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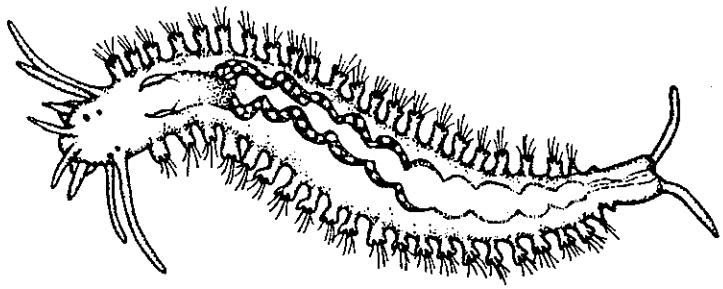
Puget Sound Estuary Program

Protocol for JUVENILE *NEANTHES* SEDIMENT BIOASSAY



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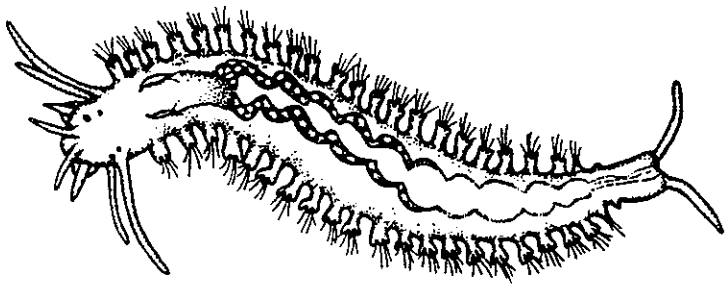

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PROTOCOL FOR JUVENILE *NEANTHES* SEDIMENT BIOASSAY

By

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For

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NEANTHES SUBLETHAL BIOASSAY

OVERVIEW

This protocol is for conducting a bioassay in which the survival and change in biomass of juvenile *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). The *Neanthes* 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.

BACKGROUND

The primary basis for this final protocol is a sublethal test demonstration study (Johns 1988) conducted for the Seattle District of the U.S. Army Corps of Engineers (Corps) and the Puget Sound Dredged Disposal Analysis (PSDDA) study. The testing procedures also incorporated information from the published literature and the testing approach developed by the Los Angeles District Corps for evaluating the acute toxicity of sediments to *Neanthes*. Following development of a draft protocol, the Washington Department of Ecology conducted an experts workshop on the development of a *Neanthes* bioassay to be used as part of the state's marine sediments management program. The general objectives of the workshop were to evaluate the draft protocol for conducting lethal and sublethal sediment bioassays using *Neanthes*, and to determine the information and research that may be needed for further test development.

As part of the workshop, the experts were asked to categorize and rank the information and research needs obtained during the review of the draft protocol in order to provide guidance to the Washington Department of Ecology on suggested changes to the protocol. Four categories of recommendations were made and each information need or research topic identified during the workshop was assigned to one of the categories. One category included recommended changes that could be incorporated into the draft protocol without the need for further testing or research. Items fitting into this category include those for which sufficient data have already been generated, or for which the experts felt a decision could be made based on current knowledge and experience. An interim protocol was then developed that was based on these workshop recommendations.

The other three categories of recommendations were concerned with research topics that might be addressed to further develop the *Neanthes* sublethal bioassay. High priority research topics identified at the workshop were addressed in work funded by the U.S. Environmental

Protection Agency, Region 10 (Johns and Ginn 1990). Results from the research by Johns and Ginn (1990) were incorporated into the interim protocol to further enhance and extend the usefulness of the test and to produce this final protocol. This protocol is now ready for widespread application and evaluation.

Research and other development documents relied upon in establishing this protocol include:

Johns, D.M. 1988. Sublethal test demonstration. Prepared for U.S. Army Corps of Engineers, Seattle District, for the Puget Sound Dredged Disposal Analysis. Submitted to E.V.S. Consultants, Seattle, WA. PTI Environmental Services, Bellevue, WA. 94 pp. + appendices.

