Southern California Bight
2008 Regional Marine Monitoring Survey
(Bight'08)

Toxicology Laboratory Manual

Prepared by:
Bight'08 Toxicology Committee

Prepared for:
Commission of Southern California Coastal Water Research Project
3535 Harbor Boulevard, Suite 110
Costa Mesa, CA 92626

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Toxicology Committee:

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Lan Wiborg	City of San Diego
Diana Young	Southern California Coastal Water Research Project

I. INTRODUCTION

This manual serves to document the methods used in the Bight'08 project 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 for the Bight'08 project or needed clarification. The body of this document is organized in three sections. Section II contains methodology for the *Eohaustorius estuarius* 10-day survival test on whole sediments. Section III has the methods for the 48-hour sediment-water interface test using mussel (*Mytilus galloprovincialis*) embryos. Section IV documents the quality assurance procedures that will be used for the toxicity portion of the Bight'08 project.

II. *EOHAUSTORIUS ESTUARIUS* 10-DAY SURVIVAL TEST: STANDARD OPERATING PROCEDURE

The whole sediment tests 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) and is summarized in Table 1. 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.

A. 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 water ($\leq$ 20 µm) for overlying water and reference toxicant exposures
- Centrifuge for pore water production

B. Experimental Design and Preparation

Sediment Handling

Samples are collected and placed into 1-L high-density polyethylene (HDPE) containers. Samples can be stored for $\leq$ 28 days in the dark at 4ºC. Although the maximum hold time for the samples is four weeks, samples held longer than two weeks and less than four 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. Prior to testing, the reference, test, and control sediment are thoroughly homogenized and sieved through a 2.0-mm mesh screen to remove organisms and debris using only the water available in the test sample.

Experimental Setup

Each treatment (test area, reference, and control materials) will be run with five replicates. Negative control sediment should consist of sediment from the area where the organisms were collected (i.e., native sediment). A grain size control will be conducted with every batch of *E. estuarius* to test the effects of fine grained sediments.

Test Organisms

Test amphipods and laboratory control sediment will be supplied by Northwestern Aquatic Sciences, 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 not be fed during the holding period or during testing.

Reference Toxicant Test

To evaluate the relative sensitivity of the test organisms, a reference toxicant test will be performed using ammonium chloride. A suggested concentration series is 0, 15.6, 31.2, 62.5, 125, and 250 mg/L. 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. On day 0, water quality measurements will be made as described below. An ammonia sample will also be measured from each concentration on day 0 in order to calculate the actual total and unionized ammonia concentrations. At test initiation, 10 amphipods per chamber will be selected as described below and randomly distributed to the test chambers. Counts and observations will be performed on days 1 - 3, and dead animals will be removed from the test chambers. At test termination, all water quality parameters will be measured on one replicate of each concentration and final survival counts will be performed at 96 ± 2 hours from test initiation.

Pore Water Ammonia

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.

C. Test Initiation

Sediment addition

Composited sediments from all test areas, reference locations, and laboratory control sediment 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 overlain. An additional surrogate replicate (no animals) for each treatment will be set up to obtain measurement of pore water ammonia at test initiation. The test will be conducted under continuous light at a temperature of 15 ± 2°C and under gentle aeration. After the sediment, water and aeration are added, the chambers are allowed to sit overnight at test conditions before addition of animals.

Amphipod Addition

After the overnight equilibration, *E. estuarius* will be selected and distributed to test chambers in a pre-determined randomized order (20 animals per chamber). Amphipods will be carefully examined a second time to ensure that they are healthy and counted correctly before adding to the test chambers. Organisms remaining in the water column and exhibiting abnormal behavior will be replaced after 1 hour. Test chambers will be covered to minimize evaporation.

Water Quality

On day 0 and day 10, water quality parameter measurements will be performed on overlying water within 1 replicate or surrogate including measurements of temperature, dissolved oxygen (DO), pH, and salinity. All instruments used for water quality measurements will be calibrated daily. For each treatment, ammonia, salinity, and pH will also be measured in both overlying water and in interstitial (or pore) water at the start of the test.

D. During Test Maintenance

Daily observations will be performed on each replicate to ensure proper aeration, animal behavior, and any obvious abnormalities (i.e., bacterial growth on the surface of the sediment). 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).

E. Test Termination

Test Breakdown

On day 10, the sediments from the chambers will be sieved through a 0.5-mm screen, and the number of survivors will be recorded. Ammonia, in addition to standard water quality measurements, will be measured in overlying water at test termination.

