outliers and heterogeneity. Preprocessing of data might be required to meet the assumptions of the analyses. All parameters measured at the end of the test (that is, whole-animal wet-weight, shell length, tissue weight, shell weight, and chemical concentrations in tissues) can be statistically analyzed. Summary statistics (for example, mean and standard deviation) can be calculated for each of these parameters on a station-by-station basis. The appropriate statistical test is a function of experimental design, hypotheses, and measurement end points. It is the investigator's responsibility to identify the appropriate statistical tests. In general, ANOVA and multiple comparison tests are used for hypothesis testing and comparison among stations. Linear regression analysis is generally used to establish relationships between bioaccumulation and growth end points along suspected chemical gradients and to establish relationships between bioaccumulation and growth. If statistical differences are found, a multiple range test can be used to determine which stations are different from the others. A textbook on statistical analyses of biological data can be referenced for appropriate tests and procedures (**96**, **97**, **98**).

8.9.1 Power analyses performed on data from caged bivalve studies in Alaska indicate that between 100 and 300 mussels per station are sufficient to detect differences in weight on the order of 0.2 and 0.1 g wet, respectively. An environmental significance, or likely adverse effect to the community, is expected when both a statistically significant difference is observed ($\alpha = 0.05$) and there is a 10 to 25 % absolute difference between the test and reference/control station(s) (**5**).

8.10 *Test Duration*—For most studies, bivalves should be exposed to site-specific conditions for a minimum of 30 days. An exposure period of less than 30 days is not generally recommended unless the chemicals of concern are low molecular weight organic compounds, such as some PAHs. Equilibrium for most other chemicals, such as metals and high molecular weight organic compounds, is generally achieved in marine and freshwater bivalves within a period of approximately 60 to 90 days (**3**, **11**,**12**, **15**, **60**, **99**, **100**, **101**, **102**, **103**, **104**). If both exposure and effects end points are being measured, it may be advantageous to continue the test for 60 to 90 days to facilitate chemical equilibrium and provide sufficient time to allow adverse effects to manifest themselves. Extending the exposure period may also increase the ability to detect statistically significant differences among effects end points. Although unlikely, it is possible that deployment of caged bivalves in or on bottom sediments may reduce concentrations of some chemicals. This may be particularly important in very small areas with restricted circulation where bivalves are removing chemicals from sediment. Consistent sediment conditions during the deployment period can be verified by sampling the sediment before and after deployment. It is the responsibility of the investigator to verify concentrations of chemicals in sediment before, during, and after deployment if this is an issue of concern.

9. Apparatus

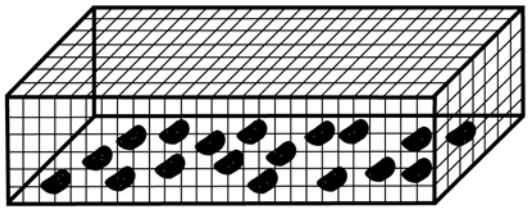
9.1 *Facilities*—Sources of water and power and the ability to be protected from rain, snow, and wind can be of considerable help in sorting the animals at the beginning of the test, making the appropriate measurements, and removing tissues for chemical analysis at the end of the test. Preparations can be made outdoors, but inclement weather can interfere with making accurate measurements. The portable analytical balance is particularly sensitive to wind although some protection can be provided by a wind barrier around the entire area, such as a lean-to, or a smaller barrier such as a box to protect the balance. Making weight measurements aboard boats or floating piers is not recommended, unless the measuring devices are specifically designed for use on unstable platforms. Length measurements made with calipers are not affected by the instability associated with boats or floating piers.

9.2 *Construction Materials*—Equipment such as cages, predator mesh, holding tubs, and ice chests, that contact the test water, sediment, and organisms should not contain substances that can be leached or dissolved by aqueous solutions in amounts that can adversely affect test organisms or be accumulated in their tissues. In addition, equipment that contacts test water, sediment, and organisms should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, high-density polyethylene, polycarbonate, and fluorocarbon plastics should be used whenever possible to minimize dissolution, leaching, and sorption, except that stainless steel should not be used in saltwater. Concrete may be used for cage anchors and rigid plastics (that is, PVC) may be used for cage frames. Plastic Frames and mesh bags should be soaked before use, preferably in flowing fresh or seawater, for at least 24 h to remove water soluble and

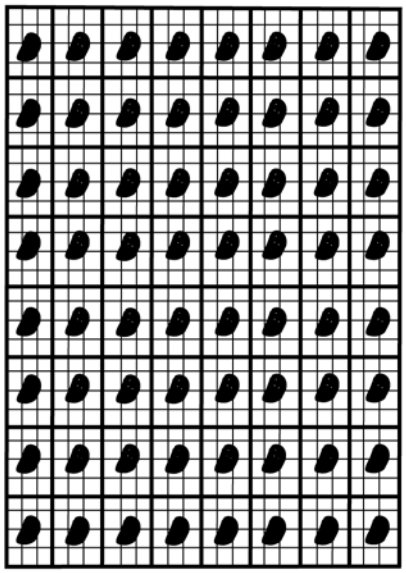
volatile chemicals. Mesh bags, tubes, or trays used to create the compartmentalized cages for holding the mussels during deployment should be made from high-density polyethylene, polycarbonate, or fluorocarbon plastic. Plastic cable ties have many applications during cage construction, such as separating the individual bivalves when mesh bags are used and attaching cages to deployment moorings and lines. Plastic cable ties should not contain metal stops as these can corrode and break upon exposure to water. This corrosion can result in detachment or addition of chemicals. Brass, copper, lead, cast iron pipe, galvanized metal, and natural rubber should not contact water, sediment, or test organisms before or during the test.

9.3 *Cages:*

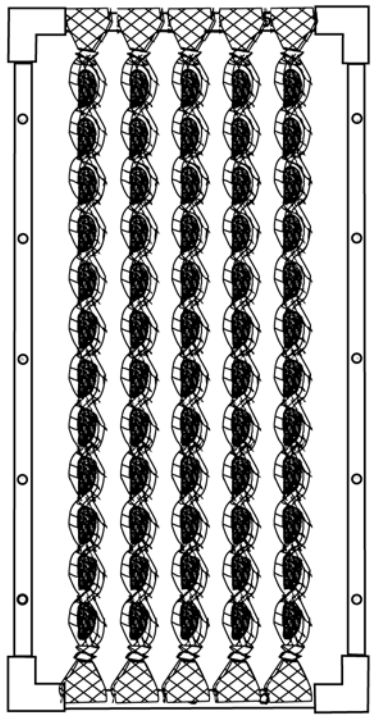
9.3.1 The basic comcept behind the cage design is to maximize water flow to the test animals. This is accomplished by using a mesh size large enough to maximize flow but small enough to contain the test animals. Cages with individual compartments are recommended for field studies with caged bivalves. The separation of individuals into individual compartments allows equal exposure to each bivalve (Fig. 1C). Compartmentalization facilitates tracking individuals throughout the test and eliminates the need to mark or notch individuals. Compartmentalization permits multiple growth measurements on individuals, ensures that an accurate record of measured end points can be maintained on individuals, and facilitates conducting tissue chemistry analysis on individuals



A. Mesh basket (front view)



B. Compartmentalized mesh tray (top view)



C. PVC frame supporting mesh bags (side view)

FIG. 1 Possible Cage Types for In-situ Field Tests With Caged Bivalves

if the individual bivalves contain sufficient biomass. Recording measurement data on an individual-by-individual basis increases the statistical power of the test. Each of the measurement end points, including tissue chemistry, can be paired during statistical analyses.

9.3.2 In its simplest form, in-situ field tests can be conducted with bivalves held in cages without compartments as shown in Fig. 1A (**105**). This approach is not recommended because it limits the ability to make multiple measurements on the same individuals throughout the course of the test. There are techniques for numbering individuals (**106, 107**), but this may be prohibitively time consuming if large numbers of animals are being caged. Numbering with different glues and epoxies could also introduce other potentially toxic chemicals. Cages can also be rigid with fixed compartments (Fig. 1B), as in plastic trays and wire baskets. Rigid cages with fixed compartments have been used in freshwater (**108, 109**) and marine (**3**) environments. Cages can also be a combination of flexible mesh material with compartments attached to a rigid frame (Fig. 1C), as with mesh bags attached to a PVC frame. This approach has been used in freshwater (**3**), estuarine (**62**), and marine habitats (**15**). The flexible mesh bags can also be attched to heavy plastic mesh that serves as protective cage and an attachment point for the bags. The mesh bags used to hold the bivalves are created from tubular oyster culch netting similar to that used in bivalve aquaculture. The bivalves are separated within the mesh bags by placing a plastic cable tie or other restricting device between individuals. Different cage designs have also been tested to compare with the performance of natural bivalve populations. These include rigid cages with and without compartments, corrals that limit the movement of sediment-dwelling bivalves, and leashes where monofilament lines were glued to each bivalve shell (**110**).

