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Final Report

on

SEDIMENT QUALITY ASSESSMENT SURVEY AND TOXICITY IDENTIFICATION EVALUATION STUDIES IN LAVACA BAY, TEXAS

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INTRODUCTION

Lavaca Bay is a secondary bay adjacent to Matagorda Bay in Calhoun County, Texas (Figure 1). A portion of Lavaca Bay was closed to the taking finfish and crabs by the Texas Department of Health in April, 1988 because of the high concentrations of mercury found in fish sampled near the ALCOA Point Comfort Operations. ALCOA operated an aluminum smelter at this site from 1948 until 1980. Currently the primary activity at the site is bauxite refining to produce alumina. Mercury contamination originated primarily from the chlor-alkali unit at ALCOA which operated from 1965 until 1979. This unit used mercury to produce chlorine gas and sodium hydroxide. As a result, approximately 67 pounds of mercury per day were discharged into the bay prior to 1970. Witco Chemical Corporation, which was also located on the ALCOA site, processed coal tar for the manufacture of electrode binder pitch and creosote between 1964 and 1985. Other operations at ALCOA have included a cryolite (sodium aluminum fluoride) plant (1962-1979), a chrome plating operation (dates of operation unknown), and the Neumin Gas Plant (1957-1989) which was an oil and gas refining and power generation facility. In 1989 the Neumin Gas Plant area and part of the smelter area were sold by ALCOA and are now owned by Formosa Plastics which has constructed a large plant on Lavaca Bay within the past decade.

In June of 1993, the fish closure area was proposed for the Superfund National Priorities List (NPL) which became effective in March of 1994 (see Figure 1). Several sediment surveys have been conducted in the vicinity of this NPL site and were designed primarily to characterize the extent of the mercury contamination. The purpose of the present study was to determine the degree and areal extent of potential toxicological impacts of the sediment contamination using several sensitive porewater toxicity tests in conjunction with a comprehensive chemical analysis of the sediments. Additional toxicity identification evaluation (TIE) studies were also conducted at several of the more toxic stations in order to determine what types of contaminates were responsible for the observed toxicity.

MATERIALS AND METHODS

Station Selection

Results of preliminary sediment toxicity studies were critically reviewed to aid in station selection. Twenty-four (24) stations were selected to include areas of concern, based on historical data, and were not replicated (Figure 1). The majority of stations (17 out of 24) were located within the fish closure area. An effort was made to concentrate on depositional sediments that had not recently been disturbed by dredging.

Field Measurements and Sample Collection

Field measurements (water temperature, salinity, dissolved oxygen (DO), and depth) and sample collections were made at all stations on one occasion during March 20-21, 1995. Composite homogenized sediment samples were subsampled for the analytical and toxicity testing components of the study to maximize statistical associations. Because the GPS receiver was malfunctioning on the day the samples were collected, sampling station locations were estimated *post priori* from know landmarks using ArcView® software.

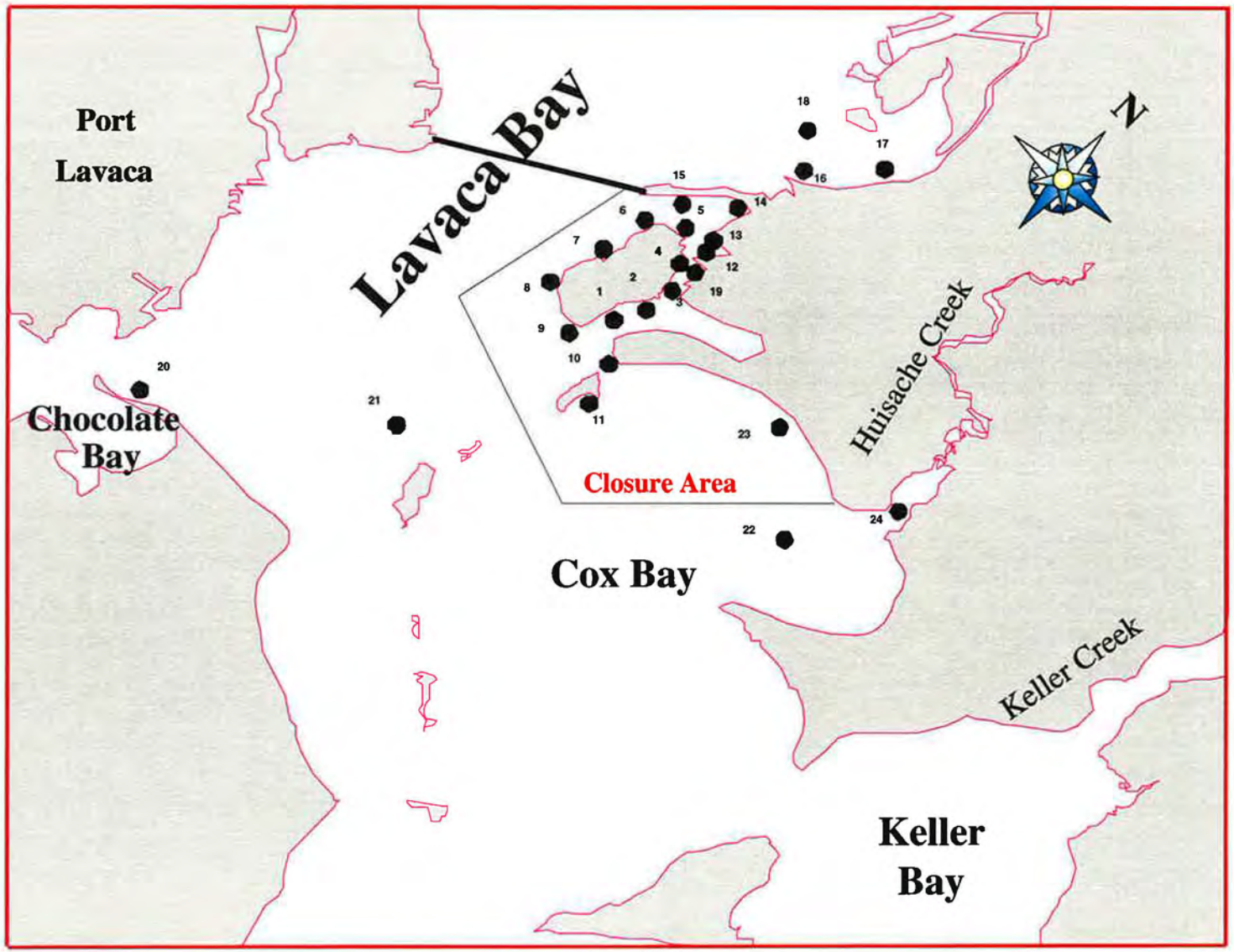

Figure 1. Sampling stations in Lavaca Bay, Texas.

Sediment samples (6 to 10 cm deep) were collected with a 10.2-cm diameter coring device equipped with a transparent PVC barrel that enabled the depth and integrity of the core to be determined before it was included in the composite sample (Onuf et al., 1996). The transparent PVC barrels were detachable, and a clean barrel was used at each sampling station. The PVC corer was equipped with a valve that closes when the sample is withdrawn and then opened manually to release the sample from the corer. The corer has multiple attachments that allow sampling at depths up to 5 m. Sediment cores (8 to 10) were placed in a Kynar®-lined stainless steel pan, and the composite sample (~5 L) was homogenized with a Teflon® spatula. Sediment subsamples for chemical analyses were placed in glass I-Chem® containers cleaned to Environmental Protection Agency (EPA) specifications (Protocol B) and kept on ice until they were frozen. The sediment subsamples for toxicity testing were placed in high density polyethylene containers (that had been pre-soaked with deionized water for several days) and held on ice or refrigerated until processed.

Sediment Chemical and Physical Analyses

Sediment chemistry analyses were conducted by the Texas A&M University Geochemical and Environmental Research Group (GERG) and Texas A&M University Trace Metal Research Laboratory. The sediment samples for chemical analyses were stored frozen until they were transported on dry ice by overnight express mail to GERG.

For the organic analyses, the sediment samples were freeze-dried and extracted in a Soxhlet extraction apparatus. A 10 g sample was weighed, freeze dried and measured into an extraction thimble. Surrogate standards and methylene chloride were added and the samples extracted for 12 hours. The extracts were treated with copper to remove sulfur and were purified by silica/alumina column chromatography (MacLeod et al., 1985; Brooks et al., 1989) to isolate the aromatic/ pesticide/PCB fractions. The quantitative analyses were performed by capillary gas chromatography with electron capture detector for pesticides and PCBs, and with a mass spectrometer detector in the SIM mode for aromatic hydrocarbons (Wade et al., 1988).

For the trace metal analyses, sediment samples (~ 200 mg dry wt.) were digested in closed Teflon® bombs using nitric and hydrofluoric acids according to procedures developed for the National Status and Trends Program (NS&T) of the National Oceanic and Atmospheric Administration (NOAA) as described in Lauenstein et al. (1993). Arsenic, cadmium, chromium, copper, lead, nickel and silver concentrations were determined by graphite furnace atomic absorption spectrophotometry (AAS) using a Perkin Elmer 3030 (with Zeeman background correction) following NOAA National Status and Trends (NS&T) protocols. Zinc concentrations were determined by flame AAS according to the same protocols. Samples were digested separately for mercury analysis using a modified EPA method 245.5 (sulfuric/nitric acid digestion) and analyzed by cold vapor AAS. Total organic carbon (TOC) was measured using a Coulometer TOC analyzer and moisture content analyses were also performed.

A complete suite of quality assurance (QA) samples was run to confirm data quality. The suite included a certified reference material (accuracy check), laboratory duplicates (precision check), matrix spikes (accuracy check), blank spikes (check of analytical control, and method blanks (contamination check). Details of the *a priori* acceptance criteria for each type of QA

sample can be found in Appendix 1. At least 95% of all QA observations must fall within the acceptance criteria. This second criteria prevents discarding data unnecessarily due to outliers which are inevitable in large data sets.

Porewater Toxicity Testing

Sediment Porewater Extraction Procedure

Pore water was extracted from the sediments using a pressurized pneumatic extraction device. This extractor is made of polyvinyl chloride (PVC) and uses a 5 μm polyester filter. It is the same device used in previous sediment quality assessment surveys (USFWS, 1992; Carr, 1993a; NBS, 1993; 1994; 1995a; 1995b; USGS, 1997a; 1997b; 1998; Carr et al., 1998, Carr and Nipper, 1998). The apparatus and extraction procedures are detailed in SOP F10.9 (Attachment 1).

Sediment samples were held refrigerated (4°C) until the pore water was extracted. Pore water was extracted within 3 days from the time of collection. After extraction, the porewater samples were centrifuged in polycarbonate bottles at 1200 g for 20 min to remove any suspended particulate material and were then frozen in precleaned (EPA protocol B) amber glass bottles.

Two days before the start of a toxicity test, the samples were moved from the freezer to a refrigerator at 4°C. One day prior to testing, samples were completely thawed in a tepid water bath. Temperature of the samples was maintained at 20 ± 1°C. Sample salinity was measured and adjusted to 30 ± 1 $^{o}/_{oo}$, if necessary, using reagent grade purified water or concentrated brine (see SOP F10.12, Attachment 2). Other water quality measurements (dissolved oxygen, pH, sulfide and ammonia concentrations) were made. Temperature and dissolved oxygen (DO) were measured with YSI® meters; salinity was measured with a Reichert® or American Optical® refractometer; and pH, sulfide (as S^{-2}), and total ammonia (expressed as nitrogen; TAN) were measured with Orion® meters and their respective probes. Unionized ammonia (expressed as nitrogen) concentrations (UAN) were calculated for each sample using the respective salinity, temperature, pH, and TAN values. Any samples containing less than 80% DO saturation were gently aerated by stirring the sample on a magnetic stir plate. Following water quality measurements and adjustments, the samples were stored overnight at 4°C but returned to 20 ± 1°C before the start of the toxicity tests.

Porewater Toxicity Testing with Sea Urchins

Toxicity of the sediment pore water was determined using the fertilization and embryological development tests with the sea urchin *Arbacia punctulata* following the procedures outlined in SOP F10.6 (Attachment 3) and SOP F10.7 (Attachment 4). The sea urchins used in this study were obtained from Gulf Specimen Company, Inc. (Panacea, Florida) or collected from the jetties at Port Aransas, TX. Each of the 24 porewater samples was tested in a dilution series design at 100, 50, and 25% of the water quality adjusted sample with 5 replicates per treatment. Dilutions were made with 0.45 μm filtered seawater.

A reference porewater sample collected from Redfish Bay, Texas, which had been handled identically to the test samples, was included with each toxicity test as a negative control. This site is far removed from any known sources of contamination and has been used as a reference station for the past 10 years. In addition, a dilution series test with sodium dodecyl sulfate (SDS) was included as a positive control.

Toxicity Identification Evaluation (TIE) - Phase 1

Two stations were chosen which exhibited strong toxicity in the screening survey for phase 1 TIE manipulations (USEPA, 1996). These stations corresponded to stations #2, located adjacent to the West Island dredged material disposal pond decant outfall, and #19, adjacent to the site of the Witco creosote plant. Two gallons of sediment were collected and processed from each station as described above to yield 650 ml of pore water for each.

Initial toxicity was determined on a water quality adjusted subsample from each station using the sea urchin fertilization test and the embryological development test to determine which test would be the more sensitive to the samples. The sea urchin fertilization test was determined to be sufficiently sensitive, and the remaining sample was thawed and water quality adjusted. Each sample was further divided and simultaneous manipulations performed on each of the subsamples. Only three manipulations were performed for this initial stage of evaluation. Filtration manipulation was excluded because the centrifugation stage of of the pore water processing procedure would eliminate the particulate matter. In addition, the oxidant reduction test with thiosulfate addition was excluded due to the low possibility of chlorine contamination at these stations. Adjustment of pH was not performed during these initial tests.

Aeration

Sixty milliliters of each test pore water, a control porewater and a dilution water blank sample was subjected to vigorous aeration for one hour using a standard aquarium pump which pumped air through a glass pasteur pipette. The samples were kept in a ventilation hood during the procedure to evacuate any volatiles. Salinity measurements were made and the salinity adjusted (30 ± 1 ‰) using HPLC pure reagent water following the procedure if necessary.

EDTA chelation

A 25 g/L stock solution of EDTA sodium salt was prepared in deionized water. Sixty milliliters of each test pore water, a control pore water and a dilution water blank was mixed with 180 µl of the stock solution for a final concentration of 75 mg/L EDTA. The samples were allowed to react for three hours prior to toxicity testing.

C_{18} column chromatography

A C_{18} column was conditioned by passing through 2 ml of HPLC grade methanol. This was followed by 10 ml of HPLC grade purified water, 20 ml of millipore (0.45 μm) filtered seawater

(MFS) and 5 ml of the pore water. An additional 60 ml of the pore water was then passed through the column and collected for toxicity testing. Separate columns were conditioned for each porewater test sample, the control porewater sample, and a blank of MFS.

Statistical Analysis

For both the fertilization and embryological development tests, statistical comparisons among treatments were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on transformed data with the aid of SAS (SAS, 1989). Prior to analysis, transformations were suggested by the SAS/LAB® Software (SAS, 1992) which would best fit the data. The fertilization data was transformed using the cubed root of the arsine square root transformation while the embryological data was transformed using the arsine square root transformation. The trimmed Spearman-Karber method (Hamilton et al., 1977) with Abbott's correction (Morgan, 1992) was used to calculate EC_{50} (50% effective concentration) values for dilution series tests. Prior to statistical analyses, the transformed data sets were screened for outliers (SAS, 1992). Outliers were detected by comparing the studentized residuals to a critical value from a *t*-distribution chosen using a Bonferroni-type adjustment. The adjustment is based on the number of observations, *n*, so that the overall probability of a type I error is at most 5%. The critical value, *cv*, is given by the following equation: $cv = t(df_{Error}, .05/(2 \times n))$. After omitting outliers but prior to further analyses, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). Three stations in the embryological development failed to meet the test for normality and homogeneity due to means and variances of zero. Despite these failures in the assumptions they were considered significantly different than the references because of the very large difference in the means.

A second criterion was also used to compare test means to reference means. Detectable significance criteria (DSC) were developed to determine the 95% confidence value based on power analysis of all similar tests performed by our lab (Carr and Biedenbach, 1999). This value is the percent minimum significant difference from the reference that is necessary to accurately detect a difference from the reference. The DSC value for the sea urchin fertilization assay at $\alpha = 0.05$ is 15.5%. At $\alpha = 0.01$, the DSC value is 19%. For the sea urchin embryological development assay, the minimum significant difference values are 16.4 and 20.6 for $\alpha = 0.05$ and $\alpha = 0.01$, respectively.

In addition, statistical associations among the chemical, physical, and toxicological parameters were analyzed for using Spearman correlation analysis (SAS, 1992). Organic constituents were normalized by the total organic carbon (TOC) values prior to analysis.

RESULTS

Field Measurements and Sample Collection

The station coordinates (estimated) and water quality parameters of the 24 stations in the survey at the time of collection are presented in Appendix 2. Surface salinity ranged from 4 to 17°/$_{oo}$ and temperature ranged from 20.9 to 24°C. Dissolved oxygen was at or near saturation for all stations and ranged from 7.2 to 10.4 mg/L. All samples were collected within two days beginning March 20, 1995.

Sediment Chemistry

A description of the *a priori* acceptance criteria used for QA of all of the chemical analyses is provided in Appendix 1. The concentrations of chlorinated hydrocarbons and pesticide residues are presented in Appendix 3. Detectable levels ($\geq$ 0.3 µg/kg) of total BHCs were found in seven treatments. PCBs in excess of 1 µg/kg were found in 21 of the 24 stations (all stations but 20, 21, and 24) with concentrations in excess of 200 µg/kg found at six stations (3, 4, 5, 12, 13, and 19). Detectable levels of HCB were found in 14 stations. Chlordanes (gamma [7 stations], alpha[1station], and/or oxy [6 stations]) were found in as many as seven samples . In addition, the pesticides dieldrin, endrin, mirex, and endosulfan II were detected in as many as six samples. However, aldrin was not found above minimum detection limits. DDT and its degradation products were detected in as many as 11 stations. The highest concentrations were found in the 2,4'-DDD form with concentrations in excess of 1 µg/kg in five stations (2, 3, 12, 13, and 19). Total DDT concentrations ranged from 0.01 to 89.55 µg/kg.

Means and standard deviations of percent recovery and Relative Percent Difference (RPD) of QC matrix spike, blank spike and matrix spike duplicate samples for pesticides and chlorinated hydrocarbons can be found in Appendix 4. In one QC duplicate (sample 8) two target analytes (HCB, and gamma chlordane) exceeded the 40% QA/QC criteria for sample analytes that are at least 10 times the minimum detection limit (MDL). In all three QA/QC standard reference material runs, trans-nonachlor, and 2,4'-DDE exceeded the reported 95% confidence intervals for the standard and exceeded the 35% QC acceptance criteria. No target analytes were detected in the procedural blank analysis.