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 %. 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. 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.

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 change 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 1 are out of range for an extended time period or degree may be considered unacceptable.

F. Data Analysis

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). Briefly, proportion data will be arcsine-square root transformed, if necessary, and statistical tests including analysis of variance (ANOVA), t-tests, or non-parametric tests for non-normally distributed data will be used to analyze data, depending on the assumptions of the individual tests.

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.

G. 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 1. Test conditions for the 10-day Solid-phase Sediment Test using *Eohaustorius estuaries*.

Sediment Sample Information	**Bight '08**
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
Grain size control sediment	To be determined
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
Test temperature	15 ± 2°C
Test salinity ranges	If 20 - 24 ppt in field, then 22 ± 2 ppt If 25 - 29 ppt in field, then 27 ± 2 ppt If ≥ 30 ppt in field, then 32 ± 2 ppt
Test dissolved oxygen	≥ 90% saturation (~8.0 mg/L)
Test pH	7.7 - 8.3
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	None.
Water renewal	None
Reference Toxicant Test	
Reference toxicant	Ammonia
Recommended range of concentrations	20 – 300 mg total NH_3/L, 4 replicates at 32 ppt
Water Quality Measurements	
Pore water: ammonia, pH, salinity	At sample receipt
Temperature in surrogate in room or bath	At a minimum daily
Overlying water: pH, temperature, DO, salinity, overlying ammonia	1 replicate or surrogate (at a minimum) on Day 0 and Day 10
Pore water: ammonia, pH, salinity	Day 0
Overlying water: pH, temperature, DO, salinity	OPTIONAL: 1 Rep/Trt - Daily measurements
Test Acceptability Criteria	**Control survival ≥ 90 %, coefficient of variation of ≤ 11.9**

III. SEDIMENT-WATER INTERFACE EXPOSURE SYSTEM FOR THE *MYTILUS GALLOPROVINCIALIS* 48-HOUR DEVELOPMENT 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 2. Any procedures or conditions not specifically covered by this SOP revert to the original protocols.

A. Specialized Equipment

- 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 – 30 µm
- 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 ± 2 ‰.

B. Experimental Design and Preparation

Sampling Procedures

Samples are collected and placed into 1-L high-density polyethylene (HDPE) containers. Samples can be stored for ≤ 28 days in the dark at 4ºC. Although the maximum hold time for the samples is four weeks, samples held longer than two weeks and less than four 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 2-mm screen to remove organisms and debris, using only the water available in the test sample.

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.

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 (ID) stock that is cut into approximately 8 cm high sections (Figure 1). The wall thickness is 3 mm. A 1cm section is cut from the bottom of the tube and serves as the pedestal that sits on the sediment surface. 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 micron screen is appropriate for the mussel development protocol. Polyethylene mesh is stronger than the typical nylon mesh and better withstands repeated use.

Test Organisms

The primary source of adult *Mytilus galloprovincialis* be from Mission Bay, CA. The animals may either be collected by the testing laboratories or purchased commercially (commercial sources are Dave Gutoff and Nautilus Environmental). 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. These held animals will be used as back-up in the case the freshly collected animals do not spawn during the summer. Fresh animals should be collected as the primary option for testing during the project. In the event that animals cannot be obtained from Mission Bay in spawning condition, it is permissible to acquire animals from another source of known good quality animals, such as Carlsbad Aquafarms.

Reference Toxicant Test

To evaluate the relative sensitivity of the test organisms, a reference toxicant test will be performed using ammonium chloride. A suggested concentration series is 0, 2, 4, 6, 8, and 10 mg/L. 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^{o}C. On day 0, water quality measurements will be conducted as described below. An ammonia sample will also be measured from each concentration on day 0 in order to calculate the actual total and unionized 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.

C. Test Initiation

Sediment Addition

Using a polypropylene spoon add 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 5 cm of sediment and 300 ml of overlying water, with a couple of cm 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 (32 ppt, 15°C) of overlying water to the test chambers. Five replicates are used per sample. Arrange the chambers 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. Before addition of the embryos, screen tubes are gently lowered to the sediment surface.

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. 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 minutes 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 0.5 M 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.