9.3.3 The final dimensions of the deployment cages depend on the size of the individual test organisms and the number of organisms per cage. One advantage of using the flexible mesh bags and a PVC frame is that the size of the individual compartments and the overall cage size can be easily adjusted. Sufficient space should be provided in each compartment to allow test animals to open their valves and grow during the exposure period; the amount of space depends on the species used, the size of individuals at the start of the test, and expected increases in growth over the deployment period. For rigid cages, investigators should make the individual compartments large enough to accommodate expected growth during the test. A 6-in. (approximately 15-cm) diameter mesh material is recommended for smaller smoothed-shelled species like mussels and clams because there is less excess mesh at the point of constriction. For larger bivalves with rough shells and irregular shapes, such as oysters, it may be necessary to use a tubing of larger diameter. Because the flexible mesh is tubular in form, it is not necessary to adjust the width/height dimensions. The length of each compartment in the mesh bag (that is, the distance between constricting cable ties) should be large enough to accommodate valve opening and expected growth during the test. The mesh bag should be long enough to accommodate the desired number of bivalves per bag plus sufficient material to allow secure attachment to the PVC frame. Approximately 30 cm of mesh netting on either end of the bag is generally sufficient for attachment to a PVC frame constructed from ¾-in. (approximately 1.90-cm) material. The PVC frame should be approximately 5 cm longer than the space occupied by the bivalves positioned in the mesh bag. The width of the frame should be about 5 cm greater than the distance occupied by all mesh bags to be attached to the frame when laid side-by-side.

9.3.4 If PVC cages are to be deployed on top of sediments, pushed a short distance into the sediments, or positioned where neutral buoyancy is desired, the PVC pipe should be drilled approximately every 24 cm with a ¼-in. (approximately 0.64-mm) hole to allow water to enter the pipe and remove trapped air. The corners of the frame should not be drilled. Drilling the corners could weaken the overall structure of the frame. For water column deployments, flotation can either be increased or decreased depending on whether the PVC frames are drilled to allow a water ballast or left undrilled to add extra flotation.

9.4 *Cage Deployment Configuration*— The methods used to deploy cages and the type of mooring system depends on the experimental design identified for the specific media being assessed and substrates of opportunity. It may be useful to conduct a reconnaissance of the deployment area prior to setting out the cages to allow identification of potential deployment impediments and potential interference from the public. If floating or fixed piers are available in the assessment area, they could provide a potentially effective substrate for attaching bivalve cages. Figs. 2-5 provide various deployment configurations, and for simplicity, only rigid cages are shown. The PVC frames supporting bivalves in mesh bags can also be used in the same deployment schemes. Fig. 2 shows caged bivalves attached to floating (**3**) and fixed piers (**5**). Under most circumstances structures such as piers may not be available and open-water, nonstructural deployments should be used as shown in Fig. 3A (**3**) and Fig. 3B. A more direct assessment of bottom sediment is possible with fixed bottom deployments as shown in Fig. 4A (**3, 61**) and Fig. 4B (**5, 111**)). Caged bivalves can be placed directly on bottom sediment or on legs used to raise the cages above the sediments. Cages with legs can also be used to stabilize the unit and maintain position in high energy areas such as the intertidal zone. The most sophisticated assessments include a gradient design with cages placed at multiple depths and distances from suspected sources, as shown in Fig. 5. Each of the preceding deployment configurations uses rigid or flexible compartmentalized cages. Similar deployment configurations have been used with rigid and flexible uncompartmentalized cages (**21, 55, 112, 113, 114**). Placing cages along suspected chemical gradients in three dimensional space helps to identify not only potential sources of chemicals, but the relative contribution of chemicals in the water column and sediments based on chemical concentrations measured in bivalve tissues.

9.4.1 *Water Column Assessment*—To evaluate the bioavailability and potential effects of chemicals within the water column, cages can be suspended from a fixed mooring (that is, floating pier, piling, or other fixed structure), suspended within the water column by attachment to a line that has an anchor or

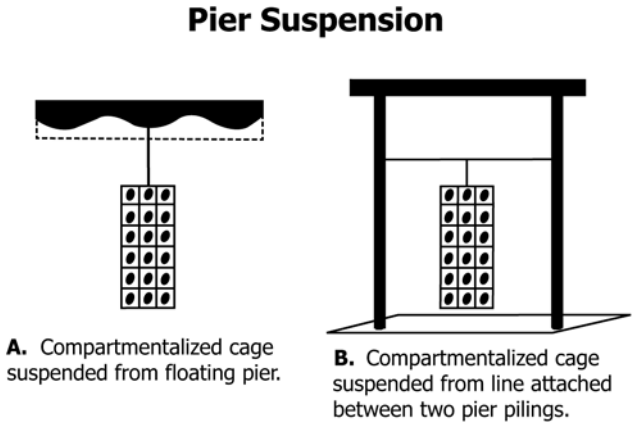


FIG. 2 Pier Deployments: Floating or Fixed

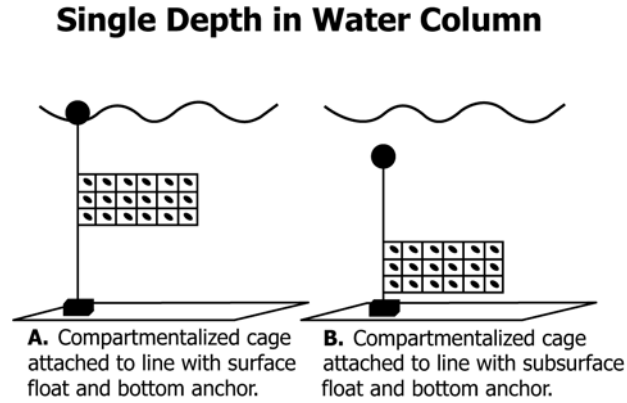


FIG. 3 Open Water, Nonstructural Deployments

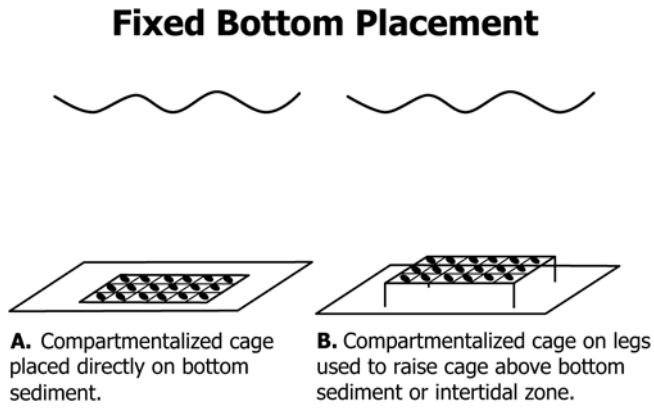


FIG. 4 Fixed Bottom Deployments

weight on one end and a surface or subsurface buoy attached to the other end, or fixed in the water column by attaching legs to the cages and pushing the legs into the sediments to hold the cages in place. Depending on the species of bivalve used, bivalves in cages deployed directly on top of the sediments can be used to assess chemicals within the water column as well. Factors that should be considered during the deployment of cages for surface water assessments include change in tidal height (that is, to ensure the cages are at the desired depth during both low and high tides), bottom slope (that is, to ensure the cages do not slide down a steep slope during the exposure period), and boating activity and recreational activity in the vicinity of the cages (that is, to avoid cages being removed by or tangled within propellers). Floats should be appropriate to accommodate the weight of the line plus cages at the depth of deployment. This type of water column monitoring has been used to evaluate PAHs, dioxins and methylmercury, tributyltin (**4**, **18**) and pulp mill effluents in marine environments. Similar methods have been used to assess freshwater sites (**110**).

