USGS
science for a changing world

Coastal Circulation and Sediment Dynamics in Hanalei Bay, Kauai

PART III:

Studies of Sediment Toxicity

U.S. Department of the Interior
U.S. Geological Survey

Open-File Report 2006-1147

QuickBird satellite image of Hanalei Bay

Coastal Circulation and Sediment Dynamics in Hanalei Bay, Kauai

PART III: Studies of Sediment Toxicity

Robert S. Carr [1], Marion Nipper [2], Michael Field [3], and James M. Biedenbach [1]

[1] U.S. Geological Survey, Marine Ecotoxicology Research Station, Corpus Christi, Tex.
[2] Texas A&M University-Corpus Christi, Center for Coastal Studies, Corpus Christi, Tex.
[3] U.S. Geological Survey, Pacific Science Center, Santa Cruz, Calif.

U.S. Geological Survey
Open-File Report 2006-1147

Corpus Christi, Texas
April 2006

TABLE OF CONTENTS

TABLES

FIGURE

APPENDICES

Appendix 1. (SOP F10.24) Extraction and Storage of Porewater Samples Using *In Situ* Devices.

Appendix 2. (SOP F10.12) Water Quality Adjustment of Samples

Appendix 3. (SOP F10.6) Sea Urchin Fertilization Toxicity Test

Appendix 4. (SOP F10.7) Sea Urchin Embryological Development Toxicity Test

ADDITIONAL DIGITAL INFORMATION

For an online PDF version of this report, please see:
http://pubs.usgs.gov/of/2006/1147/

For more information on the U.S. Geological Survey Marine Ecotoxicology Research Station please see:
http://sci.tamucc.edu/mers/index.htm

For more information on the U.S. Geological Survey Western Coastal and Marine Geology Team, please see:
http://walrus.wr.usgs.gov/

For more information on the U.S. Geological Survey's Coral Reef Project, please see:
http://coralreefs.wr.usgs.gov/

DIRECT CONTACT INFORMATION

General Project Information
Dr. Michael E. Field (Project Chief): mfield@usgs.gov

Regarding this Report:
Dr. Robert. S. Carr (Station Leader) scott_carr@usgs.gov

SUGGESTED CITATION

Carr, R.S., Nipper, M., Field, M.E. and Biedenbach, J.M., 2006, Coastal circulation and sediment dynamics in Hanalei Bay–studies of sediment toxicity: U.S. Geological Survey Open-File Report 2006-1147, 12 p. plus 4 appendices.

INTRODUCTION

Toxicity tests are commonly conducted as a measure of the bioavailability of toxic chemicals to biota in an environment. Chemical analyses alone are insufficient to determine whether contaminants pose a threat to biota (Carr and Nipper, 2003). Porewater toxicity tests are extremely sensitive to a broad range of contaminants in marine environments and provide ecologically relevant data on sensitive life stages. The inclusion of porewater toxicity testing as an additional indicator of sediment quality provides a more comprehensive picture of contaminant effects in these sensitive habitats.

In this study purple-spined sea urchin (*Arbacia punctulata*) fertilization and embryological development porewater toxicity tests were used to evaluate the sediments collected from the coastal environment around Hanalei Bay, Kauai, Hawaii. These tests have been used previously to assess the bioavailability of contaminants associated with sediments in the vicinity of coral reefs (Carr and Nipper, 1998, Carr et al. 2001; Nipper and Carr, 2000, 2001).

Project Objectives

- Collect pore water in situ from 11 sites in Hanalei Bay and from a reference site at Kë' ë Beach.

- Measure water quality parameters (salinity, dissolved oxygen, pH, temperature, and ammonia) of thawed porewater samples prior to testing and adjust salinity, temperature and dissolved oxygen, if necessary.

- Conduct the fertilization and embryological development toxicity tests with pore water using sea urchin gametes.

- Quality control assays with reference pore water, dilution blanks and a positive control dilution series with sodium dodecyl sulfate (SDS) in conjunction with each test

- Make statistical comparisons between test and reference stations.

Study Area

The study area is located in Hanalei Bay, on the north coast of Kauai, Hawaii. Most of the samples were collected near the central and east coasts of the bay, with some collected in the central part of the bay. One reference site was collected west of the bay at Kë' ë Beach. Table 1 details the geographic coordinates of the stations, dates of sampling and physical descriptions of the sites.

MATERIALS AND METHODS

This section details information about the personnel, equipment, and procedures used in the collection, handling, and testing of sediment pore water. Table 2 provides the list of personnel involved in this study and appendices 1 through 4 detail the equipment and precise

methodology used in the collection, adjustment, measurement and toxicity testing of the sample pore water.

Scientific personnel

Collection of the pore water in the field was performed by two scientists, one from the USGS Marine Ecotoxicology Research Station (MERS) and one from the Texas A&M Corpus Christi Center for Coastal Studies. A third scientist, also from MERS, was responsible for processing and testing the water samples once they were returned to Corpus Christi.

