RECOMMENDED GUIDELINES FOR

CONDUCTING LABORATORY BIOASSAYS

ON PUGET SOUND SEDIMENTS

For

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Revised July 1995

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LIST OF ACRONYMS

ASTM	American Society for Testing and Materials
DMSO	dimethyl sulfoxide
EC_{50}	median effective concentration
Ecology	Washington Department of Ecology
Eh	oxidation potential
EPA	U.S. Environmental Protection Agency
LC_{50}	median lethal concentration
PCB	polychlorinated biphenyl
PSDDA	Puget Sound Dredged Disposal Analysis
PSEP	Puget Sound Estuary Program
PSWQA	Puget Sound Water Quality Authority
PTFE	polytetrafluoroethylene (Teflon®)
QA/QC	quality assurance and quality control
RTG-2	rainbow trout gonad cell
UV	ultraviolet

ACKNOWLEDGMENTS

This chapter was prepared by PTI Environmental Services, under the direction of Dr. Scott Becker, for the U.S. Environmental Protection Agency (EPA) in partial fulfillment of Contract No. 68-D8-0085. Dr. Thomas Ginn of PTI Environmental Services was the Program Manager. Dr. Jacques Faigenblum of the Washington Department of Ecology (Ecology) and Dr. John Armstrong of EPA were the project monitors. Drs. Robert Pastorok and Scott Becker of PTI prepared the Revised July 1991 revised chapter. Michael Wheeler and Timothy Ransom of the Puget Sound Water Quality Authority coordinated a review of the chapter in 1994 and 1995, and prepared the current revised chapter.

The principal author of the original protocol chapter (prepared in 1986) was Dr. Peter Chapman of E.V.S. Consultants, Inc. The original chapter was prepared under subcontract to Tetra Tech, Inc.

INTRODUCTION

This document presents recommended guidelines for conducting the following laboratory sediment bioassays in Puget Sound:

- Amphipod bioassay
- Bivalve larvae bioassay
- Echinoderm embryo bioassay
- Anaphase aberration bioassay
- Microtox bioassay
  - Organic extraction
  - Saline extraction
- Juvenile polychaete bioassay.

The sediment bioassays considered in this document are generally the tests used most frequently by a variety of Puget Sound investigators. Each recommended guideline is based on the results of a workshop and written reviews by representatives from most organizations that fund or conduct environmental studies in Puget Sound (Table 1). The purpose of developing these guidelines is to encourage all Puget Sound investigators conducting monitoring programs, baseline surveys, and intensive investigations to use standardized methods whenever possible. If this goal is achieved, most data collected in the sound will be directly comparable and thereby capable of being integrated into a sound-wide database. Such a database is necessary for developing and maintaining a comprehensive water and sediment quality management program for Puget Sound.

The recommended guidelines for each sediment bioassay describe the use and limitations of the respective toxicity endpoints; the field collection and processing methods; and the laboratory analytical, quality assurance and quality control (QA/QC), and data reporting procedures. In developing the recommended guidelines, it was recognized that the field of sediment bioassays is relatively new and is expanding rapidly. The loose-leaf format of this document will allow modification of the recommended guidelines, if necessary, and inclusion of guidelines for additional sediment bioassays.

Although the following guidelines are recommended for most studies conducted in Puget Sound, departures from these methods may be necessary to meet the special requirements of individual projects. If such departures are made, however, the funding agency or investigator should be aware that the resulting data may not be comparable with most other data of that kind

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and will be identified as such in the Puget Sound database maintained by the Puget Sound Water Quality Authority (PSWQA). In some instances, data collected using different methods may be compared if the methods are inter-calibrated adequately.

Before guidelines for specific bioassays are described, sections are presented on 1) the criteria used to select the tests considered in this document, 2) protocols for field collection of surficial test sediments, and 3) general QA/QC procedures that apply to all sediment bioassays.

BIOASSAY SELECTION

A large number of marine sediment bioassays have been developed and used in recent years (e.g., Swartz et al. 1979, 1985; Chapman et al. 1985; Schiewe et al. 1985; Williams et al. 1986; Long and Buchman 1989; Pastorok and Becker 1990). Many of these tests have been used to evaluate the toxicity of Puget Sound sediments. Nine tests were considered for inclusion in this document based on the following criteria:

- **Sensitivity**—each test has detected biological effects in a variety of sediments
- **Usage**—each test has been used in more than one study in Puget Sound.

Of the nine sediment bioassays selected for consideration, six were identified as suitable for general application in Puget Sound for reasons outlined in Table 2. These six tests are categorized in Table 3 by kind of test and kind of effect measured. The other three tests, which represent promising techniques to be developed further, include the oligochaete respiration test (Chapman et al. 1985), the surf smelt partial life-cycle test (Chapman et al. 1985; Casillas et al. 1989), and the copepod partial life-cycle test (Misitano 1983; Chapman, unpublished data).

For the 1994 revision effort, two additional bioassays were suggested for inclusion in this chapter: solid phase Microtox and a bioaccumulation bioassay. They will be further considered in the next revision effort as addenda to this chapter.

TABLE 2. APPLICABILITY AND RELEVANCE OF SELECTED LABORATORY SEDIMENT BIOASSAYS

Bioassay[a]	*Species*	Primary Endpoint(s)	Comments
Amphipod 10-day test	*Rhepoxynius abronius* *Eohaustorius estuarius* *Ampelisca abdita*	Mortality	Simple test, reproducible, ecologically relevant, applicability verified in numerous studies, extensive usage to date in Puget Sound
Bivalve larvae 48-hour test	*Crassostrea gigas* *Mytilus edulis*	Abnormality Mortality	Simple test, relevance and applicability verified in several independent studies, extensive usage to date in Puget Sound
Echinoderm embryo test	*Strongylocentrotus purpuratus* *S. droebachiensis* *Dendraster excentricus*	Combined Abnormality Mortality	Simple sensitive test, can be conducted year-round by using different echinoderm species, extensive usage to date in Puget Sound
In vitro anaphase aberration tests with rainbow trout gonad cells	*Oncorhyncus mykiss*	Abnormal anaphases	One of the few methods to measure possible genotoxic/cytotoxic effects, minor usage to date in Puget Sound, requires a higher level of expertise than other tests but can be conducted by more than one laboratory in this region
Microtox, bacterial luminescence bioassay	*Photobacterium phosphoreum*	Luminescence	One of the few methods to measure possible effects on bacteria, shows good promise, extensive usage to date in Puget Sound
Juvenile polychaete test	*Neanthes sp.*	Biomass	Simple sublethal test, measures growth (biomass) response and mortality, applicability verified in a series of studies, minor usage to date in Puget Sound

[a] Primary references for bioassays are as follows:

Amphipod test - Swartz et al. (1985); DeWitt et al. (1989); ASTM (1993b)
Bivalve test - Chapman and Morgan (1983)
Echinoderm test - Dinnel and Stober (1985); ASTM (1993a)
Anaphase test - Landolt and Kocan (1984a,b)
Microtox test - Schiewe et al. (1985); Williams et al. (1986)
Polychaete test - Johns et al. (1990).

TABLE 3. CATEGORIZATION OF SELECTED SEDIMENT BIOASSAYS BY KIND OF TEST AND KIND OF EFFECT

	Kind of Effect[a]		
Kind of Test	Lethal	Sublethal	Genotoxic
Solid and liquid phase:			
Amphipod	X	X	
Bivalve larvae	X	X	[b]
Echinoderm embryo	X[c]	X[c]	[b]
Juvenile polychaete	X	X	
Extracts:			
Microtox		X	
Anaphase aberration			X

[a] Only the juvenile polychaete test measures long-term effects (i.e., 20 days, which is a substantial portion of the test organism's life cycle).

[b] Genotoxic endpoints have been measured by others outside the Puget Sound area.

[c] Unlike bivalve larvae, echinoderm larvae rarely disintegrate. While mortality can be calculated, it is more of a reflection of embryos lost in the sediments rather than embryos lost to disintegration. Thus a combined mortality/abnormality endpoint is more appropriate.

FIELD COLLECTION OF SUBTIDAL SURFICIAL SEDIMENTS

This section describes the protocols required to collect an acceptable subtidal surficial sediment sample for analysis by a laboratory sediment bioassay. This subject has generally been neglected in the past and sampling crews have been given relatively wide latitude in deciding how to collect samples. However, because sample collection procedures influence the results of all subsequent laboratory and data analyses, it is critical that samples be collected using acceptable and standardized techniques.

DESIGN OF SAMPLER

In Puget Sound, the most common sampling device for subtidal surficial sediments is the modified van Veen bottom grab. However, various sampling devices (e.g., Smith-McIntyre grab sampler, box corer, Kasten corer, Minisoutar) are also used. The primary criterion for an adequate sampler is that it consistently collects undisturbed samples to the required depth below the sediment surface without contaminating the samples. An additional criterion is that the sampler can be handled properly onboard the survey vessel. An otherwise acceptable sampler may yield inadequate sediment samples if it is too large, heavy, or awkward to be handled properly. It is recommended that the sampler be constructed of stainless steel to avoid metals contamination which might be likely to occur with brass or other construction materials. One potential source of sampling devices is the University of Washington Department of Oceanography.

Collection of undisturbed sediment requires that the sampler:

- Creates a minimal bow wake when descending
- Closes to form a leak-proof seal after the sediment sample is taken
- Prevents sediment washout and excessive sample disturbance when ascending
- Allows easy access to the sample surface.

Most modified van Veen grabs have open upper faces that are fitted with rubber flaps. Upon descent the flaps are forced open to minimize the bow wake, whereas upon ascent the flaps are forced closed to prevent sample washout. Some box corers have solid flaps that are clipped open upon descent and snap shut after the corer is triggered. Although most samplers seal adequately when new, the wear and tear of repeated field use eventually reduces this sealing ability (e.g., through chipped or improperly aligned jaws). A sampler should therefore be properly maintained and monitored constantly for proper operation and minimal sample leakage. If unacceptable leakage occurs or the sampler malfunctions in any manner, the
sampler should be repaired or replaced. If a sampler is to be borrowed or leased for a project, its operation and sealing ability should be evaluated prior to sampling. Also, it is prudent to have a backup sampler onboard the survey vessel if the primary sampler begins leaking during a cruise.

The required penetration depth below the sediment surface is a function of the desired sample depth (see *Penetration Depth*). Generally, it is better to penetrate below the desired sample depth to minimize sample disturbance when the sampling device closes. Penetration depth of most sampling devices varies with sediment character; it is greatest in fine-grained sediments and least in coarse-grained sediments. Sampling devices generally rely upon either gravity or a piston mechanism to penetrate the sediment. In both cases, penetration depth can be modified by adding or removing steel or lead weights from the samplers. Thus, it is optimal to use a sampler that has a means of weight adjustment. If a sampler cannot consistently achieve the desired penetration depth, an alternate device should be used.

The sampler should be brought aboard the vessel with a minimum amount of swinging, to minimize sample disturbance. Once the sampler is secured onboard the survey vessel, it is essential that the surface of the sample be made accessible without substantially disturbing the sample. Most samplers have hinged flaps on their upper face for this purpose. The openings in the upper face of the sampler should be large enough to allow convenient subsampling of the sediment surface. If an opening is too small, the sample may be unduly disturbed as the field member struggles to take a subsample.

PENETRATION DEPTH

For characterizing the toxicity of surficial sediments in Puget Sound, it is recommended that the upper 2 cm of the sediment in a sample be evaluated. The upper 2 cm of sediment is recommended for analysis because that is the sediment horizon in which most infaunal organisms reside and the horizon that is contacted most frequently by epifaunal organisms. When collecting the upper 2 cm of sediment, it is recommended that a minimum penetration depth of 4-5 cm be achieved for each acceptable sample. The portion of sample below the upper 2 cm of sediment can be discarded after the surficial sediment has been collected (unless the study design specifies otherwise).

Although the 2-cm specification is arbitrary, it will ensure that:

- Relatively recent sediments are sampled
- Adequate volumes of sediment can readily be obtained to satisfy the needs of most study objectives
- Data from different studies (historical or ongoing) can be compared validly.

Sampling depths other than the upper 2 cm may be appropriate for specific purposes. For example, when toxicity determinations are made for sediments to be dredged as part of the permitting process, sediments collected from depths as great as several meters may be tested. Additionally, some investigations may want to examine concentration gradients of contaminants

with sediment depth in order characterize the history of some contamination. It should be remembered, however, that if a sampling depth other than the upper 2 cm is used, the results based on the alternative depth may not be equivalent to results based on the upper 2 cm of sediment.

For sampling of reference sediments it may be appropriate to avoid anoxic sediments below the redox potential discontinuity (RPD) horizon. In the Puget Sound Dredged Disposal Analysis program,

reference sediments with high sulfides are suspected of producing performance problems in some bioassays.

OPERATION OF SAMPLER

The sampling device should be attached to the hydrowire using a ball-bearing swivel. The swivel will minimize the twisting forces on the sampler during deployment and ensure that proper contact is made with the bottom. For safety, the hydrowire, swivel, and all shackles should have a load capacity at least 3 times greater than the weight of a full sampler. In addition, screw-pin shackles should have wire through the eye and around one side of the shackle to prevent the pin from rotating.

The sampler should be lowered through the water column at a controlled speed of approximately 1 foot/second. Under no circumstances should the sampler be allowed to "free fall" to the bottom, as this may result in premature triggering, an excessive bow wake, or improper orientation upon contact with the bottom. The sampler should contact the bottom gently, and only its weight or piston mechanism should be used to force it into the sediment.

After the sediment sample is taken, the sampler should be raised very slowly off the bottom and then retrieved at a controlled speed of approximately 1 foot/second (0.3 meters/second). Before the sampler breaks the water surface, the survey vessel should head into the waves (if present) to minimize vessel rolling. This maneuver will minimize swinging of the sampler after it breaks the water surface. If excessive swinging occurs or if the sampler strikes the vessel during retrieval, extra attention should be paid to evaluating sample disturbance when judging sample acceptability.

The sampler should be secured immediately after it is brought onboard the survey vessel. If the sampler tips or slides around before being secured, extra attention should be paid to evaluating sample disturbance.

SAMPLE ACCEPTABILITY CRITERIA

After the sampler is secured on deck, the sediment sample should be inspected carefully before being accepted. The following acceptability criteria should be satisfied:

- The sampler is not overfilled with sample such that the sediment surface is pressed against the top of the sampler
- Overlying water is present (indicates minimal leakage)
- The overlying water is not excessively turbid (indicates minimal sample disturbance)
- The sediment surface appears to be relatively undisturbed (i.e., lack of channeling or sample washout)
- The desired penetration depth is achieved (e.g., 4-5 cm for a 2-cm-deep surficial sample).

If a sample does not meet all of these criteria, it should be rejected and discarded away from the sampling station.

SAMPLE COLLECTION

After a sample is judged acceptable, the following observations should be entered on the field log sheet:

- Date and time
- Station location at the time of bottom contact (see recommended protocol for station positioning)
- Station depth (relative to MLLW)
- Gross characteristics of the surficial sediment
  - Texture
  - Color
  - Biological structures (e.g., shells, tubes, macrophytes)
  - Presence of debris (e.g., wood chips, wood fibers, human artifacts)
  - Presence of oily sheen
  - Obvious odor (e.g., hydrogen sulfide, oil, creosote)
- Gross characteristics of the vertical profile (determined after the surficial sediments have been collected)
  - Vertical changes in sediment characteristics
  - Presence and depth of any apparent redox potential discontinuity layer
- Penetration depth.

Before subsamples of the surficial sediments are taken, the overlying water must be removed. The preferred method of removing this water is by slowly siphoning it off near one side of the sampler. Methods such as decanting the water or slightly opening the sampler to let the water flow out are not recommended, as they may result in unacceptable disturbance or loss of fine-grained surficial sediment and organic matter.

Once the overlying water has been removed, the surficial sediment can be subsampled. It is recommended that subsamples be taken using a flat scoop. This device will allow a relatively large subsample to be taken accurately to a depth of 2 cm. Coring devices are not recommended because they usually collect inadequate amounts of surficial sediment, and therefore require repeated extractions to obtain a sufficient volume of material for analysis of conventional sediment variables. A curved scoop is not recommended because it does not sample a uniform depth. Because accurate and consistent subsampling requires practice, it is advisable that an experienced person perform this task.

Finally, sample contamination during collection must be avoided. All sampling equipment (e.g., scoops, containers) should be made of noncontaminating material, and should be cleaned appropriately before use. It is recommended that all objects coming in contact with the sample be made of glass, stainless steel, or PTFE (i.e., polytetrafluoroethylene; e.g., Teflon®). To avoid contamination, all sampling equipment should be cleaned in sequence with site seawater, a detergent scrub and rinse, pesticide-grade acetone, and pesticide-grade methanol or hexane prior to initial use and between use for each station. The methanol or hexane should be allowed to evaporate prior to using the equipment. If metals samples are being taken, equipment should also be cleaned with dilute acid (10% HNO_3) and rinsed, followed by the pesticide-grade acetone steps. Where possible, innocuous or biodegradable sampling gear cleaning and decontaminating agents should be used.

SAMPLE HOMOGENIZATION

Sediment from single samples or composites of multiple collections or composites of multiple samples should be homogenized prior to collecting a subsample for bioassay analyses. Compositing and homogenization can be accomplished by transferring sediment to a clean glass or stainless steel bowl and thoroughly homogenizing by stirring with stainless steel spoons or spatulas until textural and color homogeneity are achieved. The contents of the bowl should be continuously homogenized as subsamples are taken, to prevent potential settlement of larger particles. In addition, unrepresentative material (e.g., stones, wood chips, seagrass) should be removed at the discretion of the chief scientist and noted in the field logbook. The bowl and all utensils should be cleaned in sequence with site seawater, a detergent scrub and rinse, pesticide-grade acetone, and pesticide-grade methanol or hexane between composites, and kept covered with aluminum foil to prevent airborne or other contamination. The methanol or hexane should be allowed to evaporate prior to using the bowl and utensils. If metals samples are being taken, equipment should also be cleaned with dilute acid (10% HNO_3) and rinsed, followed by the pesticide-grade acetone steps. If total sulfides are to be measured, a subsample should be taken immediately. Zinc acetate should be used as a fixative following the recommended protocol for measuring conventional sediment variables.

CONCURRENT COLLECTION OF SEDIMENT CHEMISTRY AND BIOASSAY SAMPLES

If sediment chemistry samples are being collected concurrently with sediment bioassay samples, they should be collected from the same homogenized sediment sample. Specifically, individual samples or composites of several samples should be mixed before aliquots are removed for sediment chemistry and sediment bioassay determinations. Sample homogenization and removal of bioassay aliquots should be conducted so that chemical aliquots are not contaminated in the process. The protocols for removing aliquots for analyses of organic compounds and metals should be consulted in the appropriate Puget Sound guidelines documents (PSEP 1989a,b) to ensure that removal procedures for bioassay

aliquots are compatible with those recommended for chemical aliquots.