Johns, D.M., T.C. Ginn, and J.E. Sexton. 1989. Evaluation of growth as an indicator of toxicity in marine organisms. Prepared for the Washington Department of Ecology, Olympia, WA. PTI Environmental Services, Bellevue, WA. 28 pp. + appendices.

Johns, D.M., T.C. Ginn, and D.J. Reish. 1989. Interim protocol for juvenile *Neanthes* bioassay. Prepared for the Washington Department of Ecology, Olympia, WA. PTI Environmental Services, Bellevue, WA. 14 pp. + appendices.

Johns, D.M., and T.C. Ginn. 1989. Test demonstration of a 10-day *Neanthes* acute toxicity bioassay. Prepared for U.S. Army Corps of Engineers, Seattle District. PTI Environmental Services, Bellevue, WA. 16 pp. + appendices.

Johns, D.M., and T.C. Ginn. 1990. Development of a *Neanthes* sediment bioassay for use in Puget Sound. Prepared for U.S. Environmental Protection Agency Region 10, Office of Puget Sound, Seattle, WA. EPA 910/9-90-005. PTI Environmental Services, Bellevue, WA. 51 pp. + appendices.

Johns, D.M., and T.C. Ginn. 1990. *Neanthes* long-term exposure experiment: the relationship between juvenile growth and reproductive success. Prepared for U.S. Environmental Protection Agency Region 10, Office of Puget Sound, Seattle, WA. EPA 910/9-90-010. PTI Environmental Services, Bellevue, WA. 15 pp.

Johns, D.M., R.A. Pastorok, and T.C. Ginn. 1990. A sublethal sediment toxicity test using juvenile *Neanthes* sp. (Polychaeta: Nereidae). Submitted. In: Aquatic Toxicity and Hazard Assessment: Fourteenth Symposium. American Society for Testing and Materials, Philadelphia, PA.

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 1.

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 based on worm survival and growth. Data reported by Johns and Ginn (1990) 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

TABLE 1. 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)

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.

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 having teflon-lined lids. 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 all sediment types.

Processing

Test Animals—Not applicable.

Sediment—All sediments should be stored at 4°C in the dark. Holding time should not exceed 14 days.

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. Worms of this age 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

In addition to exposure chambers containing test sediments, exposure chambers containing control and reference sediments are also prepared. Control sediment is typically collected from the site at which the test organism is found or the substrate in which the organism is cultured. The sediment provides a nontoxic sediment for evaluation of the condition of the test organisms being used in the bioassay. 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 (Johns 1988) and subsequent testing (Johns and Ginn 1990), 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.

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 (1990) 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 (1990) 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.

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 (1990) 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.

Bioassay chambers are 1-L 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.

Prior to use, all glassware is thoroughly cleaned, rinsed in distilled water, soaked in a 10 percent nitric acid ($HN0_3$) (or 10 percent hydrochloric acid) solution for 2 hours, and rinsed with distilled water.

Bioassay Procedure

Overview—The bioassays are conducted using a static renewal exposure system. Each exposure chamber consists of a 1-L jar containing 2 cm of sediment and seawater (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.

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.

