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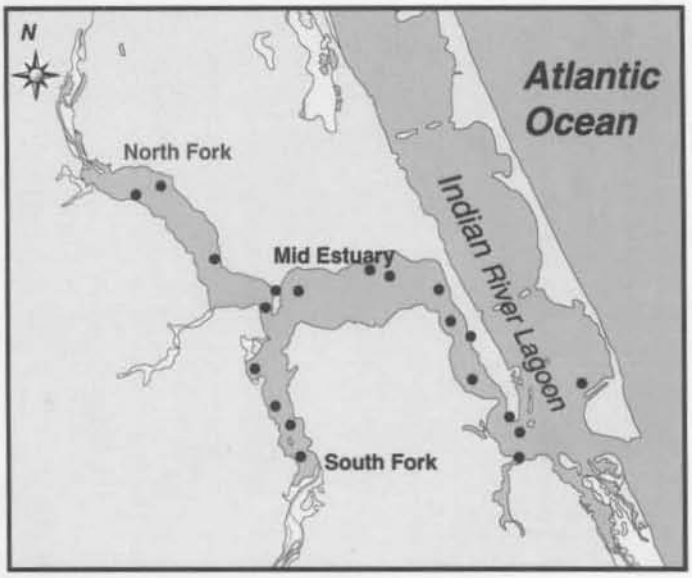
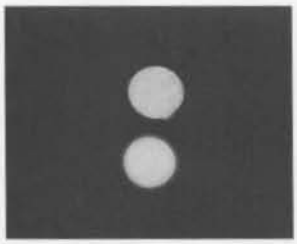
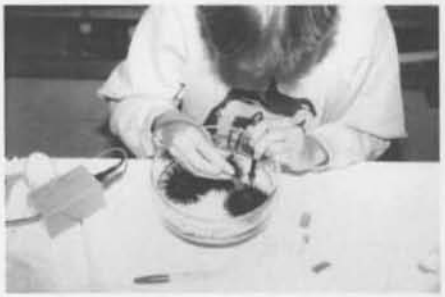
Final Report

on

TOXICITY TESTING OF SEDIMENTS FROM ST. LUCIE ESTUARY, FLORIDA







December 14, 2001

submitted to

National Oceanic Atmospheric Administration
Center for Coastal Monitoring and Assessment
1305 East-West Hwy.
Silver Springs, Maryland 20910

from

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INTRODUCTION

The National Status and Trends (NS&T) Program of NOAA has shown that some sampling sites in coastal Florida bays are relatively highly contaminated with a variety of chemicals, and the potential for adverse biological effects at these sites is among the highest of all of the sites in the USA. As part of a multi disciplinary sediment quality survey conducted to determine the severity and spatial extent of the toxicity of surficial sediments of St. Lucie, Florida and the adjoining tributaries and canals, toxicity of sediments collected from these sites was assessed using porewater in the sea urchin (*Arbacia punctulata*) fertilization. Sediment samples were collected by NOAA and shipped to the U. S. Geological Survey (USGS) Marine Ecotoxicology Research Station (MERS) in Corpus Christi, Texas where the tests were performed. Sediment pore water was extracted with a pneumatic apparatus similar to the one used in previous studies (Carr and Chapman, 1992; 1995; Carr et al., 1996a; 1996b; NBS, 1993; 1994; 1995a; 1995b, USGS, 1997a; 1997b; 1998; 1999a; 1999b; 2000a; 2000b; 2000c). Porewater samples were stored frozen until just prior to testing when water quality parameters were measured and adjusted, if necessary. A dilution series (100, 50 and 25%) test design was used to determine the toxicity of sediment porewater samples. Additional subsamples of porewater were analyzed for dissolved organic carbon.

The specific objectives of this study were to:

- Extract sediment pore water from all sediment samples as soon as possible after receipt of the samples using a pneumatic extraction device.
- Measure water quality parameters (salinity, dissolved oxygen, pH, sulfide, temperature, and ammonia) of thawed porewater samples prior to testing and adjust salinity, temperature, and dissolved oxygen, if necessary.
- Conduct the fertilization toxicity test with pore water using gametes of the sea urchin *Arbacia punctulata*. A control reference pore water and a dilution series with sodium dodecyl sulfate (SDS) as a quality assurance control was run in conjunction with each test.
- Make statistical comparisons between test and reference stations for the pore water and calculate EC_{50} values where possible.
- Measure Dissolved Organic Carbon (DOC) in pore water from all stations.

MATERIALS AND METHODS

Sediment Sample Receipt and Tracking

Surficial sediment samples were collected from 21 stations from the St. Lucie and Jupiter estuaries and sent to the USGS MERS in Corpus Christi, Texas between 5/9 and 5/15/2001. Samples were collected by personnel of the Bioeffects Assessment Branch of NOAA. Homogenized samples were placed in precleaned one gallon high density polyethylene containers, chilled, and shipped in insulated coolers with blue ice. Samples were received by the USGS MERS in Corpus Christi, Texas, the day following shipment. With the exception of one sample station, shipments were accompanied by sample tracking sheets. All samples were logged into a laboratory sample tracking system. Incoming sample temperatures ranged from 4 to 7°C. Samples were processed for porewater extraction upon receipt. Porewater samples were extracted within 5 days of the time of field collection of sediment, and within 8 hours of arrival at the Corpus Christi laboratory and frozen at - 20°C. All samples were held refrigerated at 4°C or were placed on blue ice and chilled while awaiting processing.

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, 1993; NBS, 1993; 1994; 1995a, 1995b; USGS 1997a; 1997b, 1998; 1999a; 1999b; 2000a; 2000b; 2000c). 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 as soon as possible after receipt of the samples but in no event were the sediments held longer than 24 hours from the time of receipt before they were processed. After extraction, the porewater samples were centrifuged in polycarbonate bottles at 1200 x *g* for 20 min to remove any suspended particulate material; the supernatant was collected and frozen.