REPLICATION OF BIOASSAYS

The guidelines for all of the sediment bioassays included in this document recommend that all field-collected sediments be homogenized before subsamples are removed for bioassay and chemical analyses. This technique is the one used for most studies in Puget Sound, and it ensures that the bioassay and chemical results are related as closely as possible. The replicate analyses that are subsequently conducted on the bioassay subsample assess the variability encountered in laboratory testing, rather than the variability of sediment toxicity that exists in the field. To assess field variability, an alternate sampling design could be specified that requires each bioassay replicate to be run on a separate replicate grab sample from each station. The primary drawback to this technique is that the single set of chemical concentrations usually measured at each station (i.e., primarily because of cost constraints) would not relate directly to the sediment toxicity measured in each replicate grab sample. This lack of a direct relationship between bioassay and chemical results can sometimes make data interpretation difficult for individual replicate samples. However, the mean bioassay response could be compared directly with chemical concentrations if the chemical measurements are made on a composite of equal amounts of sediment subsampled from each of the replicate samples used for bioassay analysis.

GENERAL QUALITY ASSURANCE/QUALITY CONTROL GUIDELINES

The following general QA/QC procedures apply to all sediment bioassays. QA/QC procedures specific to each of the individual bioassays addressed in this document, and that differ from the following generic guidelines, are incorporated into individual bioassay guidelines.

NEGATIVE CONTROLS

All bioassays must be conducted using well-established negative (clean) controls. Such controls are clean, nontoxic seawater and/or sediment samples taken from outside each study area. For every test series with a particular organism, one bioassay test chamber or series of chambers must contain clean, material (i.e., seawater and/or sediment). In most cases, a complete batch of bioassay analyses must be repeated if more than a specified percentage of the corresponding control animals exhibits the response of interest.

POSITIVE CONTROLS

All bioassays shall be conducted using well-established positive (toxic) controls. These controls involve the use of reference toxicants such as cadmium chloride, silver chloride, phenol, and sodium lauryl sulfate. Use of sodium pentachlorophenate as a reference toxicant should be avoided because of the potential for dioxin contamination and the difficulty encountered in disposing of this chemical. Reference toxicants are used to provide insight into mortalities or increased sensitivity that may occur as a result of disease, changes in tolerance/sensitivity, or loading density. Reference toxicants can also provide insight into nonlethal effects that occur due to acclimation, insensitivity, or stress tolerance developed during handling and acclimation. Also, the use of reference toxicants facilitates comparisons of test results among different studies. Accordingly, concurrent bioassays using a reference toxicant should be implemented for each test series. Labs are advised to investigate proper disposal of reference toxicant in their area, e.g., acceptability for disposal to sanitary sewer systems. Labs should maintain documentation on discharge practices for test solutions, sediments and culture water, including disinfection of discharged water. Finally, it is common practice for labs to establish an internal acceptance standard through control charts (for example, plus or minus two standard deviations on the EC_{50} or LC_{50}).

TEST ORGANISMS

Only healthy organisms of similar size and life history stage should be used in bioassays. Taxonomic identifications of bioassay organisms must be confirmed by a qualified taxonomist. It is recommended that a type specimen be established, with at least annual identification of organisms and maintenance of voucher specimens for each project. Labs should maintain documentation on the source of test organisms and the disposal of test organisms. Some test organisms (for example *Neanthes arenaceodentata* and *Ampelisca abdita*) are not native to Washington and must be managed in compliance with Washington State Department of Fisheries and Wildlife (WDFW) regulations regarding aquatic disease control and the importation of exotic species (Chapter 220-77 WAC, Aquatic Disease Control, and Chapter 232-12-017 WAC, Deleterious Exotic Wildlife). Also, labs are advised to have and maintain the proper WDFW permits for collecting Puget Sound marine organisms.

REFERENCE TEST SAMPLES

Laboratory negative control sediments generally are those from which infaunal test animals (e.g., amphipods) were collected. As such, physical and chemical sediment characteristics (e.g., interstitial water salinity, grain size, organic content) may be very different from those of the test sediments. Where this is the case, one or more reference sediments should be added to the test series. Reference sediments should be collected from an area documented to be free from chemical contamination and should represent the range of important natural physical and chemical characteristics of the test sediments (see section titled *Puget Sound Reference Areas*). Such a sample, if it is in fact essentially free of contamination, can provide data that can be used to separate toxicant effects from unrelated effects such as those of sediment grain size. Additionally, to further ensure the integrity of reference test samples, investigators should use experienced sampling personnel and sample from the biologically active zone, avoiding anoxic sediments below the RPD horizon. The wet-sieving method (PTI, 1991; PSDDA, 1990) should be used to match reference sediments with test sediments .

SEDIMENT HOLDING TIME

At the present time specific holding times for sediment samples used for bioassay testing have not been thoroughly evaluated. The following suggestions are recommended on an interim basis. As additional data are generated, these holding times for bioassay samples will be re-evaluated. Each study (and testing of reference and control sediments) should report the holding times with the study results.

Fresh sediment to be used in the amphipod, bivalve larvae, echinoderm embryo, juvenile polychaete, and Microtox (saline extract) bioassays should be stored in the dark at 4°C for as brief a time as possible after field collection (ideally no more than 2 week). Sediments to be used in the anaphase aberration and Microtox (organic extract) tests should be frozen at -20°C within 8 hours of collection, stored, and extracted with organic solvent within 6 months after field collection.

Although it is largely unknown whether sediment toxicity changes substantially between field collection and laboratory analysis following a holding time of 2 weeks at 4°C, the 2-week holding time is considered the minimum holding time that can be routinely achieved by most studies conducted in Puget Sound. The minimum 2-week holding time is therefore based partly on logistical considerations.

Maximum holding times are important to programs that rely on tiered testing where chemical analyses are conducted prior to toxicity testing. This tiered approach is used by the Puget Sound Dredged Disposal Analysis (PSDDA) program for evaluating dredged sediments for unconfined open-water disposal in Puget Sound. Under the PSDDA program, sediments are stored in the dark in a nitrogen atmosphere at 4°C for no longer than 8 weeks. The PSDDA program has established an 8-week maximum based on Tatem et al. (1991) and Becker and Ginn (1990). Because the results of recent studies evaluating the effects of sediment holding time on sediment toxicity have been variable, it is prudent to store sediments for as short a time as possible after field collection. If there are no other compelling reasons (such as the tiered testing schedule under PSDDA), a maximum holding time of 2 weeks is recommended for Puget Sound, based on the best professional judgment of regional investigators and on logistical constraints.

At the national level, while the American Society for Testing and Materials recommends a maximum holding time of 2 weeks for both marine sediment (ASTM 1990) and freshwater sediment (ASTM in press) prior to toxicity testing, national guidance provided by the joint EPA/Corps Ocean Testing Manual (1991) and Inland Testing Manual (draft 1994) allow longer sediment holding times similar to PSDDA.

Regardless of the holding time used for a study, it is essential that the holding time be reported along with the study results.

BLIND TESTING

For regulatory purposes, blind and random testing is required and all sediment treatment containers should be randomized and testing should be conducted without laboratory personnel knowing sample identities. Replicates of each treatment should be assigned a code number during testing and randomized in the test sequence.

For some research investigations it is recognized that blind testing may not be required.

MAINTENANCE/MEASUREMENT OF WATER QUALITY

Bioassays involving exposure of organisms in aqueous media require that the media be uncontaminated, and that proper water quality conditions be maintained to ensure the survival of the organisms and to ensure that undue stress is not exerted on the organisms unrelated to the test sediments. At a minimum, the following variables must be measured at the beginning and terminations of testing: salinity, dissolved oxygen, pH, and temperature. Other conventional water quality variables, such as sulfides and ammonia, may also influence the results of sediment toxicity testing and should be measured as routine water quality parameters at the beginning and termination of testing. For bioassay tests lasting several days, it is recommended that the following variables be measured on a periodic basis and recorded: salinity, dissolved oxygen, pH, and temperature.

EQUIPMENT CLEANING PROCEDURES

It is essential that field and laboratory equipment be properly cleaned before sediments are collected or bioassays are conducted. Proper cleaning ensures that bioassay responses are related to the contaminants present in the test sediments and not to contaminants present on improperly cleaned equipment. The following cleaning procedures are recommended for all bioassays:

- Sediment collection equipment (e.g., corers, grab samplers, homogenization bowls, and utensils):

  - Wash with laboratory detergent (non-phosphate)
  - Rinse with 10% HNO_3 followed by distilled water
  - Rinse with pesticide-grade acetone
  - Rinse with pesticide-grade methanol or hexane

- Laboratory equipment (e.g., sample containers, bioassay chambers, and utensils):

  - Wash with laboratory detergent
  - Rinse with tap water
  - Rinse with 10-percent reagent-grade hydrochloric acid
  - Rinse 5 times with deionized water
  - Rinse with pesticide-grade acetone
  - Rinse with pesticide-grade methanol or hexane
  - Allow residual methanol or hexane to evaporate

If alternate cleaning techniques are used, it should first be demonstrated that they are as effective as the above techniques, and they shall be documented as a standard operating procedure and kept with the study records.

STANDARD LABORATORY PROCEDURES

Standard laboratory procedures must be followed in all testing. These include proper documentation, proper cleaning, avoidance of contamination, and maintenance of appropriate test conditions. All unusual observations or deviations from established procedures must be recorded and reported.

PUGET SOUND REFERENCE AREAS

Performance standards for reference areas are needed to provide an objective and consistent basis for assessing contaminated sediments in various environments. Performance standards are criteria for suitable reference conditions defined in terms of habitat characteristics (e.g., sediment grain size, salinity, chemical concentrations) and biological properties (e.g., bioassay responses). The Washington Department of Ecology has developed interim performance standards for subtidal sediments in Puget Sound reference areas (Pastorok et al. 1989). Development of reference area performance standards for intertidal areas or for specific biogeographic regions (including low-salinity habitats) requires further data collection. Where quantitative performance standards could not be developed for certain variables, general objectives for locating reference area stations were provided to guide technical judgments regarding the acceptability of reference sites on a case-by-case basis. The recommended performance standards modified from Pastorok et al. (1989) are summarized below.

Reference areas used in evaluating sediments should be relatively clean (i.e., they should be consistent with the performance standards being developed by Ecology). Reference areas should not be located immediately adjacent to contaminated sites (e.g., within the central basin, within the East Passage, or within an urbanized embayment such as Elliott Bay or Commencement Bay). Pastorok et al. (1989) suggested Sequim Bay, Samish Bay, Dabob Bay, and Carr Inlet as the primary reference areas for Puget Sound based on available data. Reference areas used by other investigators of sediment conditions have included Carr Inlet (Tetra Tech 1985); Samish Bay, Dabob Bay, and Sequim Bay (Battelle 1986); Case Inlet (Malins et al. 1980; Battelle 1986); Port Madison (Malins et al. 1980); and Port Susan (Malins et al. 1982; PTI and Tetra Tech 1988a,b). Recent work suggests that other locations (e.g. in Grays Harbor and Willapa Bay) may make suitable reference areas (EPA, 1993). Additional information on specific reference areas may become available as Ecology or other agencies continue to develop performance standards. Sampling stations in reference areas should be located to achieve the following general objectives.

SEDIMENT CHEMISTRY

- Concentrations of all chemicals measured in sediments should be representative of uncontaminated areas of Puget Sound that are remote from significant sources of contaminants.

- Concentrations of all chemicals analyzed in sediments should be lower than available performance standards for Puget Sound reference areas. If such standards are not available for specific compounds, the concentrations of those compounds should be less than the state sediment quality standards.

SEDIMENT BIOASSAYS

- The bioassay response measured for sediments from each reference station should be less than the mean response that can be consistently discriminated as toxic in statistical comparisons (a=0.05) with control sediments (amphipod and juvenile polychaete bioassays), control seawater (bivalve larvae and echinoderm embryo bioassays), or solvent (anaphase aberration and Microtox bioassays).

- The physical and chemical characteristics (e.g., grain size, organic content, interstitial salinity) of reference sediments should match the corresponding characteristics corresponding to those of the test sediments as closely as possible. In some cases it may be appropriate to use the wet-sieving technique (PTI, 1991; PSDDA, 1990) to match reference sample grain sizes to test sediment.

- Quantitative performance standards for sediment bioassays from reference areas are currently being developed by Ecology.

GENERAL HABITAT

- Existing or planned development (e.g., major shoreline or water projects) should be minimal in the vicinity of reference sites

- A reference area should be accessible for sampling by routine techniques.

AMPHIPOD SEDIMENT BIOASSAY

USE AND LIMITATIONS

The amphipod (*Rhepoxynius abronius*, *Ampelisca abdita* or *Eohaustorius estuarius*) sediment bioassay is used to characterize the toxicity of marine or estuarine sediments. This bioassay may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations. Mortality is the primary endpoint in the amphipod bioassay. Sublethal endpoints, such as emergence of amphipods from the sediment during the exposure period and failure to rebury in sediment at the end of the exposure period, may also be used to assess sediment toxicity. Total effective mortality, a combined endpoint representing the sum of percent mortality and percent nonreburial, has also been used in this bioassay for *R. abronius* or *E. estuarius*. The basis for the combined endpoint is the assumption that individuals that fail to rebury in sediments at the end of the exposure period would die in nature as a result of predation.

The following constraints apply:

- For the *R. abronius* bioassay, an interstitial water salinity of ≥25 ppt is necessary to ensure that there are no salinity effects. In general, adjustment of interstitial water salinity should not be attempted because of potential effects of adjustment on toxicological properties of the sediment. However, for dredged material that will be disposed of in the marine environment (where *in situ* sediments have interstitial water salinities >25 ppt), salinity adjustment may be desirable. If interstitial water salinities of dredged material are between 15 and 24 ppt, they may be adjusted upward for use in the *R. abronius* bioassay. For other testing purposes, use of the *E. estuarius* or *A. abdita* bioassay is preferred for sediments with salinities <25 ppt.

- Grain size may have an effect on *R. abronius* or *E. estuarius* at extremes of fine and coarse material (DeWitt et al. 1988, 1989). If the clay and fines content of the test sediments exceeds 20 percent or the gravel content exceeds 35 percent, controls for the effects of particle size distribution (i.e., a reference area sediment similar in grain size to the test sediment) are recommended for interpretation of toxicity test results when using *R. abronius* or *E. estuarius*. At the coarse end of the spectrum (greater than 20 percent gravel), a grain size reference sediment is also recommended for *Ampelisca*.

- As in all bioassays using natural populations, there is a possibility that relative sensitivity of the amphipods will vary with season or other factors. Accordingly, a positive control is recommended. This should comprise a 96-hour LC_{50} measurement with a reference toxicant (e.g., cadmium chloride) conducted in the absence of sediment. The salinity and temperature of the dilution water should be equivalent to the values specified for sediment testing (i.e., 28 ppt and 15°C, respectively).

- Identification of *R. abronius*, *A. abdita* and *E. estuarius* must be confirmed by a qualified taxonomist prior to initiation of the bioassay, and representative specimens should be preserved and archived for future reference.

- Predators generally are not a problem in the bioassay, but potential problems can be avoided by observation and predator removal (if necessary).

The *R. abronius* bioassay is appropriate for sediments with interstitial water salinity of ≥25 ppt. The *E. estuarius* bioassay is appropriate for sediments with interstitial water salinity of 2-28 ppt. *A. abdita* is appropriate for sediments which have greater than 60 percent clay and silt (fines) and with interstitial water salinity < 25 ppt.

Recommended Use Matrix	Sediment fines < 60%	Sediment fines ≥ 60%
Salinity ≥ 25 ppt	*R. abronius*	*A. abdita*
Salinity < 25 ppt	*E. estuarius*	*A. abdita*

These guidelines have been adapted from Swartz et al. (1985) for *R. abronius* and DeWitt et al. (1989) for *E. estuarius*. A standard guide for conducting 10-day static sediment toxicity tests for marine and estuarine amphipods has been developed by ASTM (1990).

FIELD PROCEDURES

Collection

Test Animals—Both *R. abronius* and *E. estuarius* can be collected using benthic grabs (e.g., van Veen, Smith-McIntyre) or small dredges. *E. estuarius* can also be collected by shovel at low tide. *A. abdita* can be collected by grab or by shovel in shallow areas. *A. abdita* is an east coast species which has also been introduced into San Francisco Bay. If a dredge is used, a short haul (10 meters) will minimize potential damage to the animals during collection. *R. abronius* inhabits fine sands from the low intertidal zone to a maximum depth of 60 meters. *E. estuarius* is generally found in intertidal estuarine sediments from +0.5 meters to +2.0 meters above mean lower low water (MLLW). *A. abdita* is found mainly in fine sediments from the low intertidal to 60 m. Approximately one-third more animals than are required for the bioassay are collected. Surface and bottom seawater salinity and temperature are measured at the collection site. Sediment temperature is recorded from the first and last dredge sample. It is recommended that bioassays be conducted within 10 days of amphipod collection.

Sediment—Control, reference, and test sediments should be stored in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 0.25 liters for each bioassay chamber is recommended for each kind of sediment. Because five replicate tests are conducted for each field sample, a minimum sediment sample size of 1.25 liters is recommended for each station. As a general advisory, twice the volume of sediment needed should be collected, however, the actual amount could be project-specific.

Processing

Test Animals—Contents of the dredge or grab sampler are gently washed into a container using seawater of similar temperature and salinity to that at the collection depth. Samples that show evidence of contamination (e.g., oil sheen) are rejected. *R. abronius* and *E. estuarius* will typically bury in the sediment and if necessary can be held in the containers for several hours (at the temperature of the collection depth) prior to sieving. It is preferable to minimize the delay between collection and sieving. Screens for sieving sediments should be either stainless steel or Nitex® type plastic to avoid sediment contamination with metals. To avoid handling stress, each dredge sample is placed in a separate container. Amphipods are maintained and transported in clean coolers, should be held in sediment during transport to the laboratory, and should be kept at or below the collection site temperature. During a long transport, aeration may be required. *Ampelisca* are not as easily removed from the sediment and are sometimes transported to the lab in the tube mat and sieved there.

Sediment—Control, reference, and test sediments should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The laboratory procedures for *R. abronius* and *E. estuarius* are those described by Swartz et al. (1985) and DeWitt et al. (1989) with the following changes incorporated:

- The salinity of the overlying water is adjusted to 28 ppt for *R. abronius*
- A 1.0-mm screen is used to sieve out amphipods prior to initiation of testing
- Holding time for amphipods is standardized to between 2 and 10 days
- Additional details are provided concerning maintenance and transportation of amphipods, confirmation of taxonomic identifications, and freeing of amphipods trapped by water surface tension during testing
- A specific procedure for adjustment of interstitial water salinity for testing dredged material is included.

