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28-Day Chronic Sublethal Test Method for Evaluating Whole Sediments Using an Early Life Stage of the Marine Polychaete *Neanthes arenaceodentata*

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PURPOSE: This technical note describes a 28-day chronic sublethal test method designed to evaluate the toxicity of contaminated sediments including dredged material. The test method is a modification of the existing *N. arenaceodentata* chronic sublethal test methods (American Society for Testing and Materials (ASTM) 2007), which incorporates an early life stage of the polychaete. Using an early organism life stage enhances the capability to discern contaminated sediment while increasing the logistical ease with which the test is conducted. As with currently existing *N. arenaceodentata* test methods, the new test measures effects of contaminants on both survival and growth.

BACKGROUND: Whole sediment toxicity tests are routinely used to assess the toxicity of contaminated sediments and are required by regulation in the evaluation of contaminated dredged material (U.S. Environmental Protection Agency/U.S. Army Corps of Engineers (USEPA/USACE) 1991, 1998). Early sediment evaluations were limited to tests that measured acute effects following short-term exposures in highly sensitive species (Swartz 1989; Reish 1985). Although acute tests are generally easy to conduct and provide valuable information when evaluating highly contaminated sediment, they fail to address the potential effects that may occur following long-term exposure to low to moderately contaminated sediments (Kemble et al. 1994; McGee et al. 1993). Due to the potential for long-term low-level exposure, several estuarine and marine chronic sublethal test methods have been developed that not only measure contaminant effects on survival but also measure the effects of contaminants on sublethal endpoints such as growth and reproduction (USEPA/USACE 2001; ASTM 2007, 2008).

Test organism. The Nereid polychaete *Nereis* (*Neanthes) arenaceodentata* (Figure 1) is a widely distributed polychaeteous annelid found in shallow marine and estuarine environments throughout the world (Reish 1957; ASTM 2007). The polychaete is found in a variety of sediment types where it constructs non-permanent mucoid tubes in the upper 2 to 3 cm of sediment. Population densities as high as 1000/m^2 have been observed in some intertidal areas (ASTM 2007). *Neanthes arenaceodentata* is a relatively large polychaete (up to 70 mm in length) that deposit feeds on sediment particles up to 70 µm in diameter with a preference for sediment particles around 12 µm (Bridges and Farrar 1997; Whitlatch 1980).

The life cycle of *N. arenaceodentata* has been well documented (Reish 1957, Pesch and Hoffman 1983) and is illustrated in Figure 2. Reproduction is sexual with males and females forming pairs within a common tube. The female releases eggs into the tube, which are then fertilized by the male. The female reproduces only once. She exits the tube and dies within 1 to 2 days of egg deposition. The male incubates and guards the eggs for approximately 3 weeks. At that time,

emergent juveniles leave the parent tube and establish tubes of their own, leaving the parent male available to mate again. Development is direct with no planktonic larval stage. At about 6 weeks post-emergence, female worms will begin to develop eggs within their coelom. The life cycle is completed with the deposition of eggs at 9 to 13 weeks post-emergence. The entire life cycle can be completed in the laboratory in 12 to 17 weeks at 20°C.