Polycyclic aromatic hydrocarbons (PAHs) concentrations were found to be elevated at a number of stations. PAH and TOC concentrations are reported in Appendix 5. Seven stations (2-5, 12, 13, and 19) had the highest concentrations of PAHs. Total PAHs ranged from 65.9 to 77,309 µg/kg. For some PAHs, concentrations were as much as 1000 times that background concentrations (i.e. the lowest concentrations of samples in this study).

QC data for PAHs can be found in Appendix 6. Matrix spike analysis resulted in a total of 4 exceedences in three separate runs. Phenanthrene and anthracene in the first run and dibenzothiophene in the second and third run had percent recoveries in excess of the 120% acceptance criteria. In duplicate analysis, the relative percent difference (RPD) criteria of 40% was exceeded for five target analytes (acenaphthylene, acenaphthene, flourene, dibenzothiophene, and ethyl chrysenes) in sample 8 and one analyte (Ethyl chrysenes) in sample 16. Analysis of certified reference material on two separate runs resulted in three analytes in the first (naphthalene, perylene, and benzo-(ghi) perylene) and one analyte in the second (benzo-(k)fluoranthene) outside the 95% confidence intervals for that standard. No target analytes were detected in the procedural blank analysis.

Sediment metal concentrations and associated QA/QC analysis results can be found in Appendices 7 and 8 respectively. Elevated levels of mercury were found in a number of samples as much as 10 to 20 times that of background (i.e. 0.05 ppm dry weight). In addition, high levels of arsenic were found in several samples and an elevated zinc concentration in sample 22 was also confirmed. No exceedences were observed in the duplicate, certified reference material, or blank spike analyses. No analytes were detected in the method blank analysis.

Porewater Toxicity Testing

Water Quality Measurements

The sea urchin fertilization and embryological development tests were performed with sediment pore waters from all 24 stations. Water quality measurements made on the reference and the test pore waters prior to the assays are reported in Table 1. Initial salinity of the test pore waters ranged from 12 to 23 $^{o}/_{oo}$ which required adjusting the samples to 30 $^{o}/_{oo}$ with concentrated brine. Adjusted concentrations of pore water ranged from 80 to 90% of the initial sample. Dissolved oxygen concentrations ranged from 7.61 to 7.95 mg/L or 102 to 104% saturation. Sulfide concentrations were below the detection limit of 0.1 mg/L for all samples. Values for pH ranged from 7.15 to 8.56. Total ammonia as nitrogen (TAN) concentrations ranged from a low of 0.34 to 6.48 mg/L resulting in unionized ammonia as nitrogen (UAN) calculated concentrations from 16.3 to 485 µg/L for the test samples. The UAN lowest observable effects concentration (LOEC) determined in this laboratory for the sea urchin fertilization test (800 µg/L) was not exceeded in any of the samples. The UAN LOEC for the sea urchin embryological development test (90 µg/L) was exceeded in 4 samples (2, 20, 21 and 23).

Sea Urchin Fertilization and Embryological Development Toxicity Testing

Raw data and means from the fertilization and embryological development test are given in Tables 2 and 3, respectively. Only one data point (station 23, 100%, rep 2, fertilization assay) was determined to be an outlier in either data set. Figures 1 and 2 illustrate the stations that were found to be significantly different than the reference (Dunnett's *t*-test $p \leq 0.05$) at the 100% adjusted porewater concentration. Fourteen of the 24 stations were toxic in both tests. Furthermore, two additional stations (9 and 22) in the fertilization test and two stations (16 and 21) in the embryological development test were toxic. Toxic stations were significant at $\alpha \leq 0.01$ except for station 11 in the fertilization test and three stations (10, 16 and 18) in the embryological development test, which were significant at $\alpha \leq 0.05$. Station 18 was found to be the most toxic station in both tests and was the only station which was also significantly different than the reference at the 50% dilution.

EC_{50} values were calculable for only one station (23) in the fertilization assay and for six stations (2, 5, 14, 18, 21, and 23) in the embryological development assay (Table 4). The remaining stations had EC_{50} values in excess of 100%. The reference toxicant, sodium dodecyl sulfate (SDS) EC_{50} values were 6.07 (95% confidence intervals 5.07-6.46) and 3.37 (95% confidence intervals 3.21-3.53) for the fertilization and embryological assays, respectively.

Toxicity Identification Evaluation (TIE) - Phase 1

The raw data and means of treatments in the TIE fertilization assay are presented in Table 5. Both aeration and C_{18} column were effective in eliminating toxicity from the pore water of the two test treatments (stations 2 and 19). None of the manipulations added toxicity to the treatments as evidenced by the lack of change in the MFS blank and the pore water reference control. The SDS positive control resulted in an EC_{50} value of 6.05 (95% confidence intervals 5.70-6.42) which is within the acceptable range for this assay. An additional experiment was conducted to determine whether methyl mercury would be removed from pore water by the C_{18} column. The results of this experiment demonstrated that the C_{18} column treatment did not reduce the toxicity of the methyl mercury-spiked reference porewater sample.

Table 1. Water quality parameters after salinity adjustment and original salinity of sediment porewater samples from Lavaca Bay, Texas.

Station	Salinity[1] (‰)	DO[2] (mg/L)	% DO[3]	pH	TAN[4] (mg/L)	UAN[5] (µg/L)	Sulfide[6] (mg/L)	% OUS[7]
REF[8]	35	7.34	96.0	8.29	0.18	10.7	<0.1	86
1	16	7.9	103.7	8.06	0.67	24.0	< 0.1	83
2	21	7.61	102.1	8.15	3.63	158.7	< 0.1	88
3	15	7.87	101.9	8.44	0.5	40.9	< 0.1	82
4	16	7.82	103.1	8.13	1.32	55.2	< 0.1	83
5	16	7.95	103.1	8.26	0.45	25.0	< 0.1	83
6	14	7.93	103.7	8.34	0.55	36.4	< 0.1	82
7	15	7.82	103.2	8.52	0.36	34.8	< 0.1	82
8	15	7.93	103.6	8.25	0.39	21.2	< 0.1	82
9	15	7.89	103.5	8.23	1.36	70.8	< 0.1	82
10	18	7.84	104.2	8.25	1.19	64.8	< 0.1	85
11	20	7.89	103.9	8.19	0.94	44.9	< 0.1	86
12	15	7.83	102.9	8.31	0.34	21.1	< 0.1	82
13	16	7.82	102.8	8.19	0.83	39.6	< 0.1	83
14	17	7.88	103.6	8.19	1.19	56.8	< 0.1	83
15	19	7.72	102.8	8.38	1.17	84.3	< 0.1	86
16	13	7.91	103.7	8.39	1.07	78.7	< 0.1	80
17	12	7.91	103.4	8.17	1.77	80.8	< 0.1	80
18	16	7.87	103.4	7.15	6.48	29.5	< 0.1	81
19	19	7.9	103.1	8.06	0.97	34.7	< 0.1	86
20	18	7.91	103.1	8.56	1.2	126.2	< 0.1	85
21	23	7.82	103	8.64	3.92	485.2	< 0.1	90
22	20	7.81	102.3	7.83	0.76	16.3	< 0.1	86
23	20	7.81	103.2	8.26	2.08	115.7	< 0.1	86
24	18	7.91	102.4	8.18	1.26	58.8	< 0.1	86

[1] Salinity of sample prior to adjustment. Samples adjusted to 30±1‰.
[2] Dissolved oxygen
[3] Percent saturation of dissolved oxygen
[4] Total ammonia as nitrogen
[5] Unionized ammonia
[6] Measured as S^{-2}
[7] Percent of original sample after salinity adjustment
[8] Reference pore water extracted from sediment collected in Redfish Bay, Texas.

Table 2. Sea urchin fertilization test raw data and means for sediment porewater samples from Lavaca Bay, Texas. Asterisks denote significant differences between test and reference stations (Dunnett's *t*-test; *$\alpha \leq 0.05$, **$\alpha \leq 0.01$).

Station	% WQAS[1]	% Fertilized					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
REF[2]	100	94	97	94	91	95	95.1 ± 2.0	100
		97	94	95	98	96		
REF[2]	50	94	98	97	97	99	96.6 ± 2.3	100
		97	97	100	93	94		
REF[2]	25	93	95	94	98	98	95.1 ± 1.8	100
		95	94	96	95	93		
1	100	89	96	87	91	90	90.6 ± 3.4	95
	50	99	97	92	89	94	94.2 ± 4.0	98
	25	95	92	95	97	96	95.0 ± 1.9	100
2	100	69	66	54	78	50	63.4 ± 11.4**	67
	50	93	95	96	85	87	91.2 ± 4.9	94
	25	98	96	96	96	98	96.8 ± 1.1	102
3	100	76	88	91	80	82	83.4 ± 6.1	88
	50	92	94	93	97	99	95.0 ± 2.9	98
	25	93	92	96	95	98	94.8 ± 2.4	100
4	100	76	49	69	58	73	65.0 ± 11.2**	68.3
	50	95	92	90	93	90	92.0 ± 2.1	95
	25	96	92	96	94	96	94.8 ± 1.8	100
5	100	56	78	61	68	79	68.4 ± 10.2**	72
	50	100	96	94	96	99	97.0 ± 2.4	100
	25	97	97	95	96	96	96.2 ± 0.8	101

Table 2. Continued.

Station	% WQAS[1]	% Fertilized					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
6	100	48	72	57	75	43	59.0 ± 14.2**	62
	50	79	91	90	85	95	88.0 ± 6.2	91
	25	96	98	94	97	97	96.4 ± 1.5	101
7	100	87	94	86	87	86	88.0 ± 3.4	93
	50	95	93	100	95	98	96.2 ± 2.8	100
	25	98	97	96	91	99	96.2 ± 3.1	101
8	100	83	61	84	76	77	76.2 ± 9.2**	80
	50	98	95	94	95	96	95.6 ± 1.5	99
	25	98	95	97	96	94	96.0 ± 1.6	101
9	100	62	56	59	78	69	64.8 ± 8.8**	68
	50	91	89	91	89	93	90.6 ± 1.7	94
	25	96	97	95	95	98	96.2 ± 1.3	101
10	100	69	79	74	83	87	78.4 ± 7.1**	82
	50	98	99	95	95	98	97.0 ± 1.9	100
	25	96	94	93	91	94	93.6 ± 1.8	98
11	100	83	72	88	77	77	79.4 ± 6.2*	83
	50	93	96	94	88	91	92.4 ± 3.0	96
	25	93	97	95	96	98	95.8 ± 1.9	101
12	100	92	95	97	93	98	95.0 ± 2.6	100
	50	97	97	97	99	95	97.0 ± 1.4	100
	25	97	100	95	98	94	96.8 ± 2.4	102
13	100	94	92	96	91	91	92.8 ± 2.2	98
	50	97	96	98	96	98	97.0 ± 1.0	100
	25	97	100	96	98	96	97.4 ± 1.7	102

Table 2. Continued.

Station	% WQAS[1]	% Fertilized					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
14	100	69	83	86	52	53	68.6 ± 16.0**	72
	50	95	95	94	93	91	93.6 ± 1.7	97
	25	98	98	95	94	90	95.0 ± 3.3	100
15	100	74	70	91	90	87	82.4 ± 9.7	87
	50	95	96	98	97	93	95.8 ± 1.9	99
	25	95	95	96	91	93	94.0 ± 2.0	99
16	100	98	93	91	89	95	93.2 ± 3.5	98
	50	95	96	95	94	95	95.0 ± 0.7	98
	25	96	93	99	98	94	96.0 ± 2.6	101
17	100	87	88	83	92	91	88.2 ± 3.6	93
	50	95	95	93	92	95	94.0 ± 1.4	97
	25	92	94	90	99	98	94.6 ± 3.8	99
18	100	60	61	32	54	66	54.6 ± 13.3**	57
	50	77	70	75	67	71	72.0 ± 4.0**	75
	25	89	87	94	92	88	90.0 ± 2.9	95
19	100	52	54	87	76	82	70.2 ± 16.2**	74
	50	92	91	92	96	96	93.4 ± 2.4	97
	25	97	91	94	93	96	94.2 ± 2.4	99
20	100	80	69	82	68	60	71.8 ± 9.1**	75
	50	93	91	91	92	95	92.4 ± 1.7	96
	25	98	98	97	94	94	96.2 ± 2.0	101
21	100	66	58	69	61	67	64.2 ± 4.6**	68
	50	87	80	87	87	88	85.8 ± 3.3	89
	25	90	95	96	91	92	92.8 ± 2.6	98

Table 2. Continued.

Station	% WQAS[1]	% Fertilized					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
22	100	30	48	71	39	65	50.6 ± 17.2**	53
	50	94	96	95	91	91	93.4 ± 2.3	97
	25	95	94	94	95	97	95.0 ± 1.2	100
23	100	51	82[3]	57	35	44	53.8 ± 17.8**	57
	50	96	92	95	93	89	93 ± 2.7	96
	25	98	94	96	98	98	96.8 ± 1.8	102
24	100	69	81	83	78	73	76.8 ± 5.8**	81
	50	93	83	95	92	97	92 ± 5.4	95
	25	89	93	95	89	94	92 ± 2.8	97

[1] Percent of water quality adjusted porewater sample.

[2] Reference pore water extracted from sediment collected in Redfish Bay, Texas.

[3] Value is an outlier and was omitted from statistical analysis.

Table 3. Sea urchin embryological development test raw data and means for sediment porewater samples from Lavaca Bay, Texas. Asterisks denote significant differences between test and reference stations (Dunnett's *t*-test; *$\alpha \leq 0.05$, **$\alpha \leq 0.01$).

Station	% WQAS[1]	% Normal Development					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
REF[2]	100	91	93	98	97	92	92.7 ± 3.6	100
		92	86	93	89	96		
REF[2]	50	95	89	98	96	94	92.1 ± 4.0	100
		86	90	90	88	95		
REF[2]	25	97	89	92	100	89	91.7 ± 3.3	100
		92	95	86	92	93		
1	100	72	76	67	63	72	70.0 ± 5.0**	76
	50	93	92	93	91	88	91.4 ± 2.1	99
	25	93	85	93	90	100	92.2 ± 5.4	101
2	100	0	0	0	0	0	0.0 ± 0.0**	0.0
	50	96	93	97	91	94	94.2 ± 2.4	102
	25	84	93	89	91	94	90.2 ± 4.0	98
3	100	79	74	78	83	83	79.4 ± 3.8	86
	50	95	92	90	88	90	91.0 ± 2.6	99
	25	79	92	90	86	97	88.8 ± 6.8	97
4	100	39	70	64	52	57	56.4 ± 11.9**	61
	50	81	88	87	88	80	84.8 ± 4.0	92
	25	93	91	96	93	92	93.0 ± 1.9	101
5	100	35	35	60	40	41	42.2 ± 10.3**	46
	50	91	88	91	81	88	87.8 ± 4.1	95
	25	91	88	87	93	90	89.8 ± 2.4	98

Table 3. Continued.

Station	% WQAS[1]	% Normal Development					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
6	100	52	79	74	68	66	67.8 ± 10.2**	73
	50	91	82	89	90	93	89.0 ± 4.2	97
	25	87	88	89	89	87	88.0 ± 1.0	96
7	100	83	90	89	82	87	86.2 ± 3.6	93
	50	90	95	79	90	79	86.6 ± 7.2	94
	25	86	91	81	89	84	86.2 ± 4.0	94
8	100	71	48	76	74	73	68.4 ± 11.5**	74
	50	87	91	91	85	79	86.6 ± 5.0	94
	25	90	88	91	91	92	90.4 ± 1.5	99
9	100	86	78	70	86	75	79.0 ± 7.0	85
	50	90	91	89	96	92	91.6 ± 2.7	99
	25	91	82	91	91	87	88.4 ± 4.0	96
10	100	83	75	85	62	77	76.4 ± 9.0*	82
	50	93	93	91	91	91	91.8 ± 1.1	100
	25	93	86	99	97	79	90.8 ± 8.3	99
11	100	62	78	78	64	81	72.6 ± 8.9**	78
	50	95	93	90	91	90	91.8 ± 2.2	100
	25	86	94	91	96	85	90.4 ± 4.8	99
12	100	85	92	84	83	88	86.4 ± 3.6	93
	50	91	94	96	92	89	92.4 ± 2.7	100
	25	83	91	84	83	93	86.8 ± 4.8	95
13	100	87	92	85	79	74	83.4 ± 7.0	90
	50	86	92	88	90	94	90.0 ± 3.2	98
	25	90	92	90	90	83	89.0 ± 3.5	97

Table 3. Continued.

Station	% WQAS[1]	% Normal Development					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
14	100	62	54	43	31	36	45.2 ± 12.8**	49
	50	83	81	73	71	83	78.2 ± 5.8	85
	25	73	80	79	86	83	80.2 ± 4.9	87
15	100	86	79	80	82	88	83.0 ± 3.9	90
	50	81	76	85	90	83	83.0 ± 5.1	90
	25	75	83	80	82	95	83.0 ± 7.4	91
16	100	70	78	70	81	79	75.6 ± 5.2*	82
	50	82	86	82	79	84	82.6 ± 2.6	90
	25	85	80	85	87	83	84.0 ± 2.6	92
17	100	72	75	84	84	89	80.8 ± 7.0	87
	50	81	80	87	81	76	81.0 ± 3.9	88
	25	69	81	85	87	85	81.4 ± 7.3	89
18	100	0	0	0	0	0	0.0 ± 0.0**	0
	50	0	3	0	2	0	1.0 ± 1.4**	1
	25	89	87	89	86	80	86.2 ± 3.7	94
19	100	66	51	65	72	58	62.4 ± 8.1**	67
	50	79	91	87	87	88	86.4 ± 4.4	94
	25	85	97	91	93	90	91.2 ± 4.4	99
20	100	56	69	78	89	77	73.8 ± 12.2**	80
	50	89	97	95	90	92	92.6 ± 3.4	101
	25	91	95	93	88	86	90.6 ± 3.6	99
21	100	0	0	0	0	0	0.0 ± 0.0**	0
	50	85	92	88	92	85	88.4 ± 3.5	96
	25	89	93	88	94	86	90.0 ± 3.4	98

Table 3. Continued.

Station	% WQAS[1]	% Normal Development					MEAN ± SD	% of REF[2]
		REP 1	REP 2	REP 3	REP 4	REP 5		
22	100	82	83	74	73	77	77.8 ± 4.5	84
	50	91	95	88	81	89	88.8 ± 5.1	96
	25	91	94	93	95	89	92.4 ± 2.4	101
23	100	47	52	26	31	50	41.2 ± 11.9**	44
	50	81	82	78	93	87	84.2 ± 5.9	91
	25	85	93	91	78	95	88.4 ± 6.9	96
24	100	57	72	73	83	66	70.2 ± 9.6**	76
	50	84	84	93	88	85	86.8 ± 3.8	94
	25	97	92	91	91	91	92.4 ± 2.6	101

[1] Percent of water quality adjusted porewater sample.

[2] Reference pore water extracted from sediment collected in Redfish Bay, Texas.