Sample Collection and Tracking

Collection of samples took place on August 25, 26 and 27 of 2005. Six of the samples were collected near shore in a water depth of approximately 2 m. Five samples were taken in deeper water from a boat at depths ranging from 12 to 15 m. One reference seawater sample was taken by submerging two precleaned acid washed 290-ml polycarbonate centrifuge tubes to a depth of 1 m and removing the lids. All porewater samples were collected *in situ*, by vacuum extraction (see Appendix 1, SOP F10.24), using a device that consisted of a ground-glass airstone attached to a presoaked 60-ml polypropylene catheter type syringe. All vacuum extraction device sampling materials were presoaked for 1 week in filtered seawater (5µm) and 4 weeks in deionized water with water exchanges every 2 to 3 days. Seawater samples from the vacuum extraction devices were tested for latent toxicity following the soaking procedure and found to be nontoxic. Following collection, pore water collected in the syringes was composited in acid washed 290-ml polycarbonate centrifuge tubes, capped and kept on ice or refrigerated until they were transported back to MERS. Samples were received at MERS on August 30, 2005, where they were immediately centrifuged at 1200 x g for 20 minutes. The supernatant for each sample site was composited and then divided among 250-ml precleaned glass bottles and frozen at -20°C until testing.

Toxicity Testing

Two days before conducting the toxicity test, the samples were moved from the freezer to a refrigerator set to 4°C. One day prior to testing, samples were thawed completely in a tepid (20°C) water bath. Temperature of the sample was brought up to 20°C $\pm$ 1°C. Sample salinity was measured and adjusted to 30 $\pm$ 1‰, if necessary using purified deionized water or concentrated brine (see Appendix 2, SOP F10.12). Following salinity adjustments, the samples were returned to the refrigerator set to 4°C. They were brought up to 20°C $\pm$ 1°C before the start of the toxicity testing the next day.

On the day of testing, after temperature had been returned to 20°C $\pm$ 1°C, further water quality measurements were made on the samples. Dissolved oxygen (DO) was measured with a Yellow Springs Instrument meter; salinity was measured with a Reichert® temperature compensated refractometer; and pH and total ammonia expressed as nitrogen (TAN) were measured with Orion® meters and their respective probes. Un-ionized ammonia expressed as nitrogen (UAN) was calculated for each sample using the respective salinity, temperature, pH

and TAN values. Any samples containing less than 80 percent DO saturation were gently aerated by stirring the sample on a magnetic stir plate.

Toxicity of the sediment pore water, controls, and reference toxicants was determined using the fertilization and embryological development tests with the purple-spined sea urchin following the procedures outlined in SOP F10.6 and SOP F10.7 (appendices 3 and 4). Purple-spined sea urchins were obtained from the Marine Biological Laboratory in Woods Hole, Mass. Each of the 13 samples was tested in a dilution series design at 100, 50, and 25 percent 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 Aransas Bay, Texas 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 Nipper, 1998). In addition, a dilution water blank of filtered seawater was also included in each test. A dilution series test with sodium dodecyl sulfate (SDS) was also included as a positive control in each assay.

For both sea urchin assays, SAS (SAS Institute Inc., 1989) was used to make statistical comparisons among treatments. Analysis of variance (ANOVA) and Dunnett's one-tailed t-test (which controls the experimentwise error rate) were used on the arcsine square root transformed data. The trimmed Spearman-Karber method (Hamilton and others, 1977; Environmental Science and Technology, 1978) with Abbott's correction (Morgan, 1992) was used to calculate EC_{50} (50% effective concentration) values for dilution series tests. Prior to statistical analysis, the transformed data sets were screened for equal variance using SAS/LAB® Software (SAS Institute Inc., 1992). The SAS/LAB® software performs a Levene's test for equal variance. If a one way ANOVA (performed on the absolute deviations of the observations from their respective group mean values) revealed any statistical evidence of unequal variances, additional data transformations were performed and outliers removed if necessary. 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: $cv = t(df_{Error}, 0.05/(2 \times n))$.

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

RESULTS AND DISCUSSION

This section reviews the data collected from the water quality measurements as well as the results of the toxicity tests and discusses their possible implications. Water quality measurements can be found in Table 3, fertilization test raw data and statistical comparison

results in Table 4 and embryological development test raw data and statistical comparison results in Table 5.

To satisfy the test's salinity requirement of 30 $\pm$ 1 ‰, all 12 samples required salinity adjustment using Milli-Q® purified water. The Texas reference pore water required a salinity adjustment using concentrated brine. The initial dissolved oxygen was > 80 percent in all samples and therefore did not required aeration. The pH of test samples ranged from 7.843 to 8.112 with the reference seawater collected on site slightly higher at 8.283 and the Texas reference pore water slightly lower at 7.647. Total ammonia as nitrogen (TAN) was low for all samples and ranged from < 0.1 to 0.912 mg/L with the un-ionized fraction (the most toxic fraction) never rising above 20 μg/L. The un-ionized ammonia concentrations did not approach the lowest observed effect concentration (LOEC) for the fertilization nor the embryological development assays at 800 μg/L and 90 μg/L, respectively.

Three porewater samples in the fertilization test were found to be toxic when compared to the reference station (sample 6) from Ke´e Beach. Stations 8, 10 and 12 were all found to be toxic at the 100 percent water quality adjusted concentration with toxicity also observed in the 50% dilution from Station 10. Figure 1 identifies with colored dots the locations of the stations found to be toxic. No porewater samples were found to be toxic in the embryological development test. Reference toxicant controls were acceptable for both tests with EC_{50} values of 5.63 mg/L (5.27-6.00) for the fertilization test and 3.79 mg/L (3.59-4.00) for the embryological development test. Both of these results fall within the control charts for this laboratory. The control chart mean for the fertilization test is 5.54 mg/L (3.32-7.76). The control chart mean for the embryological development test is 4.20 mg/L (1.82-6.58).

