Bight'23 Toxicology Laboratory Manual

B'28 Toxicology Committee
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INTRODUCTION

This manual serves to document the methods used in the Bight'23 study for testing the toxicity of marine and estuarine sediments. The methods described herein are based on published manuals which contain the bulk of the details in performing the tests. This manual serves to document where the published methods have been modified or needed clarification. This document is organized in three sections. The first section contains methodology for the *Eohaustorius estuarius* 10-day survival test on whole sediments. The second section has the methods for the 48-hr sediment-water interface test using embryos of the mussel (*Mytilus galloprovincialis*). The third section documents the quality assurance plan that will be used for the toxicity testing.

10-DAY AMPHIPOD SURVIVAL TEST USING *EOHAUSTORIUS ESTUARIUS* – STANDARD OPERATING PROCEDURE

Assessment of whole sediment toxicity using an amphipod 10-day acute survival test will be conducted in accordance with the procedures outlined in the amphipod testing manual (USEPA 1994) and the American Society for Testing and Materials (ASTM) method E1367-03 (ASTM 2006). These test methods are summarized in Table 2. This SOP is meant to supplement the published protocols for this toxicity test, not replace them. Any procedures not specifically covered by this SOP revert to the original protocols.

1. Specialized Equipment
-1 L glass chambers (canning jars are recommended) for sediment exposure
-1 L glass or plastic beakers for ammonia reference toxicant exposures
-Filtered seawater (≤20 µm) for overlying water and reference toxicant exposures
-Centrifuge for pore water production
- 1mm stainless steel or Nitex sieve
- 0.5 mm stainless steel or Nitex sieve

2. Experimental Design and Preparation
2.1 Sediment Handling
Samples will be collected, placed into Teflon-lined bags, and homogenized in the field. Sediment for chemical analysis will be removed and the remainder of the sediment for toxicity tests will be kept in the Teflon-lined bag. Samples can be stored for ≤ 28 days in the dark at 4ºC. Although the maximum hold time for the samples is 4 weeks, samples held longer than 2 weeks and less than 4 weeks will be flagged in the database. The goal for the project is to initiate tests within 10 days of sampling to allow time for retesting if necessary. Prior to testing, the test and control sediments are thoroughly homogenized and sieved through a 1.0 mm mesh screen to remove organisms and debris using only the water available in the test sample.

2.2 Experimental setup

Each treatment (test sediment and control materials) will be tested using five replicates. Negative control sediment will consist of sediment from the area where the organisms were collected (i.e., native sediment).

2.3 Test organisms
Test amphipods and laboratory control sediment will be supplied by Northwestern Aquatic Sciences in Newport, Oregon. The animals will be held in the laboratory under test conditions for 2-10 days prior to being used in an assay. The amphipods will be fed once during the holding period, immediately upon receipt (0.25 g of Tetramarin slurry in 100 ml of seawater, for approximately 1000 amphipods). Weigh out the prescribed amount of Tetramarin Saltwater Flakes ("with ProCare for optimal fish health") and add approximately 100 ml of the appropriate salinity seawater. Mix on a magnetic stir plate for about 10 minutes, or until the flakes have softened and started to dissolve. Then add the entire slurry to the holding container. If the animals are split among multiple containers, the slurry may be split accordingly. The amphipods will not be fed during testing. If salinity and/or temperature adjustment of the amphipods is required during the acclimation process, salinity should not be changed by more than 5 ppt per day and temperature not by more than 3°C per day.

2.4 Reference Toxicant Test
To evaluate the relative sensitivity of the test organisms, a reference toxicant test will be performed using ammonium chloride. The concentration series that must be used by all laboratories is 0, 15.6, 31.2, 62.5, 125, and 250 mg/L (Table 1). Laboratories may expand (not substitute) the range of concentrations tested to include a 500 mg/L concentration. If a laboratory wants to use a different concentration series, they must get prior authorization from the Toxicology Committee chair. All concentrations will be made in 32 ppt laboratory seawater. The reference toxicant test will be conducted in plastic or glass containers in the dark (the entire test will be covered after organism addition). Four 800 ml replicates of each concentration will be tested for 96 hours at 15 ± 2°C.

Table 1. Ammonia reference toxicant recipe for amphipod testing.
Stock is 25,000 mg/L ammonia in DIW. (74.306 g NH_4Cl/L DIW)

Concentration (mg/L)	Volume of Stock (ml)	Volume of 32 ppt water (L)
Control	0	3.5
15.6	2.18	3.498
31.2	4.37	3.495
62.5	8.74	3.491
125	17.5	3.482
250	35	3.465

On Day 0, water quality measurements of pH, dissolved oxygen (DO), salinity, and temperature will be made as described in Section 3.3. To obtain an accurate concentration of un-ionized ammonia, subsamples of overlying water for ammonia, pH, DO, salinity, and temperature should be collected from the same test replicate or surrogate for each test concentration on Day 0 and at test termination. Samples for ammonia analysis may be preserved by EPA approved methods for later measurement. Un-ionized ammonia concentrations will be calculated at each sampling time based on concurrent measures of the concentration of total ammonia, pH, and salinity. The Day 0 water quality measurements will be used for calculation of the LC50. For consistency, a spreadsheet tool provided by SCCWRP will be used to derive un-ionized ammonia concentrations.

Ten amphipods will be added to each replicate test chamber as described in Section 3.2. Water quality parameters of temperature, pH, DO, and salinity will be performed at least every other day (T0, T2, and T4). Observations will be performed on Days 1-3, and dead animals will be removed from the test chambers. At test termination, final survival counts will be performed 96 ± 2 hours from the time of test initiation. Ammonia in overlying water will be subsampled at test termination as described above.