Two days before conducting a toxicity test, porewater samples were moved from the freezer to a refrigerator at 4°C. One day prior to testing, samples were completely thawed (if necessary) in a tepid water bath. Temperature of the samples was maintained at 20 ± 2°C. Sample salinity was measured and adjusted to 30 ± 1 $^{o}/_{oo}$, if necessary, using purified deionized 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® refractometer; and pH, sulfide (as S^{-2}), and total ammonia (TAN) were measured with Orion® meters and their respective probes. Unionized ammonia 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 test with the sea urchin *Arbacia punctulata* following the procedures outlined in SOP F10.6. (Attachment 3). The sea urchins used in this study were obtained from Gulf Specimen Company, Inc. (Panacea, Florida). Each of the 21 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 previously as a reference site (Carr and Chapman, 1992; Carr, 1993; NBS, 1993; 1994; 1995a; 1995b; USGS, 1997a; 1997b; 1998; 1999a; 1999b; 2000a; 2000b; 2000c), as noted previously. In addition, dilution blanks of filtered seawater and a reconstituted brine (brine with purified deionized water) were also included. A dilution series test with sodium dodecyl sulfate (SDS) was included as a positive control.

Statistical comparisons among treatments were made using GLM and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989). A further tranformation was necessary in analyzing the data at the 25% dilution level to correct response scaling problems. The transformation used in this instance was the arcsine sqaure root taken to the 1.5 power. 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 when possible. Prior to statistical analysis, 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 analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992).

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 laboratory (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 $\alpha = 0.05$ are 15.5% and at $\alpha = 0.01$, 19%.

Dissolved Organic Carbon Analysis

Dissolved organic carbon (DOC) was measured in the porewater samples using an OI Analytical Model 1010 Wet Oxidation Total Organic Carbon Analyzer following the model 1010 operators manual (OI Analytical, 1998). Samples of freshly extracted pore water (20 ml approx.) were immediately filtered through a 0.45 µm Whatman® nylon syringe filter and preserved with approximately 0.5 ml of phosphoric acid. Samples were stored refrigerated before analysis. Samples were analyzed in the TOC mode with 400 µl of acid (5% phosphoric acid) and 4000 µl of oxidant (200 g/L sodium persulfate). Total inorganic carbon react and detect times were 2:00 (min:sec) and 1:35 (min:sec), respectively. Total organic carbon react and detect times were 8:30 (min:sec) and 2:00 (min:sec), respectively. At least one blank was run with each batch of samples. In addition a laboratory control was run for every 10 to 15 samples. Sample analysis was repeated with dilution of the sample if concentrations were found to be in excess of the highest concentration used to calculate the calibration curve (50 mg/L). Analysis was also repeated if the percent recovery of the laboratory control failed to meet the 90-110% level.

RESULTS AND DISCUSSION

Porewater Toxicity Testing

Twenty sediment samples from St. Lucie Estuary and one from Jupiter estuary were extracted and tested for toxicity. Salinity of the pore water ranged from 22 to 38 ppt (Table 1). Initial dissolved oxygen of all samples exceeded 80% saturation and did not require aeration prior to testing. Sulfide measurements were below detection limits (0.01 mg/L) in all but three samples and pH ranged from 7.73-8.00. Total ammonia measured ranged from < 0.1 to 12.30 mg/L with the unionized fraction ranging from < 3.1 to 308.2 µg/L.

Toxicity testing of the 21 samples in the sea urchin fertilization test revealed 10 of the 21 samples (47.6%) were toxic in the undiluted adjusted pore water. Eight of 10 samples in the 50% dilution and six of the 10 samples in the 25% concentration were also found to be toxic. In four samples (SLE-15, SLE-22, SLE-27, and SLE-29) atypical dilution responses were observed. These samples became toxic as they were diluted. Dilution water blanks run with the test showed no evidence of toxicity from the dilution water itself and while there was a slight toxicity response in the reconstituted brine blank (probably due to a lack of trace elements), this would not explain the results observed as brine was not added to any of these samples. Further testing would be required to determine if pH shifts or other factors were the cause of these results. For this report, toxic responses observed in diluted samples were not considered when the undiluted sample was not toxic. Table 2 gives the raw and summarized data, respectively, for the fertilization test while Figures 1 and 2 illustrates the results in a graphical (map) format. The majority of toxic samples occurred in the north and south forks of St. Lucie Estuary with fewer toxic responses observed proceeding towards Indian River Lagoon. However the most toxic station (SLE-19) was found in the mid estuary region with an EC_{50} value < 25%. None of the samples exceeded the NOEC for ammonia (400 µg/L) for the fertilization test indicating that other contaminants are likely responsible for the observed toxicity.

EC_{50} values could be calculated for six stations (Table 3). Quality controls used in the test resulted in acceptable values. EC_{50} values for the SDS positive control was 5.51 mg/L (95% confidence intervals 5.15-5.89) which falls within the control charts for the MERS laboratory.

Dissolved Organic Carbon

Dissolved organic carbon (DOC) was measured in all porewater samples collected . Results of the (DOC) analysis can be found in Table 4. Samples were run in one batch with associated quality control standards. All analyses were run in triplicate with 1 ml autosampled volumes. Blank values were acceptable and measured 0.18 mg/L. Percent recovery from laboratory controls run in conjuction with the batch was acceptable at 99.56%. DOC values reflect the concentrations in the pore water before salinity adjustment at the time of extraction prior to freezing. Preliminary data from our laboratory indicates that DOC concentrations in freshly collected samples may range from 1 to 20% higher than samples that have been frozen. DOC concentrations ranged from 4.67 to 84.43 mg/L.

LITERATURE CITED

Carr, R.S. 1993. Sediment quality assessment survey of the Galveston Bay System. Galveston Bay National Estuary Program report, GBNEP-30, 101 pp.