Pooling Gametes

When individuals are observed to be shedding gametes, remove each spawning mussel from the tank and place them in separate 15°C chambers. 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. Do not pool eggs until they are confirmed to be of high quality. Use only high quality eggs, discard vacuolated, small, or abnormally shaped eggs. Pooled eggs are placed into a 1-L beaker and diluted using 32 ppt 15°C seawater to a concentration of 5,000 - 8,000 eggs/ml. Try to use gametes from at least three males and females, pooling the gametes before fertilization.

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 the amount of stock added to each replicate must deliver approximately 250 embryos and use between 0.1 ml and 1.0 ml of stock.

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 will be calibrated daily. Temperature measurements will be made daily in a

surrogate chamber or in the water bath. Continuous measurement of temperature is recommended.

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 change 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 will be considered unacceptable.

Inoculation of Screen Tubes

Inoculate each screen tube with the volume of embryo stock calculated above. 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.

D. During Test Maintenance

Maintain replicates at 15°C with ambient laboratory lighting (16 hour light: 8 hour dark) and light aeration for 48 hours. D.O. must not drop below 4.0, salinity remaining 32 ± 2 ppt. and the recommended pH is between 7.6 - 8.3. 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.

E. Test Termination

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 of 54 hours.

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, making a composite by taking water from the outside of the screen tube from each replicate (dissolved oxygen should be measured *in situ* from outside the screen tube in at least one replicate).

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.

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) x 100), termed % normal-alive.

Acceptability of Test Results

The mean % normal-alive must be at least 70% 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 Table 2 are out of range for an extended time period or degree may be considered unacceptable.

F. Data Analysis

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). Briefly, proportion data will be arcsine-square root transformed, if necessary and statistical tests including analysis of variance (ANOVA), t-tests, or non-parametric tests for non-normally distributed data will be used to analyze data, depending on the assumptions of the individual tests.

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.

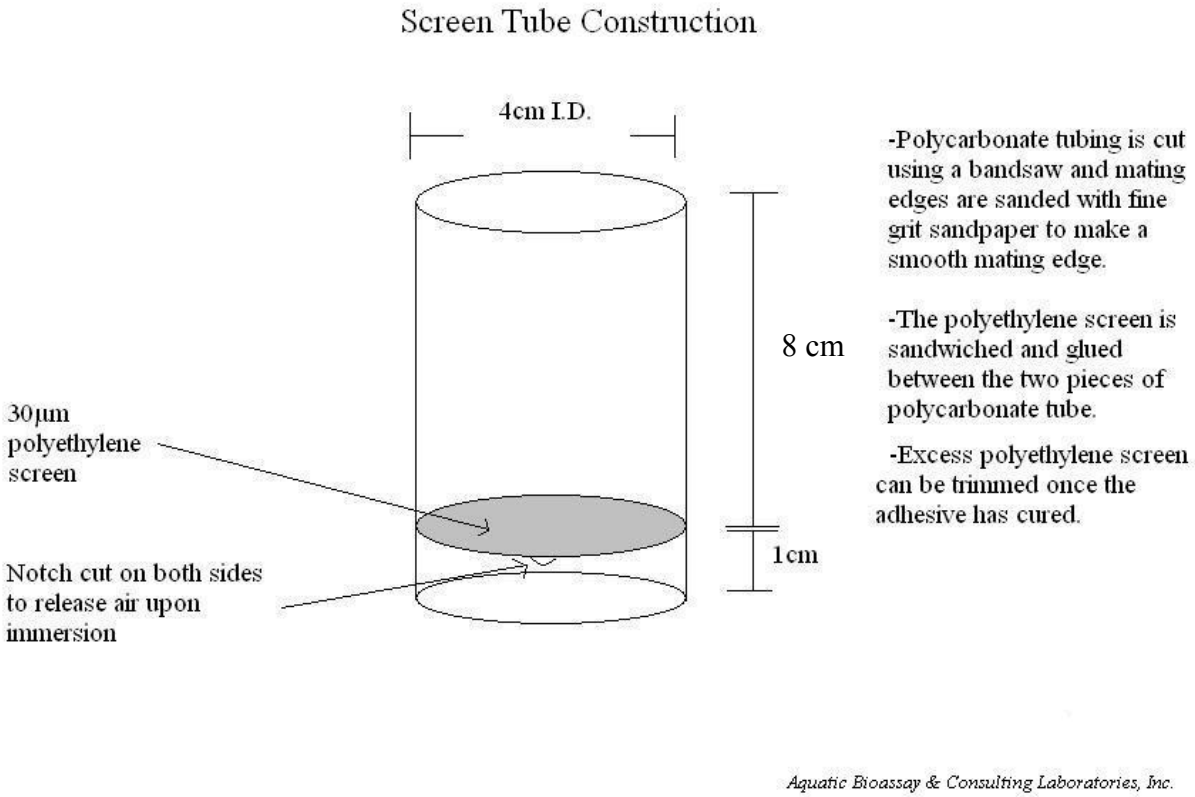

Figure 1. Screen tube construction.