At test termination, the number of surviving adult amphipods is recorded. In addition, an estimate is to be made of the total number of neonates in each replicate.

2.5 Pore Water Ammonia and Salinity
Pore water ammonia will be measured at sample receipt. If un-ionized pore water ammonia concentration in a sample is 0.8 mg/L or greater, then the ammonia reference toxicant test will be extended from 4 days to 10 days for better comparison to 10-day test sample results.

The salinity of the pore water in this sample will also be used to determine the salinity of the overlying water added to the sample during testing (Table 2). If any sample in a batch needs to be tested at an alternate salinity, then an appropriate control(s) for that salinity must also be tested in the batch.

3. Test initiation

3.1 Sediment addition
Homogenized sediments will be placed in five replicate 1 L glass jars to a thickness of 2 cm, to which approximately 800 ml of seawater will be added (see Table 2 for salinity requirements). An additional surrogate replicate (no animals added) for each treatment will be set up to obtain measurement of pore water ammonia at test initiation. Test chambers will then be placed in an environmental chamber in a pre-determined random order. The test will be conducted under continuous light at a temperature of 15 ± 2°C, under gentle aeration (approximately 1-2 bubbles per second). After the sediment, water, and aeration are added, the chambers are allowed to sit overnight at test conditions before addition of animals.

3.2 Amphipod addition
After the overnight equilibration, amphipods will be selected and distributed to test chambers in a pre-determined randomized order (20 animals per chamber). Amphipods are initially counted out into small soufflé cups or equivalent. They are then carefully examined a second time (preferably by a second technician) to verify counts and ensure they are healthy before addition to the test chambers. Aeration should be ceased during organism addition and for a period of one hour after to allow time for organisms to burrow. Organisms remaining in the water column after one hour and exhibiting abnormal behavior will be replaced. Test chambers will be covered to minimize evaporation.

3.3 Water quality
Water quality measurements of temperature, pH, DO, and salinity will be recorded at least every other day. Ammonia will be recorded on Day 0 and Day 10 in overlying water in one replicate test chamber or surrogate for each sample. All instruments used for water quality measurements will

be calibrated prior to use. For each sample, ammonia, salinity, and pH will also be measured in interstitial water at the start of the test.

4. During test maintenance

Daily observations will be performed on each replicate to ensure proper aeration and document any unusual animal behavior or obvious abnormalities (e.g. bacterial growth on the surface of the sediment). For any replicates where a biofilm is observed, water quality measurements and ammonia subsamples should also be collected. Measure water quality from the replicate with the observed deviation on the day it is discovered and on the day of the next normally scheduled water quality measurement. Temperature measurements should be made at least daily, but continuously is recommended, either in a surrogate beaker or in the water bath for the duration of the exposure. Record any unusual occurrences during the test that might serve to explain outlier type data at the end of the exposure (e.g. aeration off in a replicate).

If water quality measurements are found to fall outside of acceptable ranges, corrective actions will immediately be taken such as increasing air flow (if reduced DO) or changing temperature if it is outside of the acceptable range. Such deviations and corrective actions must be immediately noted on bench sheets and reported in the comments section of the database at the end of the project.

Those tests in which water quality measurements shown in Table 2 are out of range for an extended time period or degree may be considered unacceptable.

5. Test termination

5.1 Test breakdown
Ammonia, in addition to standard water quality measurements, will be measured in overlying water at test termination on Day 10. The sediments from the chambers will be sieved through a 0.5 mm screen, and the number of surviving amphipods will be recorded. In addition, an estimate is to be made of the number of neonates in each replicate.

5.2 Test Acceptability Criteria
Test results will be compared to the following test acceptability criteria: $\geq$ 90% mean survival in controls at test termination and a control coefficient of variation $\leq$ 11.9%. If control CV is greater than 11.9%, any samples with a mean $\geq$ 90% will be acceptable and not need to be retested, but samples with a mean <90% will need to be retested. Each laboratory must establish a control chart for their ammonia reference toxicant exposures consisting of at least three tests and no more than the 20 most recent tests. The LC_{50} for un-ionized ammonia for each test performed should fall within two standard deviations of the mean of the previous tests on the control chart. A test falling outside two standard deviations should trigger a review of all data and test procedures to ensure that the data are of high quality. The reference toxicant test can be retested during the period that the sediment is being tested if the initial test falls outside of the control chart criteria and sufficient animals remain from the original test batch. Results from both the original and retest must be submitted.

6. Data analysis
6.1 Data summarization
Data will be analyzed by statistically comparing the proportion survival of organisms in the project material to that in the control sediments using the guidance in the amphipod testing manual (USEPA 1994). A t-test assuming unequal variance will be used to analyze data. Data submission will consist of a completed Excel summary; a template will be provided by SCCWRP.

6.2 Outlier analysis

For a test where outliers are suspected among replicates, the Dixon's Test for Detecting Outliers may be used according to USEPA guidance (USEPA 2000) to statistically determine whether or not there are outliers. The Toxicology Committee will review the statistical results and ancillary data collected regarding the test batches in question to determine if data should be excluded from analyses. As a general guideline, data will not be removed from analysis unless there is corroborating evidence, beyond the statistical analysis, that indicates that a given replicate is anomalous.

7. Literature Cited

American Society for Testing and Materials (ASTM). 2006. E1367-03 Standard Guide for Conducting 10-Day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods. *Annual Book of Standards, Water and Environmental Technology, Vol. 11.05*, West Conshohocken, PA.

United States Environmental Protection Agency (USEPA). 1994. Methods for Assessing Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods. EPA/600/R-94/025. EPA Office of Research and Development, Narragansett, Rhode Island. June.

