Sediment Toxicity Test Results for the Urban Waters Study 2010, Bellingham Bay, Washington

Data Series 591

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By James M. Biedenbach

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

U.S. Department of the Interior
KEN SALAZAR, Secretary

U.S. Geological Survey
Marcia K. McNutt, Director

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Conversion Factors

SI to Inch/Pound

Multiply	By	To obtain
Volume		
liter (L)	33.82	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	.2642	gallon (gal)
liter (L)	61.02	cubic inch (in3)
milliliters (mL)	.3382	ounce, fluid (fl. oz)
microliters (μL)	.00003382	ounce, fluid (fl. oz)
Mass		
gram (g)	0.03527	ounce, avoirdupois (oz)
milligrams (mg)	.00003527	ounce, avoirdupois (oz)
micrograms (μg)	.00000003527	ounce, avoirdupois (oz)
Length		
micrometer (μm)	0.00003937	inches (in.)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F=(1.8×°C)+32$$

Concentrations of chemical constituents in water are given either in milligrams per liter (mg/L) or micrograms per liter (μg/L).

Salinities are given in parts per thousand (‰) equivalent to grams per liter (g/L).

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Introduction

The Washington Department of Ecology annually determines the quality of recently deposited sediments in Puget Sound as a part of Ecology's Urban Waters Initiative. The annual sediment quality studies use the Sediment Quality Triad (SQT) approach, thus relying on measures of chemical contamination, toxicity, and benthic in-faunal effects (Chapman, 1990). Since 2002, the studies followed a rotating sampling scheme, each year sampling a different region of the greater Puget Sound Basin. During the annual studies, samples are collected in locations selected with a stratified-random design, patterned after the designs previously used in baseline surveys completed during 1997–1999 (Long and others, 2003; Wilson and Partridge, 2007).

Sediment samples were collected by personnel from the Washington Department of Ecology, in June of 2010 and shipped to the U. S. Geological Survey (USGS) laboratory in Corpus Christi, Texas (not shown), where the tests were performed. Sediment pore water was extracted with a pneumatic apparatus and was stored frozen. Just before testing, water-quality measurements were made and salinity adjusted, if necessary. Tests were performed on a dilution series of each sample consisting of 100-, 50-, and 25-percent pore-water concentrations.

The specific objectives of this study were to:

- Extract sediment pore water from a total of 30 sediment samples from the Bellingham Bay, Washington area within a day of receipt of the samples.

- Measure water-quality parameters (salinity, dissolved oxygen, pH, sulfide, and ammonia) of thawed pore-water samples before testing and adjust salinity, temperature and dissolved oxygen, if necessary, to obtain optimal ranges for the test species.

- Conduct the fertilization toxicity test with pore water using sea urchin (*Stronylocentrotus purpuratus*) (*S. purpuratus*) gametes.

- Perform 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.

- Determine which samples caused a significant decrease in percent fertilization success relative to the negative control.

Materials and Methods

Sediment Sample Receipt and Tracking

Surficial sediment samples were collected from 30 stations in areas throughout Bellingham Bay, Washington. Samples were placed in precleaned 1-gallon high density polyethylene containers, chilled, and shipped in insulated coolers with blue ice. Samples were received by the USGS in Corpus Christi, Texas, the day following shipment. Shipments were accompanied by sample tracking sheets, and samples were logged into laboratory sample tracking systems (appendix 1). All pore-water samples were extracted within 6 days from the time of field collection of sediment, and within 30 hours of arrival at the Corpus Christi laboratory (appendix 2).

Toxicity Testing

Sediment Pore-Water Extraction Procedure

Approximately 500 milliliters (mL) of pore water was extracted from each sediment sample using a pneumatic extraction device. The extractor is made of polyvinyl chloride (PVC) and uses a 5-micrometer (μm) polyester filter. It is the same device used in previous sediment quality assessment surveys (Carr and Chapman, 1992, 1995; Carr and others, 1996a, 1996b, 1997, 2001). The apparatus and extraction procedures are detailed in CERC SOP P.649 (appendix 3). After extraction, the pore-water samples were centrifuged in polycarbonate bottles at 1,200 times gravity (g) for 20 minutes to remove any suspended particulate material; the supernatant was collected, divided into three precleaned sample bottles, and frozen at negative 20 degrees Celsius (°C).

Two days before conducting a toxicity test, one bottle from each station was moved from the freezer to a refrigerator kept at 4 °C. One day before testing, samples were thawed

and brought to room temperature in a tepid (20 ± 2 °C) water bath. Sample salinity was measured and adjusted to 30 ± 1 parts per thousand (‰), if necessary, using purified de-ionized water or concentrated brine (appendix 4; CERC SOP P.651). Following water-quality adjustments, the samples were stored overnight at 4 °C but, were returned to 12 ± 1 °C (incubated in an environmental chamber) immediately before the start of the toxicity tests.

On the day of the test, subsamples were collected, acclimated to room temperature and water-quality measurements (dissolved oxygen, pH and ammonia) were made (appendix 5; CERC SOP P.646). Additional subsamples were preserved with SAOB II reagent for sulfide measurements and refrigerated to 4 °C until the following day when they were brought to room temperature and measured (appendix 6; CERC SOP P.657). Dissolved oxygen (DO) was measured with a YSI® model 59 dissolved-oxygen meter with a YSI model 5905 BOD probe. Salinity was measured with a Reichert® temperature compensated refractometer. Sulfide (as S^{-2}), pH and total ammonia (expressed as nitrogen; TAN) were measured with Orion® model 290 A meters and the Thermo–Orion model 9616 silver/sulfide probe, Thermo-Orion model 9107BN low maintenance triode, and the Thermo-Orion model 5912 ammonia probe, respectively. Room and environmental chamber temperature were measured using a Fisherbrand 76mm Teflon encased mercury thermometer. Un-ionized ammonia concentrations (expressed as nitrogen; UAN) were calculated for each sample using the respective salinity, temperature, pH, and TAN values (Bowers and Bidwell, 1978). Any samples containing less than 80-percent DO saturation were aerated gently by stirring the sample on a magnetic stir plate before testing.

Toxicity Testing with Sea Urchins

S. purpuratus urchins were obtained from Marinus Scientific Inc., Los Angeles county, California (not shown). Each pore-water sample was tested in a dilution series at 100, 50, and 25 percent of sample (after salinity adjustment) with five replicates per treatment (appendix 7; CERC SOP P.645). A pretest was conducted, which included the reference pore water, the dilution water and a limited reference toxicant dilution series, to determine the optimum sperm dilution that would produce acceptable fertilization rates in the negative control pore water, but also would produce acceptable fertilization in the dilution water and meet the USGS Corpus Christi, Texas, laboratory standard in the reference toxicant. All pretests and tests were conducted at 12 °C in a temperature controlled chamber with an exposure time of 20 minutes each for the sperm and the sperm plus eggs. Dilutions were made with 0.45-μm filtered seawater collected from the ship channel in Port Aransas, Texas (not shown). A pore-water sample collected from Aransas Bay, Texas (not shown), which had been collected with a PVC corer, held refrigerated and extracted identically to the test samples, was included with each toxicity test as a negative control. This site is far removed from any known sources of contamination and has been used previously

as a control site (Carr and others, 2006; Carr and Nipper, 2008; Long and others, 2008).

In addition, a dilution water blank of filtered seawater was included in each test and a brine blank (control pore water diluted to the lowest salinity measured in the test samples and subsequently increased with brine) was included in those tests that contained samples that were adjusted with brine. Finally, a dilution series test with SDS was included in each assay as a positive control to evaluate overall test sensitivity. The positive control was prepared, and diluted with the same filtered seawater used to dilute the pore waters in the dilution series described above.

Sea Urchin Data Analysis

For the fertilization test, statistical comparisons among treatments were made using analysis of varience (ANOVA) and Dunnett's one-tailed *t*-test (which controls the experiment-wise error rate) on the arcsine square root transformed data with the aid of Statistical Analysis Software (SAS) (Statistical Analysis Software (SAS) Institute Incorporated, 1989). The trimmed Spearman-Karber method (Hamilton and others, 1977) with Abbott's correction (Morgan, 1992) was used to calculate 50-percent effective concentration (EC_{50}) values for the SDS dilution series.

Before statistical analysis, the transformed data sets were screened for equal variance using SAS/LAB® Software (Statistical Analysis Software (SAS) Institute Incorporated, 1992). The SAS/LAB Software performs a Levene's test for equal variance, and when there was statistical evidence (based on performing a one-way ANOVA on the absolute deviations of the observations from their respective group means) of unequal variances additional data transformations were performed and outliers removed. Outliers were detected by comparing the studentized residuals to a critical value from a *t*-distribution selected 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 percent. The critical value, *cv*, is given by the following equation:

$$cv = t\left(d\int_{Error,} .05 / (2 \times n)\right) \tag{1}$$

Replicates also were removed from statistical consideration if there were known errors in the pipetting of the gametes to the vials.