ACKNOWLEDGEMENTS

We would like to acknowledge the logistical support provided by Josh Logan during the offshore field sampling. We are also indebted to Capt. Earl Loder who provided and piloted the research vessel. Dr. Carl Berg provided insightful information to add in the selection of sampling stations.

LITERATURE CITED

Carr, R.S., and Biedenbach, J.M., 1999, Use of power analysis to develop detectable significance criteria for sea urchin toxicity tests: Aquatic Ecosystem and Health Management, v. 2, p. 413-418.

Carr, R.S., and Nipper, M.G., 1998, Preliminary survey of sediment toxicity in the vicinity of Honolulu, Hawaii: Menlo Park, Calif., U.S. Geological Survey, 18 p., 4 attachments.

Carr, R.S., and Nipper, M.G., eds., 2003, Porewater toxicity testing: biological, chemical, and ecological considerations: Pensacola, Fl, USA, Society of Environmental Toxicology and Chemistry (SETAC), 346 p.

Carr, R.S., Nipper, M.G., and Plumlee, G.S., 2001, A preliminary survey of marine contamination from mining-related activities on Marinduque Island, Philippines–porewater toxicity and chemistry, results from a field trip October 14-19, 2000: U.S. Geological Survey Open-File Report 01-441, 19 p., 3 appendices, http://pubs.usgs.gov/of/2001/ofr-01-0441/

Environmental Science and Technology, 1978, Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays [correction of original]: Environmental Science and Technology, v. 12, p. 417.

Hamilton, M.A., Russo, R.C., and Thurston, R.V., 1977, Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays: Environmental Science and Technology, v. 11, p. 714-719; Correction 12:417 (1978).

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

Nipper, M.G. and Carr, R.S., 2000, Toxicity testing of sediment pore water from the Flower Garden Banks, Gulf of Mexico: Corpus Christi, Tex., Texas A&M University–Corpus Christi, 7 p. and 3 attachments.

Nipper, M.G., and Carr, R.S., 2001, Porewater toxicity testing–a novel approach for assessing contaminant impacts in the vicinity of coral reefs: Bulletin of Marine Science, v. 69, p. 407-420.

SAS Institute Inc., 1989, SAS/STAT® user's guide, version 6, volume 2 (4th ed.): Cary, N.C., SAS Institute Inc., 846 p.

SAS Institute Inc., 1992, SAS/LAB® software–user's guide, version 6 (1st ed.): Cary, N.C., SAS Institute Inc., 291 p.

TABLE 1. Station locations and site descriptions.

Station #	Date Sampled	Location	Latitude	Longitude	Physical description
1	8/25/2005	Hanalei River Transect	22.213766	-159.4972	~100 m offshore of coordinates, 5 m water depth, obvious freshwater mixing at surface
2	8/25/2005	Hanalei River Mouth	22.201266	-159.49775	~ 50 m offshore in mixing zone, approximately 0.5m water depth
3	8/25/2005	Hanalei Pavilion Beach Park	22.2073	-159.498433	~100 m offshore of coordinates, 2 m water depth, sandy bottom with nephloid-like flock
4	8/25/2005	Waioli Beach Park	22.203033	-159.505733	~100 m offshore of coordinates, 2.3 m water depth, fine sand
5	8/25/2005	Foamy River Beach Park-Waipa Beach	22.2055	-159.514966	~ 30 m offshore from coordinates, 2 m water depth, sampled near sand/hard bottom
6	8/25/2005	Kë´ë Beach-Haena State Park	22.223216	-159.5816	~40 m offshore from coordinates, 2 m water depth, end of sand groove out into coral
7	8/26/2005	Outer Wall	22.2149477	-159.5032211	10 m west of wall, 13.2 m water depth, medium sand
8	8/26/2005	Black Hole (study designation)	22.2121235	-159.5014578	inside boat mooring area, 15.2 m water depth, flocky nephloid layer, difficult to sample
9	8/26/2005	CRAMP Monitoring Site	22.2139833	-159.5127388	near wall on western side of bay, 13.3 m water depth, coral disease and algae present
10	8/26/2005	Inner Wall	22.2133682	-159.5007138	along the wall out of visual range, flocky bottom, mixing zone on surface evident
11	8/26/2005	Mid-Bay Deep	22.2125964	-159.5083736	middle of the bay opposite Waouli Beach Park, 12 m water depth, sandy bottom
12	8/27/2005	Princeville Resort Beach	22.217916	-159.498516	~100 m offshore of coordinates, 2 m water depth, sand groove channel, flock present
Ref. SW	8/25/2005	Kë´ë Beach-Haena State Park	22.223216	-159.5816	~40 m offshore from coordinates, 2 m water depth, sample taken at 1 m depth.

TABLE 2. Scientific personnel.

Person	Affiliation	Responsibilities
Robert S. Carr	USGS	Principal Investigator, Collection of pore water
Marion Nipper	TAMU-CC/CCS	Co-Principal Investigator, Collection of pore water
Michael Field	UGSS	Project Leader
James M. Biedenbach	USGS	Pore water processing, toxicity testing

TABLE 3. Water quality parameters after salinity adjustment and original salinity of sediment porewater samples from Hanalei Bay, Kauai, Hawaii in 2005.