Carr, R.S. and J. M. Biedenbach. 1999. Use of power analysis to develop detectable significance criteria for sea urchin toxicity tests. *Aquat. Ecosys. Hlth. Mgmt.* 2:413-418

Carr, R.S. and D.C. Chapman. 1992. Comparison of solid-phase and pore-water approaches for assessing the quality of marine and estuarine sediments. *Chem. Ecol.* 7:19-30.

Carr, R.S. and D.C. Chapman. 1995. Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests-extraction, storage, and handling techniques. *Arch. Environ. Contam. Toxicol.* 28:69-77.

Carr, R.S., E.R. Long, H.L. Windom, D.C. Chapman, G. Thursby, G.M. Slone, and D.A. Wolfe. 1996a. Sediment Quality Assessment Studies of Tampa Bay, Florida. *Environ. Toxicol. and Chem.* 15:1218-1231.

Carr, R.S., D.C. Chapman, C.L. Howard, and J.M. Biedenbach. 1996b. Sediment quality triad assessment survey of the Galveston Bay, Texas system. *Ecotoxicology* 5:341-364.

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.* 11:714-719; Correction 12:417 (1978).

Morgan, B.J.T. 1992. *Analysis of Quantal Response Data,* London, England: Chapman and Hall, 511 pp.

National Biological Survey (NBS). 1993. Toxicity testing of sediments from Charleston Harbor, South Carolina and vicinity. Report submitted by the National Biological Survey to the National Oceanic and Atmospheric Administration, Ocean Assessment Division, Seattle, WA, 7 pp. + 16 tables and 4 attachments.

National Biological Survey (NBS). 1994. Survey of sediment toxicity in Pensacola Bay and St. Andrew Bay, Florida. Report submitted by the National Biological Survey to the National Oceanic and Atmospheric Administration, Ocean Assessment Division, Seattle, WA, 12 pp. + 24 tables and 5 attachments.

National Biological Service (NBS). 1995a. Toxicity testing of sediments from Biscayne Bay and Surrounding Areas. Report submitted by the National Biological Service to the National Oceanic and Atmospheric Administration, Coastal Monitoring and Bioeffects Division, Seattle, WA, 11 pp. + 17 tables, 11 figures and 4 attachments.

National Biological Service (NBS). 1995b. Toxicity testing of sediments from western Florida and coastal South Carolina and Georgia. Report submitted by the National Biological Service to the National Oceanic and Atmospheric Administration, Coastal Monitoring and Bioeffects Division, Seattle, WA, 14 pp. + 35 tables, 10 figures and 4 attachments.

OI Analytical. 1998. Model 1010 Wet Oxidation Total Organic Carbon Analyzer Operators Manual. Revision 8.0. O. I Analytical, College Station, Texas. 138 pp.

SAS Institute Inc. 1989. *SAS/STAT® User's Guide, Version 6, Fourth Edition, Version 6, Volume 2.* Cary, NC:SAS Institute Inc., 846 pp.

SAS Institute Inc. 1992. *SAS/LAB® Software: User's Guide, Version 6, First Edition*, Cary, NC: SAS Institute Inc., 291 pp.

U.S. Fish and Wildlife Service (USFWS). 1992. Amphipod solid-phase and sea urchin porewater toxicity tests of Tampa Bay, Florida sediments. Report submitted by the U.S. Fish and Wildlife Service to National Oceanic and Atmospheric Administration, Ocean Assessment Division, Seattle, WA, 9 pp. + 19 appendices.

U.S. Geological Survey (USGS). 1997a. Toxicity testing of sediments from Biscayne Bay, Florida and surrounding areas - Phase II. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Coastal Monitoring and Biological Assessment Division, Seattle, WA, 10pp. + 8 tables, 10 figures and 4 attachments.

U.S. Geological Survey (USGS). 1997b. Toxicity testing of sediments from Puget Sound, Washington and surrounding areas. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Coastal Monitoring and Biological Assessment Division, Seattle, WA, 15pp. + 13 tables, 9 figures and 7 attachments.

U.S. Geological Survey (USGS). 1998. Toxicity testing of sediments from Central Puget Sound, Washington and surrounding areas. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Coastal Monitoring and Biological Assessment Division, Seattle, WA, 11pp. + 8 tables, 6 figures and 4 attachments.

U.S. Geological Survey (USGS). 1999a. Toxicity testing of sediments from Southern Puget Sound, Washington and surrounding areas. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Coastal Monitoring and Biological Assessment Division, Seattle, WA, 11pp. + 7 tables, 5 figures and 4 attachments.

U.S. Geological Survey (USGS). 1999b. Toxicity testing of sediments from Chesapeake Bay and surrounding areas, Phase I. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Office of Ocean Resources Conservation Assessment, Bioeffects Assessment Branch, Silver Springs, MD, 9 pp. + 9 tables, 3 figures and 4 attachments.

U.S. Geological Survey (USGS). 2000a. Toxicity testing of sediments from Chesapeake Bay and surrounding areas, Year II. Report submitted by the USGS to National Oceanic and Atmospheric Administration, Office of Ocean Resources Conservation Assessment, Bioeffects Assessment Branch, Silver Springs, MD, 9 pp. + 9 tables, 5 figures and 4 attachments.

U.S. Geological Survey (USGS). 2000b. Toxicity testing of sediments from Portsmouth Naval Shipyard, Kittery, Maine. Report submitted by the USGS to Tetra Tech NUS Inc., 600 Clark Avenue, Suite 3, King of Prussia, PA, 9 pp. + 8 tables, 1 figure and 5 attachments.

U.S. Geological Survey (USGS). 2000c. Toxicity testing of sediments from the BEST/EMAP Western Estuary Group Monitoring Study. Report submitted by the USGS to the U.S. Geological Survey, Biomonitoring of Environmental Status and Trends Program, 6006 Schroeder Road, Madison, WI, 10 pp. + 22 tables, 3 figures and 4 attachments.

TABLES 1-4

Table 1. Water quality parameters after salinity adjustment and original salinity of sediment porewater samples from St. Lucie estuary, Florida.