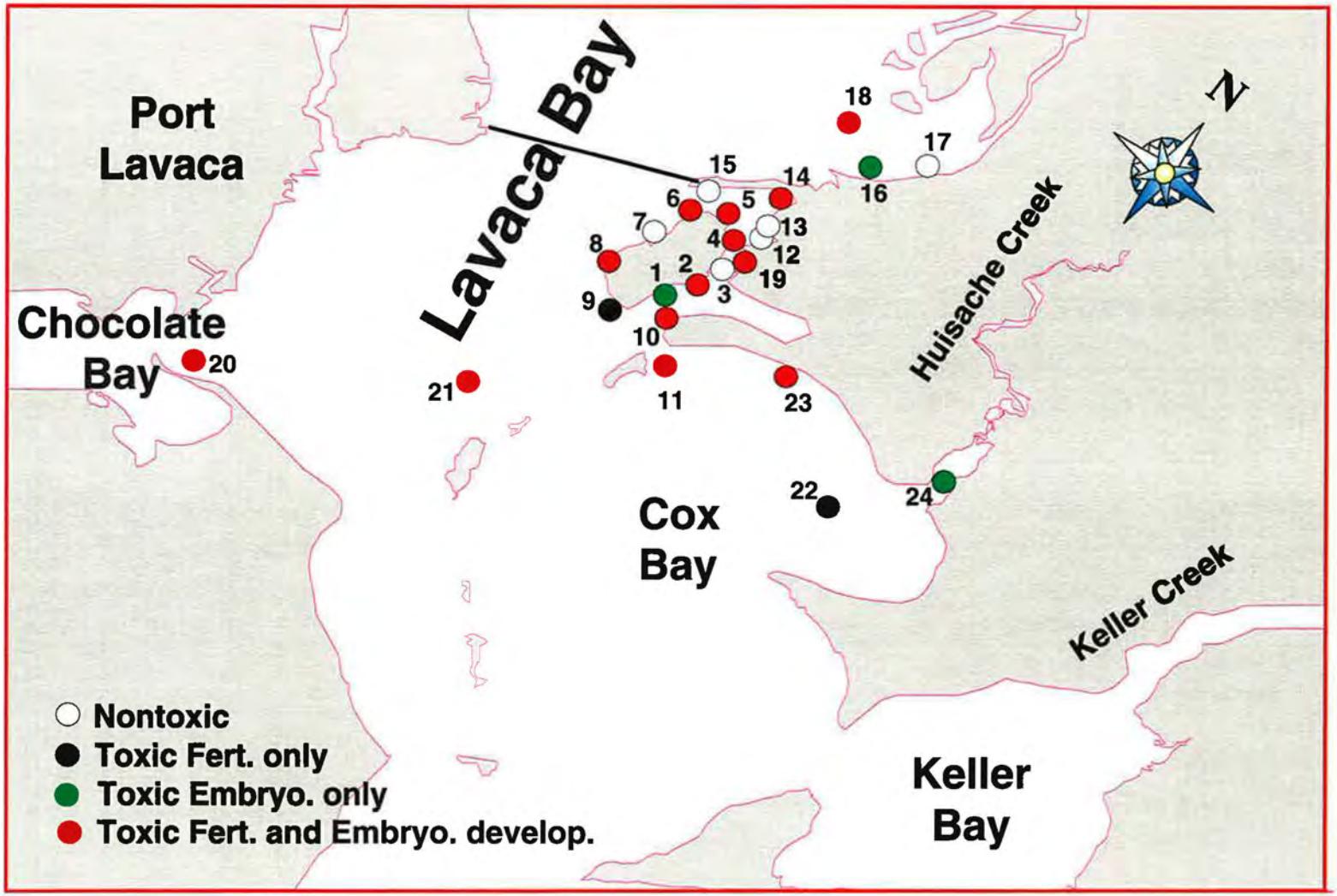

Figure 2. Results of sea urchin toxicity tests in Lavaca Bay.

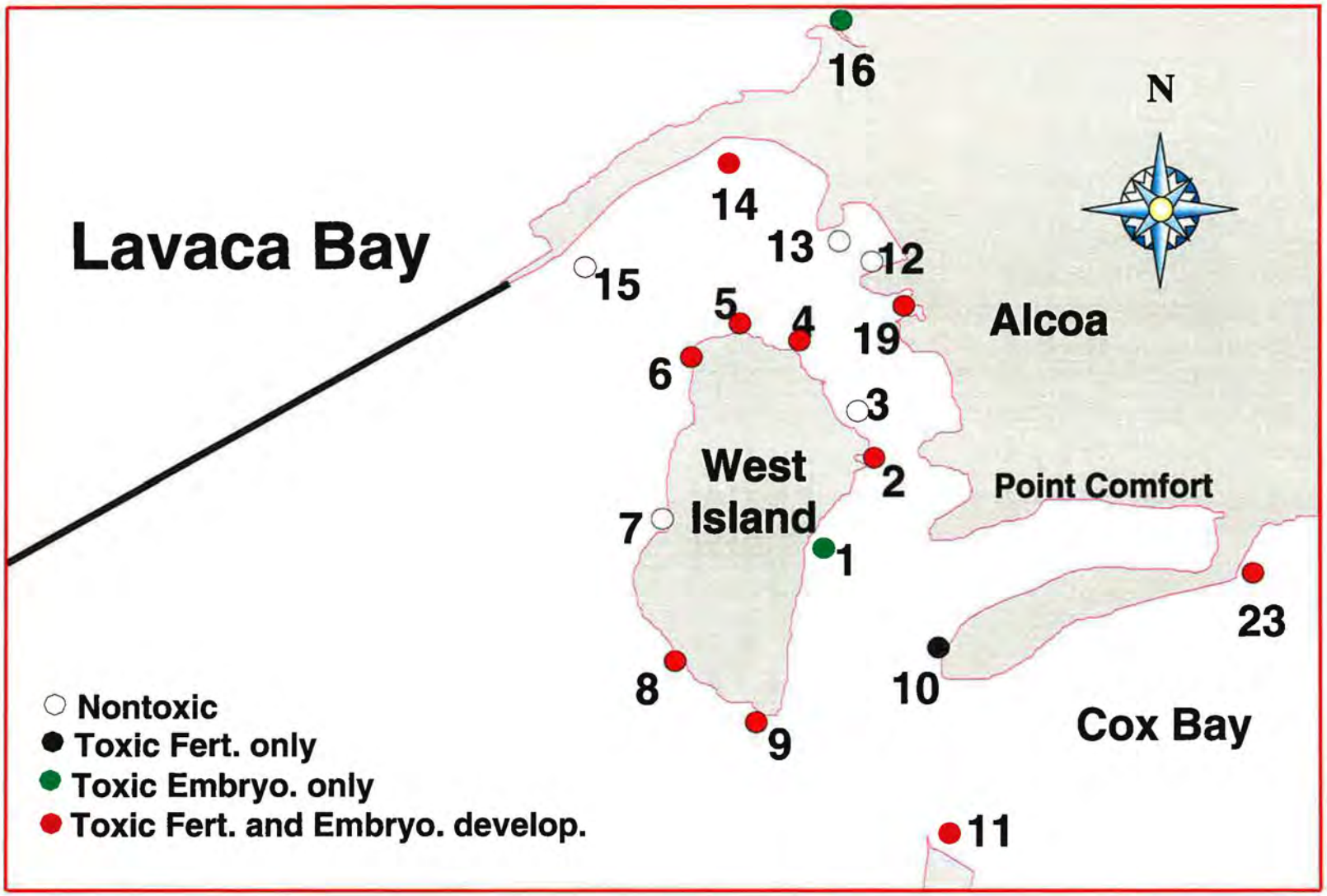

Figure 3. Expanded view of toxicity test results in the vicinity of the ALCOA facility.

Table 4. EC_{50} values of sediment porewater samples from Lavaca Bay, Texas assayed in the sea urchin fertilization and embryological development tests.

Station	Fertilization Test		Embryological Development Test	
	EC_{50} [1]	95% Confidence Limits	EC_{50} [1]	95% Confidence Limits
1	>100	-	>100	-
2	>100	-	70.71	nr[2]
3	>100	-	>100	-
4	>100	-	>100	-
5	>100	-	93.3	82.40-105.65
6	>100	-	>100	-
7	>100	-	>100	-
8	>100	-	>100	-
9	>100	-	>100	-
10	>100	-	>100	-
11	>100	-	>100	-
12	>100	-	>100	-
13	>100	-	>100	-
14	>100	-	96.22	80.22-115.41
15	>100	-	>100	-
16	>100	-	>100	-
17	>100	-	>100	-
18	>100	-	34.43	33.63-35.24
19	>100	-	>100	-
20	>100	-	>100	-
21	>100	-	68.93	66.60-71.35
22	>100	-	>100	-
23	98.6	85.84-113.25	91.36	80.17-104.10
24	>100	-	>100	-

[1] Percent of water quality adjusted porewater sample.

[2] 95% confidence limits not reliable.

Table 5. TIE treatment results for two sites and controls.

Treatment	Millipore® Filtered (0.45µm) Seawater						
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean ± SD	% of Baseline
Baseline	96	88	98	100	99	96.2 ± 4.8	100
EDTA	100	98	95	85	98	95.2 ± 6.0	99
Aeration	100	100	100	99	98	99.4 ± 0.9	103
C_{18} Column	100	100	99	100	100	99.8 ± 0.4	104
	Reference Pore Water						
Baseline	95	89	87	86	94	90.2 ± 4.1	100
EDTA	98	94	98	96	96	96.4 ± 1.7	106
Aeration	100	100	98	99	99	99.2 ± 0.8	110
C_{18} Column	99	98	99	99	98	98.6 ± 0.6	109
	Site 2 Pore Water						
Baseline	49	68	73	63	67	64.0 ± 9.1	100
EDTA	67	73	72	69	63	68.8 ± 4.0	108
Aeration	92	90	89	92	93	91.2 ± 1.6**	142
C_{18} Column	98	99	98	100	99	98.8 ± 0.8**	154
	Site 19 Pore Water						
Baseline	50	58	81	68	78	67.0 ± 13.1	100
EDTA	77	71	74	72	75	73.8 ± 2.4	110
Aeration	98	95	96	94	94	95.4 ± 1.7**	142
C_{18} Column	100	97	98	100	97	98.4 ± 1.5**	147

** Significantly different than Baseline treatment

DISCUSSION

While the widespread contamination of Lavaca Bay with mercury has been demonstrated previously (Holmes, 1986), it appears from the present survey that toxicity is now even more widespread throughout the Lavaca Bay system. Eighteen of the 24 stations sampled exhibited toxicity in one or both of the sea urchin porewater toxicity tests. Many of the stations which did not exhibit toxicity during this survey were observed to be toxic on previous surveys (e.g., stations 3, 12 and 13). Based on the composition of the sediments at these stations, it was apparent that they had been recently dredged which would account for this discrepancy. The most toxic station overall in this survey was station 18 at the Formosa Plastics Co. outfall. This station is in the upper part of Lavaca Bay which has been less impacted by the contamination from the ALCOA plant than most of the stations in this survey but is apparently receiving contaminants from a new source.

Most of the studies that had been conducted prior to this survey had focused on the distribution of mercury in Lavaca Bay specifically. This studies has demonstrated that there are other contaminates of concern, as well. Comparing the sediment chemistry concentrations with existing sediment quality guideline values (Long et al., 1995; MacDonald et al., 1996), it is apparent that a variety of other contaminants are present at elevated concentrations at a number of the stations included in this study (Table 6). The ERL and TEL values represent concentrations below which there is a low probability of adverse biological effects occuring as a result of that particular chemical. The ERM and PEL values represent concentrations above which there is a high probability that adverse biological effects may result from exposure to that chemical in a complex mixture. Only 4 stations exceeded ERM/PEL values for mercury. Two of these 4 stations (# 3 and 13) did not exhibit toxicity in either test. Therefore, sixteen stations with lower sediment mercury concentration did exhibit toxicity. Fifteen additional stations did exceed the TEL and/or ERL for mercury. The greatest number of guideline exceedances, however, were for PAHs. The stations on the eastern shore of West Island (stations 2-5) and those adjacent to the ALCOA facility (stations 12, 13 and 19) exceeded both ERMs and PELs for most of the high molecular weight PAHs from phenanthrene on up (Table 6). This same group of stations exceeded between 8 to 16 of the 31 PEL guidelines (Table 7).

Using the 31 ERM and PEL values that have been developed for specific contaminants, it was possible to calculate an ERM and PEL quotient for specific classes of contaminants or a cumulative ERM and PEL indices. The quotient for a particular ERM or PEL was calculated by dividing the whole sediment concentration by the ERM or PEL value for each contaminant and them summing the quotients for all the compounds and classes or compounds for which guidelines have been developed. This cumulative quotient is referred to as the ERM or PEL index and is specific for each station. The advantage of using the ERM or PEL index as compared with just counting the number of guideline exceedances is that the degree of the exceedance and the additive effect of all the different contaminants are taken into account in the index. Using this approach, seven stations (2-5, 12, 13, and 19) were much more highly contaminated than the remainder with PEL indices ranging between 18.3 to 59.8 (Table 7). Of these seven stations, three did not exhibit any toxicity. A possible explanation for this apparent

discrepancy is that these three stations had recently been dredged and that the contaminants were bound in a clay matrix which rendered them unbioavailable.

The two samples selected for the TIE study had the forth and fifth highest concentrations of mercury (see Appendix 7) in this study and stations 19 and 2 had the second and seventh highest concentrations of total PAHs, respectively (see Appendix 3). Both of these samples were highly toxic in both of the sea urchin assays. The toxicity of the pore water from these two sites was not reduced by the addition of EDTA which complexes with metals to rendered them unbioavailable. The toxicity was completely eliminated by the C_{18} column treatment which removes organic compounds such as PAHs and chlorinated hydrocarbons. Based on experiments performed during this study, methyl mercury was not removed by the C_{18} column treatment. This information, in conjuction with the fact that two of the four stations with the highest mercury concentrations were not toxic, suggests that the toxicity observed in this study is primarily due to organics, probably PAHs, and not mercury. We recommend that these other contaminants of concern be considered in any remedial actions that are planned for this NPL site and that the ecology of this area as well as human health issues also be considered.

Table 6. Effects Range Low (ERL), Effects Range Median (ERM), Probable Effects Level (PEL), and Threshold Effects Level (TEL) with Lavaca Bay site exceedances for various chemicals.

Substance	ERL	Stations Exceeding ERL	ERM	Stations Exceeding ERM	PEL	Stations Exceeding PEL	TEL	Stations Exceeding TEL
Acenaphthene	16	2-6,12-14,19	500	12	88.9	3-5,12,13,19	6.71	1-7,12-15,19,23
Acenaphthylene	44	3-6,12,13,19	640	-	128	4,5,12,19	5.87	2-7,12-14,19
Anthracene	85.3	2-6,12,13,19	1100	5	245	3-5,12,13,19	46.8	2-6,12,13,19
Fluorene	19	2-6,12,13,19	540	-	144	3-5,12,13,19	21.2	2-6,12,13,19
Naphthalene	160	12	2100	-	391	-	34.6	3-5,12,13,19
2-Methylnaphthalene	70	12	670	-	201	-	20.2	12,13
Phenanthrene	240	2-6,12,13,19	1500	3-5,12,13,19	544	3-5,12,13,19	86.7	2-6,12-15,19,23
LMW PAHs	552	2-6,12,13,19	3160	3,5,12,19	1442	3-5,12,13,19	312	2-6,12,13,19
Benzo(a)anthracene	261	2-6,12,13,19	1600	3-5,12,13,19	690	2-5,12,13,19	74.8	1-6,12-15,19,23
Benzo(a)pyrene	430	2-6,12,13,19	1600	2-5,12,13,19	763	2-5,12,13,19	88.8	1-6,12-15,19,23
Chrysene	384	2-5,12,13,19	2800	12,19	846	2-5,12,13,19	108	1-6,12-15,19,23
Dibenzo(a,h)anthracene	63.4	2-5,12,13,19	260	2-5,12,13,19	135	2-5,12,13,19	6.22	1-7,12-15,19,23
Fluoranthene	600	2-6,12,13,19	5100	3-5,12,19	1494	2-5,12,13,19	113	1-7,12-15,19,23
Pyrene	665	2-6,12,13,19	2600	3-5,12,13,19	1398	2-5,12,13,19	153	1-6,12-15,19,23
HMW PAHs	1700	2-6,12,13,19	9600	3-5,12,13,19	6676	2-5,12,13,19	655	1-6,12-15,19,23
Total PAHs	4022	2-6,12,13,19	44,792	5,12,19	16,770	2-5,12,13,19	1684	2-6,12-15,19,23
Total Chlordane	0.5	2-5,12,13,19,23	6	-	4.79	12	2.26	12,19

Table 6. Effects Range Low (ERL), Effects Range Median (ERM), Probable Effects Level (PEL), and Threshold Effects Level (TEL) with Lavaca Bay site exceedances for various chemicals.

Substance	ERL	Stations Exceeding ERL	ERM	Stations Exceeding ERM	PEL	Stations Exceeding PEL	TEL	Stations Exceeding TEL
Acenaphthene	16	2-6,12-14,19	500	12	88.9	3-5,12,13,19	6.71	1-7,12-15,19,23
Acenaphthylene	44	3-6,12,13,19	640	-	128	4,5,12,19	5.87	2-7,12-14,19
Anthracene	85.3	2-6,12,13,19	1100	5	245	3-5,12,13,19	46.8	2-6,12,13,19
Fluorene	19	2-6,12,13,19	540	-	144	3-5,12,13,19	21.2	2-6,12,13,19
Naphthalene	160	12	2100	-	391	-	34.6	3-5,12,13,19
2-Methylnaphthalene	70	12	670	-	201	-	20.2	12,13
Phenanthrene	240	2-6,12,13,19	1500	3-5,12,13,19	544	3-5,12,13,19	86.7	2-6,12-15,19,23
LMW PAHs	552	2-6,12,13,19	3160	3,5,12,19	1442	3-5,12,13,19	312	2-6,12,13,19
Benzo(a)anthracene	261	2-6,12,13,19	1600	3-5,12,13,19	690	2-5,12,13,19	74.8	1-6,12-15,19,23
Benzo(a)pyrene	430	2-6,12,13,19	1600	2-5,12,13,19	763	2-5,12,13,19	88.8	1-6,12-15,19,23
Chrysene	384	2-5,12,13,19	2800	12,19	846	2-5,12,13,19	108	1-6,12-15,19,23
Dibenzo(a,h)anthracene	63.4	2-5,12,13,19	260	2-5,12,13,19	135	2-5,12,13,19	6.22	1-7,12-15,19,23
Fluoranthene	600	2-6,12,13,19	5100	3-5,12,19	1494	2-5,12,13,19	113	1-7,12-15,19,23
Pyrene	665	2-6,12,13,19	2600	3-5,12,13,19	1398	2-5,12,13,19	153	1-6,12-15,19,23
HMW PAHs	1700	2-6,12,13,19	9600	3-5,12,13,19	6676	2-5,12,13,19	655	1-6,12-15,19,23
Total PAHs	4022	2-6,12,13,19	44,792	5,12,19	16,770	2-5,12,13,19	1684	2-6,12-15,19,23
Total Chlordane	0.5	2-5,12,13,19,23	6	-	4.79	12	2.26	12,19

Table 7. Probable Effects Level (PEL) and Effects Range Median (ERM) exceedances and Indices with associated toxicity results for sites in Lavaca Bay, Texas.