Station	Salinity[1] $^{o}/_{oo}$	DO[2] (mg/L)	% DO[3]	pH	TAN[4] (mg/L)	UAN[5] (ug/L)	% OUS[6]
1	35	8.7	100.4	7.948	< 0.1	< 2.8	85.7
2	32	8.78	101.0	7.988	0.258	7.9	93.8
3	35	8.91	102.6	8.078	0.0947	3.5	85.7
4	35.5	8.83	101.5	8.112	< 0.1	< 18.0	84.7
5	35	8.72	100.3	7.987	0.115	3.5	85.7
6[7]	35	8.85	101.7	7.905	0.27	6.8	85.7
7	35	8.88	102.3	7.843	0.912	20.1	85.7
8	35.5	8.42	96.6	7.853	0.179	4.0	84.7
9	35.5	8.76	100.1	7.898	< 0.1	< 2.5	84.7
10	35.5	8.81	100.6	7.912	0.137	3.5	84.7
11	35	8.29	94.7	7.944	< 0.1	< 2.8	85.7
12	35	8.35	95.6	7.877	< 0.1	< 2.4	85.7
Tex. Ref[8]	24	8.24	95.9	7.647	0.204	2.9	92.3
REFSW[9]	35.5	8.01	93.1	8.283	< 0.1	< 5.8	84.7
MFS[10]	35	7.58	88.4	8.1	< 0.1	< 3.9	85.7

[1] Salinty of sample prior to adjustment. Sample adjusted to 30 ± 1‰.

[2] Dissolved oxygen

[3] Percent saturation of dissolved oxygen

[4] Total ammonia as nitrogen

[5] Un-ionized ammonia

[6] Percent of original sample after salinty adjustment

[7] Porewater sample designated as the reference site

[8] Reference pore water extracted from sediment collected in Aransas Bay, Texas

[9] Reference seawater collected in Hanalei Bay, Kauai, Hawaii

[10] Millipore filtered seawater diluent

TABLE 4. Sea urchin fertilization test raw data and mean values for sediment porewater samples from Hanalei Bay, Kauai, Hawaii. Asterisks denote statistically significant differences (DSC) (Dunnett's *t*-test) and detectable significance criteria between test and control station 6 (*alpha < 0.05, **alpha < 0.01).

Station	% WQAS [1]	% Fertilized					Mean	SD	Sig.[2]	% of Reference
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
1	100	71	68	68	71	65	68.6	2.51		94.0
	50	68	79	69	62	71	69.8	6.14		95.4
	25	72	68	78	67	76	72.2	4.82		105.4
2	100	69	65	65	75	70	68.8	4.15		94.2
	50	76	72	87	72	77	76.8	6.14		104.9
	25	68	83	64	63	76	70.8	8.53		103.4
3	100	82	81	80	70	85	79.6	5.68		109.0
	50	68	78	83	81	70	76.0	6.67		103.8
	25	81	68	81	72	68	74.0	6.60		108.0
4	100	73	80	76	76	72	75.4	3.13		103.3
	50	72	76	72	69	75	72.8	2.77		99.5
	25	77	75	70	76	75	74.6	2.70		108.9
5	100	64	70	75	61	78	69.6	7.16		95.3
	50	62	74	78	69	73	71.2	6.06		97.3
	25	78	76	62	68	69	70.6	6.47		103.1
6 [3]	100	75	75	73	71	71	73.0	2.00		100.0
	50	68	77	73	71	77	73.2	3.90		100.0
	25	69	73	90 [5]	64	68	68.5	3.70		100.0
7	100	68	60	57	72	68	65.0	6.24		89.0
	50	73	64	71	65	71	68.8	4.02		94.0
	25	75	71	69	68	69	70.4	2.79		102.8
8	100	26	27	24	30	35	28.4	4.28	**	38.9
	50	77	81	73	75	65	74.2	5.93		101.4
	25	82	79	78	73	70	76.4	4.83		111.5
9	100	80	78	80	79	80	79.4	0.89		108.8
	50	81	83	74	68	72	75.6	6.27		103.3
	25	76	68	73	65	76	71.6	4.93		104.5
10	100	39	27	34	43	38	36.2	6.06	**	49.6
	50	52	45	58	52	50	51.4	4.67	**	70.2
	25	68	61	66	61	58	62.8	4.09		91.7
11	100	69	80	80	72	83	76.8	5.97		105.2
	50	66	75	68	69	72	70.0	3.54		95.6
	25	82	73	70	65	76	73.2	6.38		106.9

TABLE 4. Continued.

Station	% WQAS[1]	% Fertilized					Mean	SD	Sig.[2]	% of Reference
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
12	100	42	45	47	44	44	44.4	1.82	**	60.8
	50	77	84	81	72	78	78.4	4.51		107.1
	25	71	78	77	73	69	73.6	3.85		107.4
Tex. Ref. [4]	100	84	83	82	77	76	80.4	3.65		110.1
	50	70	72	62	80	73	71.4	6.47		97.5
	25	71	74	81	69	77	74.4	4.77		108.6
Ref. SW [6]	100	69	76	71	74	69	71.8	3.11		98.4
	50	78	73	72	72	68	72.6	3.58		99.2
	25	74	74	80	71	72	74.2	3.49		108.3
MFS [7]	100	76	63	75	68	69	70.2	5.36		96.2
SDS [8]	10	0	0	0	0	0	0.0	0.00	**	0.0
	5	41	53	39	58	48	47.8	7.98	**	65.5
	2.5	89	91	89	91	94	90.8	2.05		124.4
	1.25	79	78	75	74	77	77.0	2.16		105.5

[1] Percent of water quality adjusted pore water tested

[2] Significant difference from reference denoted as asterisks or plus signs

[3] Sample #6, reference porewater collected from Hanalei Bay, Kauai, Hawaii.