The laboratory procedures for *A. abdita* are those described by the ASTM (1993b). The following points should be observed when using *A. abdita*:

- *Ampelisca* are fed during the laboratory holding period before the test
- A 0.5-mm screen is used to sieve out amphipods prior to initiation of testing

- The *Ampelisca* test is run at 20°C instead of 15°C
- Holding time for amphipods is standardized to between 2 and 10 days
- Immature amphipods are used in the *Ampelisca* test because if mature male Ampelisca are used, they will likely die of senescence during the 10-day test
- There is no reburial endpoint in the *Ampelisca* test. *Ampelisca* is not indigenous to the West Coast (except for an introduced population in San Francisco Bay), therefore special care must be taken by testing laboratories not to release the animals into the environment.

Test Animals

Sieving (*R. abronius* and *E. estuarius*)—A 1.0-mm sieve is used to remove the amphipods from sediment and mature amphipods (3.0–5.0 mm total length) are used in the sediment bioassay. Gentle sieving is essential to reduce handling stress. The sieve is placed in a large tub filled with seawater at ambient salinity and temperature for the collection site sediments. The entire contents of each holding container, including water, are washed through the sieve using seawater pumped at low pressure through a fan spray nozzle. The sieve can be shaken gently, but the bottom of the screen must be beneath the water surface at all times. Material retained on the screen is washed into buckets for sorting. Large pieces of detritus and obvious predators are discarded. If there is a delay of more than 1 hour before sorting begins, the buckets should contain enough sieved sediment to allow the amphipods to bury. The buckets must be kept at or below collection site temperature. Aeration may be necessary.

Sieving (*A. abdita*)—A 2-mm mesh sieve nesting over a 0.5-mm mesh sieve is used for rinsing the sediment containing the amphipods with a forceful stream of seawater at the collection temperature and salinity. The rinsing should break up the sediment material and also force most of the amphipods out of their tubes. An additional 1-mm mesh sieve may optionally be used to sort the animals by size. Material retained on the 0.5-mm sieve is vigorously shaken and swirled so the fine sediments pass through and the amphipods are separated from tubes, sediment, and detrital material. The sieve is then lifted from the water, allowed to drain, and slowly lowered into a shallow tray of seawater so that the *Ampelisca* will be caught on the water's surface tension and can be collected with a fine mesh dip net.

Sorting—Animals should be presorted. As an example, an aliquot of detritus or sediment containing amphipods is placed in a sorting tray. Healthy, active animals are removed with a bulb pipette (5-mm opening) and placed in 10-cm-diameter finger bowls filled with seawater of appropriate salinity and a 2-cm-deep layer of 0.5-mm sieved collection site sediment. Twenty amphipods are held in each bowl and enough bowls are prepared to provide at least one-third more specimens than are required for the bioassay. Seawater temperature during sorting of amphipods must not exceed 18°C (except for *Ampelisca*). *Ampelisca* is routinely tested at 20°C, and for comparison with other *Ampelisca* test results, 20°C is recommended. Filled finger bowls are submerged in holding tanks supplied with flowing water or aeration where temperature and salinity approximate bioassay conditions. If temperature and salinity adjustments are necessary, they should be made gradually. Healthy

amphipods will remain in the finger bowl sediment and can be retrieved easily when the bioassay is set up. *A. abdita* may leave their tubes and swim. Therefore a screened overflow should be used if this species is held in flowing seawater. Amphipods should be acclimated to laboratory conditions for a minimum of 2 days and a maximum of 10 days before testing.

Control Sediment

R. abronius and *A. estuarius* typically inhabit well-sorted, fine sand. Suggestions for sieving and settling may have to be adjusted for other sediment types. *A. abdita* is a tube-dwelling amphipod found mainly in protected areas and is often abundant in sediments with a high organic content. It generally inhabits sediments from fine sand to mud and silt without shell, although it can also be found in relatively coarser sediments with a sizeable fine component.

Approximately 0.25 liters of control sediment should be collected for each bioassay chamber. This sediment is sieved twice: first, to remove the test species and other macrobenthos and second, to adjust interstitial water salinity. The entire contents of one or more sediment samples, including water and suspended particulate matter, are sieved through a 0.5-mm screen without allowing overflow from the container. After the first sieving, the sediment is allowed to settle for at least 4 hours (preferably 12-16 hours). Overlying water is then decanted and the sediment resieved through a 0.5-mm screen into water of the bioassay salinity (28 ppt for *R. abronius* and *A. abdita*, ambient salinity for *E. estuarius*). Investigators should report methods used to determine ambient salinity in cases where the interstitial salinity of the test sediments varies over a range of salinities. Again, the sediment is allowed to settle for at least 4 hours (preferably 12-16 hours), overlying water is decanted, and the control sediment is held at 4°C until the bioassay chambers are prepared.

Test and Reference Area Sediment

Approximately 0.25 liters of test sediment should be collected for each bioassay chamber. Test sediments should not be wet-sieved, but if large predators or other large organisms are present, they can be removed using forceps or by pressing the sediment through a 2.0-mm screen. The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. *R. abronius* may be adversely affected by salinity stress if the interstitial water salinity is below 25 ppt. For estuarine dredged material designated for disposal in the marine environment and for other sediments, interstitial water salinities below 25 ppt may require adjustment upwards to control for false positives. While both *R. abronius* and *E. estuarius* are tolerant of a range of sediment grain size, particle size may affect test results. For these two species, controling for the effects of particle size distribution with reference sediments is recommended if the silt and clay content exceeds 20 percent or the gravel content exceeds 35 percent. For *A. abdita*, which can be found in relatively coarser sediments with a sizeable fine component, controling for the effect of particle size distribution with reference sediments is recommended if the gravel content exceeds 35 percent. (See applicable constraints, p. 20.)

The *R. abronius* test requires a minimum water column salinity regime of 28 ppt. When the interstitial (i.e., pore water) salinity is below 25 ppt, it must be raised if this test is to be used. The following procedure is recommended. The interstitial salinity of the sediments is determined (e.g., by refractometer using interstitial water collected by centrifugation) and the sediments are placed in the

bioassay chambers with overlying water of a salinity calculated to raise interstitial salinities to a minimum of 25 ppt. The sediments are then carefully and slowly stirred by hand with a clean glass rod for 1 minute, and allowed to settle for at least 4 hours (preferably 12-16 hours). The majority (approximately 75 percent) of the overlying water is then carefully decanted and the interstitial salinity in each chamber confirmed prior to bioassay initiation. The decant water can be retained, salinity adjusted if necessary, and used as the overlying water in the bioassay. Alternatively, fresh seawater with the appropriate salinity can be used as the overlying water. Sediments are slowly mixed with a glass rod after adding the decant water. When the bioassays are terminated, the interstitial salinities are reconfirmed (e.g., by refractometer using interstitial water collected by centrifugation).

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt for the *R. abronius* and *A. abdita* tests and at ambient interstitial salinity for the sediment collection site for the *E. estuarius* test. Temperature of seawater used in bioassays of either *R. abronius* or *E. estuarius* is maintained at 15 ± 1°C. A temperature of 20°C is recommended for *A. abdita*. The bioassay seawater must be uncontaminated, which may necessitate collection of seawater at the amphipod collection site. Seawater in which algal blooms have occurred should not be used. Natural and reconstituted seawater should be held at ≤15°C for no longer than 2 days before inoculation. The quantity of seawater required is dependent on sieving and holding needs and on the number of bioassay chambers.

Bioassay seawater is passed through a filter with 0.45-µm pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salts, brine prepared by freezing or reagent grade chemicals (ASTM 1989). Seawater is prepared within 2 days of the bioassay and stored in covered, clean containers at the bioassay temperature.

Facilities and Equipment

The bioassay chamber is a standard 1-liter glass beaker (10-cm internal diameter) covered with an 11.4-cm diameter glass watchglass. The beakers are placed in a shallow water bath or temperature-controlled room with overhead aeration source. Aeration to each beaker is provided through a 1-mL glass pipette that extends between the beaker spout and watchglass to a depth not closer than 2 cm from the sediment surface. Air is bubbled into the beakers at a rate that does not disturb the sediment. The bioassay temperature is maintained by either the water bath or room temperature control.

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. Large plastic containers and plastic sieves used for preparation and storage of sediment and seawater are preconditioned initially by soaking for 24 hours in seawater and rinsed after each use with clean seawater. They are used only for bioassays and stored in a clean room. Sieves and containers used to collect and store amphipods, seawater, and control sediment are kept separate from those used for test sediment.

Bioassay Procedure

The day before the bioassay is initiated, approximately 175 mL of test sediment are placed in the bottom of the 1-liter bioassay chamber to create a 2-cm-deep layer. Five replicate tests are conducted for each field sample. Chambers are filled to 750 mL with seawater at 28 ppt for the *R. abronius* and *A. abdita* tests or ambient interstitial salinity for the *E. estuarius* test, covered with a watchglass, and placed in a water bath (15°C for *R. abronius* and *E. estuarius*, 20°C for *A. abdita*). Constant illumination is provided by overhead lights. Water in the chambers is aerated without disturbing the sediment surface. The system is allowed to equilibrate overnight before the amphipods are added. When the test is initiated, 20 amphipods are placed in each chamber and the seawater level is brought up to 950 mL. The bioassay is terminated after 10 days of exposure.

The primary endpoint is mortality after 10 days exposure to test or control sediment. The secondary endpoints that also can be measured are daily emergence of amphipods from sediment and for *R. abronius* and *E. estuarius* failure to rebury in sediment at the end of the exposure period. It is recommended that the number of amphipods reburying and the number removed from the test be recorded. This information may provide an indication of the viability of the test stock of amphipods.

Initiation—The day before the bioassay is initiated, each test sediment sample is homogenized and an aliquot of 175 mL (sufficient to make approximately a 2-cm-deep layer) is added to a bioassay chamber. For replicate bioassay samples, the volume of sediment necessary to place 175 mL in the first chamber is added to the other replicates. The same procedure applies to control and reference sediment. Treatments are randomly assigned to prenumbered bioassay chamber.

The sediment aliquot in the chamber can be settled by smoothing with a spoon, and bubbles can be removed by tapping the beaker against the palm of the hand. A disk (attached by a string for removal) is placed on the sediment surface. This minimizes sediment disruption as bioassay seawater is added up to the 750-mL mark on the chambers. This disk is removed and rinsed in bioassay water between chambers and changed between treatments. The beakers are covered with watchglasses, put into the water bath of the appropriate temperature or the temperature-controlled room, and aerated. The chambers are allowed to equilibrate overnight to bioassay conditions. Normal room lighting is maintained continuously during the bioassay. If the experimental design requires monitoring of sediment chemistry [e.g., metals, total volatile solids, oxidation potential (Eh)], additional chambers must be set up for this purpose. Monitoring the quality of seawater overlying the sediment can be accomplished in the bioassay chambers without disturbing the sediment. Temperature is recorded from a thermometer maintained in a separate chamber containing control sediment and bioassay water but no amphipods.

On the day the bioassay is initiated, amphipods are distributed among all chambers so that each receives 20 individuals. It is usually not logistically possible to distribute amphipods to all chambers at the same time, so it is necessary to select a portion of the chambers (as many as 15) to be processed together. The exact number of chambers to receive amphipods at one time is dependent on the size and design of the experiment. At least one replicate from each treatment, including control and reference area sediment, is processed at a time if possible. Otherwise, selection is random.

Amphipods are removed from the holding sediment using a sieve (1.0-mm for *R. abronius* and *E. estuarius*, 0.5-mm for *A. abdita*), and then transferred to sorting trays. About one-third more fingerbowls are removed from the holding tank at one time than are required for the number of chambers. This allows selection of active, apparently healthy animals for the bioassay. Amphipods are removed from the sorting tray and sequentially distributed among clean 10-cm fingerbowls each

containing 150 mL of bioassay seawater without sediment. The number of amphipods distributed to each fingerbowl is recounted by transferring them to a separate fingerbowl.

Amphipods are added to the bioassay chambers by placing a black plastic disk on the seawater surface and gently pouring the entire contents of the fingerbowl into the beaker. The fingerbowl is washed with bioassay water to remove adhering amphipods. The seawater level is brought up to 950 mL with bioassay water, and the disk is removed and rinsed between samples. Amphipods are allowed to bury in the sediment and any that are floating on the seawater surface are pushed down with the edge of the beaker cover or a clean glass rod. For *R. abronius* and *E. estuarius*, after 15 minutes amphipods that have not buried are removed and replaced. Normally, less than 1 percent of the animals will fail to bury in 1 hour. *A. abdita* should be given one hour to burrow into the sediments.

Monitoring—If samples for chemical analysis are desired, seawater and sediment samples can be taken from chambers at the initiation of the bioassay. A small quantity of seawater can be taken from chambers at the initiation of the bioassay, but chemistry beakers have to be sacrificed to obtain sediment samples. This is accomplished by siphoning the overlying seawater without disturbing the sediment surface and then taking appropriate sediment aliquots for chemical analyses. It is not necessary to add amphipods to chemistry beakers that are sacrificed at the initiation of the bioassay, but amphipods are added to those sacrificed later. Certain sediment and water quality variables (e.g., dissolved oxygen, pH, Eh) can be monitored by inserting analytical probes into the chemistry beakers.

During the course of the bioassay, certain observations are made daily. Temperature in the chamber set up for this purpose is monitored. Lighting and aeration systems are checked. Each chamber is carefully examined but not disturbed except for the temporary removal of the aeration pipette and watchglass. Notes are made on sediment appearance and unusual conditions. The number of amphipods that have emerged from the sediment, either floating on the water surface or lying on top of the sediment, is recorded. Amphipods that have emerged are not removed, even if they are dead. These data are used to document the temporal pattern of emergence. Amphipods trapped by surface tension at the water surface are gently pushed down with a clean instrument (e.g., pipette, glass rod, beaker cover).

Termination—The bioassay is terminated after 10 days of exposure. After daily observations are recorded, the contents of the bioassay chambers are sieved through a 0.5-mm screen. Material retained on the screen is placed in clean bioassay water in a sorting tray. The numbers of live and dead amphipods are recorded. The sum of these numbers may not always equal 20 because of death and subsequent decomposition of amphipods. An amphipod is counted as alive if there is any sign of life (e.g., pleopod twitch observed under magnification, response to gentle prodding with a clean instrument).

If the reburial endpoint is to be evaluated, amphipods that survive the test are transferred to dishes containing a 2-cm layer of negative-control sediment and observed under constant illumination. The numbers of individuals able to bury after an exposure period of 1 hour is then recorded. There is no reburial endpoint in the *Ampelisca* test.

Experimental Design

Logistics—A typical sediment bioassay involves about 50–60 bioassay chambers. Collection and preparation of animals, sediment, and seawater requires at least four people for 2 days. Three or four people are required on the days experiments are initiated and terminated. One person can monitor the experiment in progress.

Controls—Five replicates of the amphipod collection-site control sediment are included in all bioassays. These comprise a negative (clean) control that allows comparisons among experiments and among laboratories of the validity of the procedures used in individual investigations. In the negative control, mean mortality should be ≤10 percent and individual replicate mortality should be ≤20 percent for the test to be considered valid. Experiments in which contaminants are added to sediment may require additional solvent control replicates to determine effects of solvent addition.

A positive (toxic) control is also required for all testing. This involves determining 96-hour LC_{50} values for *R. abronius*, *E. estuarius*, or *A. abdita* exposed to a reference toxicant in clean, filtered seawater without sediment (following standard bioassay procedures and under the same general test conditions as the sediment bioassays). Such data are necessary to determine the relative sensitivity of the animals (e.g., seasonal difference in sensitivity) for each test series to ensure comparability of the data. The commonly used reference toxicant is reagent-grade cadmium chloride. For *R. abronius*, Swartz et al. (1986) determined a 96-hour LC_{50} of 1.61 mg/L for cadmium chloride. The LC_{50}s for *R. abronius* and *A. abdita*, using data from the Army Corps of Engineers DAIS database, are 0.79±0.48 mg/l (n=42) and 0.49±0.42 mg/l (n=9) cadmium, respectively (Army Corps of Engineers personal communication, 1994). Acute lethality results for a reference toxicant must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and one above the LC_{50}. Statistical procedures for the LC_{50} estimate are given in APHA (1985) and ASTM (1989).

Reference Area Sediment—The design of field surveys typically includes a reference sediment involving five replicate laboratory tests of samples from an area believed to be free from sediment contamination. This provides a site-specific basis for comparison of potentially toxic and nontoxic conditions while controlling for the effects of exposing amphipods to non-native sediments. The grain size composition (as measured by percent silt plus clay and percent gravel) of the reference area sediment should be as similar as possible to that of the test sediment. Organic carbon content of reference area sediment should also be matched with the test sediment as closely as possible. However, it should be recognized that matching of organic carbon content may not be warranted in cases where pollution (e.g., from pulp mills, sewage outfalls, combined sewer overflows) is responsible for high organic content of test sediments.

DeWitt et al. (1988) found that sediments having a high percentage of fine-grained material could increase the mortality rate of *Rhepoxynius abronius* in the absence of apparent chemical contamination. DeWitt et al. (1988) developed a regression model to predict the relationship between amphipod mortality and sediment grain size. In their approach, test results (i.e., mean mortality values) that lie outside the 95-percent prediction limit developed from reference area data are considered indicative of chemical toxicity. A similar model can be applied to the *E. estuarius* bioassay (DeWitt et al. 1989).

Response Criteria—Endpoints examined in the amphipod bioassay include mortality, emergence from sediment, and for *R. abronius* and *E. estuarius* failure to rebury in sediment at the end of the exposure period. Data on emergence and reburial are used to monitor sublethal behavioral responses of the amphipods during (i.e., emergence) and after (i.e., reburial) the 10-day exposure. Mortality after 10 days of exposure is the primary criterion of toxicity. An estimate of total effective mortality may also be calculated by summing percent mortality and percent failure to rebury (based on the starting number of amphipods (20), not the number alive at the end of the test). Each of these response criteria must be monitored in a "blind" fashion; that is, the observer must have no knowledge of the treatment of the sediment in the chambers. This is accomplished through randomization of beaker numbers.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Water quality measurements during testing [i.e., dissolved oxygen, temperature, salinity, pH, sulfides (optional), and ammonia (optional)]
- Daily emergence for each chamber and the 10-day mean and standard deviation for each treatment
- Failure of *R. abronius* or *E. estuarius* to rebury (optional) for each chamber and the mean and standard deviation for each treatment
- 10-day mortality and total effective mortality (optional) in each chamber and the mean and standard deviation for each treatment
- Interstitial water salinity for control, reference, and test sediments
- 96-hour LC_{50} values with reference toxicants (results for metallic compounds should be reported in terms of the metal ion rather than as the weight of the whole salt)Any problems that may have influenced data quality.