A second criterion also was used to compare test means to negative control means. Detectable significance criteria (DSC) have been developed to determine the 95-percent confidence limit based on power analysis of similar tests performed by the USGS lab (Carr and Biedenbach, 1999). At α = 0.01, the DSC value is 19 percent. The DSC is applied by multiplying the DSC percentage by the reference mean, and subtracting that value from the reference mean to obtain a cut-off value. Results below this value are considered toxic. For instance, the sea urchin fertilization DSC cut-off value at α = 0.01 for a control mean of 95 percent would be 76.95 percent

[(95-(0.19x95)]. The DSC was developed using the sea urchin *Arbacia punctulata*, but was used to evaluate these data to aid in comparison to previous studies. A DSC value has not been determined for *S. purpuratus*.

Results

Pore-Water Quality Measurements

Water-quality measurements were conducted on sediment pore water from 30 stations plus the controls. Values for all the water-quality measurements conducted are listed in table 1 (at the back of the report). One sample (#22) required salinity adjustment before testing. Salinities in test samples ranged from 25 to 31 ‰. Initial dissolved oxygen was greater than 80 percent in all the samples. Total ammonia ranged from 0.12 to 6.99 milligrams per liter (mg/L), whereas, the un-ionized ammonia (the most toxic fraction) ranged from 4.2 to 161.6 micrograms per liter (µg/L). No samples exceeded the reported No Observable Effects Concentration (NOEC) for un-ionized ammonia (170 µg/L) for this species (Bailey and others, 1995). Sulfide concentrations were measured in nearly all pore water samples and ranged from 0.01 to 0. 019 mg/L.

Sea Urchin Toxicity Testing

On 21 January 2011, 2 tests were conducted with 15 of the 30 samples in each test, plus the references and controls. The samples were divided into two separate tests to minimize sperm degradation from extended pipetting times. A dilution of eggs collected from a single female was subdivided into two aliquots and used in both tests so that animal variability would not be a factor between the two tests. Sperm from a single male also was used in both tests at the same dilution. The sperm dilutions were made just before pipetting into the samples. Pretest results indicated an optimum sperm dilution of 1 part sperm to 5,000 parts dilution water. Raw data and means from the fertilization tests are given in tables 2 and 3

(at the back of the report). All experimental variables (temperature, lighting, timing, gametes used) remained the same for both tests.

There was one statistical outlying data point in the first test (sample 299, 100-percent dilution, rep 4) (Statistical Analysis Software (SAS) Institute Incorporated, 1992). Four reps in the second test were eliminated from the analysis because of errors in the pipetting of the gametes into those vials (#53, 100-percent dilution, rep 2; #60, 100-percent dilution, rep 5; #61, 50-percent dilution, rep 4; and #61, 25-percent dilution, rep 4). The EC_{50} value for the SDS positive controls were calculated as 2.36 and 2.33 mg/L for tests 1 and 2, respectively. These results indicate that the tests were more sensitive than previous tests run but were within the 95-percent confidence limits of the historical results for the USGS Corpus Christi, Texas, laboratory for this species of 2.22 to 4.21 mg/L (appendix 8). Negative control values and dilution water values also were within historical results generated by the USGS Corpus Christi, Texas, laboratory (appendix 9).

In test 1, three samples from stations 32, 85 and 227 indicated significant differences from the reference pore water at the 100-percent pore-water concentration and met the DSC (table 2, fig. 1). Because of minimal standard deviation of the reference, several other samples (#20, 21, 31, 34, 213, 277 and 299) were significant from the reference at the 100-percent pore-water concentration, but these samples did not meet the detectable significance criteria. None of the diluted pore-water samples produced significantly different results compared to the reference. In test 2, five samples (#53, 59, 195, 379 and 42113) indicated significant differences from the negative control pore water at the 100-percent concentration and met the DSC value (table 3, fig. 1). Sample #59 just barely met the DSC value and, therefore, is only significant at an α of 0.05. As in the first test, five additional samples (# 23, 24, 25, 26 and 27) were significantly different at the 100-percent pore-water concentration, but did not meet the detectable significance criteria. In test 2, as in test 1, none of the diluted pore-water samples produced results that were significantly different than the reference.
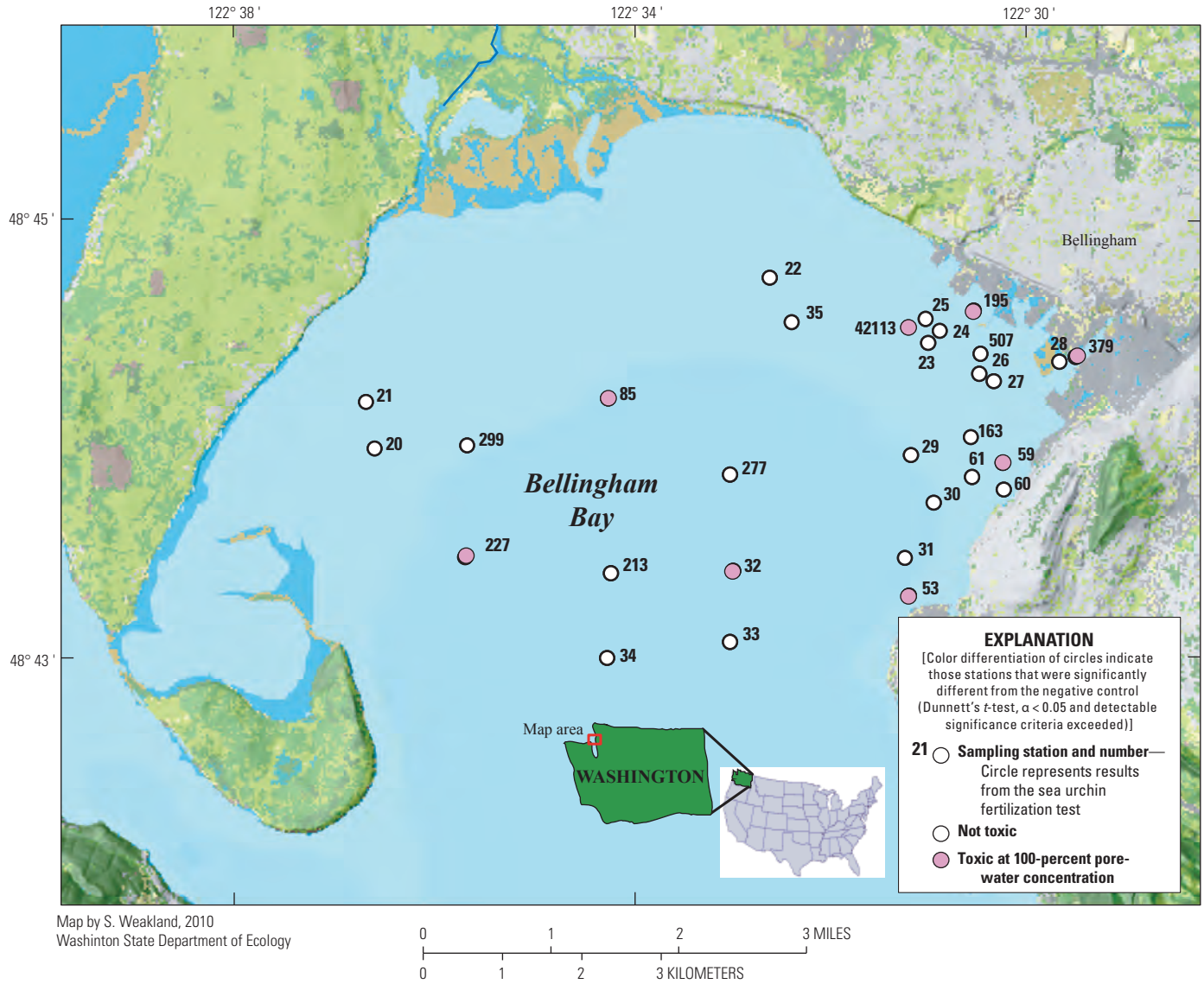
Figure 1.   Station locations of sediments and toxicity testing results for the 2010 Urban Waters study in Bellingham Bay, Washington.

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Tables 1–3

Table 1.   Water-quality measurements after salinity adjustment and original salinity of sediment pore-water samples collected for the 2010 Urban Waters study in Bellingham Bay, Washington.