[4] Reference pore water extracted from sediment collected in Aransas Bay, Texas

[5] Statistical outlier removed from analysis.

[6] Reference seawater collected in Hanalei Bay, Kauai, Hawaii.

[7] Millipore filtered seawater diluent

[8] Sodium Dodecyl Sulfate positive reference control

TABLE 5. Sea urchin embryological development test raw data and mean values for sediment porewater samples from Hanalei Bay, Kauai, Hawaii. Asterisks denote statistical significant differences (Dunnett's t-test) and detectable significance criteria (DSC) between test and control station 6 (*alpha < 0.05, **alpha < 0.01).

Station	% WQAS [1]	Percent Fertilized					Mean	SD	Sig.[2]	% of Reference
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
1	100	99	89	93	96	91	93.6	3.97		102.6
	50	90	94	90	100	96	94.0	4.24		101.5
	25	93	96	92	89	95	93.0	2.74		101.1
2	100	98	94	92	95	93	94.4	2.30		103.5
	50	97	93	91	93	95	93.8	2.28		101.3
	25	97	93	91	93	95	93.8	2.28		102.0
3	100	96	91	95	100	94	95.2	3.27		104.4
	50	92	94	96	98	96	95.2	2.28		102.8
	25	94	92	99	89	90	92.8	3.96		100.9
4	100	98	95	91	94	95	94.6	2.51		103.7
	50	95	89	93	90	89	91.2	2.68		98.5
	25	92	89	92	92	94	91.8	1.79		99.8
5	100	98	95	94	94	95	95.2	1.64		104.4
	50	87	96	92	95	93	92.6	3.51		100.0
	25	95	99	93	93	82	92.4	6.31		100.4
6 [3]	100	93	90	89	94	90	91.2	2.17		100.0
	50	93	90	94	96	90	92.6	2.61		100.0
	25	95	91	90	89	93	92.0	2.58		100.0
7	100	94	97	98	94	98	96.2	2.05		105.5
	50	96	95	98	96	97	96.4	1.14		104.1
	25	94	93	95	93	91	93.2	1.48		101.3
8	100	88	93	89	85	90	89.0	2.92		97.6
	50	84	88	94	82	93	88.2	5.31		95.2
	25	96	88	91	85	91	90.2	4.09		98.0
9	100	94	87	98	92	84	91.0	5.57		99.8
	50	91	87	88	96	91	90.6	3.51		97.8
	25	85	92	96	90	93	91.2	4.09		99.1
10	100	86	94	94	97	95	93.2	4.21		102.2
	50	91	94	91	91	94	92.2	1.64		99.6
	25	91	94	94	95	95	93.8	1.64		102.0
11	100	86	91	93	93	93	91.2	3.03		100.0
	50	91	91	90	90	92	90.8	0.84		98.1
	25	91	91	91	94	94	92.2	1.64		100.2

TABLE 5. Continued.

Station	% WQAS [2]	% Fertilized					Mean	SD	Sig.[2]	% of Reference
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
12	100	97	96	97	96	97	96.6	0.55		105.9
	50	96	95	97	86	93	93.4	4.39		100.9
	25	91	96	93	97	91	93.6	2.79		101.7
Tex. Ref. [4]	100	95	91	92	97	90	93.0	2.92		102.0
	50	89	92	89	93	94	91.4	2.30		98.7
	25	98	95	92	96	93	94.8	2.39		103.0
Ref. SW [5]	100	95	95	94	96	94	94.8	0.84		103.9
	50	95	97	91	94	85	92.4	4.67		99.8
	25	95	93	88	89	91	91.2	2.86		99.1
MFS [6]	100	88	93	94	90	91	91.2	2.39		100.0
SDS [7]	10	0	0	0	0	0	0.0	0.00	**	0.0
	5	16	11	21	11	6	13.0	5.70	**	14.3
	2.5	84	92	84	86	88	86.8	3.35		95.2
	1.25	91	94	94	90	90	91.3	1.89		100.1

[1] Percent of water quality adjusted pore water tested

[2] Significant difference from reference denoted as asterisks or plus signs

[3] Sample #6, reference porewater collected from Hanalei Bay, Kauai, Hawaii.

[4] Reference pore water extracted from sediment collected in Aransas Bay, Texas

[5] Reference seawater collected in Hanalei Bay, Kauai, Hawaii.