Figure 1. The polychaeteous annelid *Neanthes arenaceodentata.*

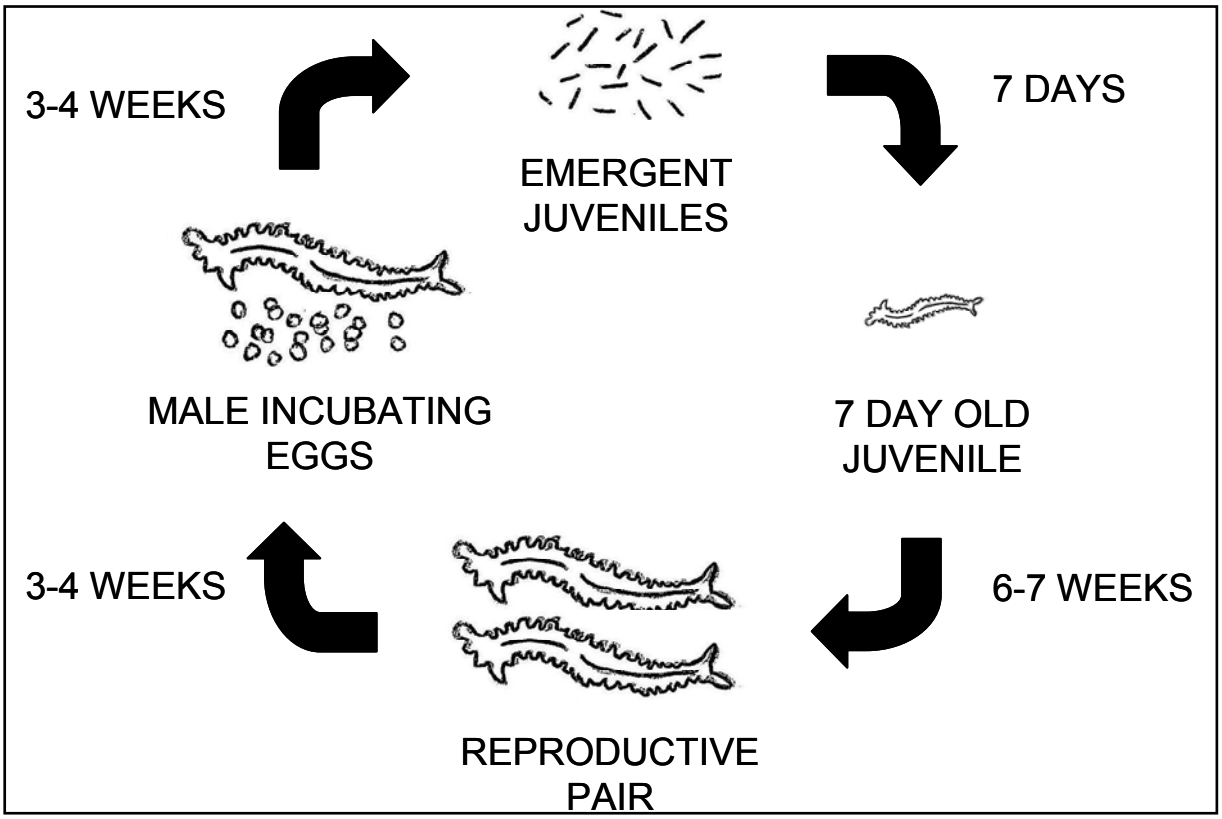

Figure 2. Life Cycle of *Neanthes arenaceodentata.*

Regulatory use. The sediment test method described in this document augments existing *N. arenaceodentata* test methods, particularly the 28-day test method described in ASTM (2007), and has applicability to the routine assessment of sediments and the evaluation of dredged material under section 103 of the Marine Protection, Research and Sanctuaries Act of 1972 (Public Law (PL) 92-532) and section 404(b)(1) of the Federal Water Pollution Control Act of 1972(PL92-500), as amended. *Neanthes arenaceodentata* is recommended by the national dredged material evaluation program as an appropriately sensitive test organism for evaluating sediment quality (USEPA/USACE 1991; 1998). The chronic test was specifically developed for use by the contracting community; therefore, great effort was made to ensure the test method was practical, logistically feasible, and required a relatively low capital investment to conduct.

Comparison to existing test methods. Current *N. arenacedentata* chronic test methods examine the effects of contaminated sediment on survival and growth following 20- and 28-day whole sediment exposures (Reish et al. 2005, Dillon et al. 1993, USEPA 1990, ASTM 2007). These tests have been routinely used for sediment evaluation particularly in the Northwest United States (e.g., USACE Northwestern Division) (Reish and Gerlinger 1997). The test method described in this technical note represents a modification of the 28-day test method described in Dillon et. al (1993) and in "Standard guide for conducting sediment toxicity tests with polychaetous annelids" (ASTM 2007). The test method was evaluated in a round robin test series in 1998 and was found to be highly precise and reproducible (Battelle Marine Sciences Laboratory 1998).

Modifications to the existing test method include:

1) The modified method utilizes < 7-day-old emergent juveniles for test initiation rather than 2- to 3-week-old juveniles. Initiating the test with a younger organism increases the overall responsiveness of the test method and maximizes the potential for detecting effects on growth. Bridges and Farrar (1997) demonstrated that emergent juveniles were more sensitive to contaminants than 2- to 3-week-old juveniles. This may be due in part to the lack of a fully developed detoxification system and a higher surface-to-area ratio, which augments uptake in younger organisms (Bridges and Farrar 1997; Rossi and Anderson 1978).