[Station, sample designation; ‰, parts per thousand; DO, dissolved oxygen; mg/L, milligrams per liter; TAN, total ammonia as nitrogen; UAN, un-ionized ammonia as nitrogen; μg/L, micrograms per liter; OUS, original unadjusted sample; MFS, Millipore® filtered seawater; <, less than; ±, plus or minus]

Station	Salinity[1] (‰)	DO[2] (mg/L)	Percent DO[3]	pH	TAN[4] (mg/L)	UAN[5] (μg/L)	Sulfide[6] (mg/L)	Percent OUS[7]
TXREF[8]	30	7.43	97.7	7.90	0.51	12.6	0.010	100.0
MFS[9]	31	6.69	86.5	8.05	.12	4.2	<.010	100.0
Brine blank[10]	30	6.70	87.6	8.01	.44	14.2	.010	100.0
20	30	7.19	93.4	7.27	4.33	25.7	.012	100.0
21	29	7.19	93.0	7.57	4.06	48.5	.015	100.0
22	25	7.56	97.6	7.68	1.11	16.9	.013	93.8
23	30	6.89	88.7	7.70	4.93	77.8	.015	100.0
24	29	6.97	89.6	7.15	3.03	13.8	.015	100.0
25	29	7.00	90.2	7.22	3.02	15.9	.016	100.0
26	30	7.19	92.5	7.25	3.72	21.3	.015	100.0
27	29	7.26	93.7	7.62	3.87	51.3	.016	100.0
28	29	7.18	92.9	7.54	4.62	51.7	.015	100.0
29	30	7.09	91.7	7.86	6.99	161.2	.016	100.0
30	30	6.81	89.1	7.59	4.68	58.7	.015	100.0
31	30	6.97	90.3	7.32	2.76	18.7	.016	100.0
32	30	7.10	92.0	7.19	4.64	23.4	.018	100.0
33	31	7.12	92.2	7.48	2.45	23.7	.015	100.0
34	30	7.42	96.6	7.37	3.77	28.6	.016	100.0
35	30	7.39	96.0	7.76	5.22	96.1	.016	100.0
53	30	7.15	93.2	7.70	6.80	109.3	.016	100.0
59	30	7.23	93.9	7.15	4.48	20.3	.016	100.0
60	30	7.36	95.5	7.30	4.25	27.1	.015	100.0
61	30	7.11	92.2	7.39	4.11	32.5	.015	100.0
85	30	7.14	92.5	7.47	5.30	50.6	.016	100.0
163	30	7.40	96.0	7.30	3.86	24.9	.015	100.0
195	29.5	7.25	94.1	7.85	5.93	131.9	.017	100.0
213	30.5	7.39	95.9	7.57	2.56	30.6	.015	100.0
227	30	7.25	94.1	7.46	4.46	41.3	.019	100.0
277	30	7.48	97.2	7.50	2.44	24.5	.015	100.0
299	30	7.32	95.0	7.71	5.77	93.3	.016	100.0
379	30	7.29	94.9	7.67	4.18	62.3	.016	100.0
507	29	7.31	95.1	7.30	3.53	22.4	.016	100.0
42113	29	6.95	90.4	7.16	3.31	15.6	.016	100.0

[1] Salinity of sample before adjustment. Sample adjusted to 30 ± 1 ‰.

[2] Dissolved oxygen.

[3] Percent saturation of dissolved oxygen.

[4] Total ammonia as nitrogen.

[5] Un-ionized ammonia as nitrogen.

[6] Measured as S^{-2}.

[7] Percent of original sample after salinity adjustment.

[8] Negative control pore water extracted from sediment collected in Aransas Bay, Texas.

[9] Millipore® filtered seawater diluent.

[10] Blank consisting of TXREF diluted to 25 ‰ with Milli-Q® purified water and then brought back to 30 ‰ with hypersaline brine.

Table 2. Sea urchin fertilization test raw data and means for sediment pore-water samples in test one of the Urban Waters 2010 study in Bellingham Bay, Washington.

[Station, sample designation; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; Sig., Significance; nc, negative control; ++, statistical difference at alpha less than or equal to 0.01; ns, not significant; **, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.01; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; ‰, parts per thousand]

| Station | Percent WQAS[1] | Percent fertilized | | | | | Mean | SD | Sig.[2] | Percent of control[3] |
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
TXREF[4]	100	98	99	100	98	98	98.3	1.06	nc	100.0
		100	97	97	98	98				
	50	99	99	100	100	100	99.0	.94	nc	100.0
		99	99	97	99	98				
	25	95	100	100	99	99	98.6	1.78	nc	100.0
		99	100	96	98	100				
20	100	92	92	89	92	94	91.8	1.79	++	93.4
	50	98	100	100	98	98	98.8	1.10	ns	99.8
	25	99	100	99	100	98	99.2	.84	ns	100.6
21	100	96	91	89	94	85	91.0	4.30	++	92.6
	50	99	100	99	100	99	99.4	.55	ns	100.4
	25	99	100	98	98	98	98.6	.89	ns	100.0
22	100	97	100	100	99	100	99.2	1.30	ns	100.9
	50	100	99	99	100	99	99.4	.55	ns	100.4
	25	100	100	100	100	99	99.8	.45	ns	101.2
29	100	95	98	99	97	96	97.0	1.58	ns	98.7
	50	99	100	99	100	99	99.4	.55	ns	100.4
	25	99	98	100	99	98	98.8	.84	ns	100.2
30	100	98	97	96	98	97	97.2	.84	ns	98.9
	50	98	100	100	98	100	99.2	1.10	ns	100.2
	25	97	99	100	100	96	98.0	1.83	ns	99.4
31	100	84	77	88	86	87	84.4	4.39	++	85.9
	50	100	98	95	100	99	98.4	2.07	ns	99.4
	25	98	100	98	100	97	98.6	1.34	ns	100.0
32	100	45	41	49	39	39	42.6	4.34	**	43.3
	50	96	100	97	98	98	97.8	1.48	ns	98.8
	25	99	97	99	98	99	98.4	.89	ns	99.8
33	100	97	96	98	97	99	97.5	1.29	ns	99.2
	50	99	96	96	99	98	97.6	1.52	ns	98.6
	25	98	98	99	99	99	98.6	.55	ns	100.0
34	100	86	85	89	90	88	87.6	2.07	++	89.1
	50	99	98	97	100	100	98.8	1.30	ns	99.8
	25	100	99	99	95	100	98.6	2.07	ns	100.0
35	100	98	96	98	97	97	97.2	.84	ns	98.9
	50	96	100	98	98	99	98.2	1.48	ns	99.2
	25	99	100	99	96	99	98.6	1.52	ns	100.0
85	100	68	84	48	66	68	66.8	12.77	**	68.0
	50	100	99	100	99	98	99.2	.84	ns	100.2
	25	99	98	100	100	100	99.4	.89	ns	100.8

Table 2.   Sea urchin fertilization test raw data and means for sediment pore-water samples in test one of the Urban Waters 2010 study in Bellingham Bay, Washington.—Continued

[Station, sample designation; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; Sig., Significance; nc, negative control; ++, statistical difference at alpha less than or equal to 0.01; ns, not significant; **, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.01; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; ‰, parts per thousand]

Station	Percent WQAS[1]	Percent fertilized					Mean	SD	Sig.[2]	Percent of control[3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
213	100	95	88	89	85	81	87.6	5.18	++	89.1
	50	99	100	95	98	99	98.2	1.92	ns	99.2
	25	100	96	99	99	100	98.8	1.64	ns	100.2
227	100	8	6	2	9	2	5.4	3.29	**	5.5
	50	96	94	99	99	94	96.4	2.51	ns	97.4
	25	99	98	96	98	97	97.6	1.14	ns	99.0
277	100	85	90	87	88	85	87.0	2.12	++	88.5
	50	100	98	97	98	96	97.8	1.48	ns	98.8
	25	98	100	98	100	99	99.0	1.00	ns	100.4
299	100	87	86	84	[5]60	83	85.0	1.83	++	86.5
	50	100	99	99	100	99	99.4	.55	ns	100.4
	25	100	100	100	98	100	99.6	.89	ns	101.0
MFS[6]	100	100	98	100	98	99	98.4	1.26	ns	100.1
		97	100	97	97	98				
Brine blank[7]	100	96	99	100	100	100	99.0	1.73	ns	100.7
SDS[8]	10	0	0	0	0	0	0.0	0.00	ns	0.0
	5	0	0	0	0	0	0.0	0.00	ns	0.0
	2.5	37	41	46	49	48	44.2	5.07	**	45.0
	1.25	93	90	88	89	91	90.2	1.92	++	91.8

[1] Percent of water quality adjusted pore-water sampled or concentration in mg/L for SDS positive control.