United States Environmental Protection Agency (USEPA). 2000. Guidance for Data Quality Assessment. EPA 600/R-96/084. Office of Environmental Information, Washington D.C. July.

Table 2. Test conditions for the 10-day whole sediment test using *E. estuarius*

Sediment Sample Information	
Test sediment holding time requirements	2 weeks, maximum 4 weeks
Test sample storage conditions	4°C, dark, minimal head space
Control sediment source	From *E. estuarius* supplier
Test Species	***E. estuarius***
Supplier	Northwestern Aquatic Sciences, Newport, OR; Record organism data that comes with amphipods to be included along with bench sheets.
Acclimation/holding time	2-10 days including holding time required to adjust to test temperature and salinity (adjust by changing <3°C per day, and < 5 ppt per day); measure water quality (DO, pH, salinity, temperature) daily while holding; if problem, change water or perform corrective action.
Age/Size class	Mature, 3 – 5 mm
Test Procedures	**USEPA 1994; ASTM E1367-03 (2006), with modifications**
Test type/duration	Acute SP / 10 days
Control water	Natural seawater, ≤20 μm filtered, salinity to match sample salinity
Test temperature	15 ± 2°C
Test dissolved oxygen	$\geq$ 90% saturation (~8.0 mg/L)
Test salinity ranges	If 0-4 ppt in initial pore water, then 2 ± 2 ppt If 5-9 ppt in initial pore water, then 7 ± 2 ppt If 10-14 ppt in initial pore water, then 12 ± 2 ppt If 15-19 ppt in initial pore water, then 17 ± 2 ppt If 20-24 ppt in initial pore water, then 22 ± 2 ppt If 25-29 ppt in initial pore water, then 27 ± 2 ppt If $\geq$ 30 ppt in initial pore water, then 32 ± 2 ppt
Test pH	7.7-8.3 (pH is not adjusted if outside the indicated range)
Test interstitial total ammonia	< 60 mg/L
Test interstitial un-ionized ammonia	< 0.8 mg/L
Test photoperiod	Constant light
Illuminance	500-1000 lux
Test chamber	1 L glass test chamber
Replicates/treatment	5
Organisms/replicate	20
Exposure volume	2 cm sediment; 800 ml water
Feeding	No feeding during testing. Organisms in holding will be fed once, immediately upon receipt. 0.25g Tetramarin slurry in 100 ml seawater, for ~1000 animals.
Water renewal	None
Reference Toxicant Test	
Reference toxicant	Ammonia
Range of concentrations	Control, 15.6, 31.2, 62.5, 125, and 250 mg total NH_3/L, 4 replicates at 32 ± 2 psu
Water Quality Measurements	
Pore water: ammonia, pH, salinity	At sample receipt and on Day 0
Overlying water: ammonia	Start and end of test (Day 0 and Day 10)
Temperature in surrogate in room or bath	At a minimum daily; continuous recommended
Overlying water: pH, temp., DO, salinity	Every other day, in 1 replicate or surrogate from each treatment
Test Acceptability Criteria	Control survival $\geq$ 90%, coefficient of variation of $\leq$ 11.9; If control CV > 11.9%, samples with mean survival $\geq$ 90% will be accepted and not need to be retested, but samples with mean survival < 90% will need to be retested.

48-HOUR EMBRYO DEVELOPMENT USING THE BIVALVE *MYTILUS GALLOPROVINCIALIS* SEDIMENT-WATER INTERFACE TEST –

STANDARD OPERATING PROCEDURE

The following procedure is for testing of homogenized surficial sediment samples. This SOP is meant to supplement the published protocols (USEPA 1995, Anderson et al. 1996) for this toxicity test, not replace them. Test conditions are summarized in Table 4. Any procedures or conditions not specifically covered by this SOP revert to the original protocols.

1. Specialized Equipment
-1mm stainless steel or Nitex sieve
-Glass chambers approximately 7.5 cm diameter and 15 cm tall (600 ml tall form beakers
 recommended)
-Polycarbonate tubing for exposure screen tubes
-Plastic cement for screen tube construction
-Polyethylene screen 25-30µm
-Sedgwick-Rafter counting cell
-Mixing plunger (for mixing gametes)
-Hemocytometer
-Tanks, trays, or aquaria for holding organisms, e.g. standard seawater aquarium with appropriate
 filtration and aeration system.
-Inverted and compound light microscope for inspecting gametes and counting developing
 embryos (recommended)
-Appropriate solution for preserving embryos and larvae (e.g. Formaldehyde, Glutaraldehyde)
-Natural ≤ 1µm filtered seawater
-Overlying water must have a salinity of 32 ±2ppt