[6] Millipore filtered seawater diluent

[7] Sodium Dodecyl Sulfate positive reference control

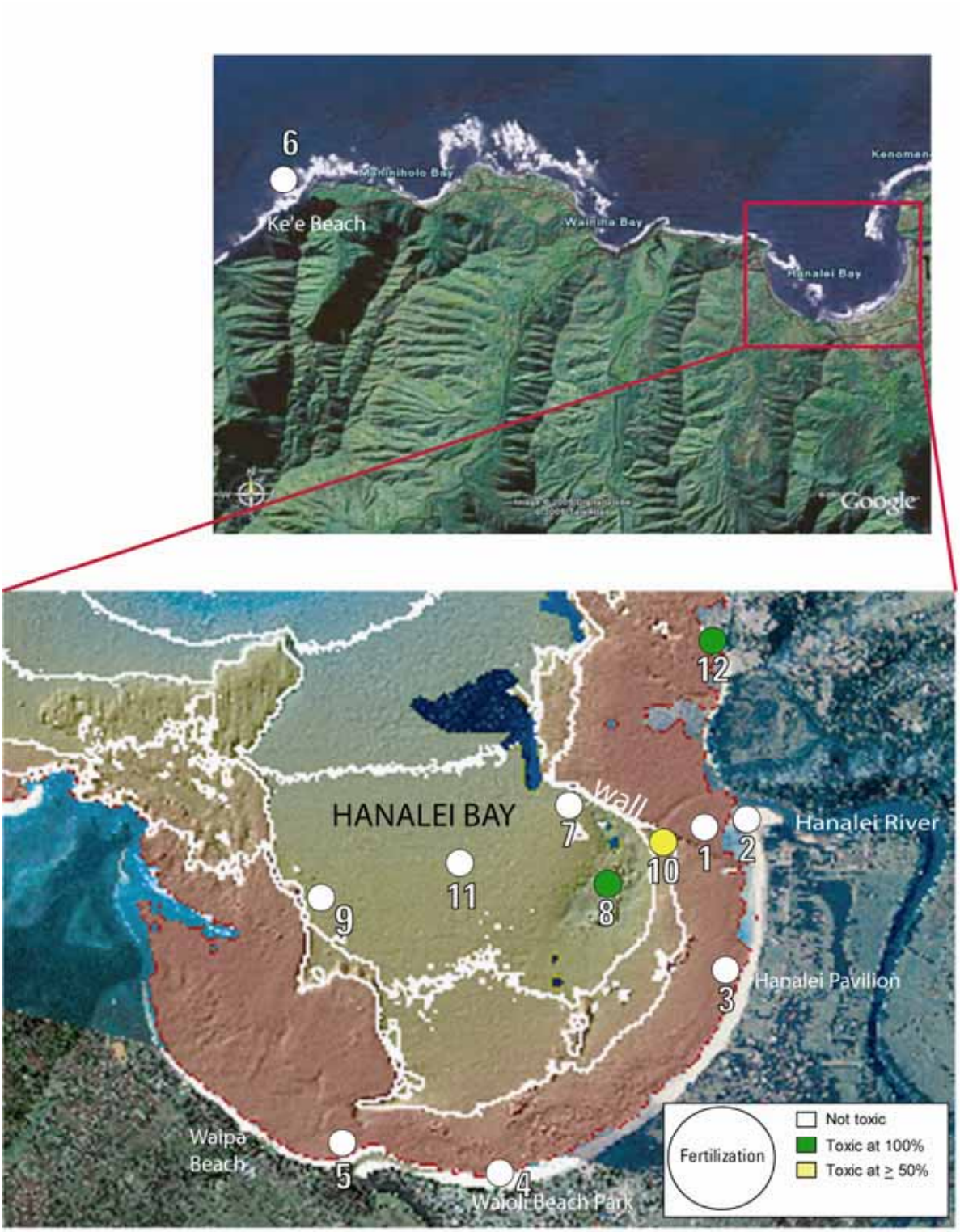

Figure 1. Toxicity test results for samples taken in Hanalei Bay, Kauai, Hawaii. Color differentiation of the circles indicates level of toxicity observed in the sea urchin fertilization test. There were no toxic samples observed for the sea urchin embryological development test.

Date Prepared: October 1, 2001

Date Revised: October 11, 2001

EXTRACTION AND STORAGE OF POREWATER SAMPLES USING IN SITU DEVICES

1.0 OBJECTIVE

This protocol describes procedures for extracting pore water samples by vacuum from marine, estuarine, or freshwater sediments, and for processing and storing pore waters for use in toxicity testing. A suction device is used to extract the pore water from sediment samples. This procedure may be performed *in situ*, or in the laboratory on samples collected with sediment coring devices.

2.0 PREPARATION

2.1 Description of the Porewater Extraction System

The extraction device consists of a filtration medium attached to a 60 mL disposable syringe (Winger and Lasier, 1991). A ground glass aquarium air stone is usually used as the filtration medium. Stainless steel cone-shaped filters with 250 μm mesh have also been used successfully as filtration media. In order to prevent breakage when inserted into the sediment, the stainless steel filters can be placed inside a 100 and 1000 μl disposable pipette tips with small holes bored throughout their surface. Figure 1 shows extraction devices with both filtration media. All sampling material is pre-soaked in deionized water for a minimum of 24 hours prior to use in the field.

Figure 1. Porewater extraction devices with two types of filtration media.

2.2 Equipment List

Supplies and equipment needed are listed in Attachment 1.

3.0 PROCEDURE

3.1 *In Situ* Procedures

In situ pore water and sediment extractions are performed by divers.

3.1.1 *In situ* porewater extraction

This procedure was developed for use at sites with coarse sands, typically in the vicinity of coral reefs. Eight to ten 60 mL syringes are usually sufficient to obtain the necessary amount of pore water for toxicity testing and water quality measurements. The syringes with attached connectors and air stones attached to a short length (~ 0.75 cm) of aquarium air tubing are placed in a mesh bag or other suitable container for transport to the sampling location by divers. Once at the sampling location, one air stone (or other selected filtration medium) is attached to each syringe. The device is gently inserted into the sediment until at least the whole filtration medium and connecting parts are buried in the sediment, ideally until the bottom 10 or 20 mL of the syringe are also buried. The total buried depth should approximate 10 cm. Once this is achieved, vacuum is applied through the syringe, promoting the suction of pore water into the syringe. When the syringe is full, the filtration medium should be carefully removed from the sediment, the whole device placed in the bag, and taken to the surface. Should the air stone be broken or lost in the sediment, the extracted pore water will still remain in the syringe and can be taken to the surface and transferred to appropriate jars as described below. If necessary, the same air stone can be removed from a full syringe and attached to an empty one for further porewater extraction.
Once on board of the research vessel, the filtration medium is removed from the tip of the syringe and the pore water is gently transferred into clean, pre-labeled polycarbonate centrifuge bottles. This procedure should be done very slowly to avoid excessive aeration of the sample, which may promote alterations of the equilibrium between water, particles, and contaminants. Samples must then be stored on ice until arrival at the laboratory. Pore water should be centrifuged as soon as possible after extraction (section 3.2).