2) Exposure chamber size has been reduced from 1 L to 300 mL. Decreasing the chamber size increases the manageability of the test by decreasing sediment and overlying water volume requirements and increasing the ease of test initiation and termination, both of which decrease labor requirements and therefore the overall cost of testing.

3) The number of organisms per replicate has been reduced from 5 to 1. Decreasing the number of animals per replicate decreases intra-chamber variability in organism size at test termination, decreases the number of organisms required to conduct a test by 60 percent, and decreases the amount of labor required at test initiation and termination.

4) The number of treatment replicates is increased from 5 to 10 per treatment. The higher number of replicates increases the probability of having an adequate sample size at test termination for evaluating the growth endpoint as well as increasing the overall statistical power of the test.

TEST METHOD: The recommended test conditions for conducting sediment evaluations with the *N. arenaceodentata* 28-day test are summarized in Table 1 and discussed in the following paragraphs. Detailed guidance for conducting the 28-day chronic sublethal test is provided in Appendix A.

Table 1. Recommended Test Conditions for Conducting 28-day Sediment Evaluations with *Neanthes arenaceodentata*

Parameter	Conditions
Substrate	2 cm sediment (75 mL mark)
Salinity	30±1 part per thousand
Aeration	Trickle flow (filtered air) 1-2 bubbles/sec
Overlying water	175 mL (250 mL mark)
Renewal of overlying water	Once weekly (50 percent)
Temperature	20±1 °C
Photoperiod	12:12 hr light:dark
Test duration	28 days
Experimental chambers	300 mL tall form beakers
Initial age of test organisms	≤7 days post-emergent juveniles
Feeding	Twice weekly (Tues and Fri) 2 mg of Tetramarin® on Tuesday and 2 mg of Tetramarin® and 1 mg of alfalfa on Friday per organism
Number of organisms/chamber	1
Number of replicate chambers/treatment	10
Water quality monitoring	Weekly (pH, DO, salinity, ammonia) Daily (temperature)
End points measured	Survival and growth
Test acceptability requirements	Minimum mean control survival of 80% and positive growth in control organisms

Laboratory Culture Methods. Cultures of *N. arenacedentata* can be maintained in the laboratory. *Neanthes arenacedentata* cultures were initiated at the U.S. Army Engineer Research and Development Center (ERDC) in 1988 and have been maintained at ERDC on a continuous basis since that time. Initial stock culture was provided by Dr. Don Reish of California State University, Long Beach, CA. Worms are maintained at 20°C in aerated (30 parts per thousand) artificial seawater. Seawater can be filtered natural seawater or prepared from a synthetic sea salt mix. Seawater at the ERDC is prepared using Instant Ocean® seasalt mix and reverse-osmosis water. Emergent juveniles (EJs) are placed in 38-L glass aquaria (100 EJs/aquaria; no sediment). The EJs are fed Tetramarin (100 mg/aquaria) and alfalfa (50 mg/aquaria) twice weekly.

At about 10 weeks, worms are paired male to female. Sexes are identified by the presence of eggs in the coelom and by the intrasexual fighting response described in Reish (1974) and "Standard Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids" (ASTM 2007). Once pairs are identified, they are placed in 600-mL beakers containing 500 mL of seawater. Each beaker receives 4 mg of Tetramarin® and 4 mg of alfalfa. Beakers are monitored daily for

the egg deposition. Once deposition occurs, the female is removed. Pairs are then monitored weekly for the presence of emergent juveniles.

Sediment Storage and Handling. Test sediment should be handled and stored as described in "Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses" (USEPA 2001). Test sediments should be sub-sampled upon arrival and analyzed for grain size, total Kjeldahl nitrogen, total organic carbon, interstitial salinity, pH, and pore water concentrations of hydrogen sulfide (H_2S) and ammonia (NH_3). Test sediments should only be sieved (e.g., <500 μm) if indigenous competing organisms are present. If testing is not initiated immediately after sediment arrival, sediments should be stored in the dark at 4°C until test initiation.