2. Experimental Design and Preparation
2.1 Sampling Procedures
Samples are collected, placed into Teflon-lined bags, and homogenized in the field. Sediment for chemical analysis will be removed and the remainder of the sediment for toxicity tests will be kept in the Teflon-lined bag. Samples can be stored for ≤ 28 days in the dark at 4ºC. Although the maximum hold time for the samples is 4 weeks, samples held longer than 2 weeks and less than 4 weeks will be flagged in the database. The goal for the project is to initiate tests within 10 days of sampling to allow time if retesting is necessary. Test sediments are thoroughly homogenized and passed through a 1 mm screen to remove organisms and debris, using only the water available in the test sample.
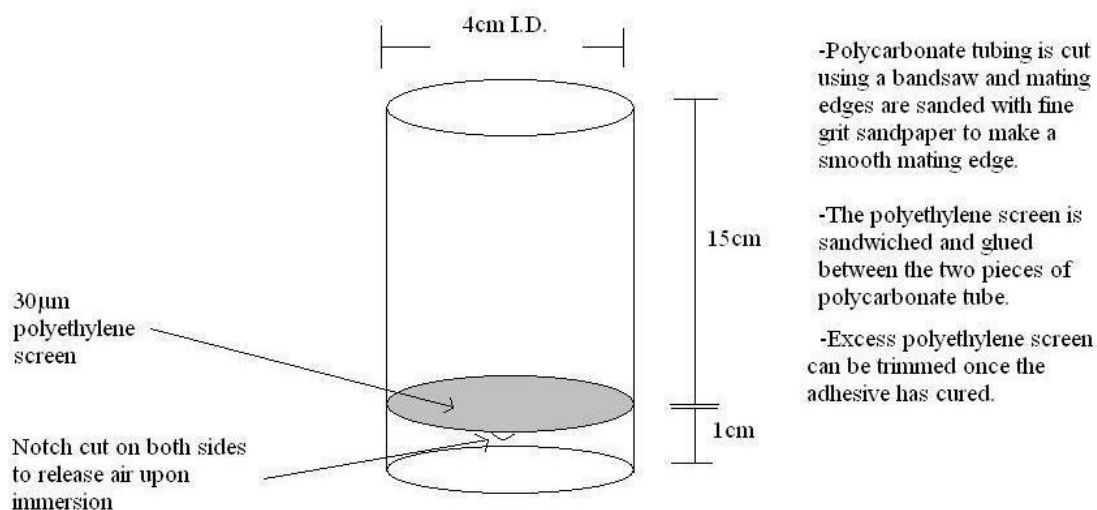
2.2 Experimental setup
Each treatment (test, reference, and control materials) will be run with five replicates. The negative control will consist of test chambers with screen tubes and 32 ppt seawater, but no sediment. This control will verify that the testing system is not causing toxicity. Another negative control to verify the health of the organisms and that the correct number of embryos were added and recovered will consist of shell vials containing 10 ml of 32 ppt seawater. The negative control from the simultaneously conducted reference toxicant can serve this purpose.

2.3 Screen Tube Construction
Screen tubes may vary slightly in size due to availability of materials. Screen tubes may be constructed from clear polycarbonate stock. The *Mytilus* embryo screen tubes are constructed from 4 cm inner diameter (I.D.) stock that is cut into approximately 8 cm high sections (Figure 1). The wall thickness is 3 mm. A 1 cm section is cut from the bottom of the tube and serves as the pedestal that sits on the sediment surface. A polyethylene screen is glued to the tube using clear-thickened acrylic plastic glue, and the pedestal is then glued back on the tube to sandwich the screen. A small hole is drilled in the side of the pedestal that is used to purge any air trapped under the screen during immersion. Twenty-five to thirty microns screen is appropriate for the mussel development protocol. Polyethylene mesh is stronger than the typical nylon mesh and better withstands repeated use. Newly made screen tubes will need to undergo an extensive washing process to leach out any residual contaminants from the construction process.

Figure 1. Screen tube construction.

4cm I.D.

15cm

30µm polyethylene screen

Notch cut on both sides to release air upon immersion

1cm

-Polycarbonate tubing is cut using a bandsaw and mating edges are sanded with fine grit sandpaper to make a smooth mating edge.

-The polyethylene screen is sandwiched and glued between the two pieces of polycarbonate tube.

-Excess polyethylene screen can be trimmed once the adhesive has cured.

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2.4 Test organisms
Laboratories can use their choice of source for adult *Mytilus galloprovincialis*. Animals may be used for spawning on the same day as receipt. It is recommended that each laboratory obtain a group of animals during the spring and hold them in the laboratory under conditions that are conducive to them remaining in spawning condition throughout the duration of the project.

Some holding conditions which have been found acceptable include: 1) hold at 15º C, under a 16:8 light cycle, and on continuous flow-through in 10-gallon glass aquaria and with 50-100 mussels per tank. The animals are fed liquid Roti-Rich at least three times a week, approximately 2-4 ml in each tank; 2) hold in 10-gallon tanks under static conditions, but with the aquarium equipped with a protein skimmer, chiller, and carbon recirculating filter for about 50 mussels. The mussels are initially kept at temperature 8º C, and ramped up 1 degree/day, so that they reach test temperature

a few days in advance of use. The animals are fed 20 ml (~3000 per ml) of artemia once/day with the filter turned off for about 30-60 minutes after feeding. Partial water exchanges are done as indicated by water quality; 3) hold at 12° C, under a 16:8 light cycle under static conditions with daily renewal in 2-gallon aquaria with 15 mussels. Fed daily with artemia hatch from one teaspoon of cysts; 4) hold at 15° C, under a 16:8 light cycle in 100-gallon aquaria with biofilter and UV sterilizer. The animals are fed monthly artemia hatch from one teaspoon of cysts.

The held animals will be used as back-up in the case when freshly collected animals do not spawn during the summer. Fresh animals should be collected as the primary option for testing during the project.

2.5 Reference toxicant test

To evaluate the relative sensitivity of the test organisms, a reference toxicant test will be performed using ammonium chloride. A concentration series of 0, 2, 4, 6, 8 10, and 20 mg/L must be tested (Table 3). If a laboratory wants to use a different concentration series, they must get prior authorization from the Toxicology Committee chair. The reference toxicant test will be conducted in glass shell vials. Five 10 ml replicates of each concentration will be tested for 48 hours at 15 ± 2°C. On Day 0, water quality measurements will be conducted as described in section 3.5. An ammonia sample will also be measured from each concentration on Day 0 and Day 2 in order to calculate the actual total and un-ionized ammonia concentrations. An extra vial for each concentration should be included at test initiation for water quality analysis at test termination. At test initiation, approximately 250 fertilized mussel eggs will be added per vial. At test termination, water quality parameters will be measured from a surrogate vial. The water quality data from Day 0 will be used for interlaboratory comparisons.