Table 2. Test conditions for the 48-hour Sediment-water Interface Test using *Mytilus 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	Mission Bay. May be collected by the laboratory or purchased commercially (Dave Gutoff or Nautilus). If good quality animals cannot be obtained from this source, then others may be used.
Acclimation/holding time	Adults should be held in laboratory under test conditions for at least 2 days. There is no upper limit for holding. It is recommended that fresh animals be obtained regularly throughout the project and that back up animals be held.
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 – 20 uE/m²/s (ambient)
Photoperiod:	16 h light 8 h dark
No. of replicates:	5
Dilution water:	Uncontaminated 1µm-filtered natural seawater
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	5 cm
Water volume	300 ml
Reference Toxicant Test	
Reference toxicant	Ammonia
Recommended range of concentrations	2 – 10 mg total NH_3/L, 5 replicates at ambient laboratory seawater salinity
Water Quality Measurements	
Overlying water	Record: D.O., pH, temp, salinity, & ammonia at start & end of test
Temperature in surrogate or bath	At a minimum daily; continuous recommended
Test Acceptability Criteria	**Control % normal-alive must be ≥ 70%**

*****Optimal pH from Rodgers (1992)*

G. 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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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.

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

IV. QUALITY ASSURANCE AND QUALITY CONTROL

A. Overview

This section describes the QA/QC procedures that will be used for the sediment toxicity assessment portion of the Bight'08 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'08 survey, each testing laboratory must document their ability to conduct tests using the selected methods. This documentation should consist of a record of at least three prior reference toxicant tests which met test acceptability criteria. The laboratory should have constructed a control chart from these tests which can serve as the documentation. Laboratories conducting only one of the two selected methods must only show competency in the method that they will be performing during the survey.

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. This 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.

For the percentage difference from the mean the following procedure will be used:

1. Pool data from all labs, treating each sample separately.
2. Remove outliers (Grubb's test).
3. Calculate grand mean.
4. Assign points to each laboratory based on the difference between their mean and the grand mean (Table 3).
5. Sum the points assigned from each sample.

Given that there are four samples for comparison, the maximum attainable score for this evaluation factor is 12.

Table 3. Summary of scoring system for percent survival or normal alive data and toxicity category.

% Survival or Normal-alive (Absolute Difference from Grand Mean)		Toxicity Category Agreement	
Result	Points	Result	Points
0 – 10 %	3	Same Category	1.5
> 10 – 20 %	2	1 Category Difference	1.0
> 20 – 30 %	1	2 Category Difference	0.5
> 30 %	0	3 Category Difference	0

The second comparison factor will be based on the sediment toxicity category. For each sample, the grand mean will be used to place the sample into a toxicity category based on California Sediment Quality Objectives thresholds (Table 4). The results for each laboratory will also be assigned to a category. The category from the grand mean and for the individual samples will be compared. The number of categories difference will then be used to assign point values (Table 3). For example, if the grand mean placed the sample in the non-toxic category and an individual laboratory was in the moderate toxicity category, then the difference would be two categories and 0.5 points would be assigned. Since there are four samples, the maximum points awarded for this category is 6.

Table 4. Threshold values for sediment toxicity test response.

Test species/endpoint	Statistical Significance	Nontoxic (%)	Low Toxicity (% Control)	Moderate Toxicity (% Control)	High Toxicity (% Control)
E. estuarius	Significant	90 to 100	82 to 89	59 to 81	< 59
Survival	Not Sig.	82 to 100	59 to 81		< 59
M. galloprovincialis	Significant	80 to 100	77 to 79	42 to 76	< 42
Normal Development	Not Sig.	77 to 79	42 to 76		< 42

The third comparison factor uses the results for the duplicate samples. The first step is to calculate the relative percent difference between the duplicates for each laboratory using the formula:

$$RPD = \frac{Abs(Dup1 - Dup2) \times 100}{Avg\ of\ Dups}$$

Abs=Absolute Value

The RPD will then be compared to the values in Table 5 to assign points. The maximum number of points for the duplicate samples is 3.