Preparation of Test Chambers. The day prior to test initiation, sediment should be added to exposure chambers (e.g. 300-mL beaker) to a depth of 2 cm (~75 mL; the approximate average burrowing depth), overlaid with seawater, placed on trickle flow aeration and brought to testing temperature. Prior to the addition of test organisms, water quality parameters are measured and recorded for three replicates of each sediment treatment. Water quality parameters measured include pH, salinity, dissolved oxygen, temperature, and pore water ammonia. All parameters should fall within the ranges specified in Table A1 prior to test initiation. Ammonia levels above 20 mg/L have been demonstrated to adversely impact *N. arenaceodentata* (Dillon et al. 1993). If pore water ammonia is greater than 20 mg/L, then all beakers (including beakers with ammonia values less than 20 mg/L) should undergo up to twice daily water exchanges until ammonia values are below 20 mg/L.

Test Initiation. To initiate a test, one worm is randomly placed into a counting chamber (e.g., 50-mL beakers). Animals can also be placed directly into an exposure beaker but care should be taken to ensure the transfer is successful. The number of chambers required will equal the total number of chambers in the test plus five additional chambers for initial weights. Initial weight chambers receive five animals each. After worms are added to all chambers, each chamber is observed to ensure the animals are present and healthy. The counting chambers are then randomly assigned to a test chamber and the animals are gently added by pouring the contents of the counting chamber into the test chamber, ensuring no animals are trapped in the surface tension of the water. Animals trapped in the surface tension of the water can be freed by gently dropping water from a pipet onto the animal. Each beaker should be carefully rinsed to ensure all animals are transferred. Worms in each of the five initial weight counting chambers should be rinsed in deionized water, placed on pans, dried in an oven at 60°C for 24 hr, and then weighed to determine individual dry weight for the calculation of growth rates.

Test Maintenance and Monitoring. Each organism is fed 2 mg of Tetramarin® on Tuesdays and Fridays and 1 mg of ground alfalfa on Fridays. Water renewals are conducted weekly and should occur before feeding if the exchange is conducted on Tuesday or Friday. Prior to water exchange, water quality parameters (i.e., pH, dissolved oxygen, salinity and temperature) are measured in three replicates per treatment. General observations of each chamber should be conducted at least twice weekly (e.g., animal activity, condition of sediment, etc.).

Test Termination. Prior to test termination, water quality parameters are measured and recorded for three replicates of each sediment treatment. Water quality parameters measured

include pH, salinity, dissolved oxygen, temperature, and pore water ammonia. Sediment from each chamber is gently poured through a 425-µm sieve and organisms are recovered.

Test End Point Measurement. *N. arenaceodentata* survival is determined by gently prodding a recovered animal with a blunt probe. If movement is observed, the animal is considered alive and included in the survival count. Animals unaccounted for likely died and decomposed or were consumed by other polychaetes and are considered dead. Only two survival outcomes are possible (i.e., 0 or 100 percent survival). Categorical statistics (e.g., Fisher's exact test) should be used for evaluating contaminant effects on the survival endpoint.

To calculate individual growth rates, all surviving animals for each replicate are placed on a preweighed pan, then dried in an oven at 60°C for 24 hr. Initial individual dry weight (test initiation) and final individual dry weight per replicate (test termination) are calculated by weighing the pan containing the animals, subtracting the pan weight, and dividing the weight value obtained by the total number of animals on the pan. Growth rate is calculated using the following equation:

$$G = \frac{DWTt_2 - DWTt_1}{t_2 - t_1}$$

where

$DWTt_2$ = estimated individual dry weight of surviving adults at test termination

DWT_1 = estimated individual dry weight of organisms at test initiation

$t_2 - t_1$ = duration of test (e.g., days)

Statistical analysis should be performed following the guidelines described in Section 16 of "Standard Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids" (ASTM 2007).

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> Farrar, D. J., and T. S. Bridges. 2011. 28-day chronic sublethal test method for evaluating whole sediments using an early life stage of the marine Polychaete *Neanthes arenaceodentata*. DOER Technical Notes Collection. ERDC TN-DOER-R14. Vicksburg, MS: U.S. Army Engineer Research and Development Center.