BIVALVE LARVAE SEDIMENT BIOASSAY

USE AND LIMITATIONS

The bivalve larvae bioassay technique is described in *Standard Methods* (APHA 1985) and by ASTM (1989) as a rapid and reliable indicator of environmental quality. Pacific oysters (*Crassostrea gigas*) and blue mussels (*Mytilus edulis*) are recommended for testing. During the first 48 hours of embryonic development, fertilized oyster and mussel eggs normally develop into free-swimming, fully shelled larvae (prodissoconch I). Failure of the eggs to survive or the proportion of larvae developing in an abnormal manner have been used as the primary indicators of toxicity. A combined mortality and abnormality endpoint may also be calculated from the number of normal surviving larvae.

This sediment bioassay can be used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The two species recommended for testing may show different levels of sensitivity to various contaminants. Therefore, the quantitative results for corresponding endpoints may not be strictly comparable between the two species. Nevertheless, results of statistical comparisons of test sediments with reference area sediments based on the two bivalve species as well as the related echinoderm embryo bioassay may be considered interchangeable for some purposes (e.g., regulatory decision-making).

The bivalve larvae bioassay probably can be used in sediments that have interstitial salinities less than 1 ppt, as the sediments are mixed and equilibrated with seawater prior to testing. However, because further testing is required to determine the validity of using this technique with such low salinity sediments, this bioassay is not recommended for sediments that have an interstitial salinity of less than 10 ppt. In addition, the following caveats apply:

- Bivalve larvae such as those of *C. gigas* normally reside in the water column and are not intimately associated with sediments. Hence, this bioassay is primarily an indicator of the relative toxicity among different samples because its direct ecological significance with respect to *in situ* sediments has not yet been established.

- Spawning of *C. gigas* occurs naturally in the Puget Sound area in summer. The natural spawning period for *M. edulis* is late spring to early summer. Both of these bivalves can be induced to spawn at other times of the year, but may show decreased viability of gametes. Gamete viability may also vary depending on the brood stock used. Accordingly, a positive control is required. This should comprise 48-hour LC_{50} and EC_{50} measurements with a reference toxicant in seawater only.

- Relative sensitivity of the three endpoints (percent mortality, percent abnormality, and

percent combined mortality/abnormality) to toxic chemicals, natural chemical factors (e.g., total organic carbon, paralytic shellfish poison) and physical factors (e.g., suspended sediment) has not been thoroughly evaluated. However, caution should be exercised in utilizing *C. gigas* larvae in sediments known to have a high proportion of clays and silts (EPA 1993).

- High mortalities in the seawater control and/or reference sediment tests may be occasionally observed. The cause of such mortality is unknown, but may be related to natural factors that reduce embryo quality.

- It is possible that abnormalities induced during testing may be underestimated due to poor recovery of living abnormal larvae from the sediments. However, investigators have found that recovery of abnormal larvae from sediment is not worth the effort.

FIELD PROCEDURES

Collection

Both test and reference area sediment should be collected in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section titled *General QA/QC Guidelines*. Each jar should be filled completely with sediment to exclude air. A minimum sediment sample size of 18 grams for each bioassay chamber is recommended for both kinds of sediment. Because five replicate tests are conducted for each field sample, and additional sediment is used for water quality monitoring, a minimum sediment sample size of 200 grams is recommended for each station.

Processing

Both test sediment and reference area sediment should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The following procedures apply equally to larvae of both *C. gigas* and *M. edulis*, and are as described by Chapman and Morgan (1983) with the following changes incorporated:

- The salinity of test water is adjusted to 28 ppt
- Exposure time can range from 48 to 60 hours and depends on larval development in the negative controls
- Replication is increased from two to five to allow adequate statistical comparisons
- Larvae of *M. edulis* are included in the bioassay protocol
- Sediment holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.
- Seawater holding time prior to testing is set at a maximum of 2 days for field-collected and reconstituted seawater. If the seawater is not filtered or UV-treated, it should be used within 8 hours.
- Sediment resuspension in the test chambers is adequately accomplished by vigorous shaking for 10 seconds; there is no need to rotate the chambers for 3 hours at 10 rpm
- Eighteen grams of sediment is suspended in 900 mL of seawater rather than 15 grams in 750 mL
- Sediments are allowed to settle in the bioassay chamber for 4 hours prior to inoculation with embryos
- pH is not adjusted before the bioassay starts and is only monitored
- An interim oyster-specific threshold value for ammonia of 0.13 mg/L unionized ammonia has been proposed (EPA 1993); data should be qualified as possible false positives if this threshold value is exceeded
- Aeration is generally recommended and is specified for test chambers in which dissolved oxygen concentrations decline below 60 percent of saturation or when ammonia or sulfides are suspected or have been measured; all chambers should be aerated if aeration is necessary
- A positive control (reference toxicant) is required.
- Additional seawater controls are added for monitoring the stage of larval development
- For test termination, three 10-mL aliquots should be taken; one is counted and two are preserved in buffered formalin for possible later use.
- Additional details provided by ASTM (1989) for conditioning and spawning adults are included.

Bioassay Species

The species selected for testing depends on the availability of brood stock and spawning success during recent bioassays or pilot tests. For a given test or series of related tests, adult bivalves (brood stock) should be obtained from the same source: either commercial rearing facilities (oysters) or a chemically uncontaminated area (mussels). If brood stock is obtained from a commercial source, the original collection area should preferably be identified. For collection of mussels, it is recommended that the bissel threads be clipped rather than pulled loose from the native substrate, as mussels are likely to die if the bissel gland is pulled out during brood stock collection. Brood stock should be sexually mature individuals with normal, well-developed shells. Within 24 hours of collection or purchase, adults should be transported to the test laboratory and placed into flowing seawater similar in character to that from which they were taken. Rough handling, extended periods of desiccation, and abrupt changes in temperature, salinity, or other water quality variables must be avoided as these may induce premature spawning or render the stock useless for later controlled spawning or both. Upon receipt, adults should be cleaned of fouling organisms and detritus and placed in flowing seawater for conditioning.

Adult bivalves are held at recommended conditioning temperatures to stimulate final maturation of the gametes. The desired conditioning temperature (20 ± 1°C for oysters and 16 ± 1°C for mussels) and salinity (28 ± 1 ppt) should be attained gradually at increments not exceeding 2°C/day and 5 ppt/day. Conditioning may extend from a few days to several weeks depending on the physiological and gametogenic status of the adults. The length of the conditioning period is determined empirically by periodic sacrificial examination and spawning of representative individuals. Adults should be spawned or discarded within 2-3 weeks after attaining acceptable maturity because gamete quality will deteriorate rapidly with excessive conditioning. Adults should be provided with an adequate supply of natural or cultured phytoplankton. Natural seawater flow should be about 28 liters/hour per individual adult. ASTM (1989) describes procedures for maintaining holding tanks. Procedures for inducing spawning in bivalves, enhancing the quality of gametes, and preparing embryos are described in ASTM (1989).

Reference Sediment

The bivalve larvae bioassay is conducted with reference area sediment in addition to seawater controls. Reference area sediment typically consists of material collected from an area documented to be free of chemical contamination and nontoxic to bivalve larvae. The reference sediment grain size should be as similar as possible to the test sediment (see section on Puget Sound Reference Areas).

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt and temperature of 20 ± 1°C for oysters and 16 ± 1°C for mussels. Seawater should be collected from uncontaminated areas (e.g., deep or offshore waters) to avoid contamination and should be held at ≤20°C for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1989) should be held at ≤20°C for no longer than 2 days before use. The bioassay seawater must be uncontaminated and of acceptably low toxicity. The biological criterion of acceptability is that the larvae, spawned by adults in the dilution water, must attain a minimum of 70% normally developed larvae.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with 0.45-µm pore diameter. If necessary, salinity of the bioassay water is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salt, brine prepared by freezing, or reagent grade chemicals (ASTM 1989). Artificial seawater is prepared within 2 days of use and is stored in clean, covered containers at the requisite temperature.

Facilities and Equipment

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. The bioassay chamber is a 1-liter glass bottle with a screw-top lid. Bioassays are conducted at 20 ± 1°C for oysters and 16 ± 1°C for mussels, with the bottles in shallow water baths, incubators, or temperature-controlled rooms.

If adults are to be conditioned for spawning out of season, a continuous supply of temperature-controlled, aerated seawater is needed. Laboratory facilities should be well-ventilated and free of organic vapors. Holding and conditioning chambers preferably should not be in a room in which toxicity tests are conducted, stock or test solutions are prepared, or equipment is cleaned. Air used for aeration should be free of organic vapors, oil, and water. Raw seawater can be used for holding and conditioning, but feeding the adults a natural or cultivated alga is necessary to deter starvation. The flow rates used for adult conditioning must be high enough (typically >28 liters/hour/individual) to prevent water quality degradation and provide as much food as possible to the adults.

Tanks and trays are necessary for holding the adults, and a water bath, incubator, or temperature-controlled room is necessary during the bioassay. Adult holding and conditioning tanks should be cleaned several times each week to prevent accumulation of organic matter and bacteria. Dead specimens should be removed immediately and the tanks cleaned. The tanks should be cleaned with detergent and rinsed with clean seawater, and if microbial growth is present, rinsed with 200 mg/L of hypochlorite and then seawater. With enriched waters and elevated conditioning temperatures, more frequent cleaning may be required.

Bioassay Procedure

Initiation—Adult bivalves, conditioned as necessary in the laboratory, are induced to spawn with selected thermal and biological (i.e., sperm) stimulation. Selected densities of the resulting embryos are exposed to the test or reference area sediments for 48 hours, during which the embryos normally will develop into prodissoconch I larvae. A slightly longer exposure period may be used if necessary to achieve adequate development of larvae in seawater controls. Exposure time should not exceed 60 hours for an acceptable test. Data from tests with longer exposures (>48 hours) may not be comparable to those from tests conducted using the standard 48-hour exposure. Toxicity test endpoints are based on abnormal shell development and larval death.

The bivalves are spawned by rapidly raising the water temperature to 5-10°C above the conditioning temperature. Individuals are additionally stimulated to spawn by the addition of sperm from a sacrificed or naturally spawned male.

Spawning is conducted by placing the bivalves in individual, clean Pyrex™ dishes containing filtered, ultraviolet (UV)-treated seawater. Fertilization is accomplished within 1 hour of spawning by combining eggs and sperm (i.e., at a concentration of 10^5–10^7 sperm/mL) in a 1-liter Nalgene beaker. The fertilized eggs are then washed through a 0.25-mm Nitex screen to remove excess gonadal material and suspended in 2 liters of filtered, UV-treated seawater at incubating temperature. The embryos are kept suspended by frequent agitation using a perforated plunger, and used in the bioassay within 2 hours of fertilization. When microscopic examination of fertilized eggs reveals the formation of polar bodies, egg density is determined from triplicate counts of the number of eggs in 1.0-mL samples of a 1:99 dilution of homogeneous egg suspension.

Sediment bioassays are conducted in clean, 1-liter glass bottles. Five replicate tests are conducted for each field sample. An additional bioassay chamber is prepared for water quality monitoring. Eighteen grams (wet weight) of the appropriate sediment is added to each bottle and a volume of 900 ml filtered or UV-treated seawater (28 ± 1 ppt salinity) is added to all containers. The reference area sediment chambers each contain 18 grams of clean sediment. In addition, negative and positive controls for determination of LC_{50} and EC_{50} are prepared consisting of clean seawater without sediment.

The sediments are suspended by vigorous shaking for 10 seconds and the suspended sediments are allowed to settle for 4 hours prior to addition of larvae. No additional agitation is provided. The seawater controls are treated similarly except for the lack of sediments.

Within 2 hours of fertilization, each container is inoculated with 20,000–40,000 developing embryos to give a concentration of about 20–40/mL. The containers are covered and incubated for 48 hours (or longer if required) at 20 ± 1°C for oysters and 16 ± 1°C for mussels under a 14-hour light:10-hour dark photoperiod. Aeration of test chambers generally is recommended during the bioassay. Moreover, if the dissolved oxygen concentration in any test chamber declines below 60 percent of saturation or if high levels of ammonia or sulfides are suspected or measured, the water in that chamber should be aerated gently for the remainder of the test. If aeration is necessary, all chambers should be aerated and at a rate of approximately 100 bubbles per minute. A random numbering method should be used to distribute the chambers in the water bath (or incubator or cold room).

The mean embryo concentration at 0 hours should be determined by collecting five replicate 10-mL samples from control containers and preserving them in 5-percent buffered formalin. This method of determining the initial embryo concentration is one of three methods recommended by ASTM (1989) for larval bioassays of water and elutriates. Because the method recommended for the present bioassay does not rely on direct assessments of embryo densities in the test chambers, the resulting density estimates have an unquantified error component associated with them. This error reduces the reliability of larval mortality estimates and may thereby influence the results of statistical analyses. However, it does not affect larval abnormality estimates because they are based on known numbers of survivors.

Monitoring—Temperature, pH, salinity, and dissolved oxygen are measured daily in the replicates prepared specifically for monitoring water quality. Measurements are taken just prior to introduction of the embryos to the bioassay chambers at the same time each day until the conclusion of the bioassay.

Measurements of conventional water quality variables (e.g., sulfides, ammonia) should be made at the start and termination of the bioassay and additionally monitored if high levels are found (EPA 1993).

Termination—The bioassay is terminated when greater than 95 percent of the embryos in the duplicate seawater control have reached the prodissoconch I stage (approximately 48–60 hours). Once this stage has been achieved in the control chambers, final water quality measurements are recorded and the test is terminated. The bioassay is terminated in the following manner. The water and larvae overlying the settled sediment in each container are carefully poured into a clean 1-liter beaker. This water is then mixed with a perforated plunger, and 10-mL aliquots of the well-mixed sample are removed by pipette and placed in 10-mL screw-cap vials. The contents of each vial are preserved in 5-percent buffered formalin.

Preserved samples (equal in volume to those containing 200–400 larvae in controls) are examined in Sedgewick-Rafter cells. Normal and abnormal larvae are enumerated to determine percent survival and percent abnormality. A minimum sample size of 20 living larvae in each of the five replicate bioassay chambers for test sediment and reference area sediment and 100 larvae in each replicate chamber for the seawater control should be scored for abnormalities. Percent survival for each replicate bioassay chamber is based on the number of normal larvae surviving relative to the mean number of normal survivors in the seawater controls. Larvae that fail to transform to the fully shelled, straight-hinged, D-shaped prodissoconch I stage are considered abnormal. Percent abnormal for each replicate bioassay chamber is based on the number of survivors that are abnormal. Definitions of normal development specified at the PSDDA larval bioassay workshop should be followed. These definitions include the following:

- An uninterrupted shell must be formed around the margin; any indication that the shell cannot close (e.g., chips or knobs) constitutes an abnormality. Classification of open shells or shells seen in other than side view should be made on best professional judgment. Empty shells that are complete count as normal, because they developed successfully to the shelled stage, no matter what happened thereafter.

- Larvae classified as normal must have a straight hinge by termination of experiment. If larvae have not reached D or prodissoconch I stage by the end of the exposure time (set by the duplicate sacrificial control vessel) they are considered abnormal.

Controls

Five replicates of the seawater control are included in all bioassays. These comprise negative (clean) controls that allow comparison among experiments and among laboratories of the validity of the procedures used in individual investigations. At least 70 percent of the larvae must the 48-hour exposure with seawater alone. Experiments in which contaminants are added to sediment may require control replicates to determine effects of solvent addition.

A positive (toxic) control is also required. This involves determining 48-hour (or longer if required) LC_{50} and EC_{50} values for bivalve larvae exposed to reference toxicants in clean, filtered or UV-treated seawater without sediment [following standard ASTM (1989) bioassay procedures and under the same general test conditions as the sediment bioassays]. Such data are necessary to determine the relative sensitivity of the larvae. Two commonly used reference toxicants are reagent-grade cadmium chloride and sodium dodecyl sulfate. Either of these reference toxicants may be used, but the results must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} or an EC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and EC_{50} and one above the LC_{50} and EC_{50}. Statistical procedures for the LC_{50} and EC_{50} estimates are given in APHA (1985) and ASTM (1989).

Reference Area Sediment

The design of field surveys may include a reference sediment from an area known to be free from chemical contamination. This provides a basis for comparison of potentially toxic and nontoxic conditions.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Source, qualitative condition, and holding time of brood stock
- Information on how the brood stock were collected
- All water quality measurements [e.g., dissolved oxygen, temperature, salinity, pH, sulfides (optional), ammonia (optional)]
- Individual replicate and mean and standard deviation data for larval percent mortality after 48-hour exposure
- Individual replicate and mean and standard deviation data for larval percent abnormality after 48-hour exposure
- Individual replicate and mean and standard deviation data for larval percent combined mortality and abnormality after 48-hour exposure (optional)
- 48-hour LC_{50} and EC_{50} values for reference toxicants (with results for metallic compounds reported in terms of the metal ion, not as weight of the whole salt)
- Data on stocking density (number/ml), stocking aliquot size (ml), and initial count data for seawater controls

- Any problems that may have influenced data quality.

Mortality = 100 x (1 - (no. of surviving test larvae / no. of control larvae))

Abnormality = 100 x (1 - (no. of abnormal larvae / no. of normal and abnormal survivors))

Combined larval mortality/abnormality = 100 x (1 - (no. of surviving normal larvae / no. of embryos inoculated))

ECHINODERM EMBRYO SEDIMENT BIOASSAY

USE AND LIMITATIONS

The echinoderm embryo bioassay is described by Dinnel and Stober (1985) as a rapid and sensitive technique for assessing the toxicity of marine sediments. Sand dollars (*Dendraster excentricus*), purple sea urchins (*Strongylocentrotus purpuratus*), and green sea urchins (*S. droebachiensis*) are the recommended species for testing. During the first 48–96 hours of embryonic development, fertilized echinoderm eggs normally develop into the pluteus stage. Failure of the eggs to survive and the proportion of larvae developing in an abnormal manner are used as indicators of toxicity. A combined mortality and abnormality endpoint is calculated from the number of normal pluteus larvae. Unlike the bivalve larval test, a "mortality" endpoint cannot be used because dead embryos rarely disintegrate during the test as do oyster or mussel embryos.

The echinoderm embryo bioassay can be used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The three species recommended for testing may show different levels of sensitivity to various contaminants. Therefore, the quantitative results for corresponding endpoints may not be strictly comparable between the three species. Nevertheless, results of statistical comparisons of test sediments with reference area sediments based on the three echinoderm species, as well as the related bivalve larvae bioassay, may be considered interchangeable for some purposes (e.g., regulatory decision-making).

The echinoderm bioassay probably can be used in sediments that have interstitial salinities less than 1 ppt, as the sediments are mixed and equilibrated with seawater prior to testing. However, because further testing is required to determine the validity of using this technique with such low salinity sediments, this bioassay is not recommended for sediments that have an interstitial salinity of less than 10 ppt. In addition the following caveats apply:

- Echinoderm larvae normally reside in the water column and are not intimately associated with sediments. Hence, this bioassay is primarily an indicator of the relative toxicity among different samples because its direct ecological significance with respect to *in situ* sediments has not yet been established.