Table 3. Ammonia Recipe for mussel testing
Stock is 1000 mg/L ammonia in DIW. (2.972 g NH_4Cl/ L DIW)

Concentration (mg/L)	Volume of Stock (ml)	Volume of 32 ppt water (ml)
Control	0	100
2.0	0.2	99.8
4.0	0.4	99.6
6.0	0.6	99.4
8.0	0.8	99.2
10	1	99
20	2	98

3.0 Test initiation

3.1 Sediment addition

Using a polypropylene spoon add approximately 5 cm of homogenized sediment to test chambers. Test chambers should be made of glass and approximately 7.5 cm in diameter and tall enough to hold 4-5 cm of sediment and 300 ml of overlying water, with a couple of centimeters of free space above that. 600 ml tall form beakers have been found to be suitable. Lower a clean, plastic disc attached to a pipette to the sediment surface, and gently add about 300 ml (32ppt, 15°C) of overlying water to the test chambers. Five replicates are used per sample. Arrange the chambers in pre-determined random order in a temperature-controlled room, cover with acrylic sheets, and add glass pipettes delivering gentle aeration (1 bubble/second). Allow 24 hours before initiation of test for the sample to equilibrate. Samples with reduced porewater salinity will need to be equilibrated for an additional 24 hours with one exchange of the overlying water. After the initial 24-hour

equilibration period, perform a 100% water change for each sample with reduced salinity and allow another 24 hours for equilibration. Before addition of the embryos, screen tubes are gently lowered to the sediment surface. The screen tube mesh should sit approximately 1 cm above the sediment surface. Take care to remove any air bubbles trapped underneath the screen. Screen tubes should be soaked in clean seawater overnight, prior to placement in the test chambers.

3.2 Spawning Induction
Several techniques for spawning mussels are available. Any suitable method can be used so long as it does not adversely affect the quality of the gametes released and is documented in the final data submission. A few common methods are described here: 1) Place the mussels into a container of 32 ppt seawater at 15°C and allow about 30 min for them to resume pumping. Over the next 15-20 minutes increase the temperature to 20°C checking for spawning; 2) A shock method of placing the mussels directly from the 15ºC tank to a separate tank at 26-28ºC works well. If no spawning occurs after 30 minutes, replace the water with 15°C water for 15 minutes and again increase the temperature to 20°C; 3) Mussels can also be induced to spawn by injection of 0.5M KCl into the posterior adductor muscle; 4) Addition of algae can induce spawning of mussels, however if this method is used organisms should be moved to clean seawater once spawning is observed; 5) Addition of heat killed sperm can also induce spawning if it is added to the water about one hour after the initial temperature increase; 6) Place mussels into a UV-treated seawater bath at 26-28°C.

3.3 Pooling gametes
When individuals are observed to be shedding gametes, remove each spawning mussel from the tank and place them in separate beakers (beakers with 100-150 ml of seawater are recommended). Examine a small sample of gametes from each spawning mussel to confirm sex and adequate gamete quality. Use only high-quality eggs; discard vacuolated, small, or abnormally shaped eggs. If good quality eggs are available from one or more females, they may be pooled. However, it is more important to use high quality eggs than it is to use a pooled population of eggs. Male gametes should be assessed under a compound microscope and chosen based on sperm density and motility and may also be pooled. Pooled eggs are placed into a 1 L beaker and diluted using 32 ppt 15°C seawater to a concentration of approximately 5,000-8,000 eggs/ml. Optimally, gametes from at least three males and females should be used, pooling the gametes before fertilization.

3.4 Fertilization
To achieve an acceptable level of sperm, several egg suspensions should be fertilized using a range of sperm volumes. Use 3 replicates of 100 ml of egg suspensions. The amount of sperm solution added to each egg suspension is at the discretion of each laboratory, but the minimum amount of sperm used should not cause polyspermy. Use the eggs with the lowest amount of sperm giving normal embryo development after 1-2 hours. Mussel embryos should show a single polar body. Prepare a final solution of the embryos to be used. The concentration of embryos in the stock is up to each laboratory's discretion, but a volume of 0.1 to 0.5 ml of stock must be added to deliver approximately 250 embryos per replicate.

3.5 Water quality analysis
Take samples for ammonia, pH, dissolved oxygen, and salinity analysis of each sample prior to introducing test organisms to the screen tubes. All instruments used for water quality measurements must be calibrated daily. Temperature measurements must be made daily in a surrogate chamber or in the water bath, though continuous measurement of temperature is recommended. Samples for ammonia analysis may be preserved by EPA approved methods for later measurement.

If water quality measurements are found to fall outside of acceptable ranges, corrective actions will immediately be taken such as increasing air flow (if reduced DO) or adjusting the temperature if it is outside of the acceptable range. Such deviations and corrective actions must be immediately noted on bench sheets and reported in the comments section of the database at the end of the project.

Those tests in which water quality measurements shown in Table 4 are out of range for an extended time period or degree may be considered unacceptable.

3.6 Inoculation of screen tubes
Inoculate each screen tube with the volume of embryo stock calculated in 3.4. Be sure to inoculate just above the water's surface, and avoid dispensing embryos along the sides of the screen tube. In addition, inoculate initial embryo count vials. Immediately preserve these vials and count them. The mean number of embryos in these 5 vials will serve as an initial count. The mean number should be no lower than 200. Record the time and date that the test is initiated. Place the air delivery tube inside the screen tube.

4. During test maintenance

Maintain replicates at $15 \pm 2°C$ with ambient laboratory lighting (16h light 8h dark) and gentle aeration for 48 hours. DO must not drop below 4.0, salinity remaining 32 ± 2ppt. and the recommended pH is between 7.6-8.3 (pH is not adjusted if outside the indicated range). Examine all replicates daily making sure that they have proper aeration. Record any abnormal conditions such as lack of air or a change in sediment condition.