[2] Significant difference determined using Dunnett's *t*-test and detectable significance criteria.

[3] Percent of TXREF negative control.

[4] Negative control pore water extracted from sediment collected in Aransas Bay, Texas.

[5] Statistical outlier removed from analysis.

[6] Millipore® filtered seawater diluent.

[7] Blank consisting of TXREF diluted to 25 ‰ with Milli-Q® purified water and then brought back to 30 ‰ with hypersaline brine.

[8] Sodium dodecyl sulfate positive control (in mg/L).

Table 3. Sea urchin fertilization test raw data and means for sediment pore-water samples in test two of the Urban Waters 2010 study in Bellingham Bay, Washington.

[Station, sample designation; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; Sig., Significance; nc, negative control; ++, statistical difference at alpha less than or equal to 0.01; ns, not significant; **, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.01; *, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.05; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; ‰, parts per thousand]

Station	Percent WQAS[1]	Percent fertilized					Mean	SD	Sig.[2]	Percent of control[3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
TXREF[4]	100	98	99	100	99	99	98.5	1.18	nc	100.0
		97	97	100	99	97				
	50	98	98	99	99	100	98.8	.79	nc	100.0
		98	99	98	100	99				
	25	99	100	98	100	100	99.5	.71	nc	100.0
		99	100	99	100	100				
23	100	94	98	97	96	92	95.4	2.41	++	96.9
	50	99	99	99	100	100	99.4	.55	ns	100.6
	25	100	100	100	100	99	99.8	.45	ns	100.3
24	100	92	95	95	91	90	92.6	2.30	++	94.0
	50	100	98	99	100	100	99.4	.89	ns	100.6
	25	99	99	100	100	99	99.4	.55	ns	99.9
25	100	94	85	85	86	80	86.0	5.05	++	87.3
	50	96	98	98	100	100	98.4	1.67	ns	99.6
	25	99	100	100	99	99	99.4	.55	ns	99.9
26	100	87	92	94	92	89	90.8	2.77	++	92.2
	50	97	99	96	99	99	98.0	1.41	ns	99.2
	25	98	95	98	100	99	98.0	1.87	ns	98.5
27	100	83	88	90	83	91	87.0	3.81	++	88.3
	50	98	99	100	98	98	98.6	.89	ns	99.8
	25	98	100	100	100	99	99.4	.89	ns	99.9
28	100	98	100	98	100	98	98.8	1.10	ns	100.3
	50	100	100	100	99	99	99.6	.55	ns	100.8
	25	98	99	96	100	100	98.6	1.67	ns	99.1
53	100	66	[5]0	80	72	75	73.3	5.85	**	74.4
	50	100	99	100	98	97	98.8	1.30	ns	100.0
	25	99	98	98	100	97	98.4	1.14	ns	98.9
59	100	83	81	80	83	88	83.0	3.56	*	84.3
	50	99	99	98	99	96	98.2	1.30	ns	99.4
	25	100	99	99	98	98	98.8	.84	ns	99.3
60	100	99	99	97	99	[5]0	98.5	1.00	ns	100.0
	50	100	98	100	96	99	98.6	1.67	ns	99.8
	25	100	98	96	99	95	97.6	2.07	ns	98.1
61	100	96	98	98	99	98	97.8	1.10	ns	99.3
	50	100	96	99	[5]0	99	98.5	1.73	ns	99.7
	25	100	96	99	[5]0	99	98.5	1.73	ns	99.0
163	100	93	99	96	99	97	96.8	2.49	ns	98.3
	50	100	100	99	99	99	99.4	.55	ns	100.6
	25	99	100	99	100	99	99.4	.55	ns	99.9

Table 3.   Sea urchin fertilization test raw data and means for sediment pore-water samples in test two of the Urban Waters 2010 study in Bellingham Bay, Washington.—Continued

[Station, sample designation; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; Sig., Significance; nc, negative control; ++, statistical difference at alpha less than or equal to 0.01; ns, not significant; **, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.01; *, statistical difference and detectable significance criteria exceedance at alpha less than or equal to 0.05; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; ‰, parts per thousand]

Station	Percent WQAS[1]	Percent fertilized					Mean	SD	Sig.[2]	Percent of control[3]
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
195	100	74	80	74	76	71	75.0	3.32	**	76.1
	50	98	100	100	100	100	99.6	.89	ns	100.8
	25	99	98	100	99	99	99.0	.71	ns	99.5
379	100	72	79	79	70	84	76.8	5.72	**	78.0
	50	100	100	99	100	97	99.2	1.30	ns	100.4
	25	97	97	100	100	99	98.6	1.52	ns	99.1
507	100	93	95	98	98	97	96.2	2.17	ns	97.7
	50	100	98	97	98	100	98.6	1.34	ns	99.8
	25	100	98	99	99	100	99.2	.84	ns	99.7
42113	100	42	44	29	25	49	41.0	8.52	**	41.6
	50	100	99	99	98	97	98.6	1.14	ns	99.8
	25	99	99	97	93	99	97.4	2.61	ns	97.9
MFS[6]	100	99	99	99	99	99	98.9	.74	ns	100.4
		99	97	99	99	100				
Brine blank[7]	100	100	100	100	100	99	99.8	.45	ns	101.3
SDS[8]	10	0	0	0	0	0	0.0	0.00	ns	0.0
	5	0	0	0	0	0	0.0	0.00	ns	0.0
	2.5	43	49	46	43	45	45.2	2.49	**	45.9
	1.25	97	93	92	95	91	93.6	2.41	++	95.0

[1] Percent of water quality adjusted pore-water sampled or concentration in mg/L for SDS positive control.

[2] Significant difference determined using Dunnett's *t*-test and detectable significance criteria.

[3] Percent of TXREF control.

[4] Negative control pore water extracted from sediment collected in Aransas Bay, Texas.

[5] Statistical outlier removed from analysis.

[6] Millipore® filtered seawater diluent.

[7] Blank consisting of TXREF diluted to 25 ‰ with Milli-Q® purified water and then brought back to 30 ‰ with hypersaline brine.

[8] Sodium dodecyl sulfate positive control (in mg/L).

Appendixes 1–9

Appendix 1

Urban Waters 2010
Urchin Fertilization Test

Sampling Date	Sampling Time		Station		Parameter
06/08/2010	00:00		34	OK	Urchin Fertilization Test
06/08/2010	00:00		20	OK	Urchin Fertilization Test
06/08/2010	00:00		21	OK	Urchin Fertilization Test
06/09/2010	00:00		29	OK	Urchin Fertilization Test
06/09/2010	00:00		30	OK	Urchin Fertilization Test
06/09/2010	00:00		31	OK	Urchin Fertilization Test
06/09/2010	00:00		33	OK	Urchin Fertilization Test
06/08/2010	00:00		34	OK	Urchin Fertilization Test
06/09/2010	00:00		85	OK	Urchin Fertilization Test
06/08/2010	00:00		213	OK	Urchin Fertilization Test
06/08/2010	00:00		227	OK	Urchin Fertilization Test
06/09/2010	00:00		277	OK	Urchin Fertilization Test
06/08/2010	00:00		299	OK	Urchin Fertilization Test

Relinquished By	Date	Received By	Date	Comments
Sandra Wea Nard	6/10/2010	FedEx Shipper	6/10/2010	
FedEx Shipper	6/10/2010		6/11/2010 10:75	Black soapy mud

Figure 1–1.   Chain of custody sheets from incoming samples arriving at the U.S. Geological Survey, Corpus Christi, Texas, laboratory from June 11–18, 2010.