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Appendix A

1. **Testing Procedure Overview.** This appendix provides guidance for conducting 28-day chronic sediment toxicity tests using the marine polychaete *Neanthes arenaceodentata*. The test method is based on modifications of a 28-day chronic sediment test method developed by the U.S. Army Engineer Research and Development Center for the evaluation of marine sediments (Dillon et. al. 1993, ASTM 2007, Bridges and Farrar 1997, and Bridges et al. 1997). Recommended test conditions are outlined in Table A1. A general testing activity schedule is provided in Table A2.

2. Required Materials for Preparation and Testing

- Biological testing material
  - *Neanthes arenaceodentata* (post-emergent juveniles ≤7 days old)
  - Control, reference and site sediment
  - Natural or synthetic seawater (e.g., Instant Ocean®) at 30 ppt using deionized/reverse osmosis water
  - Environmental chamber with temperature and photoperiod control
  - Tetramarin® Fish Flakes (ground to 0.5 mm)
  - Alfalfa (ground to 0.5 mm)
- Glassware and accessories
  - Testing chambers (e.g., ten 300-mL tall form beakers per treatment)
  - Watch glasses (for covering test chambers)
  - Glass disposable Pasteur pipettes (5-3/4 or 9 in.)
  - Aeration supply (oil free)
  - Plastic tubing for aeration (size determined by aeration design)
  - Test initiation/maintenance/breakdown materials
    - Transfer pipettes
    - HDPE cups or glass 50-mL beakers for organism counting/sorting
    - Turbulence reducers (e.g., nylon, Teflon, or polyethylene disks attached to a rod)
    - Plastic wash bottles
    - Tally counters (optional)
    - Camel hair brushes
    - Pans or bowls for counting
    - Aluminum pans for initial and final weight determination
- Instruments and equipment
  - Electric drill/mixer with stainless steel mixing rod
  - Stainless steel or high density polyethylene (HDPE) spoon/mixing rods
  - Photoperiod timers
  - Temperature controllers
  - Dissolved oxygen (D.O.) meter
  - Thermometer (°C)
  - pH meter
  - Salinity/conductivity meter or refractometer
  - ISE meter (Orion 720A or similar)
    - Ammonia-sensitive electrode (Orion 95-12 or similar)

    - 0.1 M or 1000 ppm NH3 standard, ionic strength adjuster
  - Balance/scale capable of measuring 0.1 mg
  - Desiccator/oven
- Chemicals
  - Liquid soap
  - 10 percent nitric acid
  - Acetone
  - Reference toxicant for reference test (e.g., Cadmium chloride)
- Miscellaneous
  - Data sheets: e.g., live animal acclimation, water quality, survival and growth
  - Labels for exposure chambers
  - Safety equipment (e.g., gloves, lab coats, safety glasses, safety showers and eye fountains)

3. Receipt of sediment

- The temperature upon arrival should be recorded and the bucket placed into a cold room at 4.0 ± 2°C until required for testing. A portion of the sediment should be analyzed for grain size, total Kjeldhal nitrogen, total organic carbon, interstitial salinity, pH, pore water concentrations of hydrogen sulfide (H2S) and ammonia (NH3).
- Prior to test initiation, sediment should be homogenized with appropriate homogenation equipment (e.g. electric drill or mixer equipped with a stainless steel shaft and prop/agitator). Mix sediment until it is homogenous in texture/color and has an even distribution of water. Repeat this process for all sediments following decontamination of equipment.
- Decontamination: The homogenization equipment (e.g., mixer, spatula) should be thoroughly decontaminated between the mixing of each sediment. This includes a (1) water rinse, (2) soap water scrubbing, (3) rinse with 10 percent nitric acid, (4) rinse with acetone,[1] and (5) deionized water wash. Rinse procedure is dependent on the contaminant(s) present and can be modified as needed. Rinses must be thorough to ensure all acid/solvent is removed; soaking glassware overnight in deionized water is an option.