9.4.2 *Sediment Assessment*—One way to evaluate chemicals associated with surficial sediments is to position the cages directly on top of the sediments. This facilitates positioning the

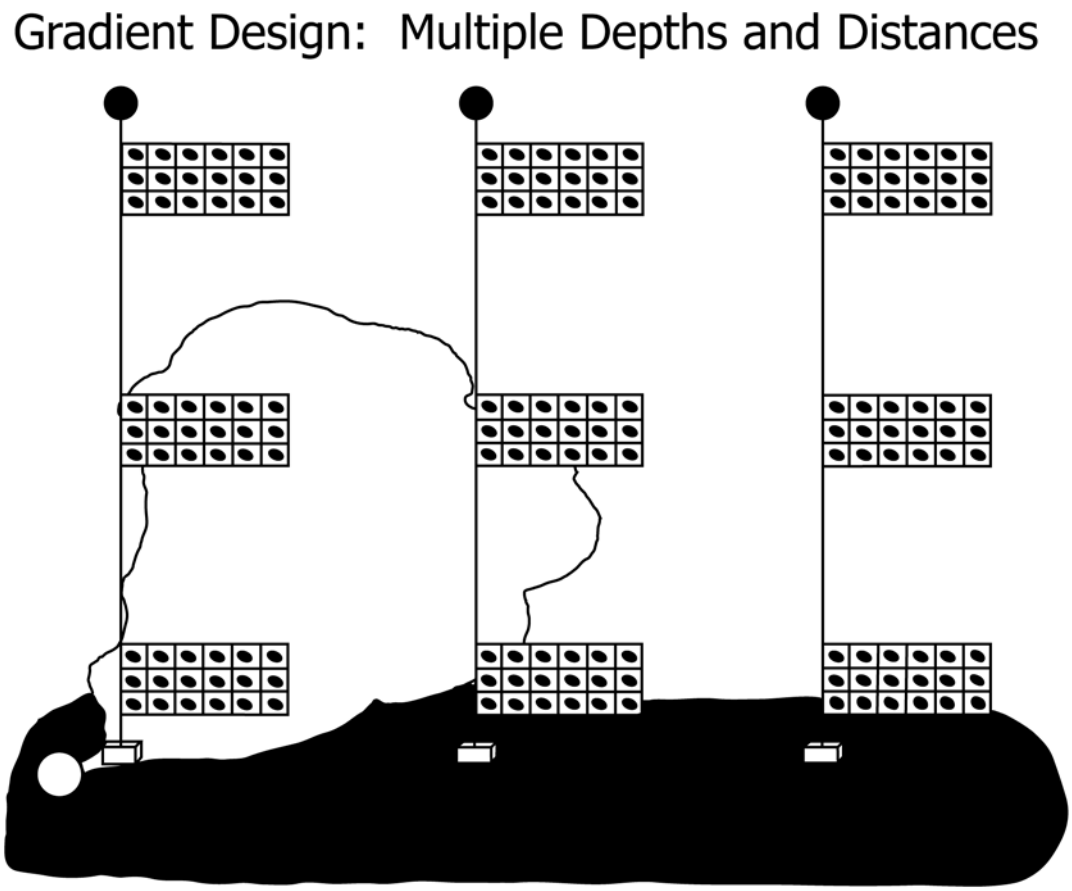


FIG. 5 Compartmentalized Cages Attached to Line at Three Depths (With Surface Float and Bottom Anchor) and at Multiple Distances from a Suspected Containment Source

test species in the upper layers of sediment where most benthic organisms are commonly found and from which sediments are most commonly collected for chemical analysis (Guides E1391 and E1525) as well as for use in laboratory bioaccumulation tests (Guide E1688) and laboratory toxicity tests (Guide E1367 and Test Methods E1706). Allowing the bivalves to bury themselves is generally less stressful than forced burial, and allows the cages to be positioned without divers in water deeper than 2 m. Placing cages directly on top of the sediments and allowing the bivalves to reposition themselves has been used successfully in freshwater (**3**, **61**) and marine (**62**) environments. The bivalves gain exposure to sediments as the sediments infiltrate the mesh material. Although confined within the mesh material, bivalves can work themselves into the sediment if sufficient room within their compartments is provided. In some cases it may be necessary to push the cage into the sediment to ensure exposure to chemicals associated with those sediments. This approach has been used before (**115**), although without compartmentalized cages. However, forcing the cages and bivalves into the sediments may induce stress, resulting in high mortalities. Forcing the cages into the sediments, or digging out sediments to bury the cages can disturb the integrity of the sediments and alter natural biogeochemical processes. Anchors, rebar, or cages with legs can be used to ensure the cages remain at the desired position. Anchors or cement blocks can be attached to the sides of the cage with line. Rebar can be used as a weight and strapped to the side of the cage or it can be bent into a "U," and pushed over the cage into the sediment to secure the cage in place. However, rebar should be coated with rubberized coatings or covered by plastic bags to prevent potential metal exposure. The cage can be constructed with legs (that is, like a table) and the legs pushed into the sediment to secure the cage in position. Factors that should be considered when deploying cages on top of the sediments include presence of natural vegetation, type of substrate, boating traffic, and recreational activity.

10. Test Organisms

10.1 *Species*—Many bivalve species have been used for assessing chemical bioavailability or effects in marine, estuarine, and freshwater environments (Table 3). The most widely used approach has been monitoring tissue chemistry of resident populations. The existing information on species life history, ability to accumulate chemicals of concern, and physiological effects can be applied to in-situ field bioassays. Species selection should be made carefully, considering conditions at the natural habitat of the species and natural factors (that is, temperature, salinity, dissolved oxygen, and pH; Table 2) at the area under investigation, presence in the area under evaluation, documented ability to accumulate chemical(s) of concern, documented sensitivity to chemical(s) of concern, life history (that is, spawning cycle, life-stage requirements, and

TABLE 3 Partial List of Bivalves Used in Transplant Studies for Monitoring Marine and Freshwater Environments

NOTE 1—Many species can be used in estuarine environments due to their ability to tolerate a wide range of salinities. "*" indicates species used in studies with compartmentalized cages; most other species have been deployed in non-compartmentalized cages, although a few benthic studies did not employ cages of any kind.

Marine Species			
Mussels	Oysters	Clams	Scallops
Arca zebra (117)	*Crassostrea gigas* (126)	*Anadara granosa* (134)	*Aequipecten opercularis* (143)
Modiolus (118)	*Crassostrea angulata* (127)	*Cerastoderma edule* (135)	*Amusium pleuronectes* (144)
Mytilopsis sallei (119)	*Crassostrea virginica** (128)	*Chione stutuchburyi* (136)	*Argopecten irradians* (145)
*Mytilus edulis** (120)	*Crassostrea rhizophorae* (129)	*Macoma nasuta* (137)	*Argopecten purpuratus* (146)
*Mytilus galloprovincialis ** (121)	*Ostrea angasi* (130)	*Macoma balthica* (115)	*Chlamys varia* (147)
*Mytilus trossulus** (122)	*Ostrea edulis* (131)	*Macoma inquinata* (138)	*Crassodoma gigantea* (148)
*Mytilus californianus** (123)	*Ostrea lurida* (132)	*Mercenaria mercenatia* (139)	*Lima hians* (149)
*Perna viridis** (124)	*Saccostrea commercialis* (133)	*Mya arenaria* (140)	*Pecten maximus* (150)
Perna bicolr (125)		*Scrobicularia plana* (141)	*Placopecten magellanicus* (151)
		Spisula solidissima (142)	
		Venerupis japonica (137)	
		Venerupis staminea (137)	
Freshwater Species			
Mussels		Clams/Cockles	
Actinonaias ligamentina (152)		*Anadara trpezium* (173)	
Actinonaias pectorosa (152)		*Corbicula fluminea** (174)	
Amblema plicata (153)		*Corbicula japonica* (175)	
Amblema perplicata (154)		*Corbicula manilensis* (176)	
Anodonta anatina (155)		*Musculium transversum* (177)	
Anodonta cygnea (156)		*Rangia cuneata* (178)	
Anodonta grandis (157)		*Sphaerium striatinum* (179)	
Anodonta implicata (158)		*Sphaerium simile** (180)	
Anodonta piscinalis (64)			
Anodontites trapesialis (159)			
*Dreissena polymorpha** (160)			
*Elliptio complanata** (161)			
Epiblasma torulosa rangiana (162)			
Epioblasma triquetra (162)			
Fusconia subrotunda (152)			
Hydridella menziesi (163)			
Lampsilis higginsi (164)			
Lampsilis radiata (165)			
Lampsilis ventricosa (166)			
Lemiox rimuosus (167)			
Margaritifera falcata (168)			
Medionidus conradicus (152)			
Proptera alata (169)			
Proptera capax (170)			
Pyganodon grandis (42, 43, 44)			
Quadrula quadrula (171)			
Unio pictorum (156)			
Villosa nebulosa (152)			
Villosa vanuxemensis (152)			
Westralunio carteri (172)			

threatened or endangered status), availability, and ease of handling in the field. Ideally, species or genera with wide geographic distributions should be selected, so that test results can be compared among different sites and different test conditions. Depending on the question being asked, it may be most important to select species that are found, have been found, or could be found in the assessment area. Species used should be identified with an appropriate taxonomic key, and identifications should be verified by a taxonomic authority wherever possible. It may be necessary to conduct a pilot study to determine if the test animals can survive under the environmental conditions at a particular site. The U.S. Fish and Wildlife Service (116) has identified the following mussels as possible surrogate species for in-situ field testing instead of threatened and endangered species in the Virginia area:

(1) Rainbow mussel (*Villosa iris*),

(2) Snuffbox mussel (*Epioblasma triquetra*),

(3) Pimpleback mussel (*Quadrula pustulosa*), and

(4) Tennessee pigtoe mussel (*Fusconaia barnesiana*). A special caution is required to prevent the introduction of exotic species (for example, zebra mussels (*Dreissena polymorpha*) and Asiatic clams (*Corbicula fluminea*) in freshwaters and clams (*Potamocorbula*) in marine waters). To prevent the spread of unwanted species, it must be verified that they already exist in the area of concern before using them in an in-situ field bioassay. Zebra mussels, Asiatic clams, and Potamocorbula are good candidates for in-situ field bioassays and have been used successfully (for example, ((181, 182,183, 184), but these species should be used with extreme caution and consultation with appropriate regulatory agencies. The introduction of zebra mussels can be particularly harmful because of their potential effects on freshwater mussels and surrounding environments. Other unwanted introductions include parasites or diseases (*Perkinsus marinus*) from infected bivalves such as oysters. Similarly, unwanted pests could be introduced from microscopic attached forms on the shells of

transplanted bivalves such as the freshwater weed *Hydrilla hydrilla*. A potential problem that is unique to freshwater bivalves is the introduction of glochidia stages that are parasitic on fish for a portion of their life cycle. Although this is common in areas where the freshwater bivalves naturally reside, potential effects should be considered during the planning of in-situ field bioassays (for example, see 10.2.1).

10.2 *Commonly Used Taxa*—The environmental requirements (Table 2) and sensitivity of new bivalve test species should be established before they are widely used in field tests. The sensitivity and bioaccumulation potential of a prospective new test species could be compared with a more commonly used species to establish its relative utility. This can be accomplished in side-by-side transplants. Monitoring variations in water quality parameters and sediment characteristics (that is, particle size, organic enrichment, and sulfides) may help distinguish the effects of these parameters from the effects of chemical exposure. The taxa most commonly used in in-situ field bioassays are described as follows:

10.2.1 *Mytilus* sp. is an intertidal bivalve that has been successfully used in transplant studies since the late 1970's (**46**). The sensitivity of this species to salinities less than 10 ppt limits its use to testing marine and estuarine areas. However, the large data base that has been developed for the response of *Mytilus* sp. to a variety of habitats and chemicals establishes its usefulness as a test species as well as a reference species for comparing the sensitivity of other species. Species of the genus *Mytilus* are widely distributed on both coasts of North America (**84**).

10.2.2 *Corbicula fluminea* is a freshwater clam that has been used extensively in field transplants and laboratory studies (**108**, **109**, **185**, **186**, **187**, **188**). Numerous laboratory studies have also been conducted on this species and several symposia have been conducted on their biology and ecology.

10.2.3 *Elliptio complanata* and *Pyganodon grandis* (formerly *Anodonta grandis*) are freshwater unionid mussels that have been used extensively for monitoring water column and sediment exposures in northern parts of the United States and in Canada (**3**, **42**, **43**, **44**, **189**). Numerous laboratory studies have also been conducted on these species.

10.2.4 *Dreissena polymorpha,* a freshwater mussel, is a relative newcomer to bivalve field bioassays. However, the rapid proliferation of this nuisance species has increased the number of laboratory and field studies that have been conducted on bioaccumulation and growth (**181**, **182**, **183**, **184**).

10.2.5 *Macoma* sp. the only commonly used facultative deposit feeder, is a marine or estuarine clam that has been used extensively in laboratory and field studies to assess bioaccumulation and growth (**11**, **12**). It is commonly found in many environments on several coasts. It has been successfully transplanted in many different areas, and there are a number of supporting laboratory studies.

10.2.6 *Rangia cuneata* is an estuarine clam that can tolerate freshwater conditions (**178**, **190**, **191**, **192**, **193**). It has been used in a number of field transplants as well as laboratory studies.

10.2.7 *Crassostrea* sp. is an oyster that has been used extensively in transplant studies in marine and estuarine studies (**194**, **195**). Oysters survive and grow better than marine mussels at lower salinities and accumulate many chemicals such as tributyltin and copper by about a factor of two above mussels. The shells of *Crassostrea* and other oyster species are usually more difficult to measure because of the irregular shell shape, sharp edges and protrusions.

10.3 *Size and Age of Test Organisms*— All bivalves used in an in-situ field study should be from the same age class and as uniform as possible in size. Age class is more difficult to determine when obtaining specimens from the wild as opposed to culturing facilities because wild populations are a composite of several age classes, with different individuals growing at different rates. In most bivalve species, size is a function of age, so if individuals from the same age cohort are selected, they will be within a fairly uniform size range. Under adverse conditions (that is, crowding, exposure to chemicals, or exposure to unfavorable natural factors), some individuals may grow very little. The age or size class of the prospective species should be chosen so that sensitivity to chemicals or bioaccumulation potential is not affected by state of maturity or reproduction. It is recommended that specimens in a sub-adult age class be used because this age class has the greatest potential for growth of somatic tissue, reproductive tissue, and shell. If adult specimens are used, the study should not be conducted during active spawning. This prevents loss of accumulated lipophilic chemicals. Investigators should note the reproductive state of the test animals including degree of gonad development by observation, mass, or volume measurements. They should also note whether spawning occurred during the beginning-of-test or end-of-test measurements.

10.3.1 Bivalves can be sorted according to size and counted to determine whether sufficient numbers have been collected in the desired uniform size range. Shell length or whole-animal wet-weight should be used to select individuals for use in a field bioassay. Although whole-animal wet-weights provide a more accurate measurement of animal size, shell lengths provide a rapid and quantitative sorting method. Shell length should be determined with vernier calipers with a measurement accuracy of 0.1 mm. Whole-animal wet-weights should be determined with an analytical balance with a measurement accuracy of 0.01 g. The final size range used should be based on the maximum number of animals in the minimum size range. As a starting point, it is recommended that the size range used in the test be approximately 5 to 10 % of the average maximum size of the species (that is, for *Mytilus* a 5 to 7-mm size range is suggested as the maximum shell length this genus is about 70 mm). By minimizing the size range of individuals, the variability in bioaccumulation and associated biological effects will also be minimized. The absolute size range used for a given species will depend on the size of the species and the availability of specimens. The decision to use juveniles, sub-adults, or adults depends on the experimental design. There is a tendency among many bivalve species for the smallest animals to grow at the greatest rates and accumulate the highest concentrations of chemicals. Various bivalve life stages have been proposed as part of an integrated environmental assessment (**196**). There are ASTM standard guides for a saltwater bivalve embryo test (Guide E724), bioconcentration

tests with adult saltwater bivalves (Guide E1022), and bioaccumulation of sediment-associated chemicals (Guide E1688). A freshwater glochidia test is also being developed (**197**). While bivalve larvae are often assumed to be the most sensitive life stage, there is a growing body of evidence suggesting they are not necessarily more sensitive than adults. Adults may be more sensitive than juveniles under certain conditions (**2, 3, 11, 198, 199**).

10.4 *Source*—Bivalves can be obtained from either natural populations or from culturing facilities. All individuals used in a field study should be from the same population, because different populations of the same species might have different sensitivities to or bioaccumulation capacities of the same chemical. Bivalves can be collected from wild populations in an uncontaminated (that is, chemicals present at concentrations lower than at the area under investigation) area although it may be easier to purchase species from field grow-out or laboratory culture facilities. The advantages of using cultured or farmed animals is that the genetic and environmental history of the test animals is well known, and the assurances of being uncontaminated are greater. A sample of the prospective test bivalves should be measured for contamination in their tissues, particularly for the chemicals of concern. Collecting permits for field collected bivalves might be required by some local and state agencies.

10.5 *Number of Specimens*—The number of bivalves collected should account for dead or dying individuals, individuals injured during handling, and the ability to minimize the size range as much as practical. Therefore, the number collected should be equal to the total number required for deployment at each of the stations plus the number required for the baseline (that is, beginning of test) tissue chemistry measurements, plus approximately 20 to 50 % more individuals to account for mortalities and size variability. Between 100 and 300 animals may be sampled at the beginning of the test to provide the initial tissue and shell weight estimates. These same individuals can be used for baseline chemical analysis. If composite tissue samples are required, see 8.8.1 for methods to determine number of animals per composite. It may be convenient to use a sample size equal to the number of individuals in each cage. Additional guidance on statistical approaches can be found in ASTM Practice E1847 and Guide E1191.