Urban Waters 2010
Urchin Fertilization Test

Sampling Date	Sampling Time	Station		Parameter	
06/10/2010	00:00	22	OK	Urchin Fertilization Test	OK
06/11/2010	00:00	28	OK	Urchin Fertilization Test	OK
06/10/2010	00:00	32	OK	Urchin Fertilization Test	OK
06/11/2010	00:00	35	OK	Urchin Fertilization Test	OK
06/10/2010	00:00	53	OK	Urchin Fertilization Test	OK
06/11/2010	00:00	59	OK	Urchin Fertilization Test	OK
06/10/2010	00:00	60	OK	Urchin Fertilization Test	OK
06/10/2010	00:00	61	OK	Urchin Fertilization Test	OK
06/11/2010	00:00	163	OK	Urchin Fertilization Test	OK
06/11/2010	00:00	379	OK	Urchin Fertilization Test	OK

All processed 6-16-10

Relinquished By	Date	Received By	Date	Comments
[signature]	6/11/2010	*[signature]*	6/11/2010	
[signature]	6/14/2010	Sandra Walland	6/14/2010	
Sandra Walland	6/14/2010	Fred Snapper	6/14/2010	
Fred Snapper	6/14 7:00	*[signature]*	6/15/2010	

Figure 1–1. Chain of custody sheets from incoming samples arriving at the U.S. Geological Survey, Corpus Christi, Texas, laboratory from June 11–18, 2010.—Continued

Urban Waters 2010
Urchin Fertilization Test

Sampling Date	Sampling Time	Station		Parameter	
06/14/2010	00:00	23	ok	Urchin Fertilization Test	OK
06/14/2010	00:00	24	ok	Urchin Fertilization Test	OK
06/15/2010	00:00	25	ok	Urchin Fertilization Test	OK
06/15/2010	00:00	26	ok	Urchin Fertilization Test	OK
06/15/2010	00:00	27	ok	Urchin Fertilization Test	OK
06/14/2010	00:00	195	ok	Urchin Fertilization Test	OK
06/15/2010	00:00	507	ok	Urchin Fertilization Test	OK
06/15/2010	00:00	42113	ok	Urchin Fertilization Test	OK

Relinquished By	Date	Received By	Date	Comments

Figure 1–1.   Chain of custody sheets from incoming samples arriving at the U.S. Geological Survey, Corpus Christi, Texas, laboratory from June 11–18, 2010.—Continued

Appendix 2. Table of relevant sample dates, pore-water volumes and holding times for the 2010 Urban Waters study in Belingham Bay, Washington.

[Station, sample designation]

Station	Date collected[1]	Date shipped[2]	Date received[3]	Date extracted[4]	Volume extracted[5]	Holding time[6]	Date tested[7]
20	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
21	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
22	6/10/2010	6/14/2010	6/15/2010	6/16/2010	450	6	1/21/2011
23	6/14/2010	6/17/2010	6/18/2010	6/18/2010	450	4	1/21/2011
24	6/14/2010	6/17/2010	6/18/2010	6/18/2010	450	4	1/21/2011
25	6/15/2010	6/17/2010	6/18/2010	6/18/2010	450	3	1/21/2011
26	6/15/2010	6/17/2010	6/18/2010	6/18/2010	450	3	1/21/2011
27	6/15/2010	6/17/2010	6/18/2010	6/18/2010	450	3	1/21/2011
28	6/11/2010	6/14/2010	6/15/2010	6/16/2010	450	5	1/21/2011
29	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
30	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
31	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
32	6/10/2010	6/14/2010	6/15/2010	6/16/2010	450	6	1/21/2011
33	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
34	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
35	6/11/2010	6/14/2010	6/15/2010	6/16/2010	450	5	1/21/2011
53	6/10/2010	6/14/2010	6/15/2010	6/16/2010	450	6	1/21/2011
59	6/11/2010	6/14/2010	6/15/2010	6/16/2010	450	5	1/21/2011
60	6/10/2010	6/14/2010	6/15/2010	6/16/2010	450	6	1/21/2011
61	6/10/2010	6/14/2010	6/15/2010	6/16/2010	450	6	1/21/2011
85	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
163	6/11/2010	6/14/2010	6/15/2010	6/16/2010	450	5	1/21/2011
195	6/14/2010	6/17/2010	6/18/2010	6/18/2010	450	4	1/21/2011
213	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
227	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
277	6/9/2010	6/10/2010	6/11/2010	6/11/2010	450	2	1/21/2011
299	6/8/2010	6/10/2010	6/11/2010	6/11/2010	450	3	1/21/2011
379	6/11/2010	6/14/2010	6/15/2010	6/16/2010	450	5	1/21/2011
507	6/15/2010	6/17/2010	6/18/2010	6/18/2010	450	3	1/21/2011
42133	6/15/2010	6/17/2010	6/18/2010	6/18/2010	450	3	1/21/2011

[1] Date sediment sample collected from the field.

[2] Date sediment sample was shipped to U.S. Geological Survey laboratory in Corpus Christi, Texas.

[3] Date sediment sample was received at the U.S. Geological Survey laboratory in Corpus Christi, Texas.

[4] Date pore water was extracted from the sediment and frozen.

[5] Volume of pore water extracted and frozen (milliliters).

[6] Number of days between sample collected in the field and porewater extraction and freezing.

[7] Date that salinity adjusted pore water was tested.

Appendix 3

Date Prepared: May 5, 1990

Date Revised: July 18, 2007

<div align="center">

**EXTRACTION AND STORAGE OF
PORE-WATER SAMPLES**

</div>

1.0 OBJECTIVE

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

2.0 PREPARATION

2.1 Description of the Pore-water Extraction System

In earlier studies (Carr and others, 1989; Carr and Chapman, 1992) pore water was extracted from sediments using a device constructed of Teflon®. Since then, the design has been improved (Carr and Chapman, 1994). The polyvinyl chloride (PVC) extractors in current use are less costly to construct and easier to operate. This device has been used in numerous sediment quality assessment surveys (Carr, and others, 1996a, 1996b, 1996c, 2000, 2001).

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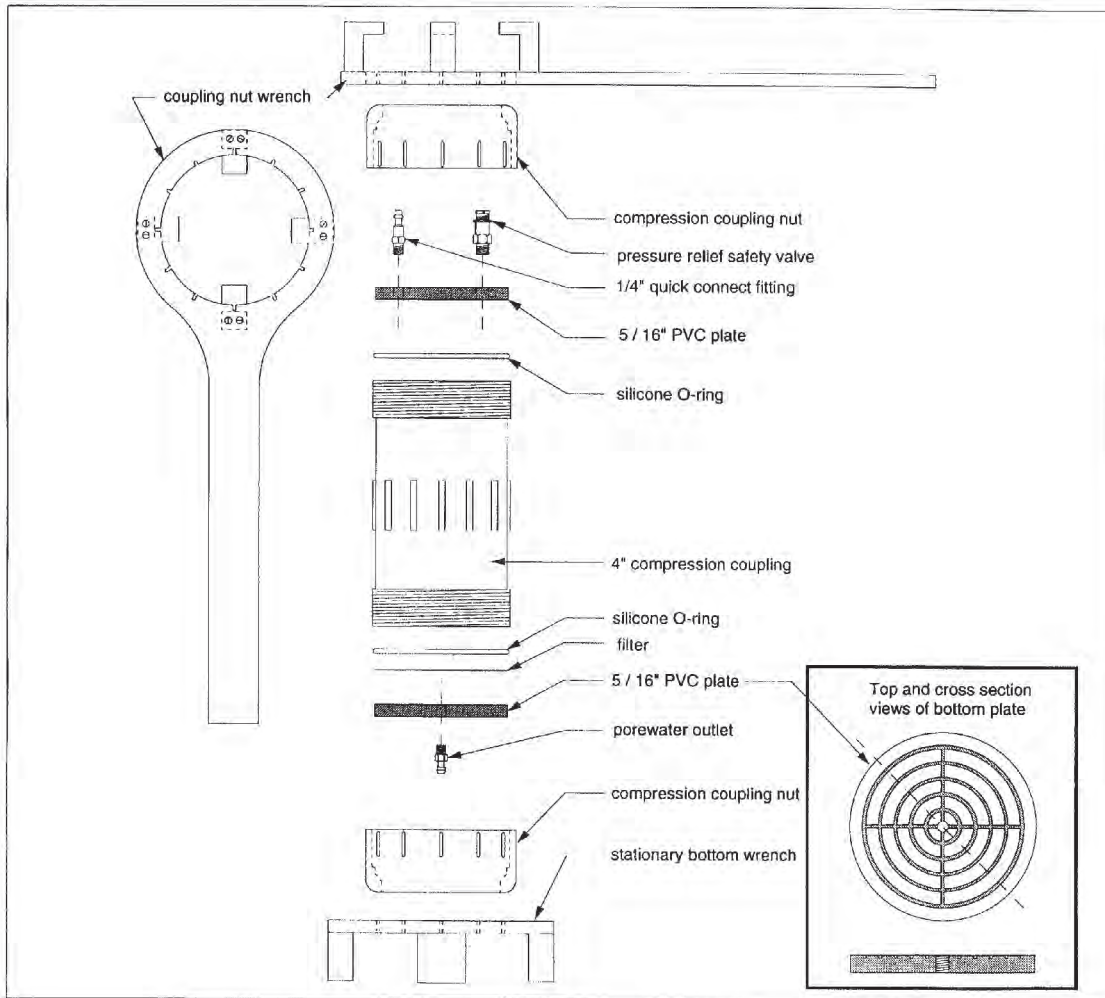
Figure 1–1. Sediment pore water squeeze extraction device.