Station	PEL Exceedances	ERM Exceedances	PEL Index	ERM Index	Fert[1]	Embryo[1]
1	0	0	2.6	1.5		**
2	8	2	18.3	9.3	**	**
3	14	9	37.6	18.1		
4	15	8	39.7	19.5	**	**
5	15	11	46.1	22.2	**	**
6	0	0	6.9	3.6	**	**
7	0	0	1.9	1.2		
8	0	0	1.5	0.9	**	**
9	0	0	1.0	0.6	**	
10	0	0	1.5	1.0	**	*
11	0	0	1.4	0.9	*	**
12	16	12	70.0	34.6		
13	14	7	32.8	16.2		
14	0	0	4.3	2.4	**	**
15	0	0	2.8	1.6		
16	0	0	2.0	1.2		*
17	0	0	2.0	1.3		
18	0	0	1.8	1.1	**	*
19	14	10	59.8	29.2	**	**
20	0	0	1.0	0.6	**	**
21	0	0	1.9	1.2	**	**
22	0	0	2.7	1.7	**	
23	0	0	3.9	2.3	**	**
24	0	0	1.5	0.9	**	**

[1] Sea urchin fertilization assay and embryological development toxicity results

* significant at $\alpha \leq 0.05$, ** significant at $\alpha \leq 0.01$

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APPENDICES 1- 8

Appendix 1. Descriptions and *a priori* acceptance criteria used for QA samples.

Appendix 2. Location and physical parameters of Lavaca Bay collection stations.

Appendix 3. Sediment chlorinated hydrocarbon concentrations (µg/kg) for the Lavaca Bay study.

Appendix 4. Quality control data for spiked chlorinated hydrocarbons for selected sediments collected in Lavaca Bay, Texas.

Appendix 5. Sediment total organic carbon (TOC; in % dry wt.) and polycyclic aromatic hydrocarbon (PAH) concentrations (µg/kg) at Lavaca Bay stations.

Appendix 6. Quality control data for spiked PAHs and average percent variation of replicate samples from sediments collected in Lavaca Bay, Texas.

Appendix 7. Sediment metal concentrations (µg/g dry weight) for Lavaca Bay study.

Appendix 8. Quality control data for spiked metals for selected sediments collected in Lavaca Bay, Texas.

Appendix 1. Descriptions and *a priori* acceptance criteria used for QA samples.

QA Sample	Description	Purpose	Acceptance Criterion
Certified Reference Material	Similar to samples with known concentrations	Accuracy check of method	Percent Recovery Organics $\pm$ 35% Metals $\pm$ 20%
Laboratory Duplicates	Two separate aliquots of the same sample analyzed independently	Precision check of method and homogenization check of sample	Relative Percent Difference (RPD) between duplicates Organics $\leq$ 40% Metals $\leq$ 20%
Matrix Spikes	Known mass of an element added to one of two relicates of unknown sample; analyzed independantly	Accuracy check of method for element and interference check	Percent Recovery of expected value Organics 40-120% Metals $\pm$ 20%
Blank Spikes	Known mass of an element added to analyte free water and analyzed as other samples	Check of analytical control	Percent Recovery of expected value Organics 40-120% Metals $\pm$ 20%
Method Blanks	Complete Digestion and analysis on sample with only reagents used in procedures	Check for contamination of samples by reagents	Organics < 3X Method Detection Limit (MDL) Metals < MDL or < 10% of lowest unknown sample

Appendix 2. Location and physical parameters of Lavaca Bay collection stations.

Station	Date of Sampling	Location[1]		Salinity (‰)	Water Temp.[2] (°C)	DO[3] (mg/L)	Water Depth (m)
		Latitude	Longitude				
1	3-21-95	28°38.68 W	96°34.07 N	15	23.5	8.73	0.8
2	3-20-95	28°38.98 W	96°33.93 N	12	22.9	9.4	2.1
3	3-20-95	28°39.16 W	96°34.00 N	12	23.3	9.5	0.8
4	3-20-95	28°39.38 W	96°34.15 N	14.5	24	8.2	0.8
5	3-20-95	28°39.47 W	96°34.35 N	14.5	23.6	7.2	0.8
6	3-21-95	28°39.35 W	96°34.48 N	10.5	22.6	9.5	0.6
7	3-21-95	28°38.73 W	96°34.57 N	11.5	22.2	9.6	0.8
8	3-21-95	28°38.27 W	96°34.50 N	15	22.7	8.9	0.8
9	3-21-95	28°38.05 W	96°34.27 N	15	22.3	9.3	0.5
10	3-21-95	28°38.10 W	96°33.80 N	14	22.2	8.9	0.4
11	3-21-95	28°37.83 W	96°33.73 N	15	22.2	9.7	0.8
12	3-20-95	28°39.53 W	96°34.03 N	12	22.3	9.5	na[4]
13	3-20-95	28°39.63 W	96°34.13 N	11	22.4	8.6	1.0
14	3-20-95	28°39.90 W	96°34.43 N	12	22.6	10.4	1.2
15	3-20-95	28°39.63 W	96°34.82 N	12	21.9	9.3	1.5
16	3-20-95	28°40.55 W	96°34.15 N	8	21.0	9.7	1.2
17	3-20-95	28°41.25 W	96°33.60 N	4	20.9	8.5	1.2
18	3-20-95	28°41.02 W	96°34.42 N	10	21.4	9.5	2.5
19	3-20-95	28°39.38 W	96°33.92 N	13	23.1	9.0	1.0
20	3-21-95	28°34.97 W	96°36.47 N	17	21.0	8.9	1.1
21	3-21-95	28°36.35 W	96°34.10 N	15	21.0	8.9	2.4
22	3-21-95	28°38.02 W	96°31.23 N	15	21.8	8.3	na[4]
23	3-21-95	28°38.62 W	96°32.77 N	16	27.3	7.8	0.7
24	3-21-95	28°38.52 W	96°30.57 N	15	21.7	7.4	0.8

[1] Estimates using ArcView® software
[2] Surface water temperature
[3] Dissolved oxygen
[4] Data not available

Appendix 3. Sediment chlorinated hydrocarbon concentrations (µg/kg) for the Lavaca Bay Study.

Station	Total BHC's	Total PCBs[2]	HCB	Hepta Chlordane	Hepta Epoxide	Oxy Chlordane	Gamma Chlordane	Alpha Chlordane	Trans-Nonachlor	Cis- Nonachlor	Aldrin
1	ND	9.45	ND	ND	ND	ND	0.16	ND	ND	ND	ND
2	0.31	74.60	0.30	ND	0.36 [3]	ND	0.54	ND	0.68	0.15	ND
3	0.72	200.00	0.17	ND	0.54 [3]	1.04	0.46	ND	1.50	0.58	ND
4	0.68	346.53	0.42	0.44	0.94	1.28	0.86	ND	1.82	0.85	ND
5	0.88	361.27	0.25	1.36	0.66 [3]	1.90	ND	ND	ND	ND	ND
6	ND	52.91	0.03 [3]	ND	0.07 [3]	0.04 [3]	ND	ND	ND	0.04 [3]	ND
7	ND	21.90	0.15	ND	ND	ND	ND	ND	ND	ND	ND
8	0.02	5.16	0.49	ND	ND	ND	0.10	ND	ND	ND	ND
9	ND	2.39	0.04 [3]	ND	ND	ND	ND	ND	ND	ND	ND
10	ND	2.41	0.02 [3]	ND	ND	ND	ND	ND	ND	ND	ND
11	ND	2.97	0.04 [3]	ND	ND	ND	ND	ND	ND	ND	ND
12	1.60	571.93	0.18	ND	ND	4.82	ND	0.05 [3]	ND	ND	ND
13	0.84	220.92	0.15	ND	ND	1.37	0.44	ND	ND	ND	ND
14	ND	24.95	0.02 [3]	ND	ND	ND	ND	ND	ND	ND	ND
15	ND	16.44	0.03 [3]	ND	ND	ND	ND	ND	ND	ND	ND
16	ND	3.07	0.05 [3]	ND	ND	ND	ND	ND	ND	ND	ND
17	0.02	2.78	0.03 [3]	ND	ND	ND	0.00 [3]	ND	ND	ND	ND
18	0.01	3.87	0.26	0.01 [3]	0.32 [3]	ND	ND	ND	ND	ND	ND
19	0.68	583.09	0.17	0.64	1.05	2.93	0.00 [3]	0.09 [3]	0.05 [3]	0.55	ND
20	0.00	0.57	0.06 [3]	ND	ND	ND	ND	ND	ND	ND	ND
21	0.00	0.92	0.38	ND	ND	ND	ND	ND	ND	ND	0.01 [3]
22	0.02	1.97	0.34	ND	ND	ND	ND	ND	ND	ND	0.01 [3]
23	0.01	36.01	0.24	ND	0.00 [3]	0.22 [3]	0.59	0.88	0.44	0.32	ND
24	ND	0.62	0.45	ND	ND	ND	ND	ND	ND	ND	ND

Appendix 3. Continued.

Station	Dieldrin	Endrin	Mirex	2,4' DDE	4,4' DDE	2,4' DDD	4,4' DDD	2,4' DDT	4,4' DDT	Total DDT	Endosulfan II
1	0.12 [3]	0.31	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	0.48 [3]	ND	ND	ND	0.26	4.07	ND	ND	ND	4.33	1.04
3	0.64 [3]	3.77	0.46	ND	ND	6.08	0.35	0.15 [3]	ND	6.58	ND
4	0.67 [3]	2.56	0.75	ND	0.23	ND	ND	0.11 [3]	ND	0.34	0.81
5	0.32 [3]	3.16	0.87	ND	ND	ND	ND	0.14 [3]	0.43	0.57	0.56
6	0.21 [3]	0.59	ND	ND	ND	ND	ND	ND	ND	ND	0.25
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.11 [3]
11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12	1.23	ND	0.96	ND	ND	89.55	ND	ND	ND	89.55	ND
13	1.25	ND	0.22	ND	ND	10.13	ND	ND	ND	10.13	ND
14	0.07 [3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	0.12 [3]	ND	ND	ND	ND	0.12	ND
17	ND	ND	ND	ND	0.23	ND	ND	ND	0.01 [1]	0.24	ND
18	ND	ND	0.00 [3]	0.08 [3]	0.39	ND	ND	ND	ND	0.47	ND
19	1.06	1.54	0.41	0.17	ND	1.40	0.59	1.03	0.16 [1]	3.35	0.52
20	ND	ND	ND	ND	0.27	ND	ND	ND	ND	0.27	ND
21	ND	ND	ND	ND	0.07 [3]	ND	ND	ND	ND	0.07	ND
22	0.01 [3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
23	ND	ND	0.00 [1]	ND	ND	ND	0.03 [3]	0.06 [3]	ND	0.09	0.51
24	ND	ND	0.00 [1]	ND	0.01 [3]	ND	ND	ND	ND	0.01	ND

[1] Sum of alpha, beta, gamma and delta forms.
[2] Sum of 71 congener groups.
[3] Below detection limit
ND = not detected

Appendix 4. Quality control data for spiked chlorinated hydrocarbons for selected sediments collected in Lavaca Bay, Texas.

Compound	Percent Recovery Spiked Samples[1] $\bar{x} \pm SD$	Percent Recovery Blank Spike[2] $\bar{x} \pm SD$	Relative Percent Difference Between Duplicates[3] $\bar{x} \pm SD$
Alpha-BHC	84 ± 4.5	85.2 ± 6.6	7 ± 3.2
HCB	99 ± 7.1	102.1 ± 6.5	7.2 ± 4.6
Beta-BHC	93 ± 7.1	92.3 ± 2.1	4.4 ± 0.5
Gamma-BHC	87 ± 5.7	87.0 ± 1.2	4.5 ± 1.5
Delta-BHC	78 ± 9.5	73.6 ± 0.0	1.4 ± 1.1
Heptachlor	84 ± 11.2	91.7 ± 11.6	6.9 ± 1.4
Hepta-Epoxide	82 ± 10.0	82.0 ± 4.6	2.4 ± 2.0
Oxychlordane	90 ± 8.5	90.0 ± 4.9	4.2 ± 1.5
Gamma-Chlordane	80 ± 14.4	84.5 ± 15.3	3.1 ± 1.1
Alpha-Chlordane	86 ± 13.4	90.5 ± 12.9	4.0 ± 2.7
Trans-Nonachlor	87 ± 10.1	93.8 ± 6.9	2.5 ± 3.3
Cis-Nonachlor	88 ± 13.1	90.2 ± 14.2	3.7 ± 1.5
Aldrin	82 ± 5.0	86.6 ± 1.8	4.6 ± 0.1
Dieldrin	87 ± 19.5	84.7 ± 17.8	3.9 ± 0.7
Endrin	85 ± 24.4	87.3 ± 16.2	2.9 ± 3.2
Mirex	90 ± 8.1	101.4 ± 10.4	1.6 ± 2.2
2,4' DDE	89 ± 10.9	92.2 ± 13.3	4.3 ± 2.8
4,4' DDE	86 ± 11.8	88.0 ± 14.0	8.8 ± 3.1

Appendix 4. Continued.

Compound	Percent Recovery Spiked Samples[1] $\bar{x} \pm SD$	Percent Recovery Blank Spike[2] $\bar{x} \pm SD$	Relative Percent Difference Between Duplicates[3] $\bar{x} \pm SD$
2,4' DDD	92 ± 19.0	90.6 ± 18.1	13.3 ± 0.3
4,4' DDD	91 ± 15.8	83.4 ± 15.2	2.9 ± 1.2
2,4' DDT	82 ± 17.2	88.8 ± 14.1	2.4 ± 1.3
4,4' DDT	76 ± 20.5	86.4 ± 25.6	5.0 ± 0.1
Endosulfan II		60.4 ± 10.2	6.2 ± 4.7

[1] Average percent recovery of spiked analyte; ($\sum$(µg/kg recoverd-baseline)/spiked quantity)/n where n=5.

[2] Average percent recovery of spiked blanks; ($\sum$(µg/kg recoverd-baseline)/spiked quantity)/n where n=2.

[3] Relative percent differences between duplicates; n=2
(((absolute value (concentration of first aliquot=C1) - (concentration of second aliquot=C2))*100)/((C1+C2)/2)).

Appendix 5. Sediment total organic carbon (TOC; in % dry wt.) and polycyclic aromatic hydrocarbon (PAH) concentrations (µg/kg) at Lavaca Bay stations.

Station	TOC	Napthalene	Methyl Naphthalene	Ethyl Naphthalene	Propyl Naphthalene	Butyl Naphthalene	Biphenyl	Acenaphthylene
1	0.14	2.6	0.8 [1]	ND	ND	ND	0.4 [1]	0.9 [1]
2	0.84	32.3	30.3	ND	ND	ND	3.8	6.9
3	0.29	43.1	34.6	ND	ND	ND	9.6	80.5
4	0.34	43.6	48.2	134.1	218.3	173.7	11.0	245.8
5	0.33	62.8	62.2	183.0	211.1	163.7	24.3	463.3
6	0.15	7.8	9.1	14.7	17.7	8.5	3.3	65.0
7	0.14	5.2	2.1 [1]	4.4	7.1	5.6	0.5 [1]	11.8
8	0.10	3.7	1.8 [1]	3.3	6.5	3.9	1.2	1.2
9	0.07	2.0 [1]	1.5 [1]	ND	ND	ND	0.5 [1]	0.9 [1]
10	0.17	3.5	3.8 [1]	ND	ND	ND	1.2	0.5 [1]
11	0.22	2.0 [1]	2.8 [1]	ND	ND	ND	1.1	1.6
12	0.65	257.8	248.6	ND	ND	ND	87.3	257.8
13	0.52	63.2	65.5	102.4	109.4	70.3	10.6	84.3
14	0.55	5.4	2.8 [1]	5.7	ND	ND	1.0	12.0
15	0.19	3.3	2.1 [1]	ND	ND	ND	1.7	4.9
16	0.57	3.7	3.1 [1]	ND	ND	ND	0.6 [1]	2.1
17	0.77	3.3	4.3 [1]	ND	ND	ND	1.7	1.4 [1]
18	1.27	5.6	6.6 [1]	ND	ND	ND	1.3	0.4 [1]
19	0.66	69.3	62.3	183.3	268.7	203.4	7.8	465.1
20	0.32	2.9	2.5 [1]	ND	ND	ND	0.6 [1]	0.7 [1]
21	0.84	3.6 [1]	3.2 [1]	ND	ND	ND	1.0 [1]	2.4
22	0.80	6.3	7.7 [1]	ND	ND	ND	2.7	2.0
23	0.51	4.3	2.8 [1]	ND	ND	ND	1.2	2.6
24	0.53	2.5 [1]	3.0 [1]	ND	ND	ND	0.7 [1]	1.1 [1]

Appendix 5. Continued.

Station	Acenaphthene	Fluorene	Methyl Flourene	Ethyl Fluorene	Propyl Fluorene	Phenanthrene	Anthracene	Methyl Phenantherene
1	7.2	6.0	2.4 [1]	ND	ND	64.4	18.6	16.6
2	66.2	40.9	ND	ND	ND	537.9	136.6	197.6
3	236.1	176.7	94.9	120.9	178.3	2742.2	644.6	756.5
4	384.5	199.6	125.9	107.7	140.7	3031.7	932.6	1010.7
5	423.6	433.4	177.6	108.7	232.2	4366.2	1248.5	1283.9
6	50.6	61.8	16.8	9.0	6.8	480.1	143.9	148.4
7	13.9	1.4	4.4	4.1	11.4	31.6	13.6	21.2
8	3.0	3.1	2.7	2.6	11.8	20.9	6.0	8.1
9	1.9	1.5	ND	ND	ND	13.6	3.3	6.6
10	2.5	1.2 [1]	ND	ND	ND	13.5	4.1	5.0
11	3.7	1.3 [1]	ND	ND	ND	19.9	6.2	9.5
12	827.1	398.4	386.8	428.2	ND	4042.6	1073.6	1627.1
13	269.1	193.4	83.8	58.9	129.0	2026.6	443.7	602.5
14	21.2	14.5	10.4	9.8	10.4	166.4	43.6	48.4
15	9.0	8.1	5.4	ND	ND	97.7	24.6	28.7
16	2.2	2.0	ND	ND	ND	5.1	1.9	ND
17	2.7	1.2 [1]	ND	ND	ND	13.3	3.9	ND
18	0.8 [1]	0.8 [1]	ND	ND	ND	5.0	1.4 [1]	ND
19	261.0	241.2	207.5	258.8	314.0	3209.4	1010.9	1435.0
20	1.1 [1]	0.5 [1]	ND	ND	ND	2.5	0.8 [1]	ND
21	0.8 [1]	1.4 [1]	ND	ND	ND	10.2	3.1	ND
22	1.0 [1]	1.2 [1]	ND	ND	ND	10.6	4.3	ND
23	9.5	9.4	ND	ND	ND	138.1	34.8	33.3
24	2.0	1.0 [1]	ND	ND	ND	4.1	0.9 [1]	ND

Appendix 5. Continued.