10.6 *Collection*—Natural populations should be collected with methods appropriate to their distribution. For intertidal marine species or freshwater species in shallow water, populations can be collected by hand. For subtidal marine species or freshwater species in deeper water, SCUBA or a small biological dredge can be used for collection. Infaunal bivalves can be separated from sediment by gentle sieving. Sieves and containers used to collect and transport bivalves should be marked "live only" and should never be used for working with formalin or any other toxic materials. Water used for sieving should be at the same temperature and salinity as bottom water at the collection site. Some species of marine and freshwater mussels produce byssal threads as an attachment mechanism. Particular care should be used when removing mussels from substrates to which they have attached to avoid damage to the byssal glands, an internal organ that secrets the byssal threads. Damage or removal of the byssal gland can lead to mortality. A knife or scissors should be used to remove the mass of byssal threads visible on the outside of the mussel shell. This process will reduce the possibility of injury to the mussel. All epiphytic growth should be removed from the exterior of the bivalve shells. Plant or animal growth can usually be removed by hand; a soft brush or scraper may be required to remove barnacles, tube worms, or other tenacious organisms.

10.7 *Handling*—Test organisms should be handled as little as possible. When handling is necessary, it should be done carefully, gently, and quickly so that specimens are not unnecessarily stressed. Every effort should be made to maintain bivalves in well aerated, flowing water for as long as possible between collection, sorting, and deployment procedures. The water used during the holding period(s) should be from a known source and free of chemical contamination. When transporting bivalves over great distances that require extended periods of time, it is better to keep them moist and cool than to maintain them in water that could become stagnant and low in dissolved oxygen. Bivalves can be kept moist and cool by placing the specimens in an ice chest with either wet ice or frozen gel packs on the bottom. Newspaper, paper toweling, or cloth toweling should be placed between the specimens and ice to prevent direct contact. Wet towels can also be placed over the specimens to provide additional moisture.

10.8 *Holding*—Test organisms should be deployed as soon as possible and holding times minimized. If necessary, test organisms can be acclimated to water quality conditions at the deployment site, as identified in 6.5. In larger studies where it may be difficult to collect a sufficient number of specimens in one day, a laboratory or field site relatively free of contamination can be used as a holding facility while the remainder are collected.

10.8.1 Some infaunal bivalves may require holding in sediment until initiating the test. Supplementary feeding for laboratory held specimens should not be necessary if holding is less than one week; specimens held in the field will continue to feed on natural sources of food.

10.9 *Animal Quality*—All bivalves used in a test need to be of acceptable quality. Before initiating a test, a qualified bivalve taxonomist should be consulted to ensure that the animals collected are all of the same species. This is particularly important with some freshwater bivalves where species differences may be extremely difficult to determine based on shell morphology. Even in the genus *Mytilus* there are subtle differences that may not be obvious, particularly in areas where two species could be found together (**200, 201, 202, 203, 204**).

10.10 Although it is extremely difficult to identify healthy animals when the shell is closed, gaping animals that close very slowly or do not close at all should not be used. A putrefied smell emanating from the batch of test bivalves indicates one or more dead specimens. Dead specimens should be removed.

11. Field Procedures

11.1 *Test Initiation: Presort*—The first step is to sort all bivalves into size groups, with each size group in its own

container. As indicated in 10.2, sorting can be based on either shell length or whole-animal wet-weight. Sorting allows identification and numeration of bivalves in a uniform size range. All bivalves collected should be retained until the test is initiated to ensure a sufficient of number of individuals. After the presort, the number of bivalves per each size category should be determined. The test should be started using the minimum size range with a target range from 5 to 10 mm (that is, from 20 to 25 mm, 33 to 38 mm, 36 to 46 mm) that contains the maximum number of individuals.

11.1.1 Unless a non-recirculating flow-through system is used, test organisms should be kept out of water to eliminate exposure to oxygen deficient conditions. If held out of water, they should be kept cool and moist by providing shade (that is, prevent exposure to direct sunlight), ice packs, or moist toweling. If air temperatures are excessively warm, it may be necessary to hold specimens in an ice chest with wet ice. Once the specimens are sorted into size groups, clean water can be added to the containers. Since the density of animals has been reduced by this time and the shells have been cleaned of epiphytic growth, it is easier to maintain them in clean water without adverse effects. Nevertheless, water temperature should be checked regularly and can be maintained within a desired range by placing plastic bags filled with ice in each container. This is particularly critical when working in a laboratory or other facility where room temperatures can exceed temperatures at the deployment site. A rapid rise in temperature of adult organisms which have ripe gametes could induce spawning, which would add another unwanted variable to the test. If in-situ temperature monitors are used during the deployment period, they can also be used during the sorting and holding period to document temperatures.

11.2 *Final Measurements and Distribution*—Once the final size range has been identified during the sorting process, shell lengths should be remeasured and weights measured for the first time. After making these measurements, the bivalves are distributed among the cages. An even distribution of test animal size can be achieved by filling the cages in order of increasing or decreasing animal size (**5**). This applies to both compartmentalized mesh bags which are attached to PVC frames to form a cage, and to rigid compartmentalized trays.

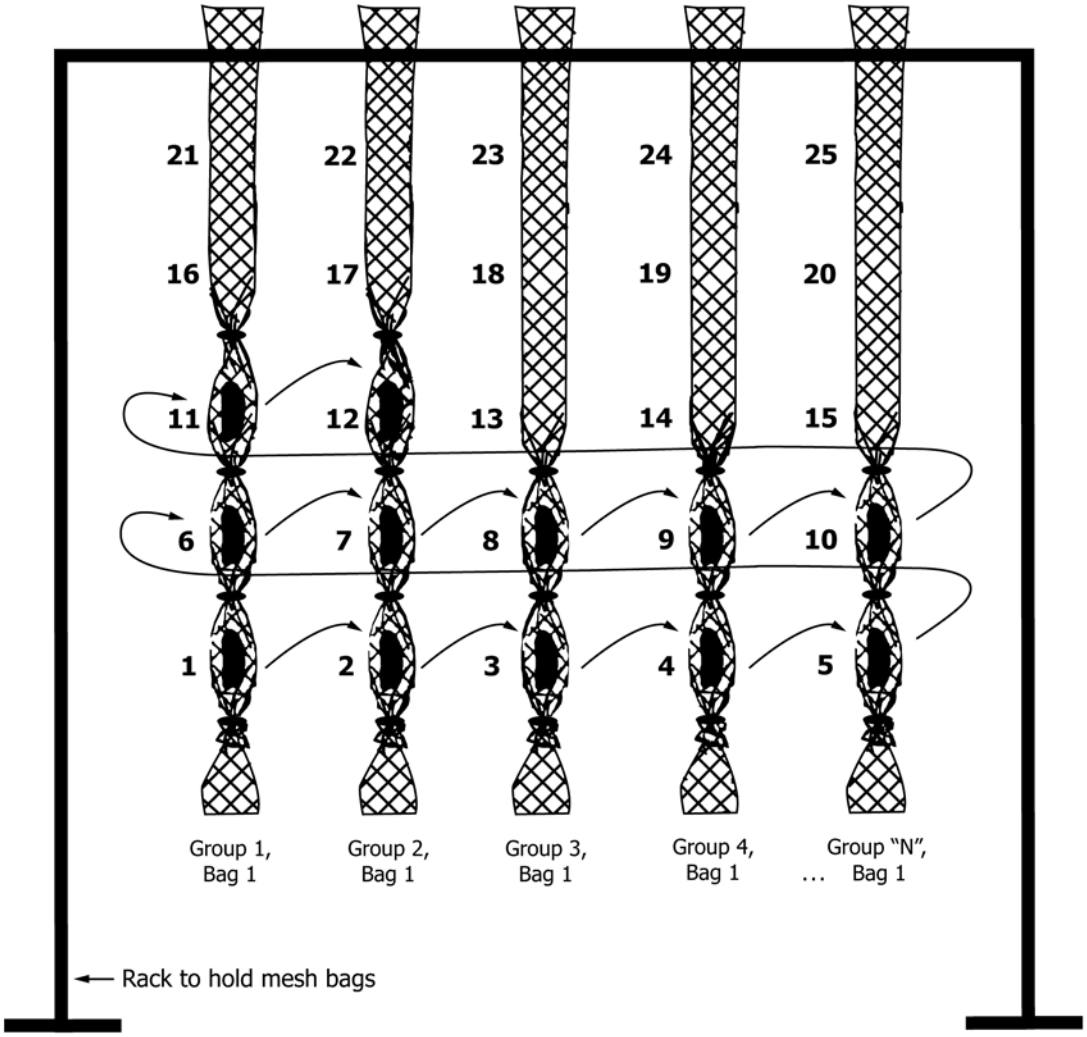
11.2.1 *Setup for Distribution*—If using mesh bags as described in 9.3.2 and 9.3.3, identification tags, made of durable plastic or other inert material, should be attached to the mesh bags, which have been knotted approximately 30 cm from the end. The identification tag should be secured with a plastic cable tie to the bag near the knot. A water-indelible, permanent marker should be used to label tags with both the cage number and the bag number: for example, a label of 2–3, indicates Cage 2, Bag #3. Color-coded beads strung through a plastic cable tie and fastened to the mesh bag can also be used for purposes of identification. This is recommended as a backup in case the identification tag is lost during the study. The colored bead can be used to identify bag number on the PVC frame, which is also labeled with an indelible marker. If using another type of compartmentalized cage, ensure that the cage is clearly identified, and each compartment within the cage is numbered.