The extractors are pressurized with air supplied from a standard SCUBA cylinder via a SCUBA first stage regulator which delivers air to a manifold with a valving system (fig 1–2). With this system, multiple cylinders can be pressurized simultaneously, using the same SCUBA cylinder.
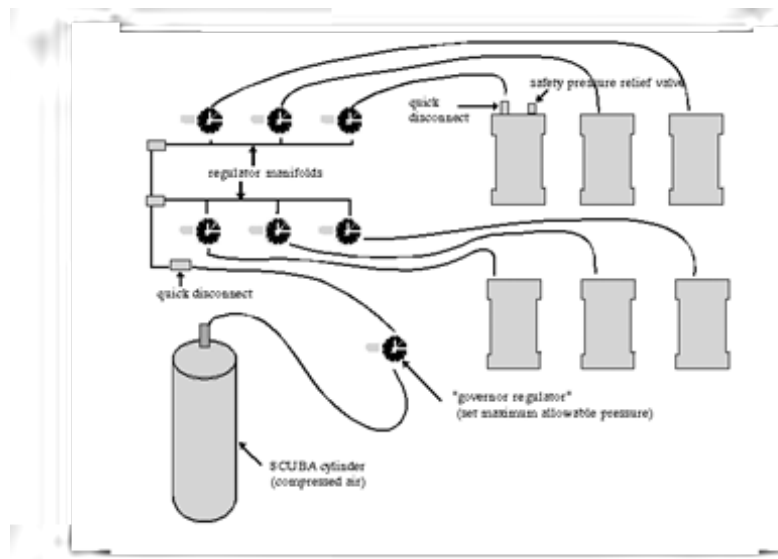

Figure 1–2. Schematic of sediment pore-water pressure extraction system.

2.2 Equipment List

Supplies and equipment needed are listed in Attachment A.

3.0 PROCEDURE

3.1 Sediment Collection and Storage Considerations

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

3.2 Load Extraction Cylinder

1. Assemble all parts of extraction cylinder except the top end compression coupling nut, top end plate and O-ring. Make sure filter is snugly in place beneath bottom O-ring (both over- and under-tightening will result in an improper seal). Place the extractor cylinder on the stand and position an appropriately labeled pore-water sample container (usually an certified pre-cleaned amber 250 mL or 125 mL glass jar cleaned to U.S. Environmental Protection Agency standards, with Teflon® lid liner) underneath the outlet.

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

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

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

3.3 Pore-water Extraction

After the extractor is sealed, a high-pressure hose is attached to the quick disconnect fitting on the top end plate, and the extractor is pressurized with air from a SCUBA tank. Pressure is controlled with a first-stage regulator on the SCUBA tank, an intermediate "governor" regulator, and final second stage regulators attached to a manifold that services multiple extractors (fig. 1–2).

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

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

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

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

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

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

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

3.4 Centrifugation of Pore-water Samples

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

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

2. Transfer the pore water from the glass sample jar to an appropriate centrifuge bottle (e.g., polycarbonate). Centrifuge at greater than or equal to 1,200 times gravity (g) for 20 minutes. Return the centrifuged sample to a rinsed and labeled glass jar, taking care not to disturb any material that may have settled on the bottom/sides of the centrifuge bottle.

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

3.5 Storage of Pore-water Samples

If the pore-water 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 (CERC SOP P.651) performed the day prior to testing. Frozen pore-water samples may be shipped with dry ice.

4.0 QUALITY CONTROL

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

5.0 TRAINING

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

6.0 SAFETY

The sediment and pore-water samples handled may contain contaminants. Care should be taken to avoid contact with the samples. Protective gloves, glasses and clothing may be worn. Waste sediment should be properly disposed.   SCUBA cylinders should be securely mounted before, during, and after use. The pressure limit (40 psi) of the extraction cylinders should not be exceeded. Before disconnecting any pressure hoses, ensure that the pressure has been released or that the controlling regulator has been closed.

7.0 ATTACHMENTS

Attachment A. Required Equipment and Materials
Attachment B. Sample History Form

8.0 REFERENCES

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Prepared by:

                                        _____

James M. Biedenbach
Biologist

Approved by:

                                          _____

R. Scott Carr
Field Station Leader

                                          _____

Laverne Cleveland
Chief, Field Stations Research Branch

                                          _____

Paul Heine
Quality Assurance Officer

Attachment A

REQUIRED EQUIPMENT AND MATERIALS

To construct a sediment pore water extraction device:

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

To use a pore water extraction device:

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

Other required supplies/equipment:

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

<u>Attachment B</u>

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): _____

_____

_____

<u>Initials</u>	<u>Date</u>	<u>Action Taken</u>
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
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Appendix 4

Date Prepared: March 14, 1991

Date Revised: July 17, 2007

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 *Schizopera knabeni*, 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 parts per thousand (‰). Although salinities used in the different toxicity tests may vary, the individual toxicity tests performed on a particular day are run at one target salinity. Since initial salinities of the porewater or water samples to be tested commonly vary, they will require salinity adjustment to within 1 ‰ of the target salinity. Additionally, DO should normally be greater than or equal to 80 percent saturation in all samples tested.

2.0 PREPARATION

2.1   Equipment and Labware

The supplies and equipment needed are listed in Attachment 1.

1.2   Source of Dilution Water

For samples lower in salinity than target salinity, concentrated brine (-100 ‰) is added to increase salinity. Concentrated brine is prepared by heating (to 35–40 °C) and gently aerating filtered natural seawater (1 micrometer (μm)) to concentrate the salts by evaporation. For samples higher in salinity than target salinity, Millipore Milli-Q® ultrapure water is added to decrease salinity.

3.0 P ROCEDURES

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 certified pre-cleaned amber 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 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

3.2.1 Reducing the salinity of aqueous samples

Refer to the formulas below to calculate the volume of Milli-Q® 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 Milli-Q ® water added as well as the final salinity.

(i)   (*Target ‰ ÷ Sample ‰*) x *Sample volume in mL = A*
(ii) *Sample volume - A = B*
(iii) *Sample volume ÷ A = C*
(iv) *B* x *C - Volume of Milli-Q® 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.

$$\text{(v.)}\quad \frac{\left[\left(Target \text{ ‰} - Sample \text{ ‰}\right) \times Sample \text{ volume in } mL\right]}{\left(Brine \text{ ‰} - Target \text{ ‰}\right)} = \quad Volume \text{ of brine to add}$$

3.3 Dissolved Oxygen Adjustment

Measure and record DO and percent DO saturation of sample (SOP P.652). Occasionally, a sample will have DO of less than 80 percent saturation. Any such samples should be gently stirred on a magnetic stirrer to increase the DO level above 80 percent.   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 greater than 80 percent saturation by stirring again if necessary before the toxicity test is performed.)

3.4 Other Water Quality Determinations

1. Measure pH (SOP P.658) and record on the Water Quality Adjustment Data Form (Attachment 2).

2. Measure and record ammonia concentration (SOP P.646).

3. Measure and record sulfide concentration if required (SOP P.657).

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. Multiple sinks are present in each of the labs, and an eye flushing station and emergency shower is present near the entrance door of each lab. 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 by donning the appropriate safety clothing and gloves.

8.0 ATTACHMENTS

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

Prepared by:

                                         _____
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Biologist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Laverne Cleveland
Chief, Field Stations
Research Branch

_____
Paul Heine
Quality Assurance Officer

<u>Attachment 1</u>

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)
Milli-Q® ultrapure water
Salinity refractometer
Dissolved oxygen meter
pH electrode, buffer solutions, and meter
Ammonia electrode, standard solutions, and meter
Sulfide electrode, standard solutions, and meter
Data sheets
Hand calculator

Attachment 2

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____   INITIALS_____

SAMPLE DESIGNATION_____   DATE_____

A. Salinity Adjustment:

    Initial volume (mL)   _____

    Initial salinity (‰)   _____

    Vol. Milli-Q® water added (mL)   _____

    Vol. ___‰ brine added (mL)   _____

    Percent of original sample   _____

    (initial volume/final volume × 100)

B. Character of Sample (after salinity adjustment):

    Volume (mL)   _____

    Salinity (‰)   _____

    pH   _____

    Dissolved oxygen (mg/L)   _____

    DO saturation (percent)   _____

    Total ammonia (mg/L)   _____

    Sulfide (mg/L)   _____

COMMENTS_____

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Appendix 5

Date Prepared: July 21, 1989

Date Revised: July 24, 2007

MEASURING DISSOLVED AMMONIA
WITH THE THERMO ORION® MODEL 95-12 AMMONIA PROBE

1.0 APPLICATION

The ammonia electrode is used to measure the concentration of ammonia in aqueous media. The detectable concentration range is from 0.01 to 14,000 milligrams per liter (mg/L) ammonia nitrogen, although in our laboratory concentrations measured usually fall within the range of 0.1 to 100 mg/L.