- Spawning of *Strongylocentrotus* spp. occurs naturally in the Puget Sound region from December to April. The natural spawning period for *D. excentricus* is from April to October. Echinoderms can be induced to spawn at other times of the year, but may show decreased viability of gametes. Gamete viability may also vary depending on the brood stock used. Accordingly, a positive control is required. This should comprise 48-hour LC_{50} and EC_{50} measurements with a reference toxicant in seawater only.

- Relative sensitivity of the two endpoints (percent abnormality and percent combined mortality/abnormality) to toxic chemicals, natural chemical factors (e.g., total organic carbon, paralytic shellfish poison) and physical factors (e.g., suspended sediment) has not been thoroughly evaluated. It has been shown that larvae of *D. excentricus* do not show an adverse response to increasing silt and clay fractions, and under conditions of expected high silts and clay, the sand dollar test is preferable to the oyster larvae test (EPA 1993).

- High mortalities in the seawater control and/or reference sediment tests may be observed occasionally. The cause of such mortality is unknown, but may be related to natural factors that reduce embryo quality.

- It is possible that abnormalities induced during testing may be underestimated due to poor recovery of living larvae from the sediments. However investigators have found that recovery of abnormal larvae from sediment is not worth the effort.

FIELD PROCEDURES

Collection

Test Animals—All recommended echinoderm species can be collected off the coast of Washington. Purple sea urchins can be found in the intertidal zone (or the shallow subtidal zone in the Strait of Juan de Fuca) and are usually ripe from December through March in Washington waters. Green sea urchins occur in the shallow subtidal zone and are usually ripe from January through April. Sea urchins should be collected with care to avoid injury from the sharp spines. Sand dollars are the preferred test species during the summer months as they are in spawning condition from about April through October. Sand dollars can be collected by hand on many Puget Sound beaches during low tide. All animals should be collected from uncontaminated areas.

Sediment—Both reference area and test sediment should be collected in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely with sediment to exclude air. A minimum sediment sample size of 18 grams for each bioassay chamber is recommended for both kinds of sediment. Because five replicate tests are conducted for each field sample and additional sediment is used for water quality monitoring, a minimum sediment sample size of 200 grams is recommended for each station.

Processing

Sediment—Both control and test sediment should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The following procedures are synthesized primarily from Dinnel and Stober (1985) and ASTM (1994). The following changes were incorporated:

- The salinity of test water is adjusted to 28 ppt
- Seawater temperature during the bioassay is maintained at 15 ± 1°C for all test species
- Exposure time can range from 48 to 96 hours and depends on larval development in the negative controls
- Replication is increased from three to five to allow adequate statistical comparisons
- Sediment holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.
- Seawater holding time prior to testing is set at a maximum of 2 days for field-collected and reconstituted seawater
- Sediment is included in each test chamber
- Sediment resuspension in each test chamber is adequately accomplished by vigorous shaking for 10 seconds
- Sediment is allowed to settle in test chambers for 4 hours before addition of fertilized eggs
- pH is not adjusted before the bioassay starts and is only monitored
- Aeration is specified for test chambers in which dissolved oxygen concentrations decline below 60 percent of saturation or if sulfides or ammonia are present. If aeration is required, all test chambers should be aerated at a rate of approximately 100 bubbles per minute
- A positive control (reference toxicant) is required
- Additional seawater controls are added for monitoring the stage of embryo development
- A single endpoint of combined mortality and abnormality should be calculated and reported (both should be measured)
- An ammonia testing criterion of 0.014 mg/L unionized ammonia has been proposed for the echinoderm test (EPA 1993); data may be qualified as a possible false positive response if

unionized ammonia values are greater than or equal to 0.04 mg/L

- For test termination, three 10-mL aliquots should be taken; one is counted and two are preserved in buffered formalin for possible later use.

Bioassay Species

The species selected for testing depends on the availability of brood stock and spawning success during recent bioassays or pilot tests. For a given test or series of related tests, adult echinoderms (brood stock) should be obtained from the same source: either commercial harvesters or a chemically uncontaminated area. If brood stock is obtained from a commercial source, the original collection area should be identified. Within 24 hours of collection or purchase, adults should be transported to the test laboratory and placed into flowing seawater similar in character to that from which they were taken. Because epidemic spawning can occur when echinoderms are transported in seawater, test animals can be transported in ice chests containing only kelp or other moist material, and kept cool. Rough handling, extended periods of desiccation, and abrupt changes in temperature, salinity, or other water quality variables must be avoided as these induce premature spawning or render the stock useless for later controlled spawning or both. Upon receipt, adults should be cleaned of detritus and placed in flowing seawater. Sand dollars are best held on a bed of sand in flowing or well-aerated seawater.

Reference Sediment

The echinoderm embryo bioassay is conducted with reference area sediment in addition to seawater controls. Reference area sediment typically consists of material collected from an area documented to be free of chemical contamination and nontoxic to echinoderm embryos.

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt. Water temperature should be maintained at 15 ± 1°C for all test species. Seawater should be collected from uncontaminated areas (e.g., deep or offshore waters) to avoid contamination and should be held at ≤15°C for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1994) should be held at ≤15°C for no longer than 2 days before use. The bioassay seawater must be uncontaminated and of acceptable low toxicity. The recommended biological criterion of acceptability is that the larvae, spawned by adults in the dilution water, must not incur more than 30-percent combined mortality/abnormality during 48-96 hours of exposure to the bioassay seawater.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with a 0.45-µm pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salt, brine prepared by freezing or reagent-grade chemicals (ASTM 1994).

Facilities and Equipment

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. The bioassay chamber is a standard 1-liter glass jar or beaker (10-cm internal diameter) covered with an 11.4-cm-diameter watchglass. The bioassay chambers are maintained at 15 ± 1°C in a shallow water bath, incubator, or temperature-controlled room with an overhead aeration source. General recommendations of ASTM (1994) should be followed for materials used for test equipment, cleaning procedures, and good laboratory practices.

If adults are to be conditioned for spawning out of season, a continuous supply of temperature-controlled, aerated seawater is needed. Laboratory facilities should be well ventilated and free of organic vapors. Holding and conditioning chambers preferably should not be in a room in which toxicity tests are conducted, stock or test solutions are prepared, or equipment is cleaned. Air used for aeration should be free of organic vapors, oil, and water. Raw seawater can be used for holding and conditioning, but feeding the adults a natural or cultivated alga is necessary to deter starvation. The flow rates used for adult conditioning must be high enough (typically >28 liters/hour/individual) to prevent water quality degradation and provide as much food as possible to the adults.

Tanks and trays are necessary for holding adults. These are placed in a water bath, incubator, or temperature-controlled room to maintain proper temperature. Adult holding tanks should be cleaned several times each week to prevent accumulation of organic matter and bacteria. Dead specimens should be removed immediately and the tanks cleaned. The tanks should be cleaned with detergent and rinsed with clean seawater, and if microbial contamination is present, rinsed with 200 mg/L of hypochlorite and then seawater. With enriched waters and elevated conditioning temperatures, more frequent cleaning may be required.

Bioassay Procedure

Initiation—Adult echinoderms, conditioned as necessary in the laboratory, are induced to spawn with chemical stimulation. Selected densities of the resulting embryos are exposed to the test or reference area sediments for 48 to 96 hours, during which the embryos normally will develop into the four-armed pluteus stage. Data from tests with longer exposures (>48 hours) may not be comparable to those from tests conducted using the standard 48-hour exposure. The toxicity test endpoint is based on failure to develop normal pluteus larvae.

Adult sea urchins are spawned by injecting 1 mL of 0.5-molar potassium chloride (KCl) through the peristomal membrane into the coelomic cavity. Sand dollars are injected with 0.5 mL of 0.5-molar KCl through the oral opening, with the syringe held at an angle. Animals are rinsed with clean seawater and inverted over individual 150–250 mL beakers filled with seawater for about 30 minutes until spawning is completed. As many as 12 females may need to be spawned to ensure an adequate quantity of eggs. The spawning beakers should be placed in a water bath or temperature-controlled room to maintain temperature at acclimation levels.

Eggs from females discharging relatively small numbers of eggs (e.g., <100,000) are discarded. The retained eggs are examined microscopically for viability and ripeness. Ripe, viable eggs are normally round, uniform in size, free of excessive debris, and appear slightly granular. Immature eggs contain a large, clear spot (the germinal vesicle) in the cytoplasm; overripe eggs are usually less circular, have inconsistent granularity of the cytoplasm, and are often associated with increased debris. If the proportion of underripe or overripe eggs in a beaker exceeds 10 percent, the eggs are discarded. Eggs that are accepted are pooled together into a 1-liter beaker and washed three times by repeatedly decanting the water above the eggs and adding 500–1,000 mL new seawater (allow the eggs to settle to the bottom of the beaker between washes). Small subsamples of eggs are counted using a dissecting microscope to determine the number of eggs per milliliter.

The solutions of sperm from males producing thick, viscous discharges are combined to provide a stock solution. Sperm density is determined by immobilizing the sperm (i.e., by heat shock or exposure to 10-percent glacial acetic acid) and counting on a hemocytometer. Fertilization should be initiated within 1 hour of spawning by adding sperm to the beaker containing the eggs, at a sperm:egg ratio of ≤2,000:1. A perforated plastic plunger is used to gently mix the contents of the beaker. Care should be taken that excessive amounts of sperm are not used. Fertilization is monitored by examining successive 1-mL aliquots microscopically and determining the percentage of eggs with a raised fertilization membrane. When greater than 90 percent of the eggs show membrane formation (about 10-15 minutes), the developing eggs are counted and the density is adjusted to 20,000–30,000 per mL, either by diluting with seawater to decrease density or decanting excess surficial water to increase density.

Test chambers should be prepared prior to spawning of the adult echinoderms to allow enough time for sediments to settle in the chambers before inoculation with the fertilized eggs. Five replicate tests are conducted for each field sample. An additional bioassay chamber is prepared for water quality monitoring. Eighteen grams of reference or test sediment is added to each chamber. Filtered or UV-treated seawater (28 ppt salinity) is added to each chamber up to 1 liter to make a final concentration in all containers of 18 grams (wet weight) of sediment per 900 ml of seawater. Each reference area sediment chamber also contains 18 grams/900ml of clean sediment. In addition, two control series are prepared consisting of clean seawater without sediment (one series is used as a duplicate, sacrificial control to monitor embryo development).

The sediments are suspended by vigorous shaking for 10 seconds and then allowed to settle for 4 hours prior to addition of the embryos. No additional agitation is provided. The seawater control chambers are treated similarly.

Within 2 hours of fertilization, a 1-mL aliquot of the solution of embryos (about 25,000 embryos) is added to each bioassay chamber using an automatic pipette. The containers are covered with a watchglass and incubated for 48 hours (or longer if required) at 15 ± 1°C under a 14-hour light:10-hour dark photoperiod. Test chambers generally are not aerated during the bioassay. However, if the dissolved oxygen concentration in any test chamber declines below 60 percent of saturation, the water in all chambers should be aerated gently for the remainder of the test. A random numbering method should be used to distribute the chambers in the water bath (or incubator or cold room).

The mean embryo concentration at 0 hours should be determined by collecting five replicate 10-mL samples from thoroughly mixed control cultures and preserving them in 5-percent buffered formalin.

This method of determining the initial embryo concentration is one of three methods recommended by ASTM (1994) for larval bioassays of water and elutriates. The other two methods include 1) direct subsampling of each test chamber after inoculation and 2) direct subsampling of the stock solution. According to ASTM (1994), the preferred method is direct subsampling of test chambers after inoculation. This method provides the best estimate of embryo densities within each chamber and the variability of densities among chambers. However, this method cannot be easily used for the present bioassay because the sediment present in each test chamber prevents the contents of each chamber from being homogenized adequately for representative subsampling. Because the method recommended for the present bioassay does not rely on direct assessments of embryo densities in the test chambers, the resulting density estimates have an unquantified error component associated with them. This error reduces the reliability of larval mortality estimates. However, it does not affect larval abnormality estimates because they are based on known numbers of survivors.

Monitoring—Temperature, pH, salinity, and dissolved oxygen are measured daily in the replicates prepared specifically for monitoring water quality. Measurements are taken just prior to introduction of the embryos to the test chambers, then at the same time each day until the conclusion of the bioassay. Measurements of conventional water quality variables (e.g., sulfides, ammonia) should be made at the start and termination of the bioassay.

Termination—The bioassay is terminated at 48 hours or when greater than approximately 90 percent of the embryos in the duplicate seawater control have reached the four-armed pluteus stage with deeply invaginated preaural arms (whichever is later and within 48–96 hours). Once this stage has been achieved in the control chambers, final water quality measurements are recorded and the test is terminated.

The bioassay is terminated in the following manner. The water and larvae overlying the settled sediment in each chamber are carefully and gently stirred to insure the larvae are suspended in the water without disturbing the sediment; and then carefully decanting up to 98 percent of the water, leaving the sediment remaining in the test chamber. The water is then mixed thoroughly using a perforated plunger and three 10-mL aliquots of the sample are removed by pipette and placed in 10-mL screw-cap vials. The contents of each vial are preserved in 5-percent buffered formalin. One 10-mL aliquot is then counted, while the other two are archived until counts are assured to be adequate for characterizing test replicates.

Preserved samples (equal in volume to those containing 200–400 larvae in controls) are examined in Sedgewick-Rafter cells. Normal and abnormal larvae are enumerated to determine percent failing to achieve a normal pluteus larva. Percent survival for each replicate bioassay chamber is based on the number of larvae surviving in each test container relative to the initial number. Percent mortality is then calculated, including correction for mortality in the seawater control. Larvae that fail to transform into clearly defined pluteus with two well-developed arms and the second pair of arms budding are considered abnormal.

Controls

Five replicates of the seawater control are included in all bioassays. These comprise negative (clean) controls that allow comparison among experiments and among laboratories of the validity of the

procedures used in individual investigations. At least 70 percent of the larvae must achieve a normal pluteus stage. Experiments in which contaminants are added to sediment may require control replicates to determine effects of solvent addition.

A positive (toxic) control is also required. This involves determining 48 to 96 hour EC_{50} values for echinoderm larvae exposed to reference toxicants in clean, filtered or UV-treated seawater without sediment [following standard ASTM (1994) bioassay procedures and under the same general test conditions as the sediment bioassays]. Such data are necessary to determine the relative sensitivity of the larvae. Two commonly used reference toxicants are reagent-grade cadmium chloride and sodium dodecyl sulfate. Either of these reference toxicants may be used, but the results must be reported along with the sediment bioassay results. Sodium dodecyl sulfate has a short shelf life, and should be made up fresh every several days. Bioassays to establish an EC_{50} involve dilution series and a control. At least one treatment should give a partial response below the EC_{50} and one above the EC_{50}. Statistical procedures for EC_{50} estimates are given in APHA (1985) and ASTM (1994). The EC_{50} for the *D. excentricus* combined endpoint, using data in the Army Corps of Engineers' DAIS database, is 10.1 ± 6.5 mg/L cadmium (n=32) (Army Corps of Engineers personnel communication, 1994).

Reference Area Sediment

The design of field surveys may include a reference sediment from an area known to be free from chemical contamination. This provides a basis for comparison of potentially toxic and nontoxic conditions. Additionally, to further ensure the integrity of reference test samples, investigators should use experienced sampling personnel and sample from the biologically active zone, avoiding anoxic sediments. The wet-sieving method (PTI, 1991; PSDDA, 1990) should be used to match reference sediments with test sediments.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this assay:

- Source, qualitative condition, and holding time of brood stock
- Stocking density (number/ml), stocking aliquot size (ml), and initial count data for seawater control
- All water quality measurements [e.g., dissolved oxygen, temperature, salinity, pH, sulfides (optional), and ammonia (optional)]

- Individual replicate and mean and standard deviation data for combined larval mortality/abnormality at termination of bioassay (should also report separate data for mortality and abnormality for comparison to previous data endpoints)

- Individual replicate and mean and standard deviation data for larval percent combined mortality plus abnormalities at termination of bioassay

- EC_{50} values for reference toxicants (with results for metallic compounds reported in terms of the metal ion, not as weight of the whole salt)

- Any problems that may have influenced data quality.

Mortality = 100 x (1 - (no. of surviving test larvae / no. of control larvae))

Abnormality = 100 x (1 - (no. of abnormal larvae / no. of normal and abnormal survivors))

Combined larval mortality/abnormality = 100 x (1 - (no. of surviving normal larvae / no. of embryos inoculated))

GENOTOXICITY: ANAPHASE ABERRATION SEDIMENT BIOASSAY

USE AND LIMITATIONS

This sediment bioassay is used to characterize the genotoxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

This bioassay can be used with any type of sediment, regardless of the interstitial salinity or grain size characteristics. However, the following caveats apply:

- The bioassay depends on a chemical extraction procedure that is specific for neutral, nonionic organic compounds. Other classes of contaminants such as metals and highly acidic and basic organic compounds are not efficiently extracted. Thus, characterization of sediment toxicity is directed towards neutral compounds such as aromatic and chlorinated hydrocarbons.

- "Natural" genotoxicity may occur in marine sediments due to the decomposition of plant species containing genotoxic substances that evolved as a means of protecting plants from parasites and predators. Thus, positive genotoxic responses may be noted in areas generally regarded as pristine.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 200 grams is recommended for each test.

Processing

Sediment samples should be stored frozen at -20°C within 8 hours of collection. Holding time should not exceed 6 months.

LABORATORY PROCEDURES

As previously mentioned, this technique depends on a chemical extraction procedure. Extraction procedures recommended are those presently used by the National Marine Fisheries Service, National Oceanic and Atmospheric Administration in Puget Sound (MacLeod et al. 1984).

The following procedure is adapted from Kocan et al. (1982), Chapman et al. (1982a,b), Kocan and Powell (1985), and Kocan et al. (1985). Accordingly, this procedure supersedes previous published methods.

Cell Cultures

Although any cell type can be used in this test, the rainbow trout gonad cell (RTG-2) is recommended because it has been used extensively for this purpose, is readily obtainable, and is easy to cultivate. It has numerous mitotic figures when growing exponentially and the mitotic cells are large in comparison to those of other species, thereby making it easy to count the damaged anaphase cells. This cell type is sensitive to a wide range of organic chemical compounds (Kocan et al. 1982) as well as complex mixtures of chemicals that may occur in marine sediments (Kocan and Powell 1985; Kocan et al. 1985). Other cells used for this purpose are the bluegill-sunfish line, human foreskin fibroblasts, newt cells, and several plant cells (in vivo). Generally these cells can be obtained from any state or federal fish disease diagnostic laboratory, from investigators at both university and federal laboratories, or from the American Type Culture Collection (Rockville, Maryland).

Sediment Extraction

Test materials must consist of substances that are compatible with the growth of cells in culture. Generally this can be accomplished using organic extracts of environmental material such as marine sediments.