Station	Ethyl Phenantherene	Propyl Phenanthrene	Butyl Phenanthrene	Dibenzothiophene	Methyl Dibenzothiophene	Ethyl Dibenzothiophene	Propyl Dibenzothiophene	Flouranthene
1	12.3	6.7	3.2	3.5	2.2 [1]	2.4	2.8	178.0
2	308.7	322.5	203.7	40.5	ND	141.9	231.4	1655.6
3	310.4	1.6 [1]	85.8	164.0	95.6	ND	ND	5777.6
4	469.0	158.9	96.9	236.6	121.9	ND	ND	6395.5
5	496.3	225.3	84.3	341.7	139.2	99.2	ND	7699.6
6	69.8	33.5	9.9	36.4	15.2	10.1	6.3	760.3
7	11.5	6.3	2.6	5.1	3.1	2.5	3.9	142.3
8	6.6	3.6	2.0 [1]	1.9	1.0 [1]	2.0 [1]	ND	53.8
9	ND	ND	ND	1.3	ND	ND	ND	33.1
10	4.1	4.6	5.2	1.1 [1]	ND	ND	ND	32.6
11	7.4	ND	ND	1.9	ND	ND	ND	52.9
12	1000.6	584.6	ND	304.3	ND	ND	ND	11380.5
13	247.7	116.1	56.6	125.8	55.5	46.5	39.7	4303.8
14	26.6	12.3	7.3	9.7	5.2	4.4	4.8	393.3
15	14.5	7.7	ND	5.9	3.6	ND	ND	224.2
16	ND	ND	ND	0.4 [1]	ND	ND	ND	14.3
17	ND	ND	ND	0.7 [1]	ND	ND	ND	37.5
18	ND	ND	ND	1.3 [1]	ND	ND	ND	6.9
19	878.9	448.6	157.3	213.9	94.4	110.7	89.4	8556.2
20	ND	ND	ND	0.6 [1]	ND	ND	ND	7.2
21	ND	ND	ND	0.8 [1]	ND	ND	ND	25.1
22	ND	ND	ND	0.8 [1]	ND	ND	ND	30.0
23	28.0	24.2	ND	7.8	ND	ND	ND	271.8
24	ND	ND	ND	0.5 [1]	ND	ND	ND	8.7

Appendix 5. Continued.

Station	Pyrene	Methyl Flouranthene + Pyrene	Benzo(a)-Anthracene	Chrysene	Methyl Chrysene	Ethyl Chrysene	Propyl Chrysene	Butyl Chrysene
1	161.0	80.9	104.5	111.7	45.6	14.8	0.8 [1]	14.9
2	1620.3	783.2	1389.0	1344.2	516.1	190.4	28.1	374.4
3	4912.7	2197.0	2416.9	2359.0	272.6	272.6	15.5	415.2
4	4745.5	2183.7	2530.9	2336.7	777.0	222.4	18.8	388.7
5	5331.8	2512.8	2950.9	2170.2	833.0	220.4	21.0	406.0
6	685.5	419.2	448.9	360.5	173.2	51.6	1.3 [1]	39.3
7	105.2	48.7	52.1	41.6	13.9	5.5	0.5 [1]	4.4
8	54.4	32.8	25.3	29.3	8.7	5.9	0.5 [1]	3.3
9	25.6	15.6	17.9	19.4	7.8	3.0	0.8 [1]	4.7
10	29.3	15.2	23.9	22.1	9.0	3.6	0.6 [1]	7.1
11	44.4	21.9	25.1	24.8	8.6	5.0	1.1 [1]	5.8
12	9620.7	5222.0	4345.6	4266.2	1661.9	596.0	63.6	833.4
13	3437.3	1808.3	2183.4	2058.1	835.9	261.6	13.2	462.1
14	324.6	155.8	243.1	229.6	82.3	32.9	1.2 [1]	51.3
15	187.2	89.7	135.9	119.9	47.8	14.8	0.7 [1]	29.6
16	15.4	11.2	8.9	10.0	7.2	10.6	1.9	4.7
17	36.5	20.5	17.3	20.9	ND	ND	ND	ND
18	6.6	ND	2.3	3.4	ND	ND	ND	ND
19	7209.6	3795.6	4604.3	4246.3	1644.6	618.4	24.6	949.3
20	6.6	5.9	4.3	4.6	ND	ND	ND	ND
21	23.9	16.3	12.8	14.4	ND	ND	ND	ND
22	28.6	22.0	15.2	21.5	ND	ND	ND	ND
23	225.4	95.5	147.8	135.5	63.2	26.5	3.2	20.9
24	8.2	ND	3.3	4.3	ND	ND	ND	ND

Appendix 5. Continued.

Station	Benzo(b)-Fluoranthene	Benzo(k)-Fluoranthene	Benzo(e)-Pyrene	Benzo(a)-Pyrene	Perylene	Ideno(123cd) Pyrene	Dibenzo(ah)-Anthracene	Benzo(ghi)-Perylene
1	168.1	157.2	79.8	144.2	35.4	83.2	17.6	67.6
2	2385.5	960.1	1264.9	2052.3	510.3	1450.7	306.3	1343.6
3	3559.1	1413.6	1791.6	3202.7	729.5	1944.8	421.1	1715.4
4	3518.4	1186.1	1557.7	2922.9	692.1	1693.9	376.7	1444.3
5	3485.5	1276.7	1534.9	2966.1	646.8	1644.0	368.5	1396.2
6	551.7	195.0	232.8	450.9	85.7	209.7	51.9	162.1
7	66.4	27.9	29.7	55.4	15.1	30.4	6.7	24.3
8	43.3	17.8	20.2	35.1	9.2	19.9	4.7	17.0
9	26.2	10.8	12.5	21.5	6.1	15.1	3.3	13.4
10	44.3	10.0	21.4	32.6	12.8	25.9	5.6	25.1
11	38.9	13.1	19.2	32.4	12.6	21.6	4.7	19.8
12	7554.7	2204.9	3508.9	6018.9	1292.3	3961.8	828.3	3717.3
13	3952.4	898.9	1810.0	3040.5	589.9	2065.9	461.8	1906.1
14	438.5	135.6	204.3	333.9	64.4	238.7	52.0	217.8
15	214.2	85.7	106.0	180.6	45.3	126.4	28.2	116.1
16	25.1	5.2	12.0	15.4	6.2 [1]	11.8	2.4	11.3
17	32.9	12.4	18.3	25.9	8.5	17.0	3.6	18.7
18	6.6	2.0	3.8	4.0	17.1	3.0	0.6 [1]	3.5
19	6985.3	2406.7	3312.4	5705.5	1108.6	3393.8	761.2	3051.3
20	8.0	2.8	4.4	6.0	4.1 [1]	4.3	0.7 [1]	4.1
21	23.2	8.8	12.8	18.5	9.3 [1]	11.9	2.3	13.1
22	31.7	11.2	16.7	21.8	10.7	15.8	3.0	14.9
23	234.9	69.7	111.3	175.2	49.2	115.2	25.6	107.6
24	7.6	1.8	3.9	4.8	2.3 [1]	3.3	0.6 [1]	3.4

Appendix 5. Continued.

Station	Total PAHs	2-Methyl-naphthalene	1-methyl-naphthalene	2,6-Dimethyl naphlalene	1,6,7-Trimethyl naphthalene	1-Methyl phenanthrene
1	1583.0	0.3 [1]	0.5 [1]	0.3 [1]	0.3 [1]	4.5
2	19965.8	13.4	16.9	14.0	12.3	45.2
3	38462.9	8.0	26.7	15.7	25.4	164.3
4	40190.4	10.8	37.4	18.3	13.7	258.8
5	45647.8	6.9	55.2	52.5	39.4	316.8
6	6018.3	2.1 [1]	6.9	6.5	4.6	33.2
7	828.4	1.0 [1]	1.1 [1]	1.0 [1]	1.6	5.6
8	468.7	1.2 [1]	0.6 [1]	2.1	1.2 [1]	1.8
9	263.5	0.8 [1]	0.7 [1]	1.1 [1]	0.5 [1]	1.2
10	358.5	2.2 [1]	1.7 [1]	1.2 [1]	0.9 [1]	1.6
11	396.6	1.3 [1]	1.5 [1]	0.6 [1]	1.1 [1]	2.2
12	77309.2	112.5	136.1	44.7	172.0	417.4
13	34499.9	25.6	39.9	19.5	14.1	130.2
14	3556.9	1.5 [1]	1.3 [1]	0.6 [1]	1.4 [1]	11.0
15	1924.3	1.2 [1]	0.9 [1]	2.1	0.6 [1]	8.1
16	188.3	1.5 [1]	1.6 [1]	0.7 [1]	1.1 [1]	0.5 [1]
17	293.8	2.0 [1]	2.3 [1]	2.4	2.2	2.3
18	65.9	4.0	2.6 [1]	2.3	2.0 [1]	1.0 [1]
19	67455.7	18.8	43.4	52.7	31.2	289.8
20	70.7	1.3 [1]	1.2 [1]	1.9	0.9 [1]	0.6 [1]
21	209.3	1.7 [1]	1.5 [1]	1.7 [1]	1.2 [1]	2.4
22	268.9	3.4 [1]	4.3	2.2	1.9 [1]	1.7 [1]
23	2125.4	1.7 [1]	1.1 [1]	1.6 [1]	1.8 [1]	8.4
24	66.3	2.0 [1]	1.0 [1]	0.5 [1]	1.7 [1]	1.4

ND = not detected
[1] Below detection limit

Appendix 6. Quality control data for spiked PAHs and average percent variation of replicate samples from sediments collected in Lavaca Bay, Texas.

Aromatic Hydrocarbon	Percent Recovery Spiked Sample[1]		Percent Recovery Blank Spikes[2]		Relative Percent Difference Between Duplicates[3]	
	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n
Naphthalene	89 ± 14.4	5	101 ± 12.0	2	9 ± 6.8	4
Methyl Naphthalene	na[4]	-	na[4]	-	na[4]	-
Ethyl Naphthalene	na[4]	-	na[4]	-	na[4]	-
Propyl Naphthalene	na[4]	-	na[4]	-	na[4]	-
Butyl Naphthalene	na[4]	-	na[4]	-	na[4]	-
Biphenyl	102 ± 11.6	5	97 ± 2.2	2	23 ± 16.0	3
Acenaphthylene	101 ± 12.2	5	90 ± 6.3	2	34 ± 45.7	4
Acenaphthene	97 ± 13.5	5	97 ± 3.1	2	28 ± 27.1	4
Fluorene	108 ± 12.6	5	106 ± 5.0	2	39 ± 37.9	3
Methyl Fluorine	na[4]	-	na[4]	-	na[4]	-
Ethyl Fluorine	na[4]	-	na[4]	-	na[4]	-
Propyl Fluorine	na[4]	-	na[4]	-	na[4]	-
Phenanthrene	93 ± 31.6	5	101 ± 16.1	2	18 ± 13.2	4
Anthracene	110 ± 15.7	5	110 ± 12.2	2	13 ± 16.0	3

Appendix 6. Continued.

Aromatic Hydrocarbon	Percent Recovery Spiked Sample[1]		Percent Recovery Blank Spikes[2]		Relative Percent Difference Between Duplicates[3]	
	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n
Methyl Phenanthrene	na[4]	-	na[4]	-	13	1
Ethyl Phenanthrene	na[4]	-	na[4]	-	27	1
Propyl Phenanthrene	na[4]	-	na[4]	-	35	1
Butyl Phenanthrene	na[4]	-	na[4]	-	na[4]	-
Dibenzothiophene	111 ± 14.4	5	91 ± 7.4	2	53	1
Methyl Dibenzothiophene	na[4]	-	na[4]	-	na[4]	-
Ethyl Dibenzothiophene	na[4]	-	na[4]	-	na[4]	-
Propyl Dibenzothiophene	na[4]	-	na[4]	-	na[4]	-
Fluoranthene	73 ± 43.6	5	88 ± 12.2	2	22 ± 16.3	5
Pyrene	69 ± 39.9	5	88 ± 12.8	2	23 ± 11.2	5
Methyl Fluoranthene + Pyrene	na[4]	-	na[4]	-	19 ± 5.9	2
Benz(a)anthracene	91 ± 20.3	5	101 ± 15.7	2	16 ± 8.2	5
Chrysene	86 ± 26.2	5	100 ± 18.1	2	23 ± 12.3	5

Appendix 6. Continued.

Aromatic Hydrocarbon	Percent Recovery Spiked Sample[1]		Percent Recovery Blank Spikes[2]		Relative Percent Difference Between Duplicates[3]	
	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n
Methyl Chrysene	na[4]	-	na[4]	-	40 ± 23.4	2
Ethyl Chrysene	na[4]	-	na[4]	-	39 ± 5.6	2
Propyl Chrysene	na[4]	-	na[4]	-	na[4]	-
Butyl Chrysene	na[4]	-	na[4]	-	15 ± 3.5	3
Benzo(b)fluoranthene	86 ± 18.3	5	95 ± 18	2	27 ± 15.3	5
Benzo(k)fluoranthene	93 ± 22.4	5	94 ± 6.2	2	14 ± 11.3	5
Benz(e)pyrene	92 ± 12.5	5	110 ± 16.5	2	12 ± 11.0	5
Benz(a)pyrene	88 ± 17.4	5	104 ± 17.6	2	15 ± 12.7	5
Perylene	91 ± 11.6	5	108 ± 6.0	2	14 ± 10.8	3
Ideno(123cd)pyrene	80 ± 18.9	5	90 ± 21.8	2	15 ± 8.3	5
Dibenz(ah)anthracene	89 ± 9.2	5	89 ± 20.4	2	8 ± 5.7	4
Benzo(ghi)perylene	78 ± 16.9	5	110 ± 26.4	2	12 ± 12.0	5
Total PAHs						
(w/o Perylene)	93 ± 14.1	5	98 ± 9.9	3	20 ± 8.3	5
2-Methylnaphthalene	97 ± 12.1	5	99 ± 2.3	3	5 ± 5.8	2

Appendix 6. Continued.

Aromatic Hydrocarbon	Percent Recovery Spiked Sample[1]		Percent Recovery Blank Spikes[2]		Relative Percent Difference Between Duplicates[3]	
	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n
1-Methylnaphthalene	106 ± 10.0	5	108 ± 12.4	3	5 ± 1.2	2
2,6-Dimethnaphthalene	96 ± 13.8	5	85 ± 10.8	3	21 ± 21.5	2
1,6,7-Trimethnaphthalene	99 ± 10.8	5	91 ± 3.7	3	18 ± 13.4	2
1-Methylphenanthrene	97 ± 10.9	5	90 ± 11.6	3	5 ± 0.7	3

[1] Average percent recovery of spiked analyte; (Σ(µg/kg recovered-baseline)/spiked quantity)/n.

[2] Average percent recovery of spiked blank; (Σ(µg/kg recovered-baseline)/spiked quantity)/n.

[3] (((absolute value (concentration of first aliquot=C1) - (concentration of second aliquot=C2))*100)/((C1+C2)/2)).

[4] Data not available; spiked analysis not performed.

Appendix 7. Sediment metal concentrations (µg/g dry weight) for Lavaca Bay Study.

Station	Ag	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
1	0.09	5.64	0.12	36.7	8.4	0.141	11.5	13.6	38
2	0.10	8.80	0.22	50.5	15.1	0.627	20.3	25.1	68
3	0.11	5.23	0.14	23.0	7.8	0.772	6.9	11.5	33
4	0.13	6.61	0.14	28.4	9.4	0.863	9.8	11.9	41
5	0.11	6.02	0.12	29.9	8.4	0.909	11.5	11.4	45
6	0.10	6.14	0.11	23.5	5.8	0.343	6.5	9.2	34
7	0.09	5.73	0.13	32.1	8.6	0.109	11.0	12.1	45
8	0.12	4.79	0.14	27.9	6.9	0.151	9.0	10.8	30
9	0.11	4.42	0.08	19.4	6.0	0.080	6.9	9.3	25
10	0.11	8.44	0.11	31.0	8.9	0.120	11.4	12.0	37
11	0.09	5.01	0.08	29.3	6.5	0.153	8.6	10.6	29
12	0.11	6.44	0.10	30.0	7.3	0.159	9.7	11.5	31
13	0.13	5.84	0.16	25.5	9.6	0.763	8.9	12.7	32
14	0.11	3.70	0.07	24.8	6.8	0.377	6.1	9.8	21
15	0.10	3.64	0.07	17.1	4.4	0.324	5.0	9.2	25
16	0.10	12.3	0.17	45.0	12.9	0.104	19.0	18.5	61
17	0.11	9.54	0.16	39.2	10.5	0.214	15.5	16.5	48
18	0.12	8.93	0.17	39.2	13.9	0.051	18.3	17.9	59
19	0.11	5.10	0.16	25.3	8.5	0.513	9.3	16.0	45
20	0.11	4.28	0.09	24.9	5.8	0.042	7.2	12.4	34
21	0.11	7.57	0.15	53.0	13.3	0.167	11.8	21.0	74
22	0.12	11.2	0.35	57.6	15.9	0.233	24.1	19.6	114
23	0.10	7.54	0.14	40.5	12.9	0.139	16.4	16.4	53
24	0.09	7.52	0.13	45.3	13.1	0.055	15.3	13.6	49

Appendix 8. Quality control data for spiked metals for selected sediments collected in Lavaca Bay, Texas.

Compound	Percent Recovery Spiked Samples[1] $\bar{x} \pm SD$	Percent Recovery Blank Spike[2] $\bar{x} \pm SD$	Relative Percent Difference Between Duplicates[3] $\bar{x} \pm SD$
Ag	92.3 ± 11.3	89.7 ± 11.4	6.6 ± 4.1
As	115.8 ± 6.7	118.2 ± 11.2	7.1 ± 0.1
Cd	87.4 ± 1.4	89.4 ± 6.4	1.8 ± 2.9
Cr	100.0 ± 9.0	100.7 ± 0.3	10.7 ± 7.0
Cu	98.8 ± 3.9	108.1 ± 1.5	3.8 ± 2.3
Hg	90.5 ± 19.6	92.9 ± 1.6	7.7 ± 3.5
Ni	101.5 ± 5.6	104.3 ± 4.8	7.3 ± 6.1
Pb	94.5 ± 13.4	99.8 ± 2.3	2.1 ± 1.5
Zn	99.3 ± 5.2	95.3 ± 0.4	3.2 ± 2.0

[1] Average percent recovery of spiked analyte; ($\sum$(µg/kg recoverd-baseline)/spiked quantity)/n where n=3.

[2] Average percent recovery of spiked blanks; ($\sum$(µg/kg recoverd-baseline)/spiked quantity)/n where n=2.

[3] Average relative percent difference between duplicates; n=3
(((absolute value (concentration of first aliquot=C1) - (concentration of second aliquot=C2))*100)/((C1+C2)/2)).