The final factor to be considered is the reference toxicant. The evaluation method involves the following steps:

1. Collect ammonia reference toxicant data from all laboratories for both *Eohaustorius* and *Mytilus* tests (historical data). Data will be formatted as mg/L un-ionized ammonia.
2. Calculate the standard deviation (SD) for all of the historical EC_{50}/LC_{50} data for each species.
3. Pool intercalibration reference toxicant EC_{50}/LC_{50} data from all labs
4. Remove outliers (Grubb's test).
5. Calculate grand mean.
6. Calculate the difference from the grand mean for each laboratory.
7. Compare the difference from the grand mean to the standard deviation from the historical data and assign points as shown in Table 5.

As an example, we will say that the SD for all historical data for one of the methods is 0.1. The mean value for the labs participating in the intercalibration we will say is 0.124 mg/L un-ionized ammonia. If Lab A found the LC 50 to be 0.263, then the difference would be 0.139 which is greater than 1 SD, but less than 2, so would therefore get a score of 2 points. The maximum achievable score for the reference toxicant evaluation factor is 3.

Table 5. Summary of scoring system for duplicate sample and reference toxicant results.

Duplicate Sample (RPD)		**Reference Toxicant SD = Deviation from Grand Mean**	
Result	Points	Result	Points
0 – 10 %	3	Within 1 SD	3
> 10 – 20 %	2	Within 2 SD	2
> 20 – 30 %	1	Within 3 SD	1
> 30 %	0	> 3 SD	0

For integration of the four comparison factors, the points will be summed for each laboratory. The proposed "grading" system for the total score is shown in Table 6. Any laboratory that falls into the "low comparability" category will be asked to examine their test procedures, make suggested changes, and retest comparison samples. After retesting, failure to receive a rating of at least "moderate comparability" will result in the addition of a cautionary data qualifier flag to that laboratory's data.

Table 6. Scoring system for sum of all factors.

Description	**% of Maximum Possible Score**	**Number of Points**
Very High Comparability	90	24.0 - 21.5
High Comparability	80	21.0 - 19.0
Moderate Comparability	70	18.5 - 16.5
Low Comparability	< 70	< 16.0

In addition to the intercalibration exercise before the beginning of the project, two split samples of sediment will be tested during the time of active testing for the project. These samples will be collected from two stations in bulk, homogenized by SCCWRP and sent to each of the testing laboratories simultaneously. Testing of these splits will be conducted within the normal holding times set forth for Bight'08 toxicity samples. Laboratories will be expected to test the sample with each of the test methods that they will be employing during the regular testing (i.e., a laboratory that will only be doing *Eohaustorius* testing will only have to test the splits with *Eohaustorius*). 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 Collection and 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 and stored in precleaned polyethylene jars. 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. 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.

On receipt, a small sample of sediment must be tested for pore water ammonia, salinity and pH. This may be achieved by either drawing off a sample of water overlying the sediment in the sample jar or mixing the water into the sediment and removing a sediment sample. In either case, the sample for water quality should be centrifuged at 3000 x g for 30 minutes or filtered through 0.45-µm filter to separate sediment particles from the water. 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

All test organisms will be obtained from a common source during the survey. 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 for each sample type at the beginning and end of the exposure. Water quality measurements on the pore water will be measured only at the beginning of the exposure. Temperature will be measured continuously in a surrogate test chamber. Water quality measurement instruments will be calibrated daily. 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, upon receipt of the sediment sample in the laboratory. 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 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 ambient 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.

Sediment Grain Size Control

There is evidence that *E. estuarius* may be negatively affected by fine grained sediments. This sensitivity seems to be seasonally influenced and somewhat unpredictable. Therefore, a grain size control will be required to be analyzed with each batch of test animals.

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 %. 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 hours, 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

Test organisms will be obtained from Mission Bay. 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 every batch of test samples in order to document amphipod 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 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 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 for each sample type at the beginning and end of the exposure. Samples for water quality will be drawn from outside the screen tube to prevent loss of embryos. Temperature will be measured continuously in a surrogate test chamber. Water quality measurement instruments will be calibrated daily. Deviations in water quality will be noted in the data files.

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-hour 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. Literature Cited

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