2.0 OPERATION

2.1 Required Equipment and Solutions

1. Thermo Orion® model 95-12 ammonia probe
2. Thermo Orion® model 290A pH/ISE meter
3. Magnetic stirrer
4. Bonded membrane caps (Thermo Orion® #951205) or loose membranes (Thermo Orion® #951204)
5. Electrode internal filling solution (Thermo Orion® #951202)
6. Standardizing Solution, NH_4Cl, 1000 mg/L as nitrogen (Thermo Orion® #951007) or prepare as described below.
7. 10 Molar (M) NaOH

2.2 Electrode Preparation and Assembly

2.2.1 Electrode Setup

When the electrode is first received or after it has been stored dry, soak the inner body in internal filling solution for at least two hours before assembling the electrode. For best results, soak the inner body overnight. Then, follow these steps:

1. Unscrew top cap and remove glass electrode inner body, and drain old filling solution. Set cap with inner body aside carefully.

2. Remove bottom cap from electrode outer body. If using bonded membrane caps, screw cap in end of electrode until finger-tight, and proceed to step 5. If using loose membranes, proceed with instructions in step 3.

3. Using tweezers, carefully grasp a white membrane from between paper separators. Hold membrane at the edge with the tweezer. Holding electrode outer body in free hand, loosely stretch the membrane across opening in bottom of probe, holding the ends against the threads with your thumb and forefinger. Avoid excessive handling of the membrane since this may affect its hydrophobic properties and reduce its life.

4. Replace cap onto probe and screw until finger-tight. The membrane should be smooth with no wrinkles.

5. Add 2.5 ml internal filling solution into the electrode outer body. If measuring low ammonia concentrations (eg. <0.06 mg/L ammonia nitrogen), the filling solution can be diluted by 1/10 to increase response time.

6. Replace inner body into outer body and screw on upper cap.

7. Shake fully assembled electrode as if it were a clinical thermometer to remove bubbles.

8. Record date of membrane and internal filling solution change in instrument record/equipment log.

9. Soak the assembled probe in 10 mg/L NH_4Cl standard for at least 2 hours before making measurements.

2.2.2 Checking Electrode Operation

Obtaining the slope value (the change in mV observed with every tenfold change in concentration) provides the best means for checking electrode operation. If using the model 290A meter and problems have not been encountered, proceed to step 6.

1. Place 100 mL deionized (DI) water into a 150 mL beaker. Add 1 mL 10 M NaOH and stir thoroughly. (The NaOH addition raises the pH above pH 11 where all of the ammonia present is in the unionized form.) Set the function switch of the meter to the mV mode.

2. Rinse electrode with DI water and place in the solution prepared in step 1.

3. Pipet 1 mL of the 1,000 mg/L ammonium chloride (NH_4Cl) standard into the beaker and stir thoroughly. Record the electrode potential in millivolts (mV).

4. Pipet 10 mL of the same 1,000 mg/L NH_4Cl standard into the same beaker and stir thoroughly. Again, record the electrode potential.

5. The difference between the first and second potential reading is the slope of the electrode. The electrode slope should fall within the range of negative 54 to negative 60 mV when the temperature of the solution is 20–25 °C. Note the electrode slope in the instrument records/equipment log. If the slope is not within this range, check

meter operation, ensure that the electrode is properly prepared and assembled, or refer to section 4.3 Troubleshooting.

6. Alternatively, the slope can be viewed after performing the normal calibration procedures undertaken before making measurements (Section 2.4 Perform Calibration Using Standard Solutions). Using the Model 290A meter (Orion®), the slope value is flashed on the LCD display after the "measure" button on the keypad is first depressed. Also, the slope can be reviewed at any time, following calibration, in the "setup" menu under category 2–1.

2.3 Preparation of Standards

1. A 1,000 mg/L ammonia as nitrogen stock solution can be purchased (Thermo Orion® #951007) or prepared. Prepare by adding 3.82 g reagent-grade NH_4Cl to 500 mL DI water in a 1,000 mL volumetric flask, stir to dissolve and dilute to volume with DI water. Make or purchase again before shelf life expires (-6 months).

2. Prepare 100, 10, 1.0, and 0.1 mg/L standard solutions by serial dilution from the 1,000 mg/L stock solution using 0.45 micrometer (μm) filtered seawater (SW). These can be made in scintillation vials:

    > 1 mL 1,000 mg/L standard + 9 mL SW = 100 mg/L
    > 1 mL 100 mg/L standard + 9 mL SW = 10 mg/L
    > 1 mL 10 mg/L standard + 9 mL SW =   1 mg/L
    > 1 mL 1 mg/L standard + 9 mL SW = 0.1 mg/L

2.4 Perform Calibration Using Standard Solutions

1. Ensure that the mode is set to "concentration". For detailed instruction on how to operate the model 290A meter, see the Thermo Orion® Model 290A pH/ISE/Temperature/mV Meter instruction manual.

2. Run calibration using three standard solutions (0.1, 1.0 and 10 mg/L) beginning with the lowest concentration. Add 2 drops 10 M NaOH to 10 mL of each respective standard solution just prior to measuring. Ensure that each sample is fully stirred during measurement.

3. Check the slope (see item 6 in section 2.2.2 Checking Electrode Operation) and record in the comments section of the Water Quality Adjustment Data Form (Attachment 1) and the equipment log.

4. If concentrations <0.1 mg/L are to be measured, another series of standard solutions for a lower range of values should be prepared, and another calibration should be performed.

5. Reproducibility of measurements, according to the instruction manual should be plus or minus ± 2 percent with calibration performed every hour. Alternatively, the calibration can be verified every 1–2 hours by measuring a standard solution or internal reference solution. If the value is similar to that expected (within ± 2 percent), recalibration is not necessary at

that time. When verification and recalibration are undertaken, the data should be noted on the Water Quality Adjustment Data Form (Appendix 1).

2.5 Measurements

Following calibration, measure the concentration of at least one standard solution for verification. If the value is not within 2 percent, repeat the calibration procedures. Additionally, an internal reference sample (standard NH_4Cl solutions previously prepared and frozen) may be measured as further verification. Once verification is complete, measure the various sample concentrations and record the data on the Water Quality Adjustment Data Form for each sample. As done with the standard solutions, 2 drops of 10 M NaOH should be added to 10 mL of each test sample just prior to measurement or 3 drops if the sample volume is 15 mL.

2.6 Measuring Hints

1. The electrode should be rinsed with DI water between measurements.

2. The electrode should be checked for bubbles on the membrane. If present, they can be removed by shaking the electrode.

3. All samples should be magnetically stirred during measurement. Magnetic stirrers may generate some heat, and a layer of rigid foam can be placed below the sample to help limit any change in temperature.

4. It is critical that the temperature be consistent for all samples and standards. This ion selective electrode is not temperature compensated. A 1 °C difference will introduce a 2 percent measurement error. Water quality measurements should generally be made at the same temperature that the test will be carried out. At the U.S. Geological Survey laboratory in Corpus Christi, Texas, most testing is done at 20 °C. Consequently, room temperature while performing water quality measurements and the various toxicity tests should be maintained at 20 °C.

5. If electrode response is slow, the membrane may contain a surface layer of contaminants. To restore performance in this case, soak the electrode in DI water for -5 minutes and then soak in a standard solution for -1 hour before use or replace the membrane and soak in a 10 mg/L NH_4Cl standard for -2 hours. For large numbers of samples, more then one probe may be assembled and utilized to reduce or eliminate the waiting time for probe equilibration.

6. If samples can not be measured on the same day they can be acidified with 1 M HCl to a pH of less then 2 and held refrigerated until the following day.

3.0 MAINTENANCE

3.1 Electrode Storage

Between measurements, do not allow the electrode tip to dry. For overnight or week-long storage, the electrode tip should be immersed in a 1,000 mg/L NaOH-free ammonia standard. For storage over one week or if the electrode is stored indefinitely, disassemble completely and rinse the inner body, outer body, and bottom cap with DI water. Allow the parts to dry, and then reassemble the electrode without internal filling solution or a membrane.

3.2 Membrane Life

According to the instruction manual, membranes will last from 1 week to several months depending on usage. At the USGS laboratory, Corpus Christi, TX, membranes may last less than one day when measuring samples with contaminants or high ammonia levels. Membrane failure is characterized by a shift in electrode potential, drift, and poor response. Membrane failure may be apparent on visual inspection as dark spots or discoloration of the membrane or carbonate deposits on the membrane and end cap.   Follow the procedures given in section 2.2.1 Electrode Setup to replace the membrane and add internal filling solution.