5. Test Termination
5.1 Determination of complete development
After 48 hours of exposure, examine one of the controls from the reference toxicant using an inverted microscope to determine if development is complete. If development is not complete, the test can be continued to a maximum duration of 54 hours.

5.2 Final water quality
Record the time and date that the test is terminated. Measure and record the ammonia, pH, temperature, and salinity of each sample, from a water quality surrogate.

5.3 Transferring embryos
Gently lift screen tubes out of each replicate and immediately rinse the embryos from the screens into counting/preservation chambers (shell vials and plastic counting chambers have been found to be acceptable) for microscopic viewing. Preserve the chambers with a minimum amount preserving solution. A staining solution such as Rose Bengal may also be added to each vial to aid in endpoint evaluation.

5.4 Endpoint evaluation
Evaluate all larvae in each test vial (an inverted light microscope works well). Carefully count and record the number of normal and abnormal embryos. A characteristic "D" shape denotes normal larvae. If completely and normally developed shells without "meat" are observed then these shells should be enumerated as normal, but should be counted and recorded separately for later quality assurance evaluation. The percentage of embryos that did survive and develop to live larvae with

completely developed shells is calculated for each treatment ((number of normal embryos/initial count)x100), termed %normal-alive.

5.5 Acceptability of Test Results
The mean %normal-alive must be at least 80% for embryos in the controls. Each laboratory must establish a control chart for their ammonia reference toxicant exposures consisting of at least three tests and no more than the 20 most recent tests. The LC_{50} for un-ionized ammonia for each test performed should fall within two standard deviations of the mean of the previous tests on the control chart. A test falling outside two standard deviations should trigger a review of all data and test procedures to assure that the data are of high quality.

If water quality measurements are found to fall outside of acceptable ranges, corrective actions will immediately be taken such as increasing air flow (if reduced DO) or increasing temperature if below acceptable range.

Those tests in which water quality measurements shown in Table 4 are out of range for an extended time period or degree may be considered unacceptable.

6. Data analysis

6.1 Data summarization
Data will be analyzed by statistically comparing the %normal-alive embryos in the project material to that in the negative control using the guidance in the West Coast testing manual (USEPA 1995). A t-test assuming unequal variance will be used to analyze data.

6.2 Outlier analysis
For a test where outliers are suspected among replicates, the Dixon's Test for Detecting Outliers may be used according to USEPA guidance (USEPA 2000) to statistically determine whether or not there are outliers. The Toxicology Committee will review the statistical results and ancillary data collected regarding the test batches in question to determine if data should be excluded from analyses. As a general guideline, data will not be removed from analysis unless there is corroborating evidence, beyond the statistical analysis, that indicates that a given replicate is anomalous.

7. Literature Cited
Anderson BS, Hunt JW, Hester M, Phillips BM. 1996. Assessment of sediment toxicity at the sediment-water interface. In: G.K. Ostrander (ed.) Techniques in Aquatic Toxicology. Lewis Publishers, Ann Arbor, MI.

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Rodgers, John H Jr., Ph.D. Species Tolerance for NPDES Bioassays, Vol. II-Marine Species, Final Report, Feb 1989.

Table 4. Test conditions for the 48-hr sediment-water interface test using *M. galloprovincialis*

Sediment Sample Information	
Test sediment holding time requirements	2 weeks, maximum 4 weeks
Test sample storage conditions	4°C, dark, minimal head space
Test Species	***Mytilus galloprovincialis***
Source	Laboratory's choice
Acclimation/holding time	Mussels may be used for spawning on the same day they are received. There is no upper limit for holding. Measure water quality daily while in holding.
Test procedures	**Anderson, 1996 (modified); USEPA 1995**
Test type:	Static non-renewal
Salinity:	32 ± 2‰
Temperature:	15 ± 2°C
Test dissolved oxygen	≥ 4.0 mg/L
Test pH	pH 7.6-8.3 suggested optimal*
Light quality:	Ambient laboratory light
Light intensity:	10-20uE/m²/s (ambient)
Photoperiod:	16h light 8h dark
No. of replicates:	5
Dilution water:	Uncontaminated 1.0µm-filtered natural seawater (should be filtered the day prior to use)
Test duration:	48-54 Hours
Endpoint	Survival & normal shell development
Test chamber	7.5 cm diameter x 14 cm high glass container; 600 ml tall form beakers recommended
Sediment depth	4-5 cm
Water volume	300 ml
Reference Toxicant Test	
Reference toxicant	Ammonia
Range of concentrations	Control, 2, 4, 6, 8, 10, and 20 mg total NH_3/L, 5 replicates at 32±2ppt
Water Quality Measurements	
Overlying water: ammonia	Start and end of test (Day 0 and Day 2)
Overlying water: pH, temperature, DO, salinity	Daily, in 1 replicate or surrogate from each treatment
Temperature in surrogate or bath	At a minimum daily; continuous recommended
Test Acceptability Criteria	**Control %normal-alive must be $\geq$ 80%**

Optimal pH from Rodgers (1992)

QUALITY ASSURANCE AND QUALITY CONTROL

A. Overview

This section describes QA/QC procedures that will be used for the assessment of sediment toxicity during the Bight '23 Survey. There will be two toxicity tests that will be employed for assessment of the sediment. The toxicity of whole sediment will be analyzed using an amphipod (*Eohaustorius estuarius*) 10-day survival test at both offshore and embayment stations. Toxicity at the sediment-water interface will be evaluated with a 48-hour mussel (*Mytilus galloprovincialis*) embryo development test only at embayment stations.