11.2.2 Once all mesh bags have been labeled, the bags can be separated into groups according to bag number, with all the bags with a –1 in one group, –2 in another group, and so forth. The distribution process is based on bag number; all bags of a common number are filled at a given time. To initiate the distribution process, gather all bags that have a "–1" on the label; there should be one for each cage number. Attach these bags to the PVC distribution frame in cage number sequence (Fig. 6). If rigid, compartmentalized cages are used, line up the cages so all cages can be filled simultaneously. Place the cage so the Number 1 compartment is in the upper left position.

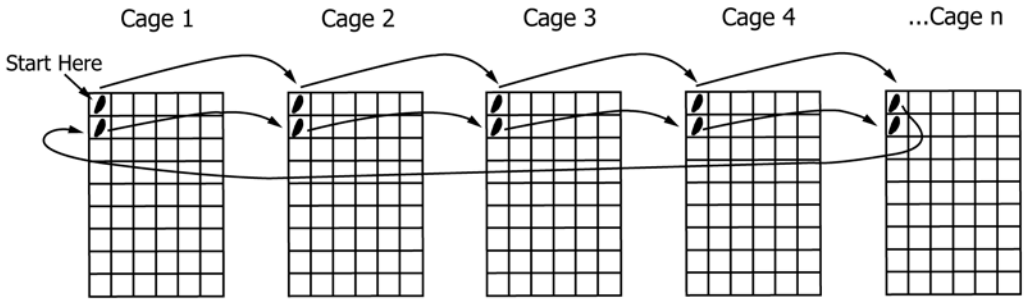
11.2.3 *Prepare Bivalves for Distribution*—Starting with either the smallest or largest size group, place all bivalves within the size group into a tray or tub containing water. The bivalves need to be maintained in water during the measurement and distribution process, and the water temperature should be maintained as close as possible (approximately ±5°C) to temperatures at the deployment site. In most temperate latitudes this can be accomplished by placing plastic bags containing wet ice in the tub with the bivalves; the temperature can be monitored with a thermometer that remains in the tray or with in-situ temperature monitors. It is essential that the bivalves be completely submerged and flat on the bottom prior to measurement. Bivalves with air between their valves either float on the surface or sit upright on the bottom. These bivalves should not be used because the air will bias the whole-animal wet-weights (water weighs more than air). Do not use individuals that float, are buoyant at one end, or do not close upon light physical stimulation (that is, agitation of the water around the bivalves or lightly tapping the shell). Bivalves that float contain air which can be released prior to use. Floating individuals can be transferred to a separate container, where, if left undisturbed, they will likely purge the trapped air.

11.2.4 *Initiate Measuring and Distribution*—Under normal conditions (that is, submerged and respiring), the bivalve shells will be slightly agape (approximately 1 mm). Most species will respond to light physical stimulation by tightly closing their shells. Bivalves that do not completely close their shells upon movement or light physical stimulation should be considered unhealthy and should not be used. In addition, bivalves that have broken shells or holes in their shells should not be used.

11.2.4.1 Initiate the distribution process by randomly selecting one specimen from the holding tray, making sure it is alive and shells are tightly closed. Using a paper towel, blot excess water from exterior of the individual. Measure its shell length with a caliper and whole-animal wet-weight with an analytical balance. Record these data to a spreadsheet created for summarizing the data. The data can be recorded either electronically or manually. If the data are entered into an electronic spreadsheet, it is recommended that a manual record also be made as a backup in a case of computer failure. Once the specimen is measured and weighed, place it into the first mesh bag (Fig. 6A) on the distribution rack or into the first cell of the first compartmentalized tray (Fig. 6B). For the mesh bags, affix a 10-cm cable tie around the mesh material above this individual. The cable tie should be adjusted so that it is tight enough to prevent the animal from passing through but the cable tie can be moved if necessary. The cable ties or other



A. Distribution process for mesh bags.



B. Distribution process for rigid compartmentalized cages.

FIG. 6 Distribution Process for Caging Bivalves

restricting devices should not be so tight that the mesh is constricted to the point that it does not allow the shell to open. There should be enough slack to allow movement and growth of the individual during the test. Randomly take another specimen from the tray and measure its shell length and weight, recording the data in the spreadsheet. Place this individual into the second mesh bag on the distribution rack or into the first cell in the second compartmentalized tray. For the mesh bag, affix a cable tie. Repeat this process until one individual has been placed into each mesh bag or each compartmentalized tray. Continue adding bivalves, one at a time to either the mesh bags or the compartmentalized trays (Fig. 6A, B). For the mesh bags, complete one "row" before another row is started, until each mesh bag contains the desired number of individuals. When all of the bags on the distribution rack have been filled, remove the bags, knot or cable tie the open end, leaving a tail length of approximately 0.3 m. For the compartmentalized trays, securely affix a mesh cover such that the bivalves can not migrate from one compartment to another. Place the completed bags or compartmentalized trays into a cooler lined with ice and moist paper towels. Repeat the above process until all the mesh bags or compartmentalized trays are filled. This process ensures that each station will have approximately the same number of individuals from the each size group.

11.2.5 Electronic spreadsheet, it can be customized to report minimum, maximum and average shell lengths and weights as they are entered. These values can be compared across cages to identify any individuals that are outside the pre- determined size range and to check for a close, even distribution. To ensure statistical similarity among stations, an Analysis of Variance (ANOVA) can be run on both shell length and whole-animal wet-weight data. The data can be analyzed by cage and by station (that is, pooled cages) if more than one cage is to be deployed at each of the test stations. If statistically significant differences are found, the test animals can be redistributed to eliminate this difference. For mesh bags, redistribution may require cutting a small opening in the mesh to replace the outlier. The opening can be secured with a plastic cable tie after inserting a replacement individual. For rigid compartmentalized trays, redistribution will require removing the mesh top.

11.2.6 *Mesh Bags Post-distribution Activities*—Once the bivalves have been distributed to all mesh bags, sort the bags by cage number. If the bags are not to be attached to the PVC cages until later, secure these bags together with a large plastic cable tie. Then, transport bivalves to a pre-identified holding area in the laboratory or field with uncontaminated water. Hold in the water overnight, or until ready to attach bags to the PVC cages.

11.2.7 *Rigid Compartmentalized Trays Post-distribution Activities*—Once the bivalves have been distributed to all the compartmentalized trays, transport bivalves to a pre-identified holding area in the laboratory or field with uncontaminated water. Hold in the water overnight, or until ready to deploy.

11.2.8 *Measuring and Distributing the Baseline (Beginning-of-Test) Tissue Chemistry Individuals*—Bivalves to be used for baseline tissue chemistry should be identified and separated during the measurement and distribution of test specimens to ensure similar sizes. This can be accomplished by assigning a cage for each baseline tissue chemistry sample. However, instead of distributing the bivalves for baseline tissue chemistry to mesh bags, they should be distributed to rigid, compartmentalized cages. The use of compartmentalized trays for the tissue chemistry specimens eliminates the need to remove individuals from the mesh bags once the distribution process is completed. The individuals should be placed into the compartmentalized trays in order, that is, with the first individual measured placed into compartment #1, the second into compartment #2, etc. At the end of the distribution process, the tissues should be removed for chemical analysis according to the procedures in 11.11.

11.3 *Attachment of Mesh Bags to PVC Frames*—A set of mesh bags attached to any more rigid frame, such as PVC or heavy protective mesh, constitutes a cage. Attach the mesh bags to the PVC frame by knotting the tail ends of the mesh directly to the PVC. If there is insufficient material to make a secure knot, use 6 in. (approximately 15 cm) cable ties to firmly attach mesh to the PVC frame. Allow a little slack in the mesh bag during attachment; the mesh should not be stretched so tightly that it restricts bivalve movement. If a temperature recording device is used, it should be attached to the frame at this time. If predators are of concern, wrap the PVC frame with a heavy duty plastic protective mesh, with a mesh size appropriate to deter predators of concern (for example, approximately 1 to 2.5-cm mesh size).