3.1.2 Sediment coring

This procedure should be used when fine grain sediments are encountered and *in situ* porewater extraction is not practicable. The upper 10 cm of

sediment are collected by multiple cores at each sampling station. Prior to leaving the research vessel, the coring tubes may be placed inside a bucket with screw cap and holes in the lid to allow water intake and easy submersion. Once the divers reach the sampling location, the lexane™ coring tubes are inserted in the sediment to a depth of approximately 10 cm. Once the insertion is complete, the stoppers are placed into the upper part of the tube and the core is extracted from the surrounding sediment. The cap is placed in the lower part of the tube as soon as it emerges from the sediment. The filled cores are placed in the bucket and taken back to the research vessel.

One liter of sediment will typically provide 100-200 mL pore water. However, a larger volume of coarse sediments may be required since they contain less water. Nine cores will provide approximately four liters of sediment, which should be sufficient to obtain the amount of pore water necessary for toxicity testing and water quality measurements. Once on board of the research vessel, the overlying water is drained from the cores and the sediment from each core is placed in a suitable pre-labeled container (e.g., clean high density polyethylene containers or Zip-Lock® bags), thoroughly shaken for sediment homogenization and stored on ice.

Pore water should be extracted from the samples as soon as possible, because the toxicity of sediments in storage may change over time. Porewater extraction is performed using the same devices described for the *in situ* extraction (section 3.1.1). A plastic or wooden rod with the length of the syringe may be used to prop up the piston while vacuum is being applied. Once the syringes are filled with pore water, they may be removed from the sediment and the pore water gently transferred to polycarbonate centrifuge bottles. Samples should be centrifuged as soon as possible after extraction (section 3.2).

A sample tracking system should be maintained for each sediment sample collected and porewater sample extracted. All manipulations made on samples should be recorded on the Sample History Data Form (Attachment 2).

3.2 Centrifugation of Porewater Samples

Porewater samples used for toxicity testing at the Marine Ecotoxicology Research Station are usually stored frozen until tested. The porewater samples should be centrifuged shortly 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. Place the polycarbonate bottle in the centrifuge and spin at •1200 g for 20 minutes. Decant the supernatant from the centrifuged sample to a pre-cleaned and labeled 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 replicate sample jars before testing or storage.

3.3 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, at a minimum: a) the date of collection or receipt, b) the date of porewater extraction, c) the date of porewater centrifugation and relevant information about this procedure, d) the volume or number of jars of pore water collected, e) date frozen and location (freezer number or other relevant information), and f) date and jar number 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 read this SOP and 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.

7.0 ATTACHMENTS

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

8.0 REFERENCES

Carr, R.S., M. Nipper, and G.S. Plumlee. 2001. A Preliminary Survey of Marine Contamination from Mining-related Activities on Marinduque Island, Philippines: Porewater Toxicity and Chemistry: Results from a Field Trip - October 14-19, 2000. 19 pp. + 3 attachments.

Nipper, M., and R.S. Carr. 2000. Toxicity testing of sediment pore water from the Flower Garden Banks, Gulf of Mexico. Prepared for the Center for Coastal Studies, Texas A & M University - Corpus Christi, 5 pp. + 3 tables, 3 figures, and 3 attachments.

Winger PV, Lasier PJ. 1991. A vacuum-operated pore-water extractor for estuarine and freshwater sediments. Arch. Environ. Contam. Toxicol. 21:321-324.

Prepared by:

_____
Marion Nipper
Senior Research Scientist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Laverne Cleveland
Chief, Field Research Division

Attachment 1

REQUIRED EQUIPMENT AND MATERIALS

For porewater extraction:

60 mL disposable syringes
Connectors
Standard aquarium air tubing
Ground glass aquarium air stone or stainless steel conic filters with 250 μm mesh
100 and 1000 μL disposable pipette tips

For sediment coring:

Lexane® cores with 5 cm inner diameter
Plastic caps for lower side of corer
Rubber stoppers for upper side of corer

Other required supplies/equipment:

Sediment sample containers or bags
Centrifuge bottles
Coolers with ice
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 $\geq$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 ($\sim$100$^{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. For samples higher in salinity than target salinity, HPLC ultrapure sterile water (J.T. Baker7 Cat. #JT4218-2) 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-Chem7 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

Prepared by:

_____
Duane C. Chapman
Fishery Biologist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Anne E. Kinsinger
Chief, Field Research Division

_____
Joseph B. Hunn
Quality Assurance Officer

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. Baker7 #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 ($^{o}/_{oo}$) _____

Vol. Milli-Q water added (mL) _____

Vol. ___$^{o}/_{oo}$ brine added (mL) _____

% of original sample
(initial vol./final vol. x 100) _____

B. Character of Sample (after salinity adjustment):

Volume (mL) _____

Salinity ($^{o}/_{oo}$) _____

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 $\pm$ 1°C when gametes are not required. Temperature is gradually increased to 19 $\pm$ 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 Millipore7 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)
0.75 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 <u>bottom</u> 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:

   Total No. Eggs - No. Eggs Unfertilized x 100 = 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.