Designation[1]	Salinity[2] (‰)	DO[3] (mg/L)	% DO[4]	pH	TAN[5] (mg/L)	UAN[6] (μg/L)	Sulfide[7] (mg/L)	% OUS[8]
REF-1[9]	35	6.01	82.5	7.99	1.02	31.3	<0.01	85
SLE-2	25	6.86	94.6	7.89	2.99	73.3	< 0.01	94
SLE-3	24.5	6.67	92.3	7.91	4.2	106.4	< 0.01	94
SLE-5	26	6.4	88.5	7.90	4.58	114.8	< 0.01	96
SLE-7	24	6.84	94.7	7.90	10.00	250.6	0.01	94
SLE-9	23	6.83	93.9	7.85	7.88	176.5	< 0.01	93
SLE-12	22	6.74	92.8	7.9	6.66	166.9	0.01	91
SLE-14	30	7.4	101.3	7.91	2.27	58.2	< 0.01	100
SLE-15	29	6.55	90.3	7.93	3.5	93.8	< 0.01	100
SLE-17	26	7.32	100.8	7.89	7.64	187.2	< 0.01	96
SLE-19	30	6.03	83.4	7.90	12.3	308.2	0.03	100
SLE-21	34	7.19	99.1	7.73	1.96	33.5	< 0.01	88
SLE-22	36	6.65	91.5	7.93	1.48	39.7	< 0.01	83
SLE-26	35	6.8	92.7	7.82	2.34	49.0	< 0.01	76
SLE-27	37	6.64	91.2	7.92	2.37	62.1	< 0.01	81
SLE-29	37	6.56	90.4	7.9	2.45	61.4	< 0.01	81
SLE-33	37	6.56	90.8	8.0	1.85	58.0	< 0.01	81
SLE-34	36	6.5	89.8	8.03	0.297	10.0	< 0.01	83
SLE-36	27	6.68	92.0	7.97	4.49	131.6	< 0.01	97
SLE-37	38	6.42	88.6	7.99	<0.1	<3.1	< 0.01	79

Table 1. Continued.

Designation[1]	Salinity[2] (‰)	DO[3] (mg/L)	% DO[4]	pH	TAN[5] (mg/L)	UAN[6] (μg/L)	Sulfide[7] (mg/L)	% OUS[8]
SLE-38	37	6.37	88.1	7.86	2.24	51.3	< 0.01	81
SLE-48	36	6.82	94.6	7.99	1.44	44.1	< 0.01	83

[1] Designation refers to station.
[2] Salinity of sample prior to adjustment. Sample adjusted to 30±1‰.
[3] Dissolved oxygen
[4] Percent saturation of dissolved oxygen
[5] Total ammonia as nitrogen
[6] Un-ionized ammonia
[7] Measured as S^{-2}
[8] Percent of original sample after salinity adjustment
[9] 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 St. Lucie estuary, Florida. Asterisks denote statistical differences (Dunnett's *t*-test) and detectable significance criteria between test and reference stations (* $\alpha \leq 0.05$, ** $\alpha \leq 0.01$). Plus signs denote only statistical differences (Dunnett's *t*-test, + $\alpha \leq 0.05$, ++ $\alpha \leq 0.01$).

Designation [1]	% WQAS [2]	% Fertilized					Mean±SD	% of REF [3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
REF [3]	100	96	97	99	98	98	97.9 ± 1.1	100
		97	98	100	98	98		
REF [3]	50	99	99	96	99	100	98.6 ± 1.4	100
		99	99	99	96	100		
REF [3]	25	99	97	100	97	99	98.5 ± 1.1	100
		98	98	98	99	100		
SLE-2	100	71	79	70	28 [4]	64	71.0 ± 6.2 **	73
	50	54	69	62	62	64	62.2 ± 5.4 **	63
	25	48	49	69	68	58	58.4 ± 10.0 **	59
SLE-3	100	22	20	31	37	21	26.2 ± 7.5 **	27
	50	66	36	78 [4]	62	65	57.2 ± 14.3 **	58
	25	70	55	51	57	50	56.6 ± 8.0 **	57
SLE-5	100	24	15	16	18	22	19.0 ± 3.9 **	19
	50	58	53	59	54	49	54.6 ± 4.0 **	55
	25	50	76	42	77	68	62.6 ± 15.8 **	64
SLE-7	100	3	2	3	2	1	2.2 ± 0.8 **	2
	50	36	20	41	35	34	33.2 ± 7.8 **	34
	25	64	69	59	70	77	67.8 ± 6.8 **	69
SLE-9	100	7	3	15	3	4	6.4 ± 5.1 **	7
	50	43	40	55	47	46	46.2 ± 5.6 **	47
	25	83	89	85	81	86	84.8 ± 3.0 ++	86

Table 2. Continued.

Designation [1]	% WQAS [2]	% Fertilized					Mean±SD	% of REF [3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
SLE-12	100	8	4	5	2	0	3.8 ± 3.0 **	4
	50	26	27	32	21	24	26.0 ± 4.1 **	26
	25	60	65	69	63	74	66.2 ± 5.4 **	67
SLE-14	100	45	56	80	54	66	60.2 ± 13.4 **	61
	50	85	88	82	83	89	85.4 ± 3.0 ++	87
	25	90	83	82	89	79	84.6 ± 4.7 ++	86
SLE-15	100	93	88	78	82	89	86.0 ± 6.0 ++	88
	50	73	91	79	89	93	85.0 ± 8.6 [5]	86
	25	78	80	70	83	77	77.6 ± 4.8 [5]	79
SLE-17	100	42	24	14	33	26	27.8 ± 10.4 **	28
	50	83	81	91	86	81	84.4 ± 4.2 ++	86
	25	91	95	100	93	94	94.6 ± 3.4	96
SLE-19	100	1	1	0	0	0	0.4 ± 0.6 **	0
	50	1	3	2	4	6	3.2 ± 1.9 **	3
	25	6	2	9	10	50 [4]	6.8 ± 3.6 **	7
SLE-21	100	94	89	98	98	97	95.2 ± 3.8	97
	50	97	91	98	96	97	95.8 ± 2.8	97
	25	95	96	96	97	99	96.6 ± 1.5	98
SLE-22	100	93	94	97	96	97	95.4 ± 1.8	97
	50	83	80	79	89	86	83.4 ± 4.2 [5]	85
	25	65	44	57	61	46	54.6 ± 9.2 [5]	55

Table 2. Continued.