4. Receipt of testing organisms (Day 1)

- An organism receipt and acclimation form should be completed upon the arrival of organisms describing organism and surrounding water conditions.
- Laboratory acclimation (at testing conditions) should proceed without sediment for ~24 hr prior to test initiation. This period is designated to gradually acclimate organisms to laboratory/testing conditions and ensure that test organisms are healthy prior to distribution into test chambers. Organisms should be acclimated in the water and container they were shipped in and gradually brought to testing temperature by partially submerging the container in an aquarium in the environmental chamber. Once the worms reach the appropriate temperature, they can be transferred to an acclimation/holding container. Neanthes may become trapped in the surface tension of the water and float. Use a drop-

[1] Attention should be given to whether all equipment is stainless steel or HDPE before using acetone rinses.

ping pipette to release a drop of water onto the floating worms to break surface tension and allow floating worms to swim to the bottom of the container. Remove and count perished organisms. If total mortality/inactivity (shipping and acclimation) exceeds 10 percent, the entire shipment may be unsuitable for testing. Aeration must be supplied using an air stone or glass pipette. During the acclimation period, the animals should be held at 20 °C with a photoperiod of 12L:12D.

5. Overlying water

- Laboratories can use natural seawater or reconstituted (synthetic) water to serve as overlying water during testing.
- Reconstituted water
  - An adequate volume of deionized, reverse osmosis or Milli-Q water should be placed in a HDPE tank equipped with a circulation pump.
  - Although other sea salt mixes are probably adequate, Instant Ocean® seasalt mix (Aquarium Systems, Inc., Mentor, OH, U.S.A.), is preferred given its history of successful use with Neanthes. The seasalt mix should be slowly added to the tank until the water reaches 30 ppt. Water must be circulated overnight (~16 hr) to fully equilibrate.

6. Test chamber and sediment preparation (day 1, 2, or 3 sediment addition)

- Glass 300-mL (tall form) beakers or other appropriate chamber (soap water/acetone/acid-washed/de-ionized water rinsed) serve as test chambers. The number required is 5 or 10 replicates, depending on the number of organisms to be added to each chamber (plus a minimum of two blank chambers), times the number of sediment treatments (including reference and controls). Each chamber should be labeled with the treatment and replicate (e.g., A, B, C, D, E) designation.
- Chambers are filled with 75 mL (~ 2 cm) of homogenized sediment (as described in this Appendix) from each site, control and reference using a decontaminated stainless steel or HDPE scoop/spatula. The sediment should be added carefully to minimize contact with the sides of the chamber and leveled by tapping the side of the chamber.
- Slowly add the overlying natural or reconstituted seawater (30 ppt) using a turbulence reducer to minimize resuspension of the sediment. Add 125 mL of overlying water to the exposure chamber. This will allow room for the additional water added with the organisms at test initiation.
- Randomly place all treatment replicates and blank chambers in the specified controlled environmental chamber at 20 ± 1°C and cover each chamber with a watch glass.
- After sediment has partially settled, gently aerate (1-2 bubbles per second) ~2-3 cm from sediment surface and allow full settling overnight. A glass pipette inserted into plastic tubing connected to an air supply will provide aeration. The aeration rate should be adequate to keep D.O. at >50 percent saturation but not disturb/resuspend the test sediment.
- Pore water ammonia concentrations will be taken from a blank chamber for all sediments. The test cannot be initiated unless pore water total ammonia is less than 20 mg/L. Water changes will be performed to flush out ammonia until the desired level is achieved in all sediments, upon which tests are conducted using the standard protocols (described

below). Water changes involve renewal of 50 percent of the overlying water with minimal sediment disturbance.

- Pore water ammonia samples are obtained by placing a 40-mL sediment sample into a 50-mL centrifuge tube. The sediment is centrifuged (4000 RPM for 15 minutes) and pore water is collected from the overlying water in the centrifuge tube.
- 10 mL of overlying water (pore water) will be taken from the centrifuge tube and placed into a beaker. Total ammonia should be measured using an ISE meter equipped with an ammonia-sensitive electrode and recorded. Maintenance and calibrations should be conducted as specified in the meter and probe manuals.