Date Prepared: May 5, 1990

Date Revised: June 10, 1994

EXTRACTION AND STORAGE OF POREWATER SAMPLES

1.0 OBJECTIVE

This protocol describes a procedure for extracting and storing porewater samples from marine, estuarine, or freshwater sediments for use in toxicity testing. A pressurized extraction device is used to force the pore water from sediment samples. This procedure may be performed in the laboratory or it may be performed at or near the site of sample collection since the sampling apparatus is portable.

2.0 PREPARATION

2.1 Description of the Porewater Extraction System

In earlier studies (Carr et al., 1989; Carr and Chapman, 1992) pore water was extracted from sediments using a device constructed of Teflon®. Since then, the design has been improved (Carr and Chapman, 1994) The polyvinyl chloride (PVC) extractors in current use are less costly to construct and easier to operate. This device has been used in numerous sediment quality assessment surveys (Carr, 1993; NBS, 1993; NBS, 1994a; NBS, 1994b; USFWS, 1992).

The extractor is constructed from a PVC compression coupling for 4" I.D. schedule 40 PVC pipe. These commercially-available couplings (Lascotite®) consist of a cylinder (25 cm height and 13 cm diameter) with threaded ends and threaded open compression nuts (Figure 1). The coupling is fitted with end plates cut from 7/16" thick PVC sheeting that are held in place by the threaded end nuts. The gaskets provided with the coupling are discarded and silicon O-rings are used to seal the top and bottom connections. The top end plate is fitted with a quick-release fitting where the pressurized air is supplied, and a safety pressure relief valve. Like the original Teflon® extractor, the bottom end plate (Figure 1) has several interconnected concentric grooves to facilitate flow of the pore water to the central exit port. A 5 μm polyester filter is situated between the bottom end plate and the silicon O-ring. Before a sediment sample is loaded, the bottom end nut is tightened in place by using the stationary bottom wrench (Figure 1) and a standard strap wrench.

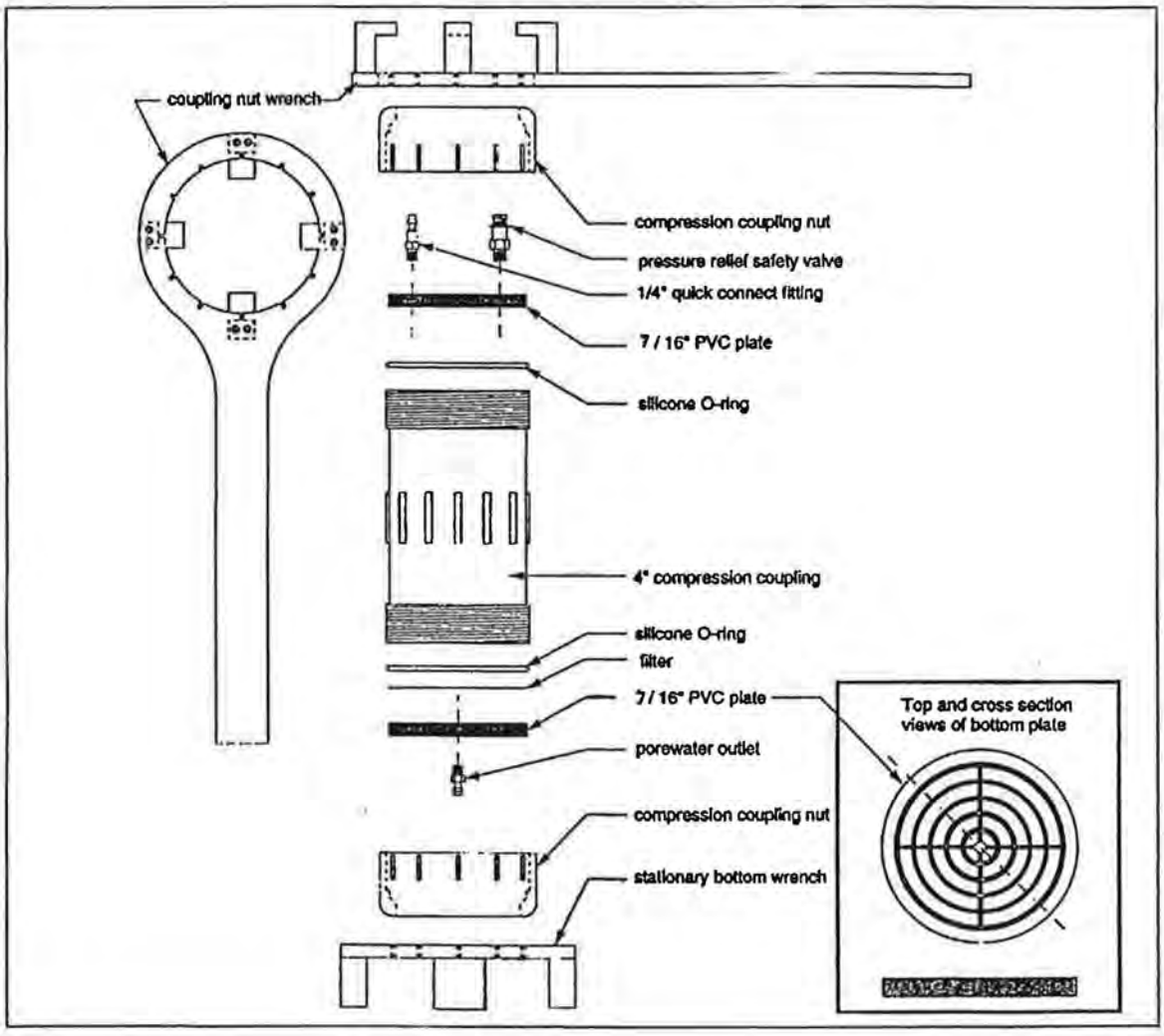

Figure 1. Sediment pore water squeeze extraction device.

The extractors are pressurized with air supplied from a standard SCUBA cylinder via a SCUBA first stage regulator which delivers air to a manifold with a valving system (Figure 2). With this system, multiple cylinders can be pressurized simultaneously, using the same SCUBA cylinder.

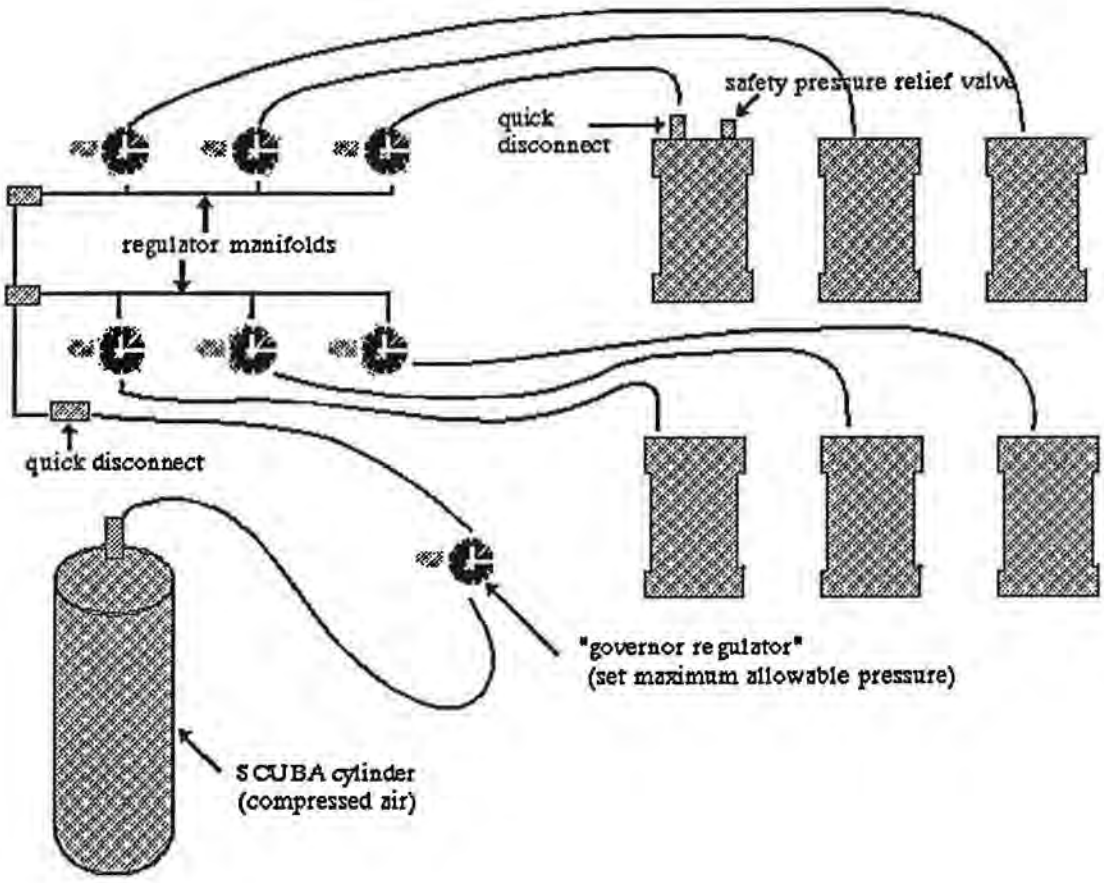

Figure 2. Schematic of sediment porewater pressure extraction system.

2.2 Equipment List

Supplies and equipment needed are listed in Attachment 1.

3.0 PROCEDURE

3.1 Sediment Collection and Storage Considerations

Generally, surficial sediment samples are collected for porewater extraction. A homogenate of the upper ~2-10 cm sediment may be collected by multiple cores or grabs at a particular sampling station. (Further details of sediment sampling procedures are not within the scope of this SOP.) One liter of sediment will typically provide 100-200 mL pore water. However, a larger volume of course sand sediments may be required since they contain less water, and a larger volume of fine clay sediments may be required since they are difficult to extract. The sample composites are kept in suitable containers (e.g., clean high density polyethelene containers or Zip-Lock® bags), labelled, and stored on ice, in a cooler, or in a refrigerator until the samples are delivered and processed. Pore water should be extracted from the samples as soon as possible because the toxicity of sediments in storage may change over time. A sample tracking system should be maintained for each sediment sample collected and porewater sample extracted. All manipulations made on samples are recorded on the Sample History Data Form (Attachment 2).

3.2 Load Extraction Cylinder

1. Assemble all parts of extraction cylinder except the top end compression coupling nut, top end plate and O-ring. Make sure filter is snugly in place beneath bottom O-ring (both over- and under-tightening will result in an improper seal). Place the extractor cylinder on the stand and positon an appropriately labelled porewater sample container (usually an I-Chem® amber 250 mL or 125 mL glass jar cleaned to EPA standards, with Teflon® lid liner) underneath the outlet.

2. Ensure that the sediment sample is homogenized, by shaking, stirring with a clean Teflon® or plastic spatula or spoon, or by both.

3. Transfer sediment from the sample container/bag to the extractor by pouring and/or using a clean Teflon® or plastic spatula or spoon. If necessary, particularly when extracting pore water from sandy or shelly sediments, the spatula may be used to compress the sample in the cylinder to eliminate channelization. The amount of sediment to be transferred will depend on the texture of the sample. The cylinder may be filled nearly full with a sandy sediment. However, when extracting pore water from a clay sediment, a relatively impermeable layer of compressed clay will eventually form on the filter, so that extraction of a large volume of clay sediment at once would take an extremely long time. When extracting pore water from extremely fine grained sediments, the cylinder should be less than one-third filled. If additional pore water is needed, this process can be repeated by removing the sediment including

sediment including removing or "peeling" the impermeable layer, and reintroducing more of the original sediment sample.

4. After sediment is loaded, the top end plate within the top compression coupling nut is installed . To tighten the top nut, the strap wrench and the coupling nut wrench (Figure 1) are used.

3.3 Porewater Extraction

After the extractor is sealed, a high-pressure hose is attached to the quick disconnect fitting on the top end plate, and the extractor is pressurized with air from a SCUBA tank. Pressure is controlled with a first-stage regulator on the SCUBA tank, an intermediate "governor" regulator, and final second stage regulators attached to a manifold that services multiple extractors (Figure 2).

1. Turn the SCUBA valve counter clockwise, pressurizing the first stage regulator and the intermediate-pressure hose (approximately 150 psi). An additional "governor" pressure regulator between the SCUBA tanks and the final second stage regulators which control pressure to the individual extractors should be set at maximum extractor pressure (~40 psi).

2. Ensure that all final pressure regulators are set to zero. Attach the hose from one of the pressure regulators on the pressure regulator manifold to the air inlet, using the quick disconnect fitting.

3. Slowly open the corresponding pressure regulator to a pressure of 5-10 psi. Check the first drops of porewater passing from the outlet for cloudiness. Occasionally, a small amount of sediment will pass through the porewater outlet, presumably around the filter. If this happens, wait until the pore water clears, discard the initial pore water collected, and continue.

4. Check the cylinder for leaks and if necessary tighten clamping nuts slightly.

5. As the flow of pore water decreases, pressure may be increased gradually to a maximum of 35-40 psi. When flow is less than or slows to less than 1-3 drops per minute, increase the pressure in 5-10 psi increments to maintain the flow. Allow the extraction to continue until sufficient pore water has been collected.

6. Disassemble the extractor, discard sediment, and rinse and wash appropriately all parts contacting sediment before placing a different sediment sample into the extractor.

7. Repeat these procedures until all available extractors are in use or until all sediment samples have been processed.

3.4 Centrifugation of Porewater Samples

Porewater samples extracted at this field station are usually stored frozen until tested. Under most circumstances, the porewater samples are centrifuged after they are collected and before they are frozen.

1. After collection, keep the porewater samples refrigerated or chilled on ice until they are centrifuged.

2. Transfer the pore water from the glass sample jar to an appropriate centrifuge bottle (e.g., polycarbonate). Centrifuge at ≥1200 *g* for 20 minutes. Return the centrifuged sample to a rinsed and labelled glass jar, taking care not to disturb any material that may have settled on the bottom/sides of the centrifuge bottle.

3. If multiple jars of pore water were collected from a single sediment sample, they should be composited after centrifugation and redistributed to the glass jars before testing or storage.

3.5 Storage of Porewater Samples

If the porewater samples are not to be used on the day of collection, they should be frozen for storage. Sufficient room for freeze expansion should be left in the jars (for example, 200 mL maximum sample in a 250 mL jar). If the volume needed for testing is known in advance, it is prudent to allocate only that specific volume plus a little excess (~10 mL) to each jar in order to conserve pore water (once thawed, the pore water cannot be refrozen and reused), and to simplify the volume measurements required for Water Quality Adjustment of Samples (SOP F10.12) performed the day prior to testing. Frozen porewater samples may be shipped with dry ice.

4.0 QUALITY CONTROL

A sample tracking system is maintained for each sediment sample collected and porewater sample extracted. All actions taken with that respective sample are recorded on the Sample History Data Form (Attachment 2). This information includes, but not exclusively, : a) the date of collection or receipt, b) the date of porewater extraction, c) the volume or number of jars (I-Chem® amber glass jars) of pore water collected, d) centrifugation information, if performed, e) date frozen and location (freezer no.), and e) date and jar no. thawed and used in which test. The Sample History Forms are kept in a three-ring binder at the same location where the samples are stored.

5.0 TRAINING

Persons who will perform this procedure should first read this SOP and then operate under the supervision of an experienced individual for at least one series of extractions.

6.0 SAFETY

The sediment and porewater samples handled may contain contaminants. Care should be taken to avoid contact with the samples. Protective gloves, glasses and clothing may be worn. Waste sediment should be properly disposed. SCUBA cylinders should be securely mounted before, during, and after use. The pressure limit (40 psi) of the extraction cylinders should not be exceeded. Before disconnecting any pressure hoses, ensure that the pressure has been released or that the controlling regulator has been closed. The pressure relief valves should be set to leak at just above maximum operating pressure, and they should be checked regularly to ensure that they are performing. Pressure relief valves should be disassembled and cleaned yearly.

7.0 ATTACHMENTS

Attachment 1. Required Equipment and Materials
Attachment 2. Sample History Form

8.0 REFERENCES

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Attachment 1

REQUIRED EQUIPMENT AND MATERIALS

To construct a sediment pore water extraction device:

1-PVC cylinder (center portion of 4" compression coupling)
2-PVC end nuts (ends of 4" compression fitting)
1-PVC top end plate (7/16" width)
1-PVC bottom end plate (7/16" width)
1-Quick disconnect brass air fitting
1-Pressure relief valve
1-Teflon® 1/8" npt male connector for exit port

To use a pore water extraction device:

1-Filter, polyester material, 5 μm pore size
1-Wooden stand (1 stand per 3 cylinders)
1-Custom wrench for 4" compression coupling end nuts
1-Custom wrench head attached to table
1-Plastic or Teflon® spatula or spoon
1-SCUBA cylinder
1-SCUBA regulator with high pressure gauge
1-SCUBA intermediate pressure hose (~10 ft length)
with governor pressure gauge set to ~40 psi
1-Air pressure control manifold that includes:
Final pressure regulator valves (several per manifold)
Pressure gauges (1 per valve)
Low pressure hose, 6' length (1 per manifold)

Other required supplies/equipment:

Sediment sample containers or bags
Pore water sample jars
Sample labels or labeling tape
Beakers
Deionized water (DI)
Wash bottles, 500 ml
Protective gloves, glasses, clothing
Pens, pencils, markers
Centrifuge and centrifugation materials
Refrigerator
Freezer

Attachment 2

SAMPLE HISTORY DATA FORM

Sample Designation: _____ Study Protocol: _____ Initials: _____

Date of acquisition: _____ Sample type: _____

How acquired (refer to sample site data sheet number, if appropriate): _____

_____

_____

_____

Initials	Date	Action Taken

Date Prepared: March 14, 1991

Date Revised: May 17, 1994

WATER QUALITY ADJUSTMENT OF SAMPLES

1.0 OBJECTIVE

In order to perform toxicity tests with saline samples, all test and reference samples should be similar in salinity so that salinity is not a factor in survival of test organisms. Additionally, dissolved oxygen (DO) concentrations should be sufficiently high to ensure that low DO is not a source of stress to the test organisms. At the Corpus Christi field station, toxicity tests are performed using a variety of marine and estuarine organisms, including the sea urchin *Arbacia punctulata*, the polychaete *Dinophilus gyrociliatus*, the harpacticoid copepod *Longipedia* sp., and the red drum *Sciaenops ocellatus*. The aqueous samples tested may be pore water, different kinds of discharges and effluents, surface microlayer, or subsurface water samples that may range in salinity from 0-36‰. Although from test to test salinities used in the different toxicity tests may vary, the individual toxicity tests performed on a particular day are run at a single target salinity. Since initial salinities of the porewater or water samples to be tested commonly vary, they will require salinity adjustment to within 1‰ of the target salinity. Additionally, DO should normally be ≥80% saturation in all samples tested.

2.0 PREPARATION

2.1 Equipment and Labware

The supplies and equipment needed are listed in Attachment 1.

2.2 Source of Dilution Water

For samples lower in salinity than target salinity, concentrated brine (~100‰) is added to increase salinity. Concentrated brine is prepared by heating (to 35-40°C) and gently aerating filtered natural seawater (1 µm) to concentrate the salts by evaporation. Prior to use, a 10% addition of reference pore water is added to the brine to replace lost trace elements. For samples higher in salinity than target salinity, Milli-Q, HPLC grade ultrapure water is added to decrease salinity.