Designation [1]	% WQAS [2]	% Fertilized					Mean±SD	% of REF [3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
SLE-26	100	99	95	98	99	97	97.6 ± 1.7	100
	50	97	95	98	93	97	96.0 ± 2.0	97
	25	99	97	99	98	93	97.2 ± 2.5	99
SLE-27	100	91	96	96	93	96	94.4 ± 2.3	96
	50	75	91	82	89	81	83.6 ± 6.5 [5]	85
	25	47	68	66	73	66	64.0 ± 9.9 [5]	65
SLE-29	100	94	94	100	98	93	95.8 ± 3.0	98
	50	78	86	78	65	83	78.0 ± 8.0 [5]	79
	25	27	69	57	36	57	49.2 ± 17.2 [5]	50
SLE-33	100	100	98	99	100	99	99.2 ± 0.8	101
	50	100	100	96	96	97	97.8 ± 2.0	99
	25	100	99	99	97	98	98.6 ± 1.1	100
SLE-34	100	95	97	97	98	92	95.8 ± 2.4	98
	50	97	99	98	95	96	97.0 ± 1.6	98
	25	92	97	99	99	98	97.0 ± 2.9	98
SLE-36	100	15	10	14	25	15	15.8 ± 5.5 **	16
	50	66	64	77	81	74	72.4 ± 7.2 **	73
	25	78	86	94	84	92	86.8 ± 6.4 ++	88
SLE-37	100	99	100	97	98	99	98.6 ± 1.1	101
	50	98	98	100	98	99	98.6 ± 0.9	100
	25	97	98	100	97	100	98.4 ± 1.5	100
SLE-38	100	96	92	94	94	90	93.2 ± 2.3 +	95
	50	99	96	97	100	99	98.2 ± 1.6	100
	25	99	99	99	98	99	98.8 ± 0.4	100

Table 2. Continued.

Designation [1]	% WQAS [2]	% Fertilized					Mean±SD	% of REF [3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
SLE-48	100	96	96	98	96	91	95.4 ± 2.6	97
	50	97	96	99	98	98	97.6 ± 1.1	99
	25	100	99	99	100	100	99.6 ± 0.6	101

[1] Designation refers to sample identification.

[2] Percent of water quality adjusted porewater sample.

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

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

[5] Atypical dilution response, not considered toxic for this report.

Table 3. EC_{50} values of sediment porewater samples from St. Lucie estuary, Florida assayed in the sea urchin fertilization test.

Designation[1]	Fertilization Test	
	EC_{50}[2]	95% Confidence Limits
SLE-2	>100	-
SLE-3	60.76	51.65 - 71.48
SLE-5	52.95	42.22 - 66.41
SLE-7	37.08	32.71 - 42.04
SLE-9	48.17	43.85 - 52.91
SLE-12	34.21	30.75 - 38.06
SLE-14	>100	-
SLE-15	>100	-
SLE-17	>100	-
SLE-19	<25	-
SLE-21	>100	-
SLE-22	>100	-
SLE-26	>100	-
SLE-27	>100	-
SLE-29	>100	-
SLE-33	>100	-
SLE-34	>100	-
SLE-36	66.39	59.48 - 74.11
SLE-37	>100	-
SLE-38	>100	-
SLE-48	>100	-

[1] Designation refers to sample site number.

[2] Percent of water quality adjusted porewater sample.

Table 4. Dissolved organic carbon (DOC) concentrations (mg/L) of porewater samples from St. Lucie estuary, Florida.

Sample type	Date analyzed	Rep 1	Rep 2	Rep 3	Mean	%RSD
SLE-2	20-May-01	12.001	13.075	12.164	12.143	4.51
SLE-3	20-May-01	13.45	14.794	13.287	13.844	5.8
SLE-5	20-May-01	12.7	13.487	13.005	13.064	2.94
SLE-7	20-May-01	22.499	24.05	22.415	22.988	3.93
SLE-9	20-May-01	23.814	23.789	23.238	23.614	1.36
SLE-12	20-May-01	16.265	16.133	15.558	15.985	2.29
SLE-14	20-May-01	9.5537	9.0735	9.0364	9.2212	2.99
SLE-15	20-May-01	9.7752	9.8915	9.6679	9.7782	1.1
SLE-17	20-May-01	16.349	17.217	16.35	16.639	2.93
SLE-19	20-May-01	16.1	18.099	16.361	16.853	6.29
SLE-21	21-May-01	7.4595	7.9657	7.4424	7.6225	3.7
SLE-22	21-May-01	6.9763	7.3692	6.8911	7.0789	3.4
SLE-26	21-May-01	5.6089	6.1903	5.655	5.8181	5.18
SLE-27	21-May-01	7.8414	8.1742	7.8865	7.9674	2.15
SLE-29	21-May-01	5.4705	6.2084	5.654	5.7776	6.2
SLE-33	21-May-01	14.206	14.622	14.085	14.304	1.91
SLE-34	21-May-01	4.5723	4.9092	4.5463	4.6759	3.97
SLE-36	21-May-01	9.9536	10.834	10.179	10.322	4.26
SLE-37	21-May-01	72.327	68.839	69.669	70.278	2.58
SLE-38	21-May-01	84.504	84.706	84.095	84.435	0.37
SLE-48	21-May-01	36.022	36.155	35.946	36.041	0.29