B. Laboratory Capability

Prior to participating in the Bight'23 survey, each testing laboratory must document their ability to conduct the test(s) using the selected methods. This documentation should consist of a record of at least three prior reference toxicant tests that met test acceptability criteria. The laboratory should have constructed a control chart from these tests which can serve as the documentation.

C. Interlaboratory Comparability

All laboratories conducting toxicity tests must participate in the interlaboratory comparison exercise prior to sample testing, for each method that they will be performing during the survey. The Toxicology Intercalibration Laboratory Manual (2023) describes the details of this intercalibration exercise including test methods and standard operating procedures, acceptable test requirements, test MQOs, and test scoring and evaluation. The intercalibration exercise will include the analysis of field collected sediments and a reference toxicant. The field samples will be distributed blindly to the participating laboratories. Successful completion of this exercise by a laboratory will be evaluated based on two criteria: 1) attainment of test acceptability criteria, and 2) comparability among laboratories.

Comparability of the labs in the intercalibration exercise will be based on four factors: the percentage difference from the mean for each sample, a comparison of the toxicity category for each sample, relative percent difference (RPD) for duplicate samples, and results from the reference toxicant test. Test scoring is a function of the difference between the laboratory mean sample results of the field collected samples delivered blind to the laboratories and the grand mean of all participating laboratories. Toxicity category is a function of the difference between the laboratory mean categorical classification of the blind samples and the category assigned to the grand mean of all laboratories. Toxicity category classification corresponds to the categories defined by the SWRCB's Sediment Quality Objectives. The RPD is a function of laboratory precision based on difference of results from blind duplicates of the field collected samples. Reference toxicant scoring is a function of the difference between the laboratory reference toxicant mean sample results and the grand mean of reference toxicity for all participating laboratories. These four factors are combined into a single score with cutoffs for acceptable performance based on comparability established by the Toxicology Committee. Laboratories unable to successfully complete the interlaboratory comparison exercise will be asked to examine their test procedures, make suggested changes, and retest the comparison samples. Failure to meet the interlaboratory comparison criteria will result in the addition of a cautionary data qualifier flag to that laboratory's data or exclusion from testing during the monitoring program.

In addition to the intercalibration before sample testing, split samples will be tested by the laboratories during the project. These samples will be used to verify that the results remain

comparable during the course of testing. The results of these additional samples will be for informational purposes only.

D. Sample Handling
Detailed methods for collection of sediment are described in the Field Operations Manual. Surface sediment (top 2 cm from offshore stations and top 5 cm from embayment stations) will be collected from Van Veen grabs, homogenized with the chemistry samples, and stored in Teflon-lined bags. A second Teflon-lined bag will be provided to split the sediment sample (3L for the mussel test and 2.5-3L for the amphipod test). If one laboratory is conducting both toxicity tests and their field crew is collecting the sample, they may choose to not split the sample into two Teflon-lined bags. A target sediment holding time of no more than two weeks has been established in order to minimize the potential alteration of the sediment toxicity due to storage; this time period is not a criterion for judging test acceptability. The goal for the project is to initiate tests within 10 days of sampling to allow time if retesting is necessary. Tests on samples that are stored from more than two weeks up to four weeks will also be considered valid, but a data qualifier will be attached to the record to indicate that the desired storage time was exceeded. Samples stored for more than four weeks before the start of toxicity testing will be considered unacceptable for testing and the data will not be included in the project database.

All samples shall be accompanied by chain of custody forms. These forms should include date of sampling and date of receipt.

Prior to the day of test setup, a small sample of sediment must be tested for pore water ammonia, salinity and pH. This will be achieved by mixing the water into the sediment and removing a sediment sample. The sediment sample for water quality should be centrifuged at 3000 x g for 30 minutes at 15 °C to separate sediment particles from the water. This analysis will be used to determine whether the overlying water salinity will need to be adjusted for the amphipod test. Records of this water quality measurement must be submitted with the other toxicity data at the end of the survey.

E. Amphipod Survival Test
An amphipod survival test will be conducted according to USEPA (1994). This test consists of a 10-day exposure of *Eohaustorius estuarius* to sediment under static conditions. Amphipods are placed in glass chambers containing a 2 cm layer of sediment overlain with seawater. The number of surviving amphipods is determined at the end of the test and used to calculate the percentage survival.

Quality of test organisms
Species identification should be verified through consultation with a taxonomist, if necessary. Individuals selected for testing should be visually inspected to confirm that they are the proper size and in good condition (i.e., no external damage). Holding time prior to testing should be no shorter than 2 days and no greater than 10 days.

Accuracy and precision
The accuracy of sediment toxicity tests of field samples cannot be determined since a reference material of known toxicity is not available. A water only reference toxicant test will be run with every batch of test samples in order to document amphipod relative sensitivity and test precision. This test will consist of a 96-hour exposure to five different concentrations of ammonia dissolved in seawater. Ammonia concentrations will be selected to provide an estimate of the LC50 and will

be verified by analysis of a sample from each of the exposure concentrations. Reference toxicant test results that fall outside of control chart limits (2 standard deviations of the mean) will trigger a review of test procedures and a possible retest of the corresponding sediment samples. A negative control consisting of amphipod home sediment will be analyzed with each test batch.

Test conditions
Water quality of the overlying water will be measured every other day for each sample type. Water quality measurements on the pore water will be measured only at the beginning of the exposure. Temperature will be measured at least daily in a surrogate test chamber. Water quality measurement instruments will be calibrated prior to use. Deviations in water quality will be noted in the data files.