3.0 PROCEDURES

The following describes the procedures required for the adjustment and determination of specific water quality parameters of a sample.

3.1 Preparation for Salinity Adjustment

1. Although fresh samples are routinely tested at the Corpus Christi field station, most of the samples tested are stored frozen in amber I-Chem® jars. If frozen, remove samples from freezer and allow them to thaw at room temperature or immerse them in a tepid water bath to thaw, ensuring that sample temperature does not exceed 25 °C. The samples may be thawed the day of water quality adjustment (WQA) or may be transferred from the freezer to a refrigerator (4 °C) the day before WQA and then completely thawed the following day. After thawing, allow the samples to come to room temperature. Generally, the samples should be maintained at the same temperature required for the toxicity test that will be conducted. The temperature requirement for most toxicity tests performed at this field station is 20±1 °C, and room temperature should be maintained accordingly.

2. Turn bottled sample end over end a few times to mix thoroughly before measuring salinity. Using a salinity refractometer, measure salinity and record on Water Quality Adjustment Data Form (Attachment 2).

3. In order to make calculations for the salinity adjustment, the volume of the sample must be known. When porewater or other water samples are collected and transferred to amber jars for storage, they are commonly measured to an approximate volume (~110 mL, for example) prior to freezing. On the day of WQA, this volume should be recorded on the WQA data form for the respective samples. If the volume is unknown at this point, it should be measured using a graduated cylinder of appropriate size, and recorded on the data sheet.

3.2 Salinity Adjustment

3.21 Reducing the salinity of aqueous samples

Refer to the formulas below to calculate the volume of HPLC water needed to reduce the initial sample salinity to the target salinity. Add the volume calculated, mix the bottle thoroughly, check the salinity with a refractometer, and record the volume of HPLC water added as well as the final salinity.

(i) (target $^{o}/_{oo}$ ÷ sample $^{o}/_{oo}$) × sample vol. in mL = A
(ii) sample vol. − A = B
(iii) sample vol. ÷ A = C
(iv) B × C = volume of HPLC water to add

3.1 Preparation for Salinity Adjustment

1. Although fresh samples are routinely tested at the Corpus Christi field station, most of the samples tested are stored frozen in amber I-Chem® jars. If frozen, remove samples from freezer and allow them to thaw at room temperature or immerse them in a tepid water bath to thaw, ensuring that sample temperature does not exceed 25°C. The samples may be thawed the day of water quality adjustment (WQA) or may be transferred from the freezer to a refrigerator (4°C) the day before WQA and then completely thawed the following day. After thawing, allow the samples to come to room temperature. Generally, the samples should be maintained at the same temperature required for the toxicity test that will be conducted. The temperature requirement for most toxicity tests performed at this field station is 20±1°C, and room temperature should be maintained accordingly.

2. Turn bottled sample end over end a few times to mix thoroughly before measuring salinity. Using a salinity refractometer, measure salinity and record on Water Quality Adjustment Data Form (Attachment 2).

3. In order to make calculations for the salinity adjustment, the volume of the sample must be known. When porewater or other water samples are collected and transferred to amber jars for storage, they are commonly measured to an approximate volume (~110 mL, for example) prior to freezing. On the day of WQA, this volume should be recorded on the WQA data form for the respective samples. If the volume is unknown at this point, it should be measured using a graduated cylinder of appropriate size, and recorded on the data sheet.

3.2 Salinity Adjustment

3.21 Reducing the salinity of aqueous samples

Refer to the formulas below to calculate the volume of HPLC water needed to reduce the initial sample salinity to the target salinity. Add the volume calculated, mix the bottle thoroughly, check the salinity with a refractometer, and record the volume of HPLC water added as well as the final salinity.

(i) (target $^{o}/_{oo}$ ÷ sample $^{o}/_{oo}$) × sample vol. in mL = A
(ii) sample vol. - A = B
(iii) sample vol. ÷ A = C
(iv) B × C = volume of HPLC water to add

3.22 Increasing the salinity of aqueous samples

Refer to the formula below to calculate the volume of concentrated brine needed to increase the initial sample salinity to the target salinity. Add the volume calculated, mix the bottle thoroughly, check the salinity with a refractometer, and record the volume of brine added as well as the final salinity.

(i) ((target ‰ - sample ‰) × sample vol. in mL) ÷ (brine ‰ - target ‰) = vol. of brine to add

3.3 Dissolved Oxygen Adjustment

Measure and record DO and percent DO saturation of sample (SOP F10.13). Occasionally, a sample will have DO of less than 80% saturation. Any such samples should be gently stirred on a magnetic stirrer to increase the DO level above 80%. Record initial DO, the elapsed mixing time, and final DO in the comments section of the Water Quality Adjustment Data Form. (On the following day, DO should be rechecked and brought to >80% by stirring again if necessary before the toxicity test is performed.)

3.4 Other Water Quality Determinations

1. Measure pH (SOP F10.21) and record on the Water Quality Adjustment Data Form.

2. Measure and record ammonia concentration (SOP F10.4).

3. Measure and record sulfide concentration if required.

4.0 DATA COLLECTION

All raw data are entered on one standardized form, the Water Quality Adjustment Data Form (see Attachment 2) at the time the determinations or adjustments are made.

5.0 QUALITY CONTROL

A data form (Attachment 2) will be used to document all sample handling procedures for each sample. The person(s) recording data on the sheet will initial each sheet. Original data forms after completion will be stored in a three-ring file in the possession of the field station leader. Copies will be kept in the lab.

6.0 TRAINING

Personnel who will perform this task should first read this protocol and then operate under supervision during the preparation of at least two samples.

7.0 SAFETY

The NaOH solution used in the ammonia determination procedure is a highly caustic liquid. Care should be taken to avoid its contact with skin or clothing. Should such contact occur, quickly flush affected with water. A sink is present along the west wall of the dry lab, another is present along the east wall of the wet lab, and an eye flushing station is present in the northwest corner of the wet lab near the entrance door. The samples handled may be pore water, effluent, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with the samples.

8.0 ATTACHMENTS

Attachment 1. Equipment List for Water Quality Adjustment
Attachment 2. Water Quality Adjustment Data Form

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ATTACHMENT 1

EQUIPMENT LIST FOR WATER QUALITY ADJUSTMENT

Graduated cylinders
Pipetters
Latex gloves
Magnetic stirrer and stir bars
10 M NaOH
Concentrated brine (See section 2.2 for preparation)
HPLC ultrapure sterile water (J.T. Baker® #JT4218-2)
Salinity refractometer
Dissolved oxygen meter
pH electrode, buffer solutions, and meter
Ammonia electrode, standard solutions, and meter
Sulfide electrode, standard solutions, and meter
Data sheets
Hand calculator

ATTACHMENT 2

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

Initial volume (mL)	_____
Initial salinity (‰)	_____
Vol. Baker® HPLC water added (mL)	_____
Vol. ____‰ brine added (mL)	_____
% of original sample (initial vol./final vol. x 100)	_____

B. Character of Sample (after salinity adjustment):

Final Volume (mL)	_____
Final Salinity (‰)	_____
pH	_____
Dissolved oxygen (mg/L)	_____
DO saturation (%)	_____
Total ammonia (mg/L)	_____
Sulfide (mg/L)	_____

COMMENTS_____

Date Prepared : April 10, 1990

Date Revised: March 10, 1995

SEA URCHIN FERTILIZATION TOXICITY TEST

1.0 OBJECTIVE

The purpose of the fertilization toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample reduces fertilization of exposed gametes relative to that of gametes exposed to a reference sample. The test may also be used to determine the concentration of a test substance which reduces fertilization. Test results are reported as treatment (or concentration) which produces statistically significant reduced fertilization or as concentration of test substance which reduces fertilization by 50 percent (EC_{50}). This test can be performed concurrently with Sea Urchin Embryological Development Toxicity Test (SOP 10.7) and/or Sea Urchin Genotoxicity/Teratogenicity Test (SOP 10.8), using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

Gametes from the sea urchin, *Arbacia punctulata* are used in the sea urchin fertilization toxicity test. Animals can be collected in the field or obtained from a commercial supplier. *A. punctulata* can be differentiated from other species of urchins which are found in Texas by the five plates surrounding the anal opening, and by round sharp spines on the dorsal surface of the test and flattened spines surrounding the Aristotle's lantern. Urchins can be maintained easily in aquaria or other tanks with running seawater or an aquarium filter. Urchins will eat a wide variety of marine vegetation. A good diet may be provided by placing rocks from jetties (which have been colonized by diatoms and macroalgae) into the tank with the urchins or romaine lettuce may be provided as a substitute. Temperature manipulations of the cultures will prolong the useful life of the urchins. Cultures are maintained at 16 ± 1°C when gametes are not required. Temperature is gradually increased to 19 ± 1°C at least one week prior to gamete collection and subsequently decreased if no further tests are planned. Photoperiod is maintained at 16 hours of light per day. Water quality parameters should be monitored weekly and salinity maintained at 30 ± 3 $^{o}/_{oo}$. Males and females should be kept in separate tanks.

2.2 Dilution Water

HPLC reagent grade purified water or concentrated seawater brine is used to adjust samples to 30 $^{o}/_{oo}$ as described in Water Quality Adjustment of Samples (SOP 10.12). Concentrated seawater brine (90-110 $^{o}/_{oo}$) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine quality will remain constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 µm) seawater adjusted to 30 $^{o}/_{oo}$ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

When testing samples for potential toxicity, five replicates per treatment are recommended. One replicate is a 5 mL volume of sample in a disposable glass scintillation vial. When conducting a dilution series test, fifty percent serial dilutions may be made in the test vials, using MFS as the diluent.

2.3.1 Equipment

A list of equipment necessary for conducting this test is given in Attachment 1 (Equipment List for Fertilization Toxicity Test).

2.3.2 Solutions

<u>10% Buffered Formalin</u>:

1,620 mL sea water
620 mL formaldehyde
6.48 g NaH_2P0_4 or KH_2PO_4 (mono)
10.5 g Na_2HPO_4 or K_2HPO_4 (dibasic)

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

Quality gametes must first be collected, and then diluted to the appropriate concentration for addition to the test vials.

2.4.1 Selection of Urchins to be Used in Toxicity Test.

1. Take two or three females and place in shallow bowl, barely covering tests with seawater.

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V transformer.

3. Collect a few eggs from between spines using a 10 mL disposable syringe with a large gauge blunt-tipped needle attached. Discard the first small quantity of eggs expelled from each gonopore and continue collecting. Place a 2 to 5 drops of eggs onto a scintillation vial containing 10ml of filtered seawater. Rinse syringe and repeat for each female.

4. Select females which have round, well developed eggs, and which do not release clumps of eggs or undeveloped ovarian tissue.

5. Place 2-4 males in shallow bowl(s) with a small amount of seawater, leaving the upper $^1/_2$ to $^1/_3$ of the animals uncovered.

6. Stimulate release of sperm from gonopores by touching test with electrodes from 12V transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

Generally, a gamete check is performed in order to ensure that both the male and the female urchins used in the test have gametes with a high degree of viability. If the gamete check is performed, two to five females (depending on confidence in the proportion of urchins in the holding facility in good reproductive status) and at least two males should be selected using the above procedures. The check is performed by adding 5 to 7 drops of a concentrated dilution of sperm to the eggs in the scintillation vials (collected as described above) and observing the eggs under the microscope after 10 minutes. The concentrated dilution of sperm is usually made by diluting 20-50µl of sperm in 10 ml of filtered seawater. If the proportion of eggs fertilized is high (95-100%), that female and male may be used in the pretest and test. Sperm from a number of males or females may be combined in the beginning if the gamete check reveals a number of high quality animals or the confidence is high in the quality of the gametes Once a good male and female are selected a pretest can be conducted to determine the correct dilution of sperm to use in the test (Attachment 2).

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V transformer.

2. Collect eggs as above using the 10 mL syringe. Remove needle before dispensing eggs into a disposable shell vial or other clean container capable of holding 25-50 mL. Collect enough eggs for pretest and test. If female stops giving eggs readily or starts giving chunky material, cease stimulation and collection of eggs from that female.

3. Add MFS to fill shell vials, gently mixing eggs. Allow eggs to settle to bottom of vial. Remove water with a pipette. Replace water, again gently mixing the eggs.

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 $^{o}/_{oo}$ MFS in a 250 mL beaker, and add enough washed eggs to bring the egg density to approximately 10,000 per mL . If more than 400 total replicates (27 treatments) are to be tested, a larger amount of water and a correspondingly larger amount of eggs should be used. Two hundred µL of this egg solution will be used per replicate, and it is easier to maintain proper mixing and uniform egg density if there is an excess of at least 50%.

2. Check egg density and adjust to within approximately 9000 to 11,000 eggs per mL, as follows. Gently swirl egg solution until evenly mixed. Using a pipette, add 1 mL of the solution to a vial containing nine mL seawater. Mix and transfer 1 mL of this diluted solution to a second vial containing 4 mL of seawater. Again, mix and transfer 1 mL of this diluted solution to a counting slide such as a Sedgewick-Rafter slide.

3. Using a microscope (either a compound microscope with a 10x objective or a dissecting scope may be used here), count the number of eggs on the slide. If the number is not between 180 and 220, then adjust by adding eggs or water. If egg count is > 220 use the following formula to calculate the amount of water to add:

   ("egg count" - 200/200) x Current Volume of Eggs = Volume seawater to add to stock (mLs)

   If egg count < 200 add a small amount of eggs. Since it is less arbitrary and more likely to arrive at an acceptable count when using the water addition formula, it is better to originally overestimate the amount of eggs to add to the 100 mL of water.

4. Repeat steps 2 and 3 until an acceptable egg count (between 180 and 220) is obtained.

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V transformer, and collect about 0.5 mL of unwetted sperm from between spines using a pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Be careful not to add any water or sperm which has contacted water to the vials. High quality sperm collected dry and kept on ice will last at least eight hours without measurable decline in viability.

2.4.5 Prepare Appropriate Sperm Dilution

It is desirable for control fertilization to be within 60-90%. Although controls outside these bounds do not automatically disqualify a test, particularly if a valuable dose response is generated, the sensitivity of the test is reduced by fertilization rates greater than 90% and good dose responses may be difficult to obtain with less than 60% fertilization in controls. Density of sperm in the sperm solution should be determined with this goal in mind. Condition of the animals and length of acclimation to the aquarium may effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:10,000 and 1:2500 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:5000 is required (as determined from the pretest), add 20 µL sperm to 10 mL MFS. Mix thoroughly, then add 1 mL of this solution to 9 mL MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 30 minutes before the test has begun, including sperm dilutions used in any pretest, should be discarded and a new dilution made from sperm kept on ice.

3.0 TEST PROCEDURES

1. Add 50 µL appropriately diluted sperm to each vial. Record time of sperm addition. Sperm should be used within 30 minutes of wetting.

2. Incubate all test vials at 20 ± 2°C for 30 minutes. At this point it is useful to set a timer for five to ten minutes prior to the end of the incubation period. This will notify the worker early enough to be ready to start the next step exactly on time.

3. While gently swirling the egg solution to maintain even mixing of eggs, use a 200 µL pipetter to add 200 µL diluted egg suspension to each vial. Pipette tips are cut back using

a clean razor blade to prevent crushing the eggs during pipetting. Record time of egg addition.

4. Incubate for 30 minutes at 20 ± 2°C. The timer may be used again at this point.

5. Using the dispenser, add 1 mL of 10% buffered formalin to each sample.

6. Vials may now be capped and stored overnight or for several days until evaluated. Fertilization membranes are easiest to see while eggs are fairly fresh, so evaluation within two to three days may decrease the time required for evaluation.

7. If it is not possible to make the evaluations within several days or the membranes are difficult to discern, an optional technique may be employed. Make up a 200 $^{o}/_{oo}$ NaCl solution (pickling salt) and add 2 to 4 drops of the solution to a 1 mL egg sample on a microscope slide. This solution causes the egg, but not the membrane, to shrink briefly thereby making the membrane easier to see. The effect only lasts for a short time (~5 min.) so the observations must be made immediately after the NaCl solution is added. If this optional technique is employed, it must be used on all samples in that test series.

4.0 DATA COLLECTION AND TABULATION

1. Transfer approximately 1 mL eggs and water from bottom of test vials to counting slide. Observe eggs using compound microscope under 100X magnification. Dark field viewing is useful here in identifying fertilization membranes.

2. Count 100 eggs/sample using hand counter with multiple keys (such as a blood cell counter), using one key to indicate fertilized eggs and another to indicate unfertilized eggs. Fertilization is defined by the presence of fertilization membrane surrounding egg.

3. Calculate fertilization percentage for each replicate test:

$$\frac{\text{Total No. Eggs - No. Eggs Unfertilized}}{\text{Total No. Eggs}} \times 100 = \text{Percent Eggs Fertilized}$$

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 3-7). Normally, percent fertilization in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea surface microlayer from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's *t*-test (Sokal and Rohlf 1981) on the arc sine square root transformed data. For multiple comparisons among treatments, Ryan's Q test (Day and Quinn 1989) with the arc sine square root transformed data is recommended. The trimmed Spearman-Karber method with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Quality control tests may be run using both positive and negative controls with multiple replicates (as many as desired). Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is tested with each test to evaluate the effectiveness of the sperm dilution chosen. Negative controls may include a reference porewater, filtered seawater, and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining egg concentrations and fertilization counts are test specific activities. These functions can be performed independently after a trainee has demonstrated he or she can accurately reproduce the test.

8.0 SAFETY

The sea urchin fertilization toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10% buffered formalin solution; use a hood if available, but make sure the test area is well ventilated. Protective gloves can be worn when pipetting or dispensing formalin or potentially toxic samples.

Care should be taken when collecting or otherwise handling sea urchins. Urchin spines are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

Attachment 1. Equipment List for Fertilization Toxicity Test
Attachment 2. Pretest to Insure Selection of Quality Gametes
Attachment 3. Water Quality Adjustment Data Form
Attachment 4. Sea Urchin Pretest Data Sheet
Attachment 5. Sea Urchin Pretest Continuation Data Sheet
Attachment 6. Sea Urchin Fertilization/Embryological Development Toxicity Test Gamete Data Sheet
Attachment 7. Sea Urchin Fertilization Toxicity Test Fertilization Data Sheet

10.0 REFERENCES

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Attachment 1

EQUIPMENT LIST FOR FERTILIZATION TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL KIMBLE scintillation vials (These should be type shipped with caps off, and without cap liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 µL pipetter
50-200 µL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 µm) seawater, adjusted to 30 $^{o}/_{oo}$
Data sheets
Baker reagent grade water
Approximately 100 $^{o}/_{oo}$ concentrated brine

Attachment 2
PRETEST TO INSURE SELECTION OF QUALITY GAMETES

1. Using the procedure in section 2.4.1, select 2 to 5 females and at least 2 male urchins to be used in the pretest.

2. Fill pretest vials with five mL of **reference** water. There should be at least two vials for each combination of male, female, and pretest sperm concentration (step 4 below). For example, in a pretest with two females, one male, and six pretest sperm concentrations, 24 vials (2 X 2 X 6) would be needed. Arrange and mark vials accordingly in a rack.