FIGURES

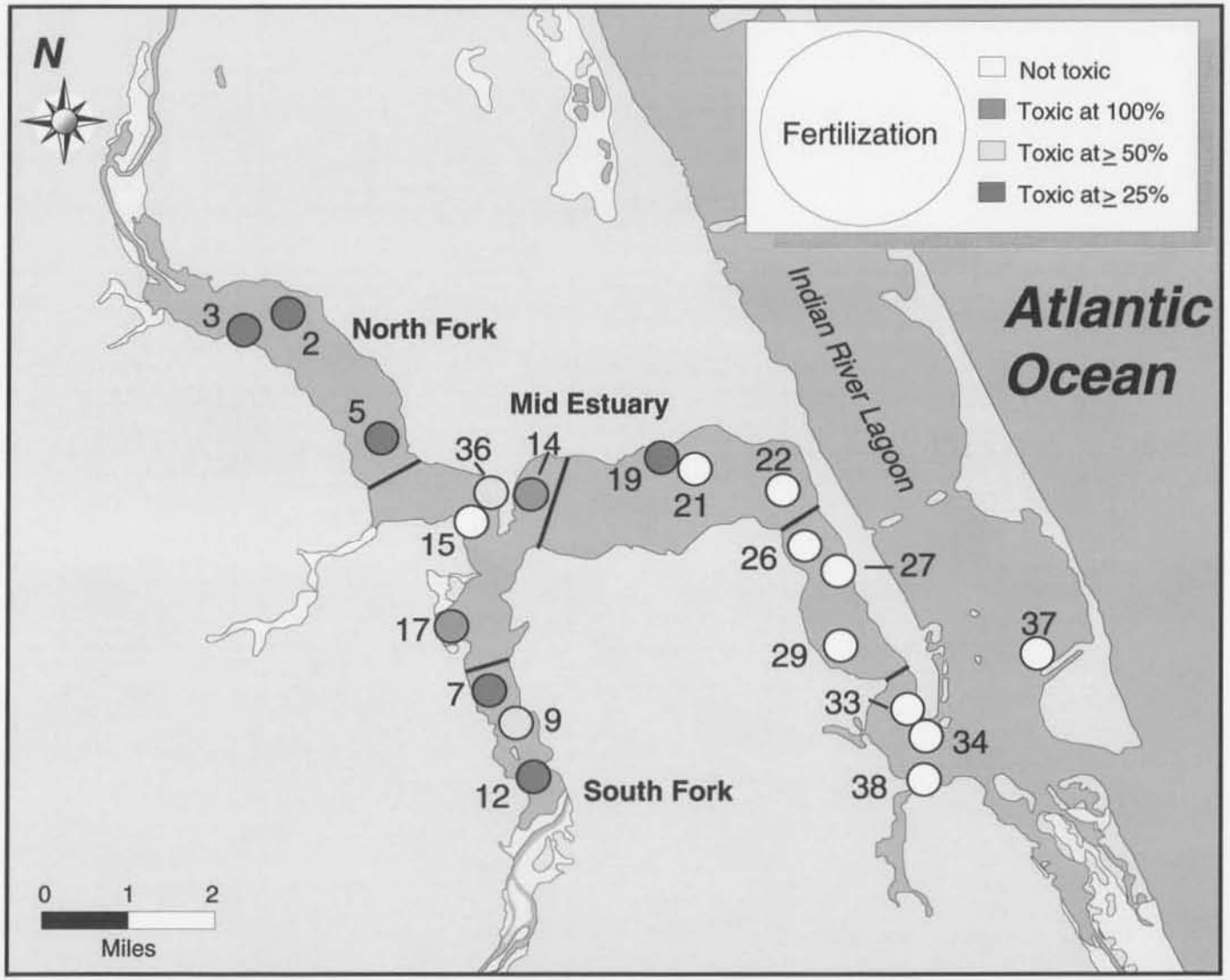

Figure 1. Sea urchin (*Arbacia punctulata*) fertilization test results for stations in St. Lucie estuary, Florida. Color differentiation of symbol indicates those stations that were significantly different than the reference (Dunnett's *t*-test $\alpha \leq 0.05$ and detectable significance criteria applied).