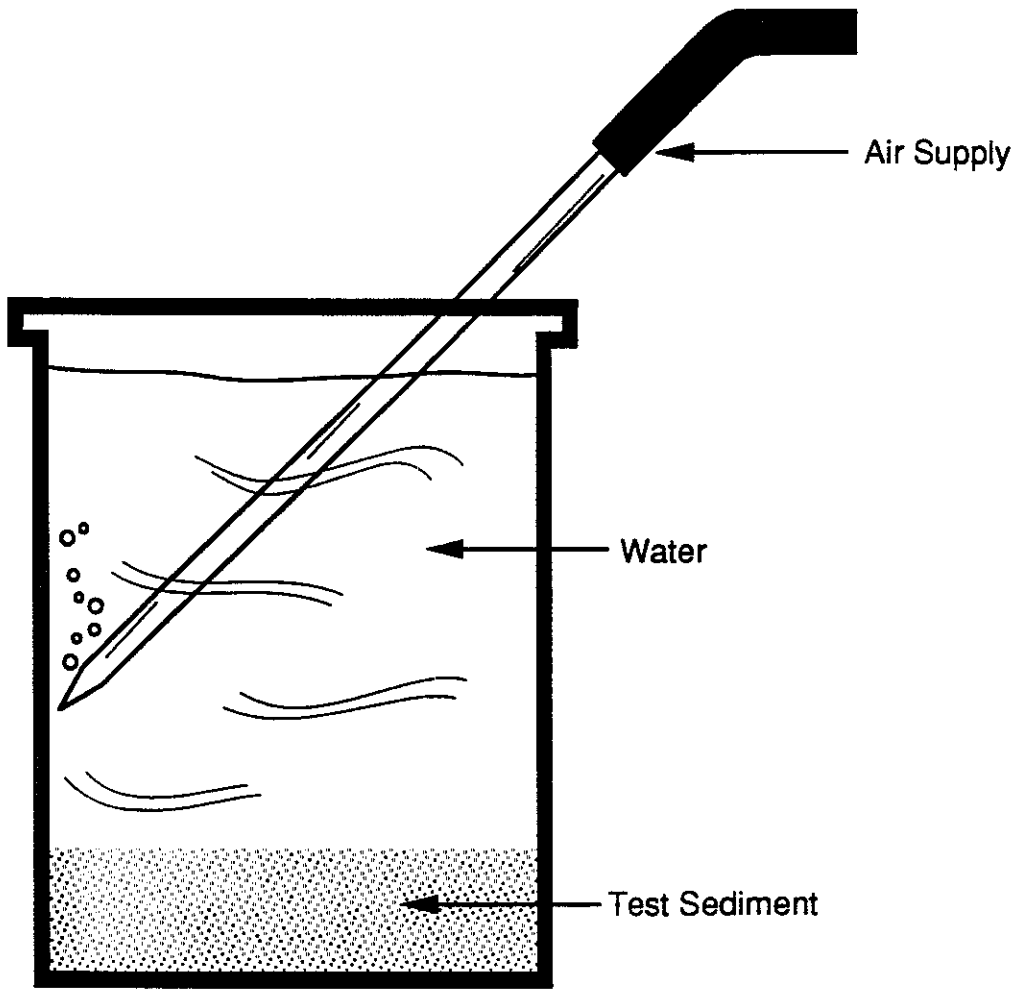

Figure 1. Static exposure system used for the *Neanthes* sublethal bioassay

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 an ammonium formate solution, 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 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 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. 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).

Prior to seawater replacement, dissolved oxygen, salinity, and pH are determined for each exposure chamber. 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. Typically, five replicate exposure chambers should be included for each sediment tested including all control and reference sediments. Five replicates provide sufficient observations per treatment to allow for statistical differentiation between treatments. However, the number of replicates used in any experiment should be based on the objectives of the study rather than on the need to meet the statistical testing requirements recommended in this protocol. For example, if the bioassay is to be used during a reconnaissance survey to identify potentially contaminated sediments, the replicates may not be needed to meet study objectives.

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 chloride range between 12.0 and 22.0 mg/L (Reish 1984; Johns and Ginn 1990).

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 American Public Health Association (1985).

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

One of the three 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. 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 beakers.

DATA REPORTING REQUIREMENTS AND STATISTICAL ANALYSIS

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

- Water quality measurements during testing (i.e., dissolved oxygen, temperature, salinity, pH)
- 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
- Interstitial salinity values of control, reference, and test sediments (both initial and final)
- 96-hour LC_{50} values with reference toxicant
- Any problems that may have influenced data quality.

Data resulting from the *Neanthes* bioassay can be statistically analyzed using a number of procedures depending upon study objectives and test design (i.e., number of replicates used). Statistical procedures that might be used include the *t*-test and an analysis of variance.

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