3. Perform steps 2.4.2 (egg collection) and 2.4.3 (egg dilution) for each female urchin. Make enough volume of the egg suspension to perform the pretest and the test.

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90% fertilization rate in the test. Sperm dilution will depend on the health and reproductive status of the male urchin, but in most cases the following "standard dilution" should be used:

1: 250 (20 µL dry sperm added to 5 mL MFS. This concentration is used only as stock solution to make up the rest of the dilution series and is not used full strength in the pretest.)
1: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

Sperm must be used within 30 minutes of dilution. Leave undiluted sperm on ice and retain, because a new sperm dilution of the concentration determined in this pretest will be needed for the toxicity test. **Sperm diluted for use in the pretest may not be used in the toxicity test, because the time elapsed since the addition of water is too great.**

5. As in section 3.0 add 50 µL of the diluted sperm to each pretest vial. Incubate for 30 minutes at approximately 20°C, and add 200 µL of the egg suspension. Incubate for another 30 minutes, then fix with 1 mL of the buffered formalin solution.

6. As in section 4.0, obtain a fertilization rate for the vials. There is no need to count all vials, enough vials should be counted to determine a good male/female combination, and an appropriate sperm dilution factor. If more than one male/female combination is acceptable, this is a good opportunity to choose a female which exhibits easily visible fertilization membranes or in cases where there are many samples, to combine eggs from different females . The appearance of the fertilization membranes may vary among female urchins, and presence of easily visible membranes facilitates counting.

Attachment 3

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

Initial volume (mL)	_____
Initial salinity (‰)	_____
Vol. Milli-Q water added (mL)	_____
Vol. ___‰ brine added (mL)	_____
% of original sample (initial vol./final vol. x 100)	_____

B. Character of Sample (after salinity adjustment):

Volume (mL)	_____
Salinity (‰)	_____
pH	_____
Dissolved oxygen (mg/L)	_____
DO saturation (%)	_____
Total ammonia (mg/L)	_____
Sulfide (mg/L)	_____

COMMENTS_____

_____

_____

_____

_____

Attachment 4

SEA URCHIN PRETEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

Female number:	____	____	____	____
Collection time:	____	____	____	____
Count:	____	____	____	____

SPERM

Male number:	____	____	____	____
Collection time:	____	____	____	____
Dilution start time:	____	____	____	____

TEST TIMES

Sperm in:_____ Eggs in:_____ Formalin in:_____

SPERM DILUTION _____

COMMENTS _____

_____

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

Sperm Dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 5

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID________________________ INITIALS____________

STUDY PROTOCOL____________________ DATE____________

% FERTILIZATION Reference sample designation:____________

Female #____________ Male #____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #____________ Male #____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #____________ Male #____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #____________ Male #____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 6

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT

TOXICITY TEST GAMETE DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

Collection time:_____

Initial count/volume:_____

Final count:_____

SPERM

Collection time:_____ Dilution start time:_____

Sperm dilution:_____

_____

Test start temperature:_____

TEST TIMES

Box #	Sperm in:	Eggs in:	Formalin in:

COMMENTS_____

_____

_____

Attachment 7

SEA URCHIN FERTILIZATION TOXICITY TEST

FERTILIZATION DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

PERCENT FERTILIZED

	Replicate						
Treatment	1	2	3	4	5	Mean±SD	Unfert.

COMMENTS _____

Date Prepared : April 10, 1990

Date Revised: August 15, 1995

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

1.0 OBJECTIVE

The purpose of the embryological development toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample affects development of exposed embryos (development arrested at an early stage or a developmental abnormality) relative to that of embryos exposed to a reference sample. The test may also be used to determine the concentration of a test substance which affects development. Test results are reported as treatment (or concentration) which produces statistically significant developmental effect. This test can be performed concurrently with Sea Urchin Fertilization Toxicity Test (SOP 10.6) and/or Sea Urchin Genotoxicity/Teratogenicity Test (SOP 10.8), using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

Gametes from the sea urchin, *Arbacia punctulata* are used in the sea urchin embryological development toxicity test. Animals can be collected in the field or obtained from a commercial supplier. *A. punctulata* can be differentiated from other species of urchins which are found in Texas by the five plates surrounding the anal opening, and by round sharp spines on the dorsal surface of the test and flattened spines surrounding the Aristotle's lantern. Urchins can be maintained easily in aquaria or other tanks with running seawater or an aquarium filter. Urchins will eat a wide variety of marine vegetation. A good diet may be provided by placing rocks from jetties (which have been colonized by diatoms and macroalgae) into the tank with the urchins or romaine lettuce may be provided as a substitute. Temperature manipulations of the cultures will prolong the useful life of the urchins. Cultures are maintained at 16 ± 1°C when gametes are not required. Temperature is gradually increased to 19 ± 1°C at least one week prior to gamete collection and subsequently decreased if no further tests are planned. Photoperiod is maintained at 16 hours of light per day. Water quality parameters should be monitored weekly and salinity maintained at 30 ± 3 $^{o}/_{oo}$. Males and females should be kept in separate tanks.

2.2 Dilution Water

HPLC reagent grade purified water or concentrated seawater brine is used to adjust samples to 30 $^{o}/_{oo}$ as described in Water Quality Adjustment of Samples (SOP 10.12). Concentrated seawater brine (90-110 $^{o}/_{oo}$) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine quality will remain constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 μm) seawater adjusted to 30 $^{o}/_{oo}$ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

When testing samples for potential toxicity, five replicates per treatment are recommended. One replicate is a 5 mL volume of sample in a disposable glass scintillation vial. When conducting a dilution series test, fifty percent serial dilutions may be made in the test vials, using MFS as the diluent.

2.3.1 Equipment

A list of equipment necessary for conducting this test is given in Attachment 1 (Equipment List for Embryological Development Toxicity Test).

2.3.2 Solutions

<u>10% Buffered Formalin</u>:

1,620 mL sea water
620 mL formaldehyde
6.48 g NaH_2P0_4 or KH_2PO_4 (mono)
10.5 g Na_2HPO_4 or K_2HPO_4 (dibasic)

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

Quality gametes must first be collected, and then diluted to the appropriate concentration for addition to the test vials.

2.4.1 Selection of Urchins to be Used in Toxicity Test.

1. Take two or three females and place in shallow bowl, barely covering tests with seawater.

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V transformer.

3. Collect a few eggs from between spines using a 10 mL disposable syringe with a large gauge blunt-tipped needle attached. Discard the first small quantity of eggs expelled from each gonopore and continue collecting. Place a 2 to 5 drops of eggs onto a scintillation vial containing 10mL of filtered seawater. Rinse syringe and repeat for each female.

4. Select females which have round, well developed eggs, and which do not release clumps of eggs or undeveloped ovarian tissue.

5. Place 2-4 males in shallow bowl(s) with a small amount of seawater, leaving the upper $^1/_2$ to $^1/_3$ of the animals uncovered.

6. Stimulate release of sperm from gonopores by touching test with electrodes from 12V transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

Generally, a gamete check is performed in order to ensure that both the male and the female urchins used in the test have gametes with a high degree of viability. If the gamete check is performed, two to five females and at least two males should be selected using the above procedures. The check is performed by adding 5 to 7 drops of a concentrated dilution of sperm to the eggs in the scintillation vials (collected as described above) and observing the eggs under the microscope after 10 minutes. The concentrated dilution of sperm is usually made by diluting 20-50µL of sperm in 10 mL of filtered seawater. If the proportion of eggs fertilized is high (95-100%), that female and male may be used in the pretest and test. Sperm from a number of males or eggs of females may be combined if the gamete check reveals a number of high quality animals or the confidence is high in the quality of the gametes Once a good male and female are selected a pretest can be conducted to determine the correct dilution of sperm to use in the test (Attachment 2).

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V transformer.

2. Collect eggs as above using the 10 mL syringe. Remove needle before dispensing eggs into a disposable shell vial or other clean container capable of holding 25-50 mL. Collect enough eggs for pretest and test. If female stops giving eggs readily or starts giving chunky material, cease stimulation and collection of eggs from that female.

3. Add MFS to fill shell vials, gently mixing eggs. Allow eggs to settle to bottom of vial. Remove water with a pipette. Replace water, again gently mixing the eggs.

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 $^{o}/_{oo}$ MFS in a 250 mL beaker, and add enough washed eggs to bring the egg density to approximately 10,000 per mL . If more than 400 total replicates (27 treatments) are to be tested, a larger amount of water and a correspondingly larger amount of eggs should be used. Two hundred µL of this egg solution will be used per replicate, and it is easier to maintain proper mixing and uniform egg density if there is an excess of at least 50%.

2. Check egg density and adjust to within approximately 9000 to 11,000 eggs per mL, as follows. Gently swirl egg solution until evenly mixed. Using a pipette, add 1 mL of the solution to a vial containing nine mL seawater. Mix and transfer 1 mL of this diluted solution to a second vial containing 4 mL of seawater. Again, mix and transfer 1 mL of this diluted solution to a counting slide such as a Sedgewick-Rafter slide.

3. Using a microscope (either a compound microscope with a 10x objective or a dissecting scope may be used here), count the number of eggs on the slide. If the number is not between 180 and 220, then adjust by adding eggs or water. If egg count is > 220 use the following formula to calculate the amount of water to add:

   ("egg count" - 200/200) x Current Volume of Eggs = Volume seawater to add to stock (mL)

   If egg count < 200 add a small amount of eggs. Since it is less arbitrary and more likely to arrive at an acceptable count when using the water addition formula, it is better to originally overestimate the amount of eggs to add to the 100 mL of water.

4. Repeat steps 2 and 3 until an acceptable egg count (between 180 and 220) is obtained.

5. Just before the eggs are to be used, add 2 mL of a penicillin-G stock solution (5000 units/mL) per 100 mL of eggs in the egg suspension. The addition of penicillin to the embryological development test has been shown to be beneficial in evalution of the stages of development by inhibiting bacterial growth which can cause the embryos to disintegrate before the test is terminated.

   The penicillin stock solution is prepare by diluting 296 mg of Penicillin-G sodium salt (1690 units/mg) in 100 mL of MFS and mixing until dissolved. The addition of 2 mL/100 mL of eggs will result in a final concentration of 4 units/mL in each replicate. The number of units of penicillin per mg of penicillin-G sodium salt is variable with each lot. Thus, the quantity added to the stock will change in order to keep the final concentration at 4 units/mL.

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V transformer, and collect about 0.5 mL of unwetted sperm from between spines using a pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Be careful not to add any water or sperm which has contacted water to the vials. High quality sperm collected dry and kept on ice will last at least eight hours without measurable decline in viability.

2.4.5 Prepare Appropriate Sperm Dilution

As in the Sea Urchin Fertilization Test, it is desirable for control fertilization to be within 60-90%. Although controls outside these bounds do not automatically disqualify a test, particularly if a valuable dose response is generated, the chance of inducing polyspermy is increased with increased concentrations of sperm, and good dose responses may be difficult to obtain with less than 60% fertilization in controls. Density of sperm in the sperm solution should be determined with this goal in mind.' Condition of the animals and length of acclimation to the aquarium may effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:10,000 and 1:2500 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:5000 is required (as determined from the pretest), add 20 µL sperm to 10 mL MFS. Mix thoroughly, then add 1 mL of this solution to 9 mL MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 30 minutes before the test has begun, including sperm dilutions used in any pretest, should be discarded and a new dilution made from sperm kept on ice.

3.0 TEST PROCEDURES

1. While gently swirling the egg solution to maintain even mixing of eggs, use a 200 μL pipetter to add 200 μL diluted egg suspension to each vial. Record time of egg addition.

2. Add 50 μL appropriately diluted sperm to each vial. Record time of sperm addition. Sperm should be used within 30 minutes of wetting.

3. Incubate all test vials at 20 $\pm$ 1°C for 48 hours.

4. Using the dispenser, add 1 mL 10% buffered formalin to each vial.

5. Vials may now be capped and stored overnight or for several days until evaluated.

4.0 DATA COLLECTION AND TABULATION

1. Transfer approximately 1 mL embryos and water from bottom of test vials to counting slide. Observe embryos using a compound microscope under 100X magnification.

2. Count 100 embryos/sample using hand counter with multiple keys (such as a blood cell counter), using one key to indicate normally developed pluteus larvae and others to indicate unfertilized eggs, embryos arrested in earlier developmental stages, and other abnormalities. Attachment 3 has a list of developmental stages and drawings of each.

3. Calculate the proportion of normal plutei for each replicate test:

$$\frac{\text{Number normal plutei X 100}}{\text{Total no. eggs/embryos}} = \text{Percent normal plutei}$$

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 4-9). Normally, percent normal development (normal plutei) in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea surface microlayer from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's *t*-test (Sokal and Rohlf 1981) on the arc sine square root transformed data. For multiple comparisons among treatments, Ryan's Q test (Day and Quinn 1989) with the arc sine square root transformed data is recommended. The trimmed Spearman-Karber method

with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Quality control tests may be run using both positive and negative controls with multiple replicates (as many as desired). Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is tested with each test to evaluate the effectiveness of the sperm dilution chosen. Negative controls may include a reference porewater, filtered seawater, and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining egg concentrations, embryological stages and counts are test specific activities. These functions can be performed independently after a trainee has demonstrated he or she can accurately reproduce the test.

8.0 SAFETY

The sea urchin embryological development toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10% buffered formalin solution; use a hood if available, but make sure the test area is well ventilated. Protective gloves can be worn when pipetting or dispensing formalin or potentially toxic samples.

Care should be taken when collecting or otherwise handling sea urchins. Urchin spines are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

Attachment 1. Equipment List for Embryological Development Toxicity Test
Attachment 2. Pretest to Insure Selection of Quality Gametes
Attachment 3. Development of Sea Urchin Eggs to Pluteus Larvae
Attachment 4. Water Quality Adjustment Data Form
Attachment 5. Sea Urchin Pretest Data Sheet
Attachment 6. Sea Urchin Pretest Continuation Data Sheet
Attachment 7. Sea Urchin Fertilization/Embryological Development Toxicity Test Gamete Data Sheet
Attachment 8. Sea Urchin Embryological Development Test Data Sheet
Attachment 9. Sea Urchin Embryological Development Test Continuation Data Sheet

10.0 REFERENCES

Day, R.W. and G.P. Quinn. 1989. Comparisons of treatments after an analysis of variance in ecology. Ecol. Monogr. 59:433-463.

Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7):714-719; Correction 12(4):417 (1978)

Sokal, R.R., and F.J. Rohlf. 1981. Biometry. 2nd edition. W.H. Freeman and Company, San Francisco, CA 859 pp.

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Attachment 1

EQUIPMENT LIST FOR EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL KIMBLE scintillation vials (These should be type shipped with caps off, and without cap liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 µL pipetter
50-200 µL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 µm) seawater, adjusted to 30 $^{o}/_{oo}$
Data sheets
Baker reagent grade water
Approximately 100 $^{o}/_{oo}$ concentrated brine

Attachment 2
PRETEST TO INSURE SELECTION OF QUALITY GAMETES

1. Using the procedure in section 2.4.1, select 2 to 5 females and at least 2 male urchins to be used in the pretest.

2. Fill pretest vials with five mL of **reference** water. There should be at least two vials for each combination of male, female, and pretest sperm concentration (step 4 below). For example, in a pretest with two females, one male, and six pretest sperm concentrations, 24 vials (2 X 2 X 6) would be needed. Arrange and mark vials accordingly in a rack.

3. Perform steps 2.4.2 (egg collection) and 2.4.3 (egg dilution) for each female urchin. Make enough volume of the egg suspension to perform the pretest and the test.

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90% fertilization rate in the test. Sperm dilution will depend on the health and reproductive status of the male urchin, but in most cases the following "standard dilution" should be used:

1:250 (20 µL dry sperm added to 5 mL MFS. This concentration is used only as stock solution to make up the rest of the dilution series and is not used full strength in the pretest.)
1: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

Sperm must be used within 30 minutes of dilution. Leave undiluted sperm on ice and retain, because a new sperm dilution of the concentration determined in this pretest will be needed for the toxicity test. **Sperm diluted for use in the pretest may not be used in the toxicity test, because the time elapsed since the addition of water is too great.**

5. As in section 3.0 add 50 µL of the diluted sperm to each pretest vial. Incubate for 30 minutes at approximately 20°C, and add 200 µL of the egg suspension. Incubate for another 30 minutes, then fix with 1 mL of the buffered formalin solution.

6. As in section 4.0, obtain a fertilization rate for the vials. There is no need to count all vials, enough vials should be counted to determine a good male/female combination, and an appropriate sperm dilution factor. If more than one male/female combination is acceptable, this is a good opportunity to choose a female which exhibits easily visible fertilization membranes or in cases where there are many samples, to combine eggs from different females . The appearance of the fertilization membranes may vary among female urchins, and presence of easily visible membranes facilitates counting.

Attachment 4

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

Initial volume (mL)	_____
Initial salinity (‰)	_____
Vol. Milli-Q water added (mL)	_____
Vol. ___‰ brine added (mL)	_____
% of original sample (initial vol./final vol. x 100)	_____

B. Character of Sample (after salinity adjustment):

Volume (mL)	_____
Salinity (‰)	_____
pH	_____
Dissolved oxygen (mg/L)	_____
DO saturation (%)	_____
Total ammonia (mg/L)	_____
Sulfide (mg/L)	_____

COMMENTS_____

_____

_____

_____

_____

Attachment 5

SEA URCHIN PRETEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

Female number:	____	____	____	____
Collection time:	____	____	____	____
Count:	____	____	____	____

SPERM

Male number:	____	____	____	____
Collection time:	____	____	____	____
Dilution start time:	____	____	____	____

TEST TIMES

Sperm in:_____ Eggs in:_____ Formalin in:_____

SPERM DILUTION _____

COMMENTS _____

_____

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

Sperm Dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 6

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID____________________ INITIALS____________

STUDY PROTOCOL____________________ DATE____________

% FERTILIZATION Reference sample designation:____________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 7

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT

TOXICITY TEST GAMETE DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

Collection time:_____

Initial count/volume:_____

Final count:_____

SPERM

Collection time:_____ Dilution start time:_____

Sperm dilution:_____

_____

Test start temperature:_____

TEST TIMES

Box #	Sperm in:	Eggs in:	Formalin in:
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____
____	_____	_____	_____

COMMENTS_____

_____

_____

Attachment 8

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

Test Start (date & hour)_____ Test stopped (date & hour)_____

Treatment	Rep.	Eggs	Blastula	Early Gastrula	Late Gastrula	Pluteus	% Normal Development	%Non-Norm

COMMENTS_____

Attachment 9

EMBRYOLOGICAL DEVELOPMENT TEST CONTINUATION DATA SHEET

Test Id_____ Initials _____

Study protocol_____ Date_____

Treatment	Rep.	Eggs	Blastula	Early Gastrula	Late Gastrula	Pluteus	% Normal Development	%Non-Norm

COMMENTS_____