[ck the location of site 36] SJ

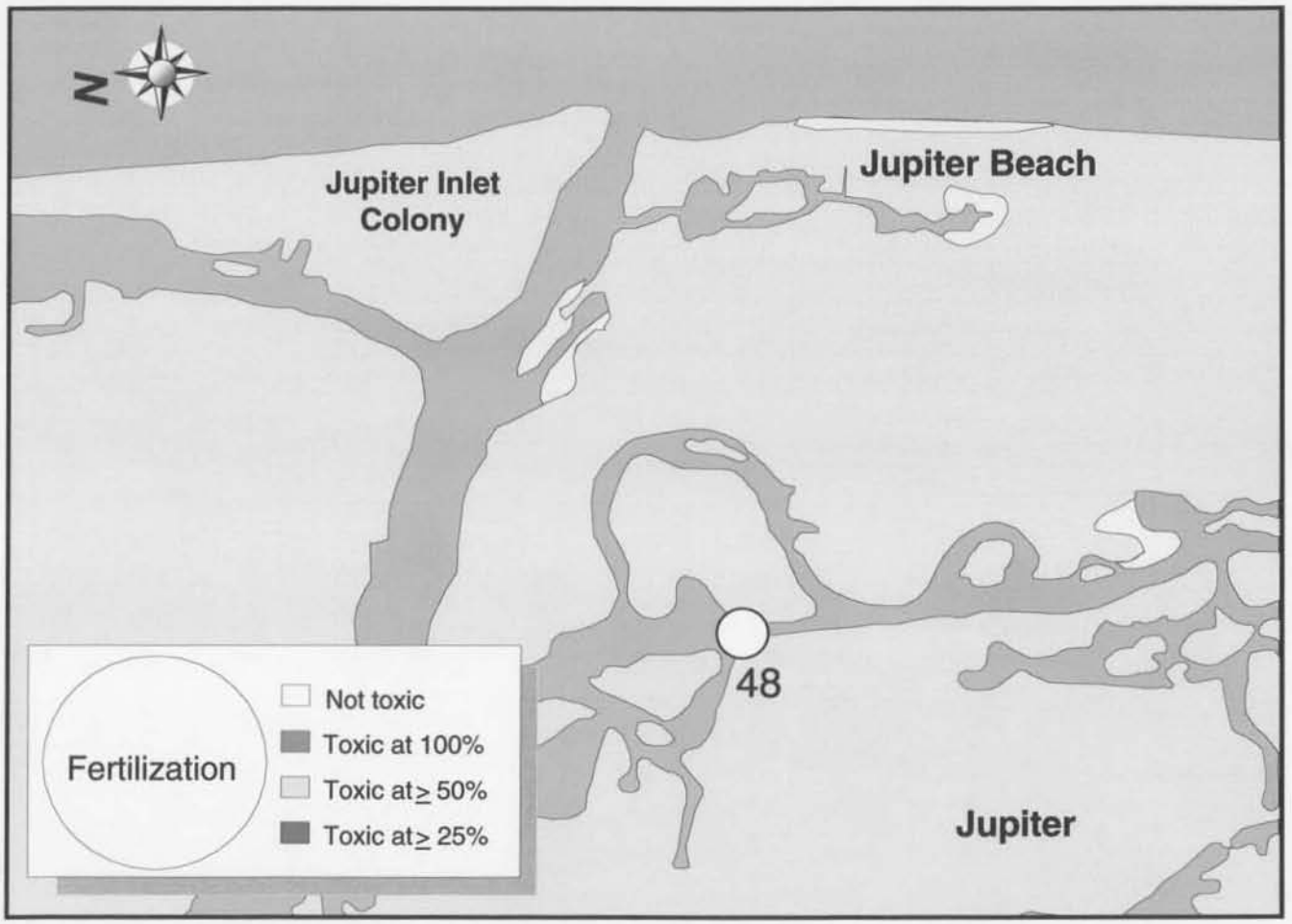

Figure 2. Sea urchin (*Arbacia punctulata*) fertilization test result for station near Jupiter, Florida. Color differentiation of symbol indicates significant difference from the reference (Dunnett's *t*-test $\alpha \leq 0.05$ and detectable significance criteria applied).

ATTACHMENTS 1-3

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.

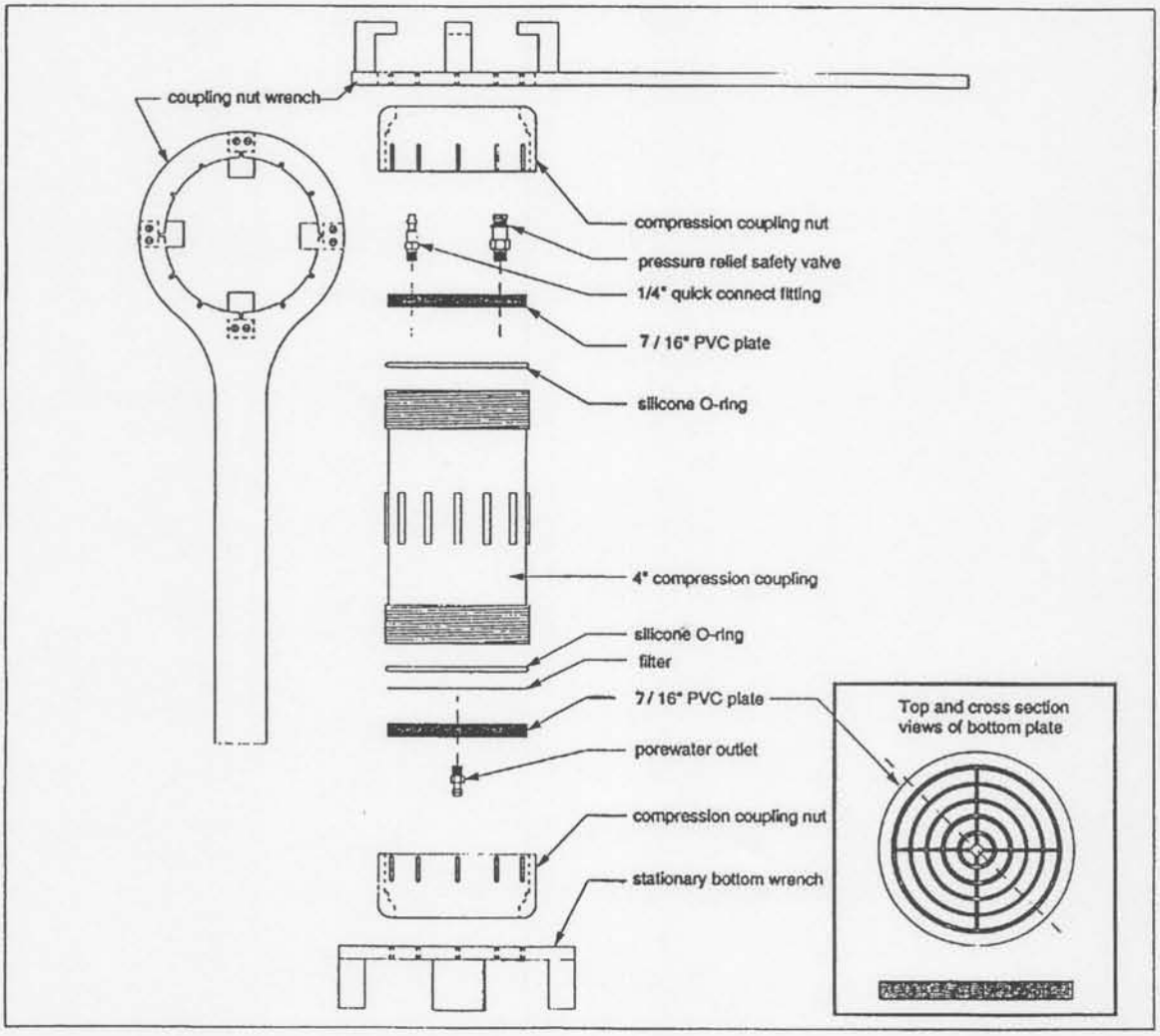

Figure 1. Sediment pore water squeeze extraction device.