Extractions should be made using pesticide-grade reagents that have been tested for toxicity to the cells prior to their use for extraction. This initial toxicity testing can be done by evaporating a volume of the reagent equivalent to that which would be used for the actual extraction, and adding this to the cultures in varying amounts dissolved in the solvent [e.g., dimethyl sulfoxide (DMSO)] to be used for cell exposure. If the maximum anticipated amount of the solvent blank to be used in the final test does not significantly affect the cell cultures, the extraction can proceed without concern about the possible toxic effect of the solvents. Once the extracts have been made, gravimetric determinations of their absolute organic content must be made so that comparable organic concentrations from each site can be used in cell cultures exposed to extracts from different locations. This is accomplished by first weighing the tube to be used for extract storage to the nearest 0.001 gram, adding the extract in the solvent, evaporating the solvent with nitrogen, and reweighing the tubes. The difference between the original tube weight and the final tube weight is the weight of the sample extract. The sample extract is then redissolved in spectral-grade DMSO so that each sample contains the same weight/volume ratio of extract (e.g., mg/mL). In this way it is possible to expose each set of cultures to exactly the same amount of organic extract, thereby

making comparisons possible on an organic content basis. If relative toxicities from site to site are

needed, one can extrapolate back to the original organic content of each sample and compare sites based on total amounts of organic compounds present. The following detailed procedures for the preparation of sediment extracts follow those of Chapman et al. (1982a,b) and MacLeod et al. (1984).

Sediment samples are frozen and stored until just prior to extraction. Each sample is then thawed and rehomogenized by thorough stirring. An aliquot of approximately 20 grams wet weight is transferred to a clean, tared beaker, dried to constant weight (80°C), desiccated, and reweighed to determine the percent water. A second aliquot (approximately 150 grams wet weight) is transferred to a tared, 315-mL stainless steel centrifuge bottle with a PTFE-lined screw-cap, and weighed. The sample is then serially extracted with pesticide-grade solvents.

Methanol (50 mL) is added to each centrifuge bottle. The bottle is tightly capped, shaken vigorously for 2 minutes, and centrifuged at 2,000 rpm for 5 minutes. The clean solvent is decanted into a 1-liter separatory funnel. The procedure is performed twice more and the methanol extracts are combined in the separatory funnel, which is then closed and covered with aluminum foil.

Next, 100 mL of a dichloromethane/methanol (2:1 v/v) solution is added to the centrifuge bottle, the cap is closed tightly, and the bottle is shaken vigorously for 2 minutes to ensure complete mixing. The bottle is then placed in a shaker table overnight (approximately 18 hours), following which the sediment is settled by centrifuging at 2,000 rpm for 5 minutes and the solvent decanted into the separatory funnel with the methanol. A second 100-mL aliquot of the dichloromethane/methanol (2:1) solution is added, the bottle is shaken vigorously, and the bottle is then placed on the shaker table for 6 hours. The sediments are again settled by centrifuging and the solvents are decanted.

The remaining sample is shaken vigorously for 2 minutes with approximately 30 mL of dichloromethane. It is then centrifuged and the solvent is decanted into the separatory funnel. Another 100 mL of dichloromethane is added to the bottle, the cap is secured, the bottle is shaken vigorously, and the bottle is placed on the shaker table overnight. The sediments are again settled by centrifuging and the solvent is decanted into the separatory funnel. A final 30-mL rinse of dichloromethane is added, the bottle is shaken vigorously, centrifuged, and decanted. The sediment is then discarded.

Approximately 500 mL of clean, distilled water is added to the combined solvents in the separatory funnel. The funnel is carefully swirled and inverted (with frequent venting) for 2 minutes. The liquid phases are allowed to separate and the dichloromethane (lower) layer is drained into a 500-mL separatory funnel. The aqueous layer is re-extracted twice with 20 mL of dichloromethane and the remainder is discarded. The dichloromethane fractions are combined in the 500-mL funnel, transferred with rinsing, back to the 1-liter funnel, and re-extracted with another 500 mL of distilled water. The dichloromethane is drained into the 500-mL funnel and the aqueous layer is extracted once more with 20 mL of dichloromethane. The latter solvent is added to the 500-mL funnel and the aqueous layer is discarded.

The dichloromethane is drained from the 500-mL separatory funnel through approximately 20 grams of combusted and washed anhydrous sodium sulfate that is held in a 30-mL glass conical centrifuge tube with the tip cut off. The effluent from this mini-column is discharged into a 500-mL Kuderna-Danish flask with a 15-mL receiver. When empty, the 500-mL separatory funnel is rinsed with 20 mL of dichloromethane, which is drained through the sodium sulfate column into the flask. The column is washed a final time with 10 mL of dichloromethane and drained into the flask.

Boiling chips are added to the Kuderna-Danish flask and a three-ball Snyder column is placed on top. The solvent volume is reduced to about 5 mL using a hot water bath. When cooled, the sides of the flask are rinsed into the receiver with dichloromethane. The receiver is removed and the contents are quantitatively transferred to a tared conical centrifuge tube with a ground glass stopper. The sample is taken almost to dryness using the hot water bath, and stored wrapped in aluminum foil in a desiccator with the stopper open slightly until a constant weight is achieved upon reweighing the tube. This weight is the amount of extractable organic material. Samples should never be taken completely to dryness, because low molecular weight volatile compounds could be lost in the process.

After weighing, the tube is closed and wrapped fully in aluminum foil, ready for anaphase aberration testing. Extracts are treated with 1 mL of spectrophotometric-grade DMSO for 24 hours with frequent stirring on a vortex mixer. The DMSO is then removed to a glass vial and used as "stock" solution. Because all extracted material is not dissolved in the DMSO during testing, the centrifuge tubes are dried and reweighed to determine the exact amount used in testing (fraction soluble). Both stock and extract solutions are stored in the dark under nitrogen until applied to the cell cultures.

Culture Conditions

RTG-2 cells as well as most fish cell lines grow in a variety of commercially available culture media. The Leibovitz L-15 medium was found to be most consistent in terms of ease of preparation, use without special buffers or carbon dioxide incubators, and long-term storage capability. This medium can be obtained from any scientific supply house that carried cell/tissue culture materials. The medium comes as a dry powder that is added to distilled water, autoclaved or filter-sterilized, and stored in a refrigerator at 4°C until it is used. Full instructions are available with the medium when it is purchased. Generally, heat-deactivated fetal calf serum is added to any culture medium at 10-percent concentration to ensure that the proper growth factors are present. This serum can also be purchased in 100-mL to 1-liter lots already sterilized from the scientific suppliers. Before the cells are placed in the medium, pH should be adjusted to 7.1–7.3 using either sodium bicarbonate or HCl. Some laboratories use HEPES buffer (either acid or base in a commercially available product) in place of the bicarbonate or HCl. This culture system differs from that originally described by Kocan et al. (1982) only because it has been modified to simplify the laboratory procedures.

Bioassay Procedure

Cells are grown and tested at 18°C on standard, clean microscope slides or on 1×5-cm coverslips in Leighton tubes, depending on the amount of test material available. The cells are placed into the culture system 1 day prior to the actual exposure to ensure that they have had a chance to attach to the glass substrate and begin growing. On the following day (18-24 hours later), the culture medium is removed and the test material is added. This should consist of normal L-15 medium dissolved in DMSO, to which the organic extract has been added. The DMSO should be from a pretested lot to ensure that it is nontoxic to the system and should not exceed 0.5 percent (v/v) of the culture medium (e.g., 5 µL/mL). This can be reduced to 0.1-percent DMSO if toxicity is a problem or if minimal DMSO is required for conservation of extract. Exposure time should be 48 hours from the time of addition of the treated medium until fixation. Damaged cells can be observed for longer periods even after the toxic substance has been removed, but the maximum response does not increase beyond 48 hours.

An initial screening test must be conducted to determine the actual extract dilutions to be used for this bioassay. Ideally, dilutions tested for anaphase aberrations comprise both the highest concentration of extract (mg/L) that permits continued cell proliferation (i.e., is nontoxic) and a second concentration one dilution lower. This method ensures that a sufficient number of mitotic figures are present to score for chromosome damage. Based on previous experience in Puget Sound, the following six extract dilutions should be prepared: 50, 25, 15, 5, 2, and 1 µg/mL. Cells are first exposed to these concentrations for each sediment extract tested, and then the concentrations that inhibit mitosis are determined. Although identical extract dilutions may not be used to test each sediment sample, all results are normalized to organic content which has been previously determined.

To determine mitotic effects and anaphase aberrations, the slides or coverslips containing the cells are removed from the culture medium and fixed in methanol:acetic acid (3:1). The methanol is absolute and the acetic acid is glacial (undiluted). Following 15-60 minutes in the fixative (no adverse effects occur if they are left longer), the slides are air dried, then placed into 3-percent Gurr's R66 Geimsa stain for 15-30 minutes. This stain is made up in Sorensen's buffer (pH 6.8). Optimum staining is determined empirically by examining the slides with a microscope at various intervals after placing them in the stain. Problems usually occur because of too little staining time rather than excessive staining. If excessive staining does occur, the cells can be destained in Sorensen's buffer. The staining system selectively stains the condensed chromosomes undergoing mitosis and has very little effect on the cytoplasm of the cell, which allows good resolution of the small fragments of chromosome that are not associated with the main chromosome bundles.

Once the cells have been stained, they are mounted on microscope slides (if on coverslips) or are covered with coverslips (if grown on slides) to facilitate microscopic examination and scoring. Slide identification labels are covered with a piece of tape to prevent observer bias while scoring. Three replicate slides are made of each exposure concentration with two concentrations for each sediment extract. Each slide is then examined at 500X to 1,000X until a minimum of 100 anaphase cells is observed and scored. In this way, there will be three replicates per dose with 100 anaphase cells per replicate.

The numbers and percents of normal and abnormal anaphases are recorded. Cells are scored as

abnormal if they contain any of the previously described chromosomal lesions reported for this test (Nichols et al. 1977; Kocan et al. 1982; Chapman et al. 1982a; Kocan and Powell 1985; Kocan et al. 1985).

Controls

Controls consist of 1) untreated cultures used as negative controls to ensure that the culture conditions (e.g., medium, serum) are not toxic to the cells, 2) a solvent blank to ensure that the residue from the solvents used during the extraction procedure is not cytotoxic or genotoxic (generally done prior to actual testing and includes the DMSO that will be used in the final solution), and 3) a positive control consisting of cultures exposed to several concentrations of a known genotoxic agent to indicate that the test system is functioning properly and does indeed respond to genotoxic substances. One possible positive control is a 0.25-µg/mL concentration of benzo(a)pyrene. This level of exposure should result in an anaphase aberration frequency of 50-65 percent (Chapman et al. 1982a).

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Initial screening data for the determination of extract concentrations
- Individual replicate and mean and standard deviation data for numbers (and percentages) of normal and abnormal anaphases observed
- Types of anaphase aberrations observed
- Frequency of anaphase aberrations observed with the positive control
- Any problems that may have influenced data quality.

MICROTOX SEDIMENT BIOASSAY - ORGANIC EXTRACT

USE AND LIMITATIONS

The Microtox bioassay is a rapid, sensitive method of toxicity testing based on light emission by the luminescent bacterium *Photobacterium phosphoreum* in the presence and absence of aqueous toxicants. The emitted light is a product of the bacterial electron transport system and thus directly reflects the metabolic state of the cells. Accordingly, decreased luminescence following exposure to chemical contaminants provides a quantitative measure of toxicity. The assay was developed for use in freshwater habitats to assess the toxicity of waterborne pollutants (Bulich et al. 1981) and has been adapted for use in the marine environment to assess toxicity of organic sediment extracts (Schiewe et al. 1985).

This sediment bioassay is used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The Microtox bioassay can be used with any type of sediment, regardless of interstitial salinity or grain size characteristics. However, the following caveats apply:

- The bioassay depends on a chemical extraction procedure that is specific for neutral, nonionic organic compounds. Other classes of contaminants such as metals and highly acidic and basic organic compounds are not efficiently extracted. Thus, characterization of sediment toxicity is directed towards neutral compounds such as aromatic and chlorinated hydrocarbons.

- Extraction by an organic solvent results in exposure of test organisms to concentrations of contaminants much higher than those expected to occur in sediment interstitial water. The use of an organic extract may not mimic pore-water composition and natural exposure routes.

- Naturally occurring toxic substances may be present in and extracted from marine sediments. Hence, relatively high toxicity occasionally may be noted in areas generally regarded as free from chemical contamination.

- Luminescence can increase, rather than decrease, in some samples. At present, these samples are considered nontoxic. However, additional research is needed to determine the meaning of an increase in luminescence.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 500 grams is recommended for each station.

Processing

Sediment samples should be stored frozen at -20°C within 8 hours of collection. Holding time should not exceed 6 months.

LABORATORY PROCEDURES

Facilities and Equipment

The bioassay is performed using a Microtox toxicity analyzer system, a temperature-regulated photometer equipped with a photomultiplier. Freeze-dried bacteria, reconstitution solution (i.e., organic-free distilled water), diluent, and other necessary materials can be purchased from commercial suppliers. The test procedure is conceptually quite straightforward. However, the methodology requires careful attention to detail and requires 1-2 weeks to become technically proficient.

Sediment Extraction

As previously mentioned, this technique depends on a chemical extraction procedure. Extraction procedures recommended are those presently used by the National Marine Fisheries Service, National Oceanic and Atmospheric Administration in Puget Sound (MacLeod et al. 1984).

- Thaw sediment, decant and discard excess water. Homogenize sediment with stainless steel spoon. Discard large pebbles, shells, seaweed, wood, crabs, etc.
- Weigh out 10 ± 0.5 grams sediment to the nearest 0.01 gram and place in a dichloromethane-rinsed centrifuge bottle.
- Set aside approximately 10 grams of the homogenized sediment for the determination of dry weight.
- Centrifuge sediment sample for 5 minutes at 1,000 × G and discard water. Centrifuge bottles should be made of glass or stainless steel.
- Add 100 mL spectral-grade dichloromethane and 50 grams sodium sulfate to sediment sample. Note that spectral-grade solvents may contain some trace contaminants that

may be toxic to the test organism. Pesticide-grade solvents are preferred.

- Manually shake bottle until contents are loose and free-flowing and then roll 16 hours (overnight) on a tumbler.
- Centrifuge sediment 5 minutes at 1,000 × G and save the dichloromethane extract.
- Add another 100 mL dichloromethane to sediment and tumble for 6 hours (during day).
- Centrifuge for 5 minutes at 1,000 × G and save the dichloromethane extract.
- Repeat extraction a third time with 100 mL dichloromethane for 16 hours (overnight) and collect extract by centrifugation.
- Combine the three 100-mL portions of the dichloromethane extract.
- Add three or four boiling chips to flask containing the dichloromethane extract and attach to Snyder column.
- Concentrate extract to 10-15 mL in a 60°C water bath and then transfer it to a concentrator tube.
- Wash down the flask 2 times with 3-4 mL dichloromethane and add washings to the tube.
- Add a boiling chip to the tube and, using a tube heater, concentrate the extract to greater than 0.9 but less than 1.0 mL.
- Adjust volume to 1.0 mL with dichloromethane (at this point 0.1 mL may be removed for gas chromatography/mass spectrometry analyses).
- Add 3 mL of hexane to the remaining 0.9 mL of extract and concentrate to 2 mL.
- Remove 100 µL of extract and add to a concentrator tube containing 3 mL nondenatured ethanol.
- Place tube in heater block and concentrate to 2 mL.
- Adjust volume to 3.0 mL with nondenatured ethanol (the standard sediment extract used in Microtox testing).
- Extraction blanks are prepared using an identical procedure but without the sediment.

Bioassay Procedure

The approach to testing organic extracts uses the basic Microtox method described in the *Microtox Operating Manual* (Beckman Instruments 1982) and by Bulich et al. (1981).

- Reconstitute lyophilized bacteria with 1-mL double-distilled, charcoal-filtered water in a

Microtox cuvette and place in 4°C holding well. Bacterial suspensions should be used within 2 hours of reconstitution.

- Prepare three or more widely spaced primary dilutions [e.g., 5.0-, 0.5-, and 0.05-percent extract (v/v)] in double-distilled, charcoal-filtered water.

- Adjust concentration of each primary dilution to 2-percent NaCl by adding 0.1 mL of 22-percent NaCl per mL of diluted extract.

- Use these diluted extracts in a range-finding assay to determine an appropriate primary dilution for the definitive assay described below. The primary dilution should cause a 65-90 percent decrease in bioluminescence in 15 minutes. Methods for the range-finding assay are the same as those for the definitive assay except usually only three concentrations are tested without replication.

- For the definitive assay, two-fold serial dilutions (i.e., 6.0, 2.5, 1.25, and 0.625 percent) of extract are prepared in 2-percent NaCl. A 2-percent NaCl blank is also prepared for testing to measure spontaneous decay of light production which occurs naturally independent of treatment.

- In each of 10 test cuvettes, a 10-µL aliquot of bacterial suspension is added to 500 µL of diluent and incubated for 15 minutes in the incubation wells. This assures temperature equilibration and stability of bioluminescence.

- After 15 minutes, initial levels of light emission are measured in each of the 10 test cuvettes.

- At 30-second intervals, 500-µL aliquots of each concentration of extract are added to two of the cuvettes (i.e., two replicates each of the four extract dilutions and the saline blank). Timing is critical because bioluminescence gradually decreases over time.

- Exactly 5 minutes after addition of the sediment extract, light emission is measured at 30-second intervals and in the same sequence used for extract additions in the preceding step. Light emission is measured again at 15 minutes; additional measurements are sometimes made at 30 minutes.

- Immediately after testing each sediment extract, an ethanol-only control is assayed using the same primary dilution sequence used in the sediment extract test. The ethanol-only data are used to adjust the sediment extract data for the contribution of the solvent vehicle.

Controls

- Ethanol, sodium lauryl sulfate, or other suitable reference toxicants should be used as

positive controls to assess daily bioassay performance and to determine differences in response among lots of bacteria

- Clean sediment can be evaluated as a negative control

- Bioassay repeatability is evaluated by duplicate testing (i.e., extraction and analysis) of 10 percent of the sediment extracts.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Range-finding assay results.

- Raw light emission data for each test series.

- Determination of a significant dose-response relationship by least-squares regression of the natural log of gamma values for percent decrease in luminescence on the natural log of extract concentration based on the 15-minute data.

- 15-minute EC_{50} values and 95-percent confidence intervals for each test series and for controls. Estimates of the 15-minute EC_{50}s (i.e., the concentration of extract causing a 50-percent reduction in bioluminescence) are obtained using linear regression analyses [see *Microtox Operating Manual* (Beckman Instruments 1982)]. Briefly, the percent inhibition of light emitted at each test concentration and time point is converted to a gamma value which is defined as the ratio of light lost to light remaining. Gamma values are normalized for natural decline in light production measured over time as described by Bulich et al. (1981) and further adjusted for the contribution of the ethanol vehicle. The natural log of gamma is regressed on the natural log of extract concentration and the EC_{50} is calculated from the regression equation. A statistical procedure based on Fieller's theorem (Finney 1984) is used to calculated a 95-percent confidence interval.