Revised by:

James M. Biedenbach
Biologist

Prepared by:

Duane Chapman
Fishery Biologist

Approved by:

R. Scott Carr
Field Station Leader

Anne E. Kinsinger
Chief, Field Research Division

Joseph B. Hunn
Quality Assurance Officer

Attachment 1

EQUIPMENT LIST FOR FERTILIZATION TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL Wheaton scintillation vials (These should be type shipped with caps with polyseal cone 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 ($^{o}/_{oo}$) _____

Vol. Milli-Q water added (mL) _____

Vol. ___$^{o}/_{oo}$ brine added (mL) _____

% of original sample
(initial vol./final vol. x 100) _____

B. Character of Sample (after salinity adjustment):

Volume (mL) _____

Salinity ($^{o}/_{oo}$) _____

pH _____

Dissolved oxygen (mg/L) _____

DO saturation (%) _____

Total ammonia (mg/L) _____

Sulfide (mg/L) _____

COMMENTS_____

_____

_____

_____

_____

_____

Attachment 4
SEAURCHIN PRETEST DATA SHEET

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

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

Comments:_____

Date Prepared : April 10, 1990

Date Revised: February 29, 2000

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 $\pm$ 1°C when gametes are not required. Temperature is gradually increased to 19 $\pm$ 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 $\pm$ 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 Millipore7 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)

0.75 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. 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 70-90%. 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 70% normal pluteus 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 1:1250 and 1:7500 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. The quantity of sperm to be added to the egg dilution is calculated by dividing the total volume of eggs by five and adding 50 µL of sperm dilution per that number. Sperm should be allowed to incubate with the eggs for 10 minutes to allow fertilization to take place. After 10 minutes, eggs should be evaluated under 100 X magnification for fertilization membranes. If 70-90% of the eggs are fertilized, the embryos can be pipetted into the test vials. If the percentage is lower than 70%, additional sperm may be added and/or more time allowed for fertilization. If the fertilization does not increase above 70% after 30 minutes, the embryos should be discarded and new gametes selected for use. Embryos should not be allowed to undergo division before pipetting them into the test vials.

3.0 TEST PROCEDURES

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

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

3. Using the dispenser, add 0.75 mL 10% buffered formalin to each vial.

4. 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 <u>bottom</u> 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 or for more efficient data collection, stages other than pleuteus and abnormalities may be lumped together and counted on one key. 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 gametes 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 I. 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

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.

Revised by:

James M. Biedenbach
Biologist

Prepared by:

Duane Chapman
Fishery Biologist

Approved by:

R. Scott Carr
Field Station Leader

Anne E. Kinsinger
Chief, Field Research Division

Joseph B. Hunn
Quality Assurance Officer

Attachment 1
EQUIPMENT LIST FOR EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL Wheaton scintillation vials (These should be type shipped with caps with polycone 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

DEVELOPMENT OF SEA URCHIN EGGS TO PLUTEUS LARVAE

The development of sea urchin eggs from fertilization to pluteus larvae normally occurs in approximately 48 hours. Although development is a continuous process of mitosis and cellular differentiation, developmental biology defines distinct stages of development by gross morphological characteristics. For the purpose of the Sea Urchin Embryological Development Test, six stages are defined and used in the characterization of embryos (Drawings on following page).

1. Unfertilized egg - single cell which appears dense and lacks a fertilization membrane.

2. Fertilized egg - egg with a distinct fertilization membrane which appears as a thin band lying slightly away from the central egg. The early stages of cell division are included in this group.

3. Blastula - spherical, "hollow-ball" stage which is ciliated and becomes free-swimming by breaking out of the fertilization membrane.

4. Early gastrula - beginnings of invagination of the blastula wall are evident. Cells move inward (invaginate) to form a central cavity (archenteron). Early gastrula includes embryos with the earliest stages of invagination and continues until the archenteron reaches approximately two-thirds of the diameter of the embryo.

5. Late gastrula - gastrula in which archenteron has developed in length to two-thirds of the embryo diameter and has begun to differentiate and bend towards and break through the embryo wall. Included are the later stages (prism) with primitive gut (complete digestive system), early skeletal rod development, and beginnings of deltoid shape formation.

6. Pluteus - deltoid-shaped larval stage with complete digestive system, skeletal rods, and growth of projecting arms.

Attachment 4
WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

Initial volume (mL) _____

Initial salinity ($^{o}/_{oo}$) _____

Vol. Milli-Q water added (mL) _____

Vol. ___$^{o}/_{oo}$ brine added (mL) _____

% of original sample
(initial vol./final vol. x 100) _____

B. Character of Sample (after salinity adjustment):

Volume (mL) _____

Salinity ($^{o}/_{oo}$) _____

pH _____

Dissolved oxygen (mg/L) _____

DO saturation (%) _____

Total ammonia (mg/L) _____

Sulfide (mg/L) _____

COMMENTS_____

_____

_____

_____

_____

_____

Attachment 5
SEAURCHIN PRETEST DATA SHEET

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ 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 TOXICITY TEST

DATA SHEET

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

PERCENT NORMAL PLUTEUS

	Replicate						
Treatment	1	2	3	4	5	Mean±SD	% Not Plut.

Comments:_____