7. Test initiation (Day 0)

- Take pore water ammonia concentration. Testing cannot be initiated unless total ammonia is less than 20 mg/L (see Section 6 above). Pore water ammonia should also be measured at the end of the test using the second blank chamber.
- Overlying water quality measurements (temperature, salinity, pH, and D.O.) will be taken in three replicate chambers after water exchange and recorded. If parameters are within the specified ranges (Table A1), the test may be initiated. Neanthes emergent juveniles will be placed in a culture bowl or counting tray. The required number of organisms should be randomly placed into HDPE cups or glass 50-mL beakers. Organism transfer should be carried out using wide-bore pipettes or camel hair brushes, fully submerging the pipette or camel hair brush in the water contained in the counting chamber to minimize injury (forceps are not to be used). Only apparently healthy individuals should be selected at random for testing. Any organisms that are dropped or contact a dry surface cannot be used in testing. After the appropriate number of counting containers are filled, confirm that each container contains the appropriate number of healthy worms. During test initiation, add five worms to each of five counting containers containing seawater for initial biomass measurements. Select worms randomly and at regular intervals during test initiation so as not to bias the initial size measurements.
- Aeration to test chambers will be suspended and each counting chamber is selected at random to be gently poured into a test chamber. Ensure that each chamber receives an organism and that all individuals are removed from the counting chamber. Wash bottles containing 30 ppt seawater can be used to dislodge worms that are stuck to the sides. Special attention should be paid to floating worms, which should be submerged using a dropping pipette.
- Test organisms should also be added to each remaining blank chamber designated for pore water determination at day 28. The blank chambers should receive the same maintenance as the treatment chambers (i.e., water exchange and feeding).
- Upon addition of test organisms, the time of test initiation must be recorded on the appropriate data sheet, it must be confirmed that chambers are filled to the 250 mL mark, and aeration must be re-supplied.
- Approximately 1 hr following the addition of test organisms, chambers should be observed for injured worms that did not burrow or appear unhealthy. At this time, individuals that are injured, unhealthy, or failed to burrow may be replaced if the response does not appear to be specific to the particular sediment treatment. Floating worms should be submerged using a dropping pipette.

- To make initial weight measurements, individual worms should be "scooped" with a camel hair brush rinsed in deionized water (approximately 5 seconds), blotted dry with a kimwipe or paper towel, and transferred onto a pre-weighed premarked pan. Place all five worms from two transfer chambers into a single pan (total of ten animals per pan). Fold boat/pan to prevent the loss of worms. Oven dry worms at 60 °C for 24 hr prior to weighing. Remove boats/pans from the oven and place them in a desiccator for approximately 1 hr to cool to room temperature. Measure weights on a balance that can measure to 0.01 mg and record.
- Feed the worms as described in Section 8 below. If the test is initiated on a non-feeding day, then feeding is not required.
- Record observations or any other experimental notes.

8. Test monitoring and maintenance (Days 1–27)

- Water quality: Record environmental chamber temperature (min/max) daily. Record temperature, salinity, pH, and D.O. once per week (e.g., Wed.) prior to water exchange in three replicates per treatment. An effort should be made to ensure that measurements are rotated among treatment replicates.
- Exchange the water (50 percent) in all chambers once per week following water quality measurements using a method that minimizes sediment disturbance and ensures no worms are lost from the chamber (e.g., syringe, siphoning).
- Provide each chamber with 2 mg of Tetramarin® each Tuesday and 2 mg of Tetramarin® and 1 mg of alfalfa each Friday. Food should be delivered in a seawater slurry. Prepare a slurry containing 2 mg of Tetramarin® (or 2 mg of Tetramarin® and 1 mg of alfalfa) per mL. Deliver 1 mL to each chamber.
- Check light cycle and ensure that each test chamber is adequately aerated daily.

9. Test termination/breakdown (Day 28)

- Water quality: record temperature, salinity, pH, and D.O. in three chambers for each treatment. Also take pore water ammonia from a blank chamber for all treatments. This ammonia measurement indicates whether ammonia values remained below 20 mg/L during testing.
- Survival and growth endpoint determination.
    - Gently pour off all but 75 mL of overlying water through an 8- or 12-in.-diam, 0.425-mm or smaller, ASTM testing sieve. Swirl and suspend sediment with the remaining overlying water for easier passing of sediment through the sieves. If using an 8-in. sieve, a 12-in.-diam sieve (1-mm mesh) can be placed over the bucket receiving the waste. A wash bottle can be used to wash sediment through the sieve but care must be taken not to injure the worms. Transfer small amounts of the material retained on each sieve to a counting bowl or tray for examination. Count and record surviving worms. All missing individuals or worms that fail to move following close observation (e.g., under dissection scope) and gentle prodding with a blunt probe are considered dead. Once removed from the sieve the worm should be placed in a container containing clean 30-ppt seawater. The original exposure chamber, void of sediment, can be used for this purpose.