- Any problems that may have influenced data quality.

MICROTOX BIOASSAY - SALINE EXTRACT

USE AND LIMITATIONS

The use of saline extracts of sediment for the Microtox bioassay has been described by Williams et al. (1986). The organic and saline extract approaches both use the basic Microtox method described in the Microtox Operating Manual (Beckman Instruments 1982) and by Bulich et al. (1981). The major difference is in the preparation of test samples. Each procedure is specific with regard to the classes of contaminants that are tested for toxicity and, in general, the results for each approach can be viewed as complementary. Additional variations of the Microtox test are being developed that are based on the direct exposure of the test organisms to interstitial water or sediments (i.e., in Microtox diluent).

The saline extract Microtox bioassay procedure removes only the water-soluble fraction of sediment-adsorbed trace metals and organic pollutants from the sediments. Thus, contaminants with extremely low water solubilities [polychlorinated biphenyls (PCBs)] will tend to be partitioned almost exclusively onto sediment particles and are unlikely to occur in high concentration in the saline extract used in toxicity testing. This is a limitation of the test if exposure is primarily through ingestion of sediment, rather than contact with pore-water. Limitations of the Microtox saline-extract bioassay include the following:

- A correction factor needs to be established for changes in bacterial luminescence caused by variation among samples in sediment pore-water salinity. Although Williams et al. (1986) showed that salinity-induced changes in luminescence were negligible for sediments taken from Commencement Bay, other estuarine sediments may have a greater range of pore-water salinities and may require a salinity correction factor.

- The use of a standardized dilution series limits the calculation of EC_{50} in some cases. The 100-percent dilution presently specified in the protocol consists of approximately 58-percent sediment extract and 42-percent Microtox diluent, thereby limiting the sensitivity of the test. A range-finding test could be conducted to determine the appropriate dilution series to calculate an EC_{50}.

- The use of a saline extract may not mimic the actual pore-water composition. Alternatively, True and Heyward (1989) conducted the Microtox test on undiluted interstitial water and demonstrated a greater sensitivity than that achieved with the saline extract protocol. The assay requires further research to demonstrate the efficiency and precision of the extraction procedure and the stability of toxicity during the allowable sediment holding time (i.e., 2 weeks) and the allowable extract holding time (i.e., 2 hours). Further research into alternative techniques (i.e., analysis of full-strength saline extract or sediment pore water) is recommended.

- Luminescence can increase, rather than decrease, in some samples. At present, these samples are considered nontoxic. however, additional research is needed to determine

the meaning of an increase in luminescence.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 200 grams is recommended for each station.

Processing

Sediment samples should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

With slight modification, sample processing follows procedures described by Williams et al. (1986). Glass test tubes are filled with a minimum of 60 grams of sediment, sparged with high-purity nitrogen gas, tightly sealed with PTFE-lined caps, stored in the dark at 4°C, and assayed within 14 days following collection.

Preparation of Sediment Extract

- Remove 30 grams of sediment from each test tube with a stainless-steel spatula, place in 30-mL glass containers equipped with a fitted glass cap, and add 10 mL of Microtox diluent (2.0-percent NaCl w/v in double-distilled, organic-free water).

- Mix the sediment-diluent slurry for 24 hours in the dark at 4°C by gentle agitation (100 rpm) on a rotary shaker table.

- Transfer the sediment slurry to 30-mL Corex tubes and centrifuge for 15 minutes at 9,000 rpm (9,770 × G) in a refrigerated (4°C) centrifuge.

- Draw the supernatant off by pipette, place it in a clean test tube, cool on ice, and use immediately in preparation of serial dilutions for the Microtox bioassay. Supernatant may be held at 4°C for no longer than 2 hours before use.

Bioassay Procedure

- Rehydrate a vial of freeze-dried bacteria with 1.0 mL of reconstitution solution, cover with parafilm, store in a 4°C well on the Microtox analyzer, and use within 2 hours of rehydration.

- Prepare 100-, 50-, 25-, 12.5-, and 0-percent serial dilutions of the sediment supernatant in Microtox diluent. The 0-percent dilution is a reagent blank needed to measure spontaneous decay in bacterial luminescence independent of any treatment.

- In each of 10 test cuvettes, add 10 µL of the rehydrated bacterial suspension to 350 µL of diluent and incubate for 15 minutes in one of the 15°C wells on the analyzer. This ensures temperature equilibration of the bacterial suspension and stability of luminescence.

- After 15 minutes, measure initial luminescence in each of the 10 test cuvettes.

- At 30-second intervals, add 500-µL aliquots of each supernatant dilution to two of the cuvettes (e.g., two replicates each of the four test dilutions and the saline blank). Timing is critical because toxicant-induced decrease in luminescence begins as soon as the sediment supernatant is added to the bacterial suspension.

- Exactly 15 minutes after addition of the sediment supernatants, measure luminescence at 30-second intervals and in the same sequence used for supernatant additions in the preceding step.

- Calculate percent decrease in luminescence relative to the reagent blank using the formula:

$$\text{Percent Decrease} = [(Ri_o - I_t)/(RI_o)] \times 100$$

where:

I_o = initial luminescence
I_t = luminescence at the end of 15 minutes
R = blank ratio.

The blank ratio is calculated by:

$$R = B_t/B_o$$

where:

B_o = initial luminescence of the reagent blank

B_t = luminescence of the reagent blank after 15 minutes.

Controls

- Clean reference sediments used as negative controls

- Construction of a calibration curve to determine salinity-induced changes in bacterial luminescence (Bechman Instruments 1982)

- Use of a reference toxicant (i.e., phenol, sodium arsenate) to assess day-to-day performance of the bioassay and to determine differences in toxic response among lot number of bacteria. The EC_{50} for phenol using data in the Army Corps of Engineers' DAIS database is 20.1 ± 4.7 mg/L (n=56) (Army Corps of Engineers personnel communication, 1994).

- Verification of a dose-response relationship between bacterial luminescence and sediment extract concentration.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Initial and final luminescence and percent decrease in luminescence after 15-minute exposure for each concentration of supernatant (e.g., saline sediment extract) tested.

- Determination of a significant dose-response relationship by least-squares regression of the natural log of gamma values for percent decrease in luminescence on the natural log of extract concentration based on the 15-minute data.

- Fifteen-minute EC_{50} values and 95-percent confidence limits for each test series and the reference toxicant. Estimates of the 15-minute EC_{50} values (i.e., the concentration of extract causing a 50-percent reduction in bioluminescence) are obtained using linear regression analyses [see *Microtox Operating Manual* (Beckman Instruments 1982)]. Briefly, the percent inhibition of light emitted at each test concentration and time point is converted to a gamma value which is defined as the ratio of light lost to light remaining. Gamma values are normalized for natural decline in light production measured over time as described by Bulich et al. (1981). The natural log of gamma is regressed on the natural log of extract concentration and the EC_{50} is calculated from the regression equation. A statistical procedure based on Fieller's theorem (Finney 1984) is used to calculate a 95-percent confidence interval.

- Any problems that may have influenced data quality.

JUVENILE POLYCHAETE SEDIMENT BIOASSAY

OVERVIEW

This protocol is for conducting a bioassay in which the survival and change in biomass of juvenile polychaetes *(Neanthes* sp.) are determined following a 20-day exposure to test sediments. Parameters measured to determine the effects of exposure include mortality, total biomass, and average individual biomass. Sediments can be either naturally occurring, field-collected samples, or sediments that have been experimentally modified (e.g., sediment mixed with other sediment to form a gradient of sediment types or sediment to which chemicals have been added). This bioassay is conducted as a static renewal exposure, and food (i.e., TetraMarin®) is provided to the test organisms during the exposure period to promote body tissue increases. Following the 20-day exposure period, all surviving worms are collected, dried to a constant weight, and total and average individual biomass are determined.

INTRODUCTION

Neanthes sp., a marine nereid polychaete, is widely distributed throughout the world, and has been collected in New England, Florida, California, Europe, and the central Pacific Ocean (Reish 1980). Laboratory cultures of *Neanthes* have been successfully maintained since 1964. Pesch et al. (1988) reported a difference in chromosome numbers from two populations (collected in Connecticut and California, respectively) of *Neanthes*. In addition to the differences observed in chromosome numbers, differences were also noted in the morphology indicating that these populations represent different species. Although specimens from both populations have been used in testing, almost all the testing data are associated with experiments conducted with specimens from the California population. The procedures discussed in this protocol are for *Neanthes* originating from the California population.

Since 1966, various life stages of *Neanthes* have been used as bioassay organisms for a wide variety of investigations including evaluating the effects of dissolved oxygen concentrations, nutrients, salinity, temperature, metals, pesticides, hydrocarbons, and contaminated sediments on survival, growth, and reproduction. In addition, *Neanthes* has also been used to investigate the effects of mutagens (Pesch et al. 1981; Pesch and Pesch 1980) and irradiation (Jones et al. 1983) on marine organisms; an interlaboratory comparison has been conducted with a *Neanthes* 28-day flow-through seawater toxicity test (Pesch and Hoffman 1983); and a 96-hour acute sediment bioassay using *Neanthes* is currently being used for dredged material testing by the U.S. Army Corps of Engineers, Los Angeles District (Reish and Lemay 1988).

Species Sensitivity

Neanthes has been used to evaluate the toxicity of a wide variety of contaminants including metals, hydrocarbons, and multicontaminated media (i.e., sediments). Examples of the types of toxicity tests conducted with *Neanthes* and species sensitivity are presented in Table 4.

Reish (1984) summarized data on the sensitivity of *Neanthes* to metals. In comparison to other polychaetes, *Neanthes* appears to be moderately sensitive to most metals tested. Studies indicate that mercury and copper are the most toxic to *Neanthes*, followed by aluminum, cadmium, chromium, zinc, lead, and nickel.

Ecological Importance

Neanthes is distributed on the west coast from Mexico to southern California (Reish 1980). *Neanthes* has not been collected from Puget Sound. The family Nereidae is widely distributed and is a dominant taxa in intertidal and subtidal habitats. In Puget Sound, the nereid *Platynereis bicanaliculata* is a dominant member of the polychaete fauna at many sites (Lie 1968; PTI and Tetra Tech 1988a,b). *P. bicanaliculata* is morphologically similar to *Neanthes* in jaw structure and is also recorded to be an omnivore, feeding on algae and other detritus (Fauchald and Jumars 1979). Both species also build similar tubes of organic material and display similar aggressive behavior patterns (Gray 1974).

USE AND LIMITATIONS

The *Neanthes* sublethal bioassay is used to characterize the toxicity of marine sediments primarily based on worm growth. Data reported by Johns and Ginn (1990b) indicate that the level of contamination affecting juvenile growth in *Neanthes* is similar to the level of contamination that affects reproductive success. The bioassay may be used alone (e.g., as a screening tool in broad-scale sediment surveys), in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments to address various sediment and water quality manipulations. The following constraints apply:

- The bioassay should be conducted with laboratory-cultured juvenile *Neanthes*
- Modification of the protocol may be required for tests conducted at salinities (both interstitial and overlying water) less than 20 ppt.

TABLE 4. SENSITIVITY OF *NEANTHES* TO VARIOUS CONTAMINANTS

Contaminant	Lowest Concentration for Observed Effect (mg/L)	Endpoint	Test Duration	Reference
Aluminum	>2.0	Mortality	4 days	Petrich and Reish (1979)
Cadmium (as $CdCl_2$)	3	Mortality	28 days	Reish (1980)
	1	Reproduction	Life cycle	Reish and Gerlinger (1984)
Chromium (as CrO_3)	0.6	Mortality	28 days	Reish (1980)
Hexavalent chromium (as $K_2Cr_2O_7$)	0.0125	Reproduction	Life cycle	Oshida (1976)
Copper	0.1	Mortality	29 days	Pesch and Morgan (1978)
Lead [as $Pb(CH_3CO)_2$]	3.2	Mortality	28 days	Reish (1980)
	0.97	Reproduction	Life cycle	Reish and Gerlinger (1984)
Mercury (as $HgCl_2$)	0.17	Mortality	28 days	Reish (1980)
Nickel	49.0	Mortality	4 days	Petrich and Reish (1979)
Silver (as $AgNO_3$)	0.165	Mortality	28 days	Pesch and Hoffman (1983)
Zinc (as $ZnSO_4$)	1.4	Mortality	28 days	Reish (1980)
	0.32	Reproduction	Life cycle	Reish and Gerlinger (1984)
DDT	0.1	Mortality	28 days	Reish (1985)
No. 2 fuel oil	2.7	Mortality	4 days	Rossi and Anderson (1976)
South Louisiana crude oil	12.5	Mortality	4 days	Rossi and Anderson (1976)

FIELD PROCEDURES

Collection

Test Animals—*Neanthes* are not indigenous to Puget Sound and test organisms must be obtained from laboratory cultures. (See *Laboratory Procedures* section for a discussion on culturing and obtaining test organisms.)

Sediment—Control, reference and test sediments should be collected in solvent-cleaned glass containers that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample collection size of 0.25 liters for each bioassay chamber is recommended for all sediment types. Because five replicate tests are conducted for each field sample, a minimum sample size of 1.25 liters is recommended for each station.

Processing

Test Animals—Not applicable.

Sediment—All sediments should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

Test Animals

Culturing—Almost all *Neanthes* used for laboratory tests come from laboratory cultures. Culturing techniques have been described by Reish (1980) and Pesch and Schauer (1988). Under laboratory conditions, the *Neanthes* life cycle is completed in 3–4 months at 20–22°C.

Cultures of adult *Neanthes* are maintained in glass aquaria under static (with monthly renewal) or flow-through water conditions. After sexually mature males and females pair, the pairs can be isolated in jars and maintained until juveniles are ready to be removed and used for testing. Eggs are laid within the worm tube, and the female dies within 2–3 days. The zygotes are cared for by the surviving male. Larvae emerge from the worm tube in approximately 3 weeks (at 20–22°C) following fertilization. Hatched larvae feed on yolk reserves until emergence from the adult worm tube. Following emergence, the juvenile worms are capable of feeding and building independent tubes. Until testing, the juvenile worms are maintained without sediment and are provided TetraMarin® (a food source) and powdered alga (either *Enteromorpha* or *Ulva* sp.). Enough powdered alga (sieved to less than 0.3 mm) should

be provided to cover the bottom of the aquarium. The powdered alga provides material for tube construction and increases survival (Pesch and Schauer 1988).

Shipping and Holding—Juvenile *Neanthes* are obtained from laboratory cultures. If test organisms are obtained from an outside source, enough time should be allotted to allow the worms to acclimate prior to starting a test. *Neanthes* can be shipped by overnight courier without significant mortality. Worms are typically packed in plastic bags containing seawater with 50 organisms per bag. Each bag should contain several fronds of dried *Enteromorpha*. This alga can be collected, dried, and stored for extended periods. Prior to use, the alga should be soaked in seawater. The bags are shipped in a hard-sided container (e.g., cardboard box). When the shipment arrives at the laboratory, the worms, still in the plastic bags, are placed in a holding aquarium containing seawater at the proper test temperature. The worms are released from the bags after temperature equilibration. The worms are maintained in the holding aquarium for 1-2 days prior to initiation of the bioassays. The holding time will provide for acclimation between the culture temperature and the anticipated testing temperature and for observation of the condition of the test organisms to ensure that the bioassay is conducted with healthy individuals.

Neanthes juveniles should be held in all-glass aquaria containing clean seawater and provided with gentle aeration [see Pesch and Schauer (1988) if flowing seawater is available]. Water temperature is maintained at 20 ± 1°C, and salinity is maintained at the salinity at which the bioassay will be conducted. Enough powdered green alga (*Enteromorpha* or *Ulva* sp.) should be provided to cover the bottom of the holding tank.

During the holding period, organisms are provided with TetraMarin® on an every-other-day basis. The amount of food provided should be calculated at approximately 8 mg (dry weight) per juvenile, but the tank should be observed following feeding to determine if the food is being consumed. If it is not being consumed, then the amount of food provided should be reduced in order to avoid potential water fouling problems. If the entire amount of food provided is being eaten, then an increase in the food ration might be appropriate.

No water changes in the holding tank are required if the worms are being maintained in the aquaria for less than 1 week. If the worms are to be maintained for a longer period, then the water should be replaced with fresh seawater once every 2 weeks. Rising salinity, due to evaporative losses during the holding period, can be compensated for by adding sufficient distilled water to lower the salinity to the desired level.

Test Animal Size—The size of juvenile worms used in the bioassays is potentially a critical factor to the eventual success of the bioassay. Worms should be 0.5–1.0 mg (dry weight) (i.e., 2-3 weeks post-emergence) to ensure that they are in a rapid growth phase during the exposure period. Under PSDDA testing, a minimum initial size of 0.5 mg is considered a warning flag if not achieved. Worms of the appropriate size are large enough to be easily handled to avoid errors in placing the correct number of worms in each exposure chamber. For consistency in aging test organisms, initiation of emergence should be considered as the point when feeding juveniles emerge from the egg case. Commencement of feeding can be identified by the presence of food particles in the digestive tract.

Feeding Requirements—Several different types of food have been used in culturing *Neanthes*, including alfalfa flour, powdered alga (*Enteromorpha* or *Ulva* sp.), TetraMarin®, and prawn flakes. Of these foods, prawn flakes and TetraMarin® appear to provide the best and most consistent growth throughout the life cycle. Because of potential problems in obtaining a consistent supply of prawn flakes, TetraMarin® should be used. TetraMarin® should be provided to juveniles maintained in holding tanks prior to testing and during the exposure period. In both cases, the worms should be fed on an every-other-day basis. The amount of food provided should be calculated at approximately 8 mg (dry weight) per juvenile *Neanthes*.

Control and Reference Sediments

Five replicates of the polychaete collection-site control sediment are included in all bioassays. These compromise a negative (clean) control that allows comparisons among experiments and among laboratories of the validity of the procedures used in individual investigations. For the *Neanthes* bioassay, sand should be used as the control sediment.

Sand was initially chosen as an appropriate control sediment based on the work of Pesch and Hoffman (1982), who used sand as a substrate in a series of experiments with *Neanthes*. They reported no significant mortality associated with maintaining the worms in sand. For the sublethal bioassay test demonstration study and subsequent testing (Johns and Ginn 1990a,b), sand collected from West Beach on Whidbey Island, Washington, was used as the control sediment. *Neanthes* maintained in West Beach sand exhibited low mortality and high percentage increases in biomass during the exposure period, indicating that West Beach sand is a suitable material for a control sediment. In addition, West Beach sand was selected because it was used as a control sediment for a number of the regulatory bioassays conducted in Puget Sound and is known to be relatively free of contaminants. However, West Beach sand was adequate only when food was supplied.