11.3.1 It may be necessary to adjust the space between bivalves in the mesh bags so that the mesh bags can be attached to the PVC frame without being too taut or too loose. During the attachment process, slide the cable ties as necessary to increase or decrease the space between individuals without compromising the space available for each individual (that is, do not decrease the space between animals so that there is insufficient space for them to open their valves during respiration).

11.4 *Deployment*—Deploy caged bivalves at stations in accordance with the procedures in 9.4.

11.5 *Retrieval and End-of-Test Measurements*—Foreign material, if present can be removed, by dipping into the water. The exterior of the shells and the mesh bags can be wiped with paper towels to remove fouling or other coatings not removed by dipping. If the bags of bivalves are removed from the PVC frames, a separate ice chest lined with wet ice and moist paper towels should be used to transport the bivalves to the processing site. Otherwise, place a tarp or other protective covering under and over the bivalve cages during transportation to prevent contact with contaminated surfaces ans desiccation.

11.5.1 Guide E1688 recommends a 24-h gut purging period for deposit-feeding bivalves tested under laboratory conditions. Guide E1688 also gives suggestions when not to purge the gut. It is up to the investigator to determine if the experimental design requires gut purging. However, in the field a 24-h gut purging may not be convenient or necessary due to time and cost constraints. A number of studies have been conducted in which gut purging ranged from 0 to 24 h, such as site-specific bioassays (**3, 15**) and national, state, and regional mussel watch

monitoring programs (**45**, **46**, **205**). Gut purging can be accomplished by suspending the caged bivalves at the reference station(s), or at another location known to be relatively free of contamination when compared to the test stations, for appropriate time periods.

11.5.2 Bivalves from all bags constituting a cage or all bivalves in one compartmentalized tray should be processed together. It is critical to retain the order of bivalves during the end-of-test measurements. For mesh bags, it is recommended to remove the bivalves from the mesh bags and place them in a rigid compartmentalized tray to maintain order and facilitate end-of-test measurements. Start with Bag–1 from a given cage. Starting at the end of the bag with the plastic label, remove the bivalves and place them, in sequential order, into a compartmentalized plastic tray. Trays with holes to allow water circulation are recommended. After all individuals from Bag–1 have been transferred to the compartmentalized tray, repeat the process with the remaining bags, maintaining bag sequence (that is, process Bag–2, then Bag–3, and so forth). If a dead or missing individual is encountered, leave its corresponding compartment in the tray empty, or place a marker (that is, a bead or other device) in the compartment. This will ensure the order of individuals is maintained. Depending on the number of bivalves used, it may be necessary to use more than one compartmentalized tray to hold all the bivalves from a given cage. For bivalves deployed in rigid compartmentalized trays, remove the mesh cover. Approximately 5 to 10 min before initiating length and weight measurements, set the tray(s) into a tub or larger tray containing clean water. The water can be collected from the reference station, the holding facility, or another source of clean water. Upon placement of the compartmentalized tray containing bivalves into the tub of water, some individuals may "float," indicating air trapped between their valves. It is essential that the bivalves do not float prior to making the weight measurements since the presence of air will compromise the whole-animal weight measurements. It may be necessary to leave the bivalves undisturbed for approximately 5 to 10 min in the water in order for them to open their valves and release the trapped air. Once the air has been released, the bivalves can be taken from the tray and measured.

11.5.3 Starting with the bivalve in the Number 1 compartment, begin the shell length and whole-animal wet-weight measurements. Make sure the individual is alive and the shells are tightly closed before removing it from the compartmentalized tray. The end-of-test measurement procedures are similar to those in 11.2.4: blot the excess water from the exterior of the individual's shells, measure shell length along the longest axis, and obtain a whole-animal wet-weight measurement. Record the data, either electronically or manually onto spreadsheets. After the individual is measured, place it into a separate compartmentalized tray in the Number 1 compartment. Do not put these compartmentalized trays in larger tubs containing water as it is not necessary to keep bivalves in water once the growth measurements are made. Continue the end-of-test measurements, measuring one individual at a time and retain the order of individuals. For dead or missing individuals, transfer the marker from one compartmentalized tray to the other and enter a "M" or "D" into the spreadsheet to indicate "missing" or "dead." After all bivalves in a cage are measured and weighed, begin the tissue removal process as described in accordance with 11.7.

11.6 *Analysis of Tissues for Background Contamination*—For in-situ field studies with an exposure component, the initial or background concentration of chemicals in tissues of the test organisms should be analyzed for the chemicals of concern. It may also be necessary to characterize tissue chemistry of the source population well in advance of initiating a test to confirm their appropriateness as an uncontaminated source. However, exposure studies may be conducted without prior chemical analysis of tissues if the bivalves are collected from an area that is monitored for chemical contamination and known to be free of toxicants, or if the tissues of those bivalves have been monitored regularly as in culture facilities. Bivalves collected from unmonitored or potentially contaiminated areas should be used caution. It is recommended that their tissues be analyzed for chemical concentrations to confirm they are not contaminated. For in-situ studies that only assess effects, it is not necessary to analyze tissues for baseline, beginning-of-test chemical concentrations. However, as beginning- and end-of-test tissue chemistry data can aid in the interpretation of effects data, these analyses are recommended.

11.7 *Collection and Preparation of Bivalve Tissues for Chemical Analysis*—All equipment used for tissue extraction/collection should be of corrosion resistant stainless steel, anodized aluminum, or borosilicate glass. If corrosion resistant stainless steel is unavailable, use regular stainless steel products, carefully checking before each use for signs of rust, pitting, or corrosion. Do not use if rust, pitting, or corrosion is evident. Before each use, all instruments (that is, cutting board, shucking knife, and weigh pans) should be cleaned in accordance with the minimum following process: wash with a soap-free cleaning solution, hot tap water rinse, or deionized water rinse. If deemed necessary by the investigator, an acetone, hexane, or 95 % ethanol rinse can follow the last water rinse. Allow the instruments to air dry to remove the potential for adding water to the tissues being collected.

11.7.1 During tissue collection, the order of bivalves should be maintained; tissue weights are recorded by the individual and will be paired with whole-animal wet-weights and other size metrics. Use the compartmentalized trays for holding bivalves prior to shucking, and maintain order after tissues are removed.

11.7.2 If using cutting boards made of a material other than corrosion resistant stainless steel, anodized aluminum, or borosilicate glass, cutting boards should be covered with aluminum foil and cleaned as indicated in 11.7. If gloves are worn during the shucking process, they should be powder free. Wash hands thoroughly with a soap-free cleaning solution, or replace gloves between processing a cage of bivalves.

11.7.3 Tissues are removed in accordance with the following process. Start with the first individual in the compartmentalized tray, work with one individual at a time, and retain the order of individuals. Place bivalve on the cutting board. Slide the knife blade between bivalve shells, severing posterior and anterior adductor muscles. Spread the shells apart to reveal soft tissues. If preparing tissues from clams or oysters, it may be

necessary to notch the shell prior to inserting the knife blade between their shells. Use a separate knife designated only for the purpose of shell notching. Be sure that none of this shell material is combined with the soft tissue material. Using the tip of the knife blade, separate tissue from the shell, scraping as much of the adductor muscle from points of attachment as possible. Holding tissues to the shell with the blade of the shucking knife, tip the shell to drain excess liquid (that is, the water that was trapped between the shells during the measurement process).

11.7.4 After complete separation, keep the tissues in the shell and use the shell as a "holding dish" until tissue weights are measured. If the two shell halves should become separated, place one half under the other. Place the shell(s) containing the separated tissue on a tray lined with aluminum foil, keeping bivalves in order and sufficient space between the individuals to prevent the shell of one individual from touching the soft tissue of another. Minimize exposure of bivalve tissue to hands, aluminum foil, and any other surface other than the interior of the specimen's original shell. Repeat this process until all bivalves constituting a "chemical replicate" are shucked.

11.7.5 Place the weigh pan on the analytical balance; tare balance. Pick up the first specimen, and using the shucking knife blade tip, slide the tissue onto the weigh pan. Allow the balance to stabilize. Record the weight, either electronically or manually. Tare material on balance. Continue adding tissues, one at a time, recording the weights of each individual. Tare after each addition. When all tissues of a "chemical replicate" have been weighed, transfer tissues from the weigh pan to the prepared sample jar by gently sliding them off the foil. Tightly cap the sample jar, affix prepared label, and place the tissue samples in a cool location (that is, ice chest containing gel packs, wet or dry ice, or a freezer) depending on the specifications of the analytical laboratory performing the tissue chemistry analyses. If using aluminum foil to line surfaces, discard foil and clean all sampling equipment in accordance with 11.11 before proceeding to the next sample.