- Surviving worms should be "scooped" with a camel hair brush rinsed in deionized water (approximately 5 seconds), blotted dry with a kimwipe, and transferred onto a pre-weighed, pre-marked pan. Dry in oven at 60 °C for 24 hr. Remove from oven and allow pans to cool. Record total weight of each pan. Report growth as growth rate. Growth rate is determined using the following equation:

$$G = \frac{DWTt_2 - DWTt_1}{t_2 - t_1}$$

where

$DWTt_2$ = estimated individual dry weight of surviving adults at test termination
DWT_1 = estimated individual dry weight of organisms at test initiation
$t_2 - t_1$ = duration of test (e.g., days)

10. Acceptability

- The test is not acceptable if any of the following occur:
  - Less than 80 percent average survival in the control.
  - Positive growth in control organisms.
  - Water quality parameters fall outside prescribed limits (depending on the magnitude of the variation, the data may still be valid but should be interpreted with caution).

11. Statistical Analysis

- Only two survival outcomes are possible (i.e., 0 or 100 percent survival). Categorical statistics (e.g., Fisher's exact test) should be used for evaluating contaminant effects on the survival endpoint. Statistical analysis on growth data should be performed following the guidelines described in Section 16 of "Standard guide for conducting Sediment Toxicity Tests with Polychaetous Annelids" (ASTM 2007).

Table A1. Summary of conditions for 28-day chronic toxicity test method with *Neanthes arenaceodentata*

Description	Condition
Test type	Static renewal
Test duration	28 days
Temperature	Daily instantaneous 20 ± 3 °C Test average 20 ± 2 °C
Salinity	Daily instantaneous 30 ± 3 ppt Test average 30 ± 2 ppt
pH	7-9
Light quality	Wide spectrum fluorescent lights
Light intensity	500-1000 lux
Photoperiod	12:12 L:D
Test chamber size	300 mL
Sediment depth/volume	2 cm (75 mL)
Overlying water volume	Fill to 250 mL
Sediment settling time	Overnight
Overlying water quality measurements	Temperature D.O., pH, salinity on three chambers at test setup and breakdown. Daily temperature in environmental chamber (min/max). Temperature, pH, D.O., and salinity in three replicates per treatment once per week prior to water exchange
Renewal of overlying water	50% renewal once weekly
Age of test organisms	≤7-day-old post-emergent juveniles
Organisms/chamber	1
Replicates/treatment	10
Organisms/treatment	10
Feeding regime	Feed 2 mg of Tetramarin® per chamber on Tuesdays and 2 mg of Tetramarin® and 1 mg of alfalfa per chamber on Fridays
Test chamber cleaning	None
Test solution aeration	Trickle flow (<100 bubbles/min); >50 percent saturation
Dilution water	30 ppt natural or reconstituted seawater (Instant Ocean®)
Test concentrations	Site sediment, reference, control
Endpoint(s)	Survival and growth

Table A2. Summary of Tasks for 28-day Chronic Toxicity Tests using *Neanthes arenaceodentata*

Day	Task
-X	Receive and begin acclimating *Neanthes.* Feed daily with Tetramarin® as needed.
-1	Homogenize the sediment and add to the test chambers. Add overlying water and aerate overnight.
0	Test initiation: Measure pore water ammonia and initiate if value is ≤ 20 mg/L. Record temperature, salinity, pH, D.O. in three replicates per treatment. Add worms to test chambers. Place worms on pans for initial weight determination.
1-27	Measure temperature in the environmental chamber (min/max). Perform test chamber observations and temperature, pH, D.O., and salinity from three replicates per treatment prior to water exchanges. Water exchange each chamber (400 mL per chamber) once weekly (e.g., Wed.). Feed 2 mg of Tetramarin® (slurry 2 mg/mL; 1 mL per chamber) on Tuesdays and 2 mg of Tetramarin® and alfalfa on Fridays (slurry 2 mg Tetramarin® and alfalfa/mL; 1 mL per chamber).
28	Test termination: Measure temperature, pH, D.O., and salinity in three replicates per treatment. Measure pore-water ammonia from a blank exposure chamber for each treatment. Sieve sediment for *Neanthes* and record survival. Place surviving adults on pans for mass/growth determination.