Because control sediments may differ greatly from the test sediments with respect to physical and chemical sediment characteristics (e.g., grain size and organic content), a reference sediment is also included in the bioassay series. Data from the reference sediment can be used to partition contaminant effects associated with a test sediment from those relating to the physical and chemical characteristics of the test sediment. Johns and Ginn (1990a) evaluated the influence of sediment grain size on *Neanthes* survival and growth following exposure to sediments having differing granulometry (expressed as a percentage of the silt/clay fraction in the sediment). The results of this experiment indicate that *Neanthes* are able to survive and grow in a wide range of sediment types. Johns and Ginn (1990a) also noted that statistical differences in growth could occasionally be detected in *Neanthes* exposed to widely differing sediment types, and cautioned that reference sediment used in *Neanthes* bioassays should have a similar grain size and organic content as the test sediments to avoid potential differences in organism response related to the physical characteristics of the sediment.

Nontreatment factors such as ammonia and sulfides can affect the results of sublethal bioassays such as the *Neanthes* biomass test. When such nontreatment effects occur, water quality monitoring measurements are necessary for determining the factors contributing to the expressed effect. For the *Neanthes* biomass test it is recommended that ammonia and total sulfides be measured at the beginning and end of the test. Further ammonia and sulfides monitoring is recommended prior to the first and second water renewals when initial monitoring reveals greater than 0.7 mg/L unionized ammonia or

greater than 5 mg/L total sulfides (Dillon et al. 1993).

Test Sediments

The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. Johns and Ginn (1990a) determined the 96-hour LC_{50} for *Neanthes* exposed to seawater of different salinity to be 15 ppt. Caution should be used when performing and interpreting the results of *Neanthes* bioassays conducted with sediments with an interstitial salinity of less than 20 ppt. Modification to the test sediment (e.g., mixing the sediment with high salinity water to raise interstitial salinity) or test protocol (e.g., use of high salinity seawater in the exposure chamber) might be considered when testing sediment collected from low salinity areas.

Bioassay Seawater

Seawater used in the bioassay should be maintained at a salinity of 28 ± 2 ppt and at a temperature of 20 ± 1°C. If a series of experiments is planned, then the test temperature and salinity should be the same throughout the series. The bioassay seawater must be uncontaminated.

Facilities and Equipment

Bioassay chambers are 1-liter glass containers with an internal diameter of approximately 10 cm. The chambers are covered with lids to reduce contamination of the contents and evaporation of the seawater or loss of volatiles. The bioassay chambers are maintained at 20 ± 1°C in either a shallow waterbath or in a constant-temperature room. Exposure chambers are gently aerated with air that is free of fumes, oil, and water. This air is delivered to the exposure chamber by nontoxic tubing with a glass Pasteur pipette suspended 3–4 mm below the water surface. The aeration rate should be between 150 and 300 mL/minute, or approximately 100 bubbles per minute.

Prior to use, all glassware is thoroughly cleaned, following the General QA/QC guidelines for equipment cleaning procedures.

Bioassay Procedure

Overview—The bioassays are conducted using a static renewal exposure system. Five replicate tests are conducted for each field sample. Each exposure chamber consists of a 1-liter jar or beaker containing 175 mL of sediment (which should be approximately 2 cm in depth) and sufficient seawater to bring the level up to 950 ml (Figure 1). Prior to testing, all exposure chambers are cleaned and rinsed in turn with distilled water, 10-percent nitric acid ($HN0_3$), and distilled water.

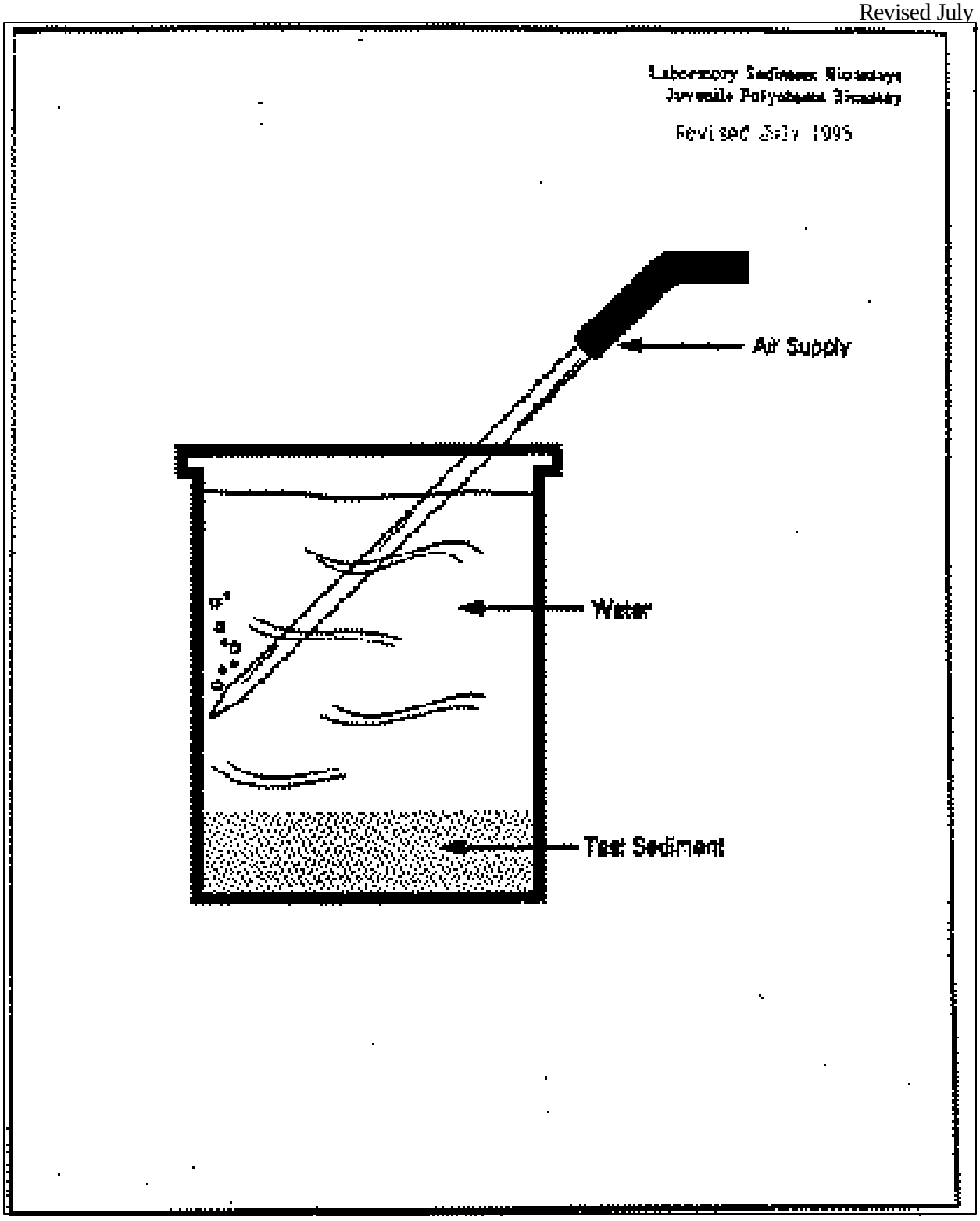

FIGURE 1. Static exposure system used for the juvenile polychaete bioassay

At the beginning of each test, five juvenile worms are randomly placed into each exposure chamber. During setup, three subsamples of worms (five worms per subsample) are randomly selected to provide an estimate of initial worm biomass.

During the exposure period, each exposure chamber is provided with 40 mg of food (i.e., 8 mg per individual) on an every-other-day basis. Every third day, one-third of the seawater in each exposure chamber is exchanged with fresh seawater. Water quality measurements taken during the exposure period include dissolved oxygen concentration, salinity, and pH. These measurements are made for each exposure chamber just prior to the seawater exchange.

Following the exposure period, the contents of each replicate chamber are sieved through a 0.5-mm screen and the number of living worms is recorded. Surviving worms are then placed in a vial containing clean seawater. After all chambers have been sieved, the surviving worms in each vial are quickly rinsed with deionized water, placed on a preweighed aluminum pan, and dried at 50°C to a constant weight. Total weights are then determined to the nearest 0.1 mg.

Initiation—Prior to initiation of a bioassay, all exposure chambers are cleaned as described above, and test organisms are acclimated. On the day before test initiation, test sediments are placed in each of the five replicate exposure chambers. Each chamber should be filled with 175 mL of sediment so that a 2-cm sediment layer is formed in the bottom of the chamber. Sediment placed in the chamber is smoothed by tapping the jar against the palm of the hand. Once the sediment is smoothed, the chamber is filled with seawater to the 950 mL level by gently pouring the water down the side of the chamber. Filled chambers are placed in a 20 ± 1°C waterbath and capped. An air line is inserted through a hole in the cap. The exposure chambers are allowed to equilibrate overnight to bioassay conditions. The photoperiod during testing should be continuous, using ambient light of low to moderate intensity. Although the intensity does not have to be measured, light levels should be similar to that obtained from fluorescent or incandescent light sources that are not placed directly over the water bath.

On the day of test initiation, juvenile worms are collected from the holding tank for distribution to the exposure chambers. The worms should be handled as little as possible. Handling should be conducted quickly and carefully so that the worms are not unnecessarily stressed. Any worms that are accidentally dropped onto hard surfaces or are injured during handling should be discarded. To prevent possible damage to the worms during handling, various handling procedures can be employed. One procedure is to use a small, fine-point paint brush to remove the organisms from the holding tank. Because *Neanthes* produce mucus over the body surface, individual worms are easily captured and transferred with this procedure. Another handling procedure is to use a wide-bore pipette with an attached bulb. Individual organisms can be collected in the pipette through suction and can be removed from the pipette using a gentle flushing action.

Individual worms, in excess of the number needed to conduct the bioassay, are transferred from the holding tank to a shallow dish containing seawater maintained at the test temperature and salinity. Worms placed in the shallow dish should be as similar in size as possible, given the size range of worms available from the holding tank. The mean size of worms used should be no smaller than 0.5 mg. Worms transferred to the shallow dish should be observed to determine that they represent the best worms available for testing (e.g., all appear healthy and represent the smallest range in size of test organisms).

Individual worms are removed from the dish and randomly placed in a plastic cup (five worms per cup) containing seawater. Enough cups are used to equal three more than the number of exposure chambers that will be used during the bioassay. Once this procedure has been completed, worms within a cup are randomly transferred to an exposure chamber by pouring the contents into the chamber. A squirt bottle containing seawater maintained at the test temperature and salinity can be used to free any worms adhering to the cup. During the transfer process, three of the cups containing worms are randomly selected and set aside. Worms from these cups are used to estimate initial total biomass. To determine initial total biomass, worms from these three cups are quickly rinsed with an isotonic, 0.9-percent (w/v) ammonium formate solution of distilled water, placed on a preweighed aluminum pan, dried at 50°C to a constant weight, and weighed to the nearest 0.1 mg.

Once worms have been placed in all of the exposure chambers, each chamber is checked to ensure that air is flowing to the chamber and that the worms have begun to burrow into the sediment. Following setup, food (e.g., TetraMarin®) is provided to each chamber. To ensure adequate distribution of the food within the exposure chamber, a small volume of seawater (i.e., 5 mL) at test temperature and salinity is added to the cup containing the preweighed food ration. Once wetted, the food is poured into the exposure chamber. Water from a squirt bottle is used to rinse the cup of any remaining food.

Following placement of the worms in the exposure chamber, initial (i.e., 1 hour) observations of burrowing should be made. If a worm, or group of worms, do not appear to be burrowing and the observer believes that the nonburrowing behavior results from factors other than sediment toxicity (e.g., reduced viability or damage to test organisms), then those organisms should be replaced.

Monitoring—During the 20-day exposure period, the test chambers are observed on a daily basis to ensure that adequate aeration is provided and to note the general status of each chamber (e.g., presence of accumulated food, burrowing activity of worms, and presence of fouling on sediment surface). On an every-other-day basis, worms in each exposure chamber are provided with food. As discussed earlier, 40 mg of food are provided to each exposure chamber. This food ration is maintained throughout the exposure period, even though mortality may occur during the test.

Every third day, one-third of the seawater in each exposure chamber is replaced. Water replacement is achieved by removing the aeration line, then siphoning one-third of the volume and carefully replacing it with fresh seawater that has been maintained at 20 ± 1°C and at the appropriate test salinity. Steps should be taken during seawater replacement to ensure that test sediments are not disturbed. One method of replacement is to add the fresh seawater by allowing the water to slowly flow down the inside wall of the exposure chamber. When the chamber is filled, the aeration line is placed back in the chamber and the air flow is adjusted to the specified level (i.e., 150 to 300 mL/minute or approximately 100 bubbles per minute).

Prior to seawater replacement, dissolved oxygen, salinity, and pH are determined in one randomly selected exposure chamber for each sample. Dissolved oxygen is determined using a dissolved oxygen electrode. Following determination of dissolved oxygen in each chamber, the electrode is thoroughly rinsed with 20 ± 1°C seawater. Salinity is determined on a small sample of seawater using a hand-held refractometer. The seawater sample for the salinity measurement is obtained with a Pasteur pipette. The pipette should be thoroughly rinsed with seawater between samples. The pH is determined with a portable pH meter and probe. As with the dissolved oxygen electrode, the pH probe is rinsed between

readings.

Termination—Following the exposure period, worms from each exposure chamber are removed from the test sediment. Two methods can be used to collect worms from each exposure chamber. In the first, surviving worms are collected by sieving the sediment through a 0.5-mm screen. The sieve should be gently shaken in a water bath rather than sprayed with water to remove the sediment. In the second method, the sediment is placed in a white enamel pan containing seawater and searched for surviving worms. Worms collected from sediment often remain in their tubes. A worm can be removed from the tube by gently prodding either end of the tube to force the worm to leave. Once out, the worms are removed using either the tip of a small paint brush or a wide-bore pipette. Following collection, the number of worms surviving is noted on data sheets.

To determine total biomass, surviving worms are quickly rinsed in isotonic 0.9-percent (w/v) ammonium formate or distilled water, placed on a preweighed drying pan, and dried at 50°C until a constant weight is attained. Total biomass is determined to the nearest 0.1 mg as the difference in weight of the aluminum pan with and without the worms. Prior to rinsing the worms, observations should be made to determine if food or sediment is present in the digestive tract. Such information may be useful in explaining changes in individual biomass occurring during the exposure period.

During the sublethal test demonstration study, a constant dry weight was attained within 24 hours. To determine when a constant weight has been achieved, several aluminum pans containing worm samples are removed from the drying oven, placed in a desiccator, and allowed to reach room temperature. Following cooling, each aluminum pan is placed on the balance and the weight is determined. Following dry weight determinations, all samples are placed back in the drying oven. After additional drying (i.e., at least 1 hour), the same samples are again removed from the drying oven, allowed to cool in the desiccator, and reweighed. When the dry weights for the samples are the same for consecutive readings (i.e., within 0.1 mg of each other), a constant weight has been attained.

Experimental Design

Logistics—A typical *Neanthes* bioassay for testing 10 sediment samples involves about 50 to 60 exposure chambers. Collection and preparation of test organisms, sediment, and seawater requires at least four people for 2 days. Three or four people are required on the days tests are initiated and terminated. One or two people can monitor a test in progress.

Controls—A control sediment and a reference sediment should be included as part of every test. The control sediment provides a nontoxic sediment to evaluate the condition of the test organisms being used in the bioassay. The reference sediment provides a test reference to partition contaminant effects associated with the treatment sediment from those relating to noncontaminant characteristics (e.g., grain size and total organic carbon).

A positive (toxic) control is also required for all testing. This involves determining 96-hour LC_{50} values for *Neanthes* juveniles exposed in clean, filtered seawater without sediment to reference toxicants (following standard bioassay procedures and under the same general test conditions as the sediment bioassays). Such data are necessary to determine the relative sensitivity of the animals (e.g., seasonal difference in sensitivity) for each test series to ensure comparability of the data. The commonly used reference toxicant is reagent-grade cadmium chloride. Reported 96-hour LC_{50} values for *Neanthes* exposed to cadmium range between 5.0 and 22.0 mg/L (Reish 1984; Johns and Ginn 1990a; Dillon et al. 1993). The LC_{50} calculated using data in the Army Corps of Engineers' DAIS database is 12.5±5.4 mg/L cadmium (n=30) (Army Corps of Engineers personal communication, 1994).

The positive control should be conducted with 10 juveniles per exposure chamber. The worms should not be fed during the 96-hour LC_{50} exposure.

The acute lethality results must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and one above the LC_{50}. Statistical procedures for the LC_{50} estimate are given in APHA (1985).

Response Criteria—Survival, total biomass (dry weight), average individual biomass (i.e., total biomass divided by the number of surviving worms), and average individual growth rate are the four response criteria that can be determined for the *Neanthes* bioassay.

Of the four endpoints, data collected to date indicate that the survival endpoint is the least sensitive to changes in level of contamination. Although survival rates of worms in each replicate have generally been similar, it should be noted that variability in percent survival within replicates could be high since each worm in a replicate represents 20 percent of the replicate survival. The total biomass endpoint is an estimate of the biomass produced by the group of worms in the exposure container. Total biomass represents an integrated measurement of lethal and sublethal effects. Thus, a reduction in total biomass could indicate that one or more worms had died during the exposure or that the growth of all worms had been reduced. Average individual biomass is an estimate of the biomass of each surviving worm. Unlike the survival and total biomass endpoints, worm survival is not integrated into the determination of individual biomass. Dillon et al. (1993) recommend normalizing to initial weight to estimate average individual growth rates. Worm survival is an important ancillary measurement and should always be considered in the interpretation of either biomass endpoint. Each of these response criteria should be monitored in a "blind" fashion; that is, the observer must have no knowledge of the treatment of the sediment in the chambers.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Water quality measurements during testing [i.e., dissolved oxygen, temperature, salinity, pH, sulfides (optional), ammonia (optional)]

- 20-day survival in each exposure chamber and the mean and standard deviation for each treatment

- Initial total biomass (dry weight) for three groups of five worms
- 20-day total biomass (dry weight) in each exposure chamber and the mean and standard deviation for each treatment
- 20-day average individual biomass (dry weight) in each exposure chamber and the mean and standard deviation for each treatment
- Average individual growth rate (dry weight/day) in each exposure chamber and the mean and standard deviation for each treatment
- Interstitial salinity values of control, reference, and test sediments (both initial and final)
- 96-hour LC_{50} values with reference toxicant (results for metallic compounds should be reported in terms of the metal ion rather than as the weight of the whole salt)
- Any problems that may have influenced data quality.

The growth endpoint is calculated according to the following expression:

$$G = (DW_t - DW_t)/T$$

where

G = estimated individual growth rate (milligrams dry weight/day)
DW_t = estimated individual dry weight at termination (milligrams)
DW_i = mean estimated individual dry weight at initiation (milligrams)
T = exposure time (days)

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