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.

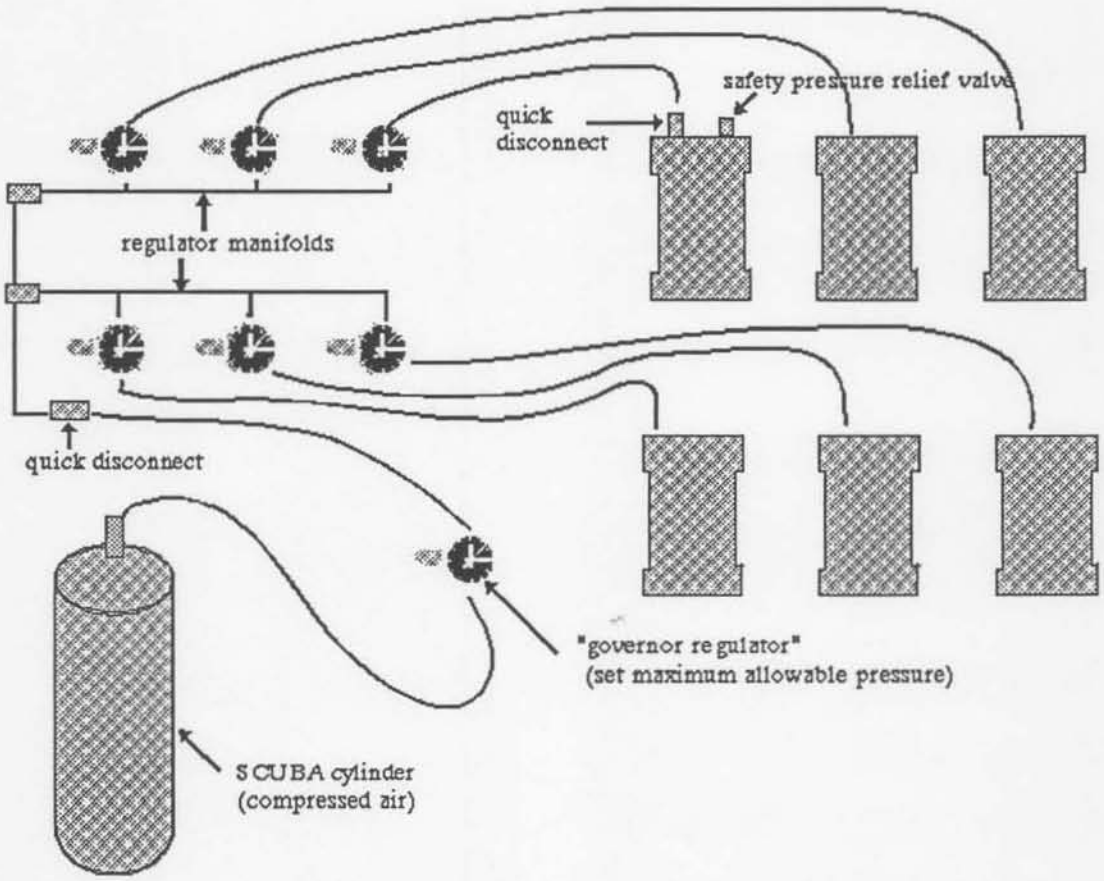

Figure 2. Schematic of sediment porewater pressure extraction system.

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

Carr, R.S. 1993. Sediment quality assessment survey of the Galveston Bay System. Galveston Bay National Estuary Program report, GBNEP-30, 101 pp.

Carr, R.S., J.W. Williams, and C.T.B. Fragata. 1989. Development and evaluation of a novel marine sediment pore water toxicity test with the polychaete *Dinophilus gyrociliatus*. Environ. Toxicol. Chem. 8:533-543.

Carr, R.S. and D.C. Chapman. 1992. Comparison of solid-phase and pore-water approaches for assessing the quality of marine and estuarine sediments. Chem. Ecol. 7:19-30.

Carr, R.S. and Chapman. 1994. Improved device for extracting sediment pore water. National Biological Survey, Research Information Bulletin No. 38.

National Biological Survey (NBS). 1993. Toxicity testing of sediments from Charleston Harbor, South Carolina and vicinity. Report submitted by the National Biological Survey to the National Oceanic and Atmospheric Administration, Ocean Assessment Division, Seattle, WA, 7 pp. + 16 tables and 4 attachments.

National Biological Survey (NBS). 1994a. Survey of sediment toxicity in Pensacola Bay and St. Andrew Bay, Florida. Report submitted by the National Biological Survey to the National Oceanic and Atmospheric Administration, Ocean Assessment Division, Seattle, WA, 12 pp. + 24 tables and 5 attachments.

National Biological Survey (NBS). 1994b. Toxicity testing of sediments from Boston Harbor, Massachusetts. Final report submitted to National Oceanic and Atmospheric Administration, 6 pp. + 10 tables and 4 attachments.

US Fish and Wildlife Service (USFWS) 1992. Amphipod solid-phase and sea urchin porewater toxicity tests with Tampa Bay, Florida sediments. Final report submitted to National Oceanic and Atmospheric Administration, 9 pp. + 16 tables and 3 attachments.

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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$^{o}/_{oo}$. 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$^{o}/_{oo}$ 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$^{o}/_{oo}$) 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.

3.1 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

3.0 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

3.22 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 $^{o}/_{oo}$ - sample $^{o}/_{oo}$) × sample vol. in mL) ÷ (brine $^{o}/_{oo}$ - target $^{o}/_{oo}$) = 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

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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 l. 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

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 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 _____