Interference by ammonia
The presence of high concentrations of ammonia in pore water may be a confounding factor for sediment toxicity tests with *E. estuarius*. Laboratories will be required to measure and record the concentration of un-ionized ammonia in the pore water from each station, after receipt of the sediment sample in the laboratory and at test initiation. If the pore water concentration exceeds the limit of 0.8 mg/L un-ionized ammonia for any station within a batch, the laboratory will be required to extend the 4-day ammonia reference toxicant to 10 days. The decision on extending the test will be based on the sample from test initiation. The data will be recorded for both time points. The results of the 10-day ammonia reference test will be compared to the concentrations of ammonia in the test samples to determine if the levels are high enough to account for any observed toxicity in the sediment samples.

Salinity adjustment
Samples will be tested at salinities close to the ambient sample salinity. Samples having pore water salinities between 20 and 24 ppt will be tested at 22 ppt; those having salinities greater than 24 up to 29 ppt will be tested at 27 ppt; samples with salinities greater than 29 ppt will be tested at 32 ppt. The salinity of each test sample will be determined by measuring the salinity of the pore water upon receipt of the sample in the laboratory. For each salinity range tested in a batch, an appropriate salinity control for each must be included.

Test acceptability
The *Eohaustorius* test procedure is considered unacceptable if survival in the negative control is less than 90%, or if the coefficient of variation among the control replicates is > 11.9%. If control CV is greater than 11.9%, any samples with a mean ≥ 90% will be acceptable and not need to be retested, but samples with a mean <90% will need to be retested. Reference toxicant results should also be within two standard deviations of the mean response specific to the laboratory. Water quality parameters (salinity, temperature, pH, and ammonia) should also be within the tolerance range of the test organism, as specified in EPA (1994) guidance.

F. Sediment-water Interface Test with Mussel Embryos
A sediment-water interface test using mussel (*Mytilus galloprovincialis*) embryos will be conducted on a subset of stations following a modification of published methods (USEPA 1995, Anderson *et al.* 1996). This modified method consists of adding screened and homogenized sediment to a glass chamber to a depth of 5 cm and overlain with ambient salinity seawater. A screen tube is then rested on the sediment surface to which the fertilized mussel eggs are added. After 48 hr, the screen tube is removed, and the developed embryos are rinsed into a vial and preserved. The embryos are then examined microscopically to determine normal development.

The number of normally developed embryos is compared to the number of embryos added at the start to determine the endpoint of %normal-alive.

Quality of test organisms

The animals may either be collected by the testing laboratories or purchased commercially. Fresh animals will be acquired as needed throughout the project. It is recommended that a group of test organisms will be obtained from beginning of the survey and held under conditions conducive to keeping them in spawning condition throughout the survey (USEPA 1995). This group of animals will be used in the event that fresh animals in spawning condition are not available during the survey. Previous experience has indicated that there can be difficultly purchasing mussels in spawning condition during the summer.

Accuracy and precision

The accuracy of sediment toxicity tests of field samples cannot be determined since a reference material of known toxicity is not available. A water only reference toxicant test will be run with each batch of test samples in order to document mussel relative sensitivity and test precision. This test will consist of a 48-hour exposure to five different concentrations of ammonia dissolved in seawater. Ammonia concentrations will be verified by analysis of a sample from each of the exposure concentrations. Ammonia concentrations will be selected to provide an estimate of the LC_{50} and will be verified by analysis of a sample from each of the exposure concentrations. Reference toxicant test results that fall outside of control chart limits (2 standard deviations of the mean) will trigger a review of test procedures and a possible retest of the corresponding sediment samples. A negative control consisting of a test chamber with a screen tube and laboratory seawater, but no sediment will be analyzed with each test batch. This control will test for the presence of any toxicity caused by the exposure system. A second negative control consisting of a shell vial with laboratory seawater will also be analyzed with each test batch to ensure the health of the organisms. The control from the simultaneously tested reference toxicant exposure can serve this purpose.

Test conditions

Water quality of the overlying water will be measured every other day for each sample type. Samples for water quality will be drawn from outside the screen tube or from a surrogate chamber to prevent loss of embryos. Temperature will be measured at least daily in a surrogate test chamber. Water quality measurement instruments will be calibrated daily. Deviations in water quality will be noted in the data files.

Salinity adjustment

Samples with reduced porewater salinity will need to be equilibrated for an additional 24 hours with one exchange of the overlying water. After the initial 24-hour equilibration period, perform a 100% water change for each sample with reduced salinity and allow another 24 hours for equilibration.

Interference by ammonia

The presence of high concentrations of ammonia in the overlying water may be a confounding factor for sediment-water interface toxicity tests with *M. galloprovincialis*. The no effect concentration results from the 48-hr ammonia reference test will be compared to the concentrations of ammonia in the test samples to determine if the levels can account for any observed toxicity in the sediment samples. The Toxicology Committee will review the information and decide on the necessity for data qualifiers.

Test acceptability

The *M. galloprovincialis* sediment-water interface test procedure is considered unacceptable if survival in the screen tube control has a %normal-alive value of less than 70%. Reference toxicant results should also be within two standard deviations of the mean response specific to the laboratory. Water quality parameters (salinity, temperature, pH, and ammonia) should also be with the tolerance range of the test organism, as specified in EPA (1995) guidance.

G. Contingencies

If a laboratory suspects that it will be unable to test a sample within the hold time, the laboratory should contact SCCWRP who will reach out to the other B'23 participating laboratories and identify a backup laboratory.

H. Literature Cited

Anderson, B.S., J.W. Hunt, M. Hester and B.M. Phillips. 1996. Assessment of sediment toxicity at the sediment-water interface. pp. 609-624 *in*: G.K. Ostrander (ed.), Techniques in aquatic toxicology. CRC Press Inc. Boca Raton.

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