11.8 *Quality Assurance/Quality Control Procedures*—Quality assurance is a program designed to provide accurate and precise results. Included are the selection of proper technical methods, sample collection, selection of limits, and qualifications and training of personnel. Quality control are specific actions required to provide information for the quality assurance program. Included are standardization, calibration, replicates, and control and check samples suitable for statistical estimates of confidence of the data (**206**).

11.8.1 To ensure that the measuring instruments (that is, calipers and balance) are providing accurate readings, the instrument can be tested by measuring a standard weight or shell length. For the balance, one or more from a series of standard weights (that is, 10, 50, 100 and 200 g) can be applied to the balance at intervals throughout the measurement process. For example, after every 100 measurements made on the balance, a standard 100-g weight can be applied to the balance. If the balance is off by more than 1 % (1 g), the balance should be recalibrated; it may be necessary to reweigh some of the previous individuals depending on the degree of off-calibration. The measurement accuracy of the calipers can be checked by completely closing the device and recording the displayed measurement, which should be 0.000 mm. If the caliper displays a value greater than 0.5 mm, the unit should be re-zeroed. If available, a standard length can be measured to check the accuracy of the calipers. Depending on the degree of off-calibration, it may be necessary to remeasure some of the previous individuals.

11.8.2 Quality assurance/quality control (QA/QC) procedures of bivalve measurements should be used primarily in the development process as practitioners refine their methodology. Once the methods have become routine, it may only be necessary to use the QA/QC procedures on a yearly basis to confirm that no artifacts have inadvertently entered the methodology.

11.8.2.1 One suggested approach for QA/QC procedures for the bivalve measurements is to remeasure and reweigh 5 % of the animals. These QA/QC measurements can be performed during the initial and end-of-test measurement processes. The QA/QC shell length measurements and whole-animal wet-weight measurements outside ±5 % of the original measurements may be considered unacceptable error measurements. The remeasuring of animal shell length and weight occurs throughout the measurement process as each series of bags is processed to ensure that all measurements are within the limits defined as acceptable. As an example, suggested limits for *Mytilus* sp. approximately 30 to 40 mm in length are 1.0 ± 0.5-mm variance in shell length and a 0.5 ± 0.25-g variance in weight. If the results of the remeasurements fall outside of these limits, it is recommended that the previous batch of 100 individuals be remeasured. The hard copy data sheets can contain a separate row for the QA/QC measurements. To facilitate the process, it is suggested that these QA/QC measurements be made on the last "row" of bivalves to be entered into a series of bags.

11.9 *Sample Containers, Handling, and Preservation*—Precleaned sample containers should be purchased from a supplier or provided by the analytical laboratories. Each jar should be sealed, affixed with a completed label, assigned a unique tag number, and stored under appropriate conditions. Sample labels should be made of self-adhering, waterproof material; an indelible pen should be used to fill out each label. Each sample label should contain the project number, sample identification, preservation technique, analyses, date and time of collection, and initials of the person(s) preparing the sample. A completed sample label should be affixed to each sample container. In addition, a unique numbered tag can be affixed to each sample container. Chain-of-custody forms and tamper-proof tape can be used for projects that are litigation sensitive. The preservation of tissue samples is a function of chemical analytes and methods used by the analytical laboratory. The analytical laboratory should provide guidance on proper handling and preservation of tissue samples. For most analyses, samples should be protected from light and refrigerated at 4° ± 2°C from the time of receipt until they are extracted and analyzed. For some chemical analyses or longer holding times, it may be necessary to freeze the samples at -20°C (Guide E1688).

12. Ancillary Measurements

12.1 It is recommended that supplementary measurements be made on those factors most likely to affect bioaccumulation and growth. These include factors such as chemicals in water and sediment, temperature, food, dissolved oxygen, pH, salinity, suspended particulate matter, turbidity, and water current velocity. These measurements provide site-specific information that can be used to help explain the measured exposure and associated effects. These measurements are more easily made at shallower depths but may become problematic at the greater depths where caged bivalves have been deployed (for example, 650 m). Nevertheless, it is important to measure the chemicals in water or sediment, and this has been accomplished even at these great depths with discrete samples (for example, (**13**, **14**)). In-situ temperature monitoring devices are available to measure temperatures for periods up to 1 year at depths up to 330 m. Standardized procedures should be used to measure chemicals of concern in water and sediment.

12.2 *Chemical Exposure*—It is highly recommended that the concentration of chemicals in water sediment be determined, in addition to measuring the concentration of chemicals in tissues, to characterize total potential exposure; that is, exposure from both water and sediment. These data can be used to establish relationships between chemicals in various environmental compartments as a form of field validation or to establish first-order approximations of these relationships that are validated in the laboratory. Results of the bivalve field bioassay can be used to predict bioaccumulation and biological effects likely to occur in other aquatic organisms under comparable field conditions. While this may only represent first order approximations in some cases, equilibrium partitioning theory, quantitative structure activity relationships, and critical body residue theory suggest that tissue burdens of chemicals associated with adverse effects may be similar across species (**6**, **7**).

12.3 *Temperature*—Marine and freshwater species should be selected to match the site-specific temperatures in the area of concern. Ideally, if species are naturally found in the area or have been found in the area in the past, it is a good indication that temperature tolerances are appropriate. Since temperature could influence bioaccumulation and growth (**2**, **51**), it is important to monitor temperature during the course of the test using in-situ temperature monitors.

12.4 *Food*—As with temperature, if indigenous populations of the bivalves of choice are found in the area of concern, it is a good indication that there is adequate food to support caged bivalves in the area. Since food could also influence bioaccumulation and growth (**2**, **51**), it may be helpful to measure parameters such as chlorophyll-a, particulate or total organic carbon, and suspended solids during the course of the test.

13. Acceptability of Test

13.1 An acceptable test should meet both survival and growth criteria because the data are intended for different purposes. Survival is a generic indication of overall health and test acceptability. Growth may be a more sensitive indicator of health than survival, and tissue weights should also be monitored. There should be no significant loss in tissue weight during the exposure period. This is necessary for meaningful bioaccumulation potential even if significant growth does not occur. It may be necessary to use different criteria for exposure and effects end points. If survivors have not lost significant tissue mass, a survival criterion of >45 % may be acceptable to interpret the bioaccumulation data. Conversely, since more individuals may be necessary to interpret effects end points like growth, a 45 % survival criterion may not be acceptable.

13.2 There should not be significant loss in either end-of-test tissue weights or whole-animal wet-weights when compared to measurements made at the beginning of the test, particularly at reference station(s), if they are used. If tissue weights decrease by more than 20 %, this could provide valuable effects information. However, this loss in tissue weight could be accompanied by loss in chemicals and represents a biased estimate of potential chemical bioavailability. Therefore, results may be considered unacceptable for interpreting the environmental significance of bioaccumulation if (*1*) the end-of-test tissue weights are more than 20 % lower than the beginning-of-test estimates, and (*2*) the end-of-test tissue weights are significantly less ($\alpha = 0.05$) than the beginning-of-test estimates.

14. Report

14.1 A record of the results of an acceptable caged bivalve exposure and effects test should include the following information:

14.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of the test;

14.1.2 Source of test animals, scientific name and how verified, initial whole-animal wet-weights, shell lengths, and estimates of tissue weights as well as end-of-test percent survival, whole animal wet-weights, shell lengths, and estimates of tissue weights. Means, ranges, and standard deviations of all measurements.

14.1.3 Description of the experimental design and cages, including any attached instrumentation and predator deterring devices, water depth and depth of cages at time of deployment, the number of animals per station, station coordinates, and any other outstanding features of the area to assist in station identification.

14.1.4 Averages and ranges of the acclimation temperature during the measurement and distribution process as well as the time spent out of water while in transit to the measurement location at the beginning of the test and while in transit to the deployment locations at the beginning and end of the test.

14.1.5 Reproductive state of the test animals including degree of gonad development by observation, mass, or volume measurements. Note whether bivalves spawned during the beginning-of-test or end-of-test measurements.

14.1.6 A table of data on concentrations of chemicals in water, sediment, and tissues (including percent lipids and percent moisture in tissues) should be included with sufficient detail to allow independent statistical analyses. The table should also include analytical methods and laboratory qualifiers.

14.1.7 A table of survival, effects, and tissue chemistry data (including percent lipids and percent moisture of the tissues) in sufficient detail to allow independent statistical analyses.

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

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

15. Keywords

15.1 bioaccumulation; bivalve; exposure effects; field bioassay; growth; in-situ

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