3.3 Troubleshooting

Generally, if the meter is operating properly, and if the electrode has been prepared and assembled properly and has passed the electrode checkout procedures, the electrode slope should fall within the range of negative 54 to negative 60 mV when the temperature of the solution is 20–25 °C. When the temperature of the solution is 20 °C, the electrode slope should be approximately negative 58.2 mV. For problems encountered that this SOP does not address, refer to the Model 290A meter instruction manual (Thermo Orion®, Inc. 1991) and the Model 95-12 ammonia electrode instruction manual (Thermo Orion®, Inc. 2002).

4.0 TRAINING

Personnel who will perform this task should first read this protocol and then operate under supervision until proper technique and accuracy of measurements is ensured.

5.0 SAFETY

10 M NaOH is a highly caustic liquid. Care should be taken to avoid its contact with skin or clothing. Should such contact occur, quickly flush affected area with water. Sinks are present along 2 walls of the wet lab, and an eye flushing station and shower is present near the entrance door of all labs. 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 by donning gloves, lab coats and other necessary safety equipment.

6.0 ATTACHMENTS

Attachment 1. Water Quality Adjustment Data Form

7.0 REFERENCES

Thermo Orion® Inc., 1991, Portable pH/ISE Meters Instruction Manual (Models 230A, 250A, and 290A): Beverly, MA., Thermo Electron Corporation, 60 p.

Thermo Orion® Inc., 2002, Model 95-12 Ammonia Electrode Instruction Manual: Beverly, MA., Thermo Electron Corporation, 42 p.

Revised by:
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_____

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Quality Assurance Officer

Attachment 1

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

    Initial volume (mL)   _____

    Initial salinity (‰)   _____

    Vol. Milli-Q® water added (mL)   _____

    Vol. ___‰ brine added (mL)   _____

    Percent of original sample   _____

    (initial volume/final volume × 100)

B. Character of Sample (after salinity adjustment):

    Volume (mL)   _____

    Salinity (‰)   _____

    pH   _____

    Dissolved oxygen (mg/L)   _____

    DO saturation (percent)   _____

    Total ammonia (mg/L)   _____

    Sulfide (mg/L)   _____

COMMENTS_____

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Appendix 6

Date Prepared: October 13, 1993

Date Revised: July 26, 2007

MEASURING SULFIDE WITH THE THERMO ORION® MODEL 9616 SUREFLOW COMBINATION SILVER/SULFIDE PROBE

1.0 APPLICATION

The silver/sulfide probe is used to measure the concentration of silver or sulfide ion in solution by measuring an electrode potential which develops across the sensing element. This potential, which depends on the level of free silver or sulfide ion in solution, is measured against a constant reference potential with a digital pH/millivolt (mV) or specific ion meter.

2.0 OPERATION

2.1 Required Equipment and Solutions

1. Thermo Orion® model 9616 Sure-flow Combination silver/sulfide electrode
2. Thermo Orion® "A" Optimum Results™ Reference Electrode Filling Solution (# 900061)
3. Thermo Orion® model 290A pH/mV/specific ion meter or comparable meter
4. Sodium Sulfide stock solution (see 2.1.1)
5. Weekly Sulfide standard (see 2.1.2)
6. Standard sulfide solutions: 100, 10, 1, 0.1, .08, .06, .05, .04, .03, .02, .0150, .0125, .01, .009 milligrams per liter (mg/L) (see 2.1.3)
7. Magnetic stir bars and stirrer
8. Thermo Orion® Sulfide Antioxidant Buffer (SAOB II®) solution (see 2.1.4 below)
9. De-aerated de-ionized (DI) water and de-aerated filtered seawater

2.1.1 Sodium sulfide stock solution

Prepare a stock solution of saturated sodium sulfide by dissolving approximately 100 g reagent grade $Na_2S=9H_2O$ in 100 milliliters (mL) de-ionized, de-aerated water. De-ionized water (DI) may be de-aerated by passing through a .45 micrometer (μm) filter under vacuum suction. Shake the solution and let stand capped overnight in a fume hood.

2.1.2 Weekly sufide standard

Prepare a weekly sulfide standard by pipetting 1 mL of the stock solution into a 100 mL volumetric flask. Add 50 mL of SAOB II® solution and dilute to volume with de-ionized, de-aerated water.

Determine the exact concentration, by titrating 10 mL of the standard with 0.1 molar (M) Lead Perchlorate, using the electrode pair as the end point indicator. Additions of Lead Perchlorate are best accomplished using a 1 cubic centimeter (CC) syringe with a fine needle. The end point is the point at which the mV reading drops rapidly with the addition of one more drop of Lead Perchlorate. In other words, if the mV readings were being graphed versus volume of Lead Perchlorate added, then the electrode pair would be the point on the graph with the greatest absolute slope.

Calculate the concentration (C) of the weekly standard using the equation:

(i.) $C = 3206 \, (Vt \div Vs)$
    where:
        C = concentration as mg/L sulfide
        Vt = volume of Lead Perchlorate at end point (mL)
        Vs = volume of standard titrated (10 mL)

2.1.3 Standard sulfide solutions

Prepare other standards daily by dilution of the weekly standard with equal volumes of 0.45 µm filtered, de-aerated seawater (FDS) and SAOB II. For example, to do a tenfold dilution, pipet 1 mL of the standard into a small beaker and add 4.5 mL of SAOB II® and 4.5 mL of FDS. It is recommended that the first dilution from the weekly stock solution be 100 mg/L followed by serial dilutions to achieve concentrations to the 0.1 mg/L concentration and then calculate the lower dilutions until the desired working range (ie. .009 to 10 mg/L) is achieved. <u>Remember: all dilutions must be made with both SAOB II® and FDS.</u>

2.1.4 SAOB II®

SAOB II® solution is commercially available from Thermo Orion® (# 941609) or can be prepared by dissolving 40 grams (g) of Sodium Hydroxide and 29.2 g of Disodium Ethylenediamine Tetraacetate (EDTA) in de-aerated de-ionized water in a 500 mL volumetric flask. Prior to use this solution is "activated" by the addition of 18 g of Ascorbic acid. The activated solution will last up to one month if stored in a dark bottle.

2.2 Instrument Setup

1. Remove the rubber cap covering the electrode tip.
2. Fill the electrode with "A" Optimum Results™ reference electrode filling solution.
3. Connect the electrodes to the meter soak the probe in reference electrode filling solution for 10 minutes before calibrating the probe

2.3 Calibration

Measure 5 mL of the most dilute standard (0.009 mg/L) into a small beaker. Add 5 mLs of SAOB II® solution and a stir bar and place on the automatic stirrer. Rinse electrodes, blot dry, and place in beaker. Wait for a stable millivolt reading and plot the reading (linear axis)

against concentration (log axis) on standard 4-cycle semi-log paper. Repeat this process with the progressively higher concentration standards. Calculate the slope of the linear portion of the graph. The linear portion of the graft is that portion above 0.5 mg/L. The slope of the curve should be between negative 25 and negative 30 mv/decade. If the slope is out of range, perform the electrode slope check described in the probe instruction manual and/or consult the troubleshooting section of the manual.

2.4 Sample Measurement

Measure 5 mL of sample into a small beaker. Add 5 mL of SAOB II® solution and a stir bar and place on the automatic stirrer. Place probes in the solution and press the measure button on the meter. When the reading stabilizes, record the reading and read concentration from the calibration curve. Remove the probes, rinse with DI and blot. Continue with any additional samples.

Recheck the accuracy of the measurements by measuring the concentration of a known standard periodically. If the reading deviates more than 5 percent from the known value, recalibrate the probe.

2.8 Sample Requirements and Measuring Hints

1. Samples and standards should be at the same temperature. Temperature must be less than 100 °C.

2. Sulfide samples must be buffered at pH greater than 12 with SAOB II® so that HS^- and H_2S are converted to $S^=$.

3. Always use fresh standards for calibration mixed with de-aerated water.

4. Rinse electrodes with DI water between measurements and blot electrode dry.

5. For high ionic strength samples, (e.g., seawater) prepare standards with composition similar to that of sample (de-aerated seawater of similar salinity).

3.0   MAINTENANCE

1. If electrode response is slow, the sensing element may contain a surface layer of contaminants. Restore performance by polishing with polishing strips (included with the probe) and deionized water. Cut off 1-inch of the strip and polish the sensing element with a circular motion for 30 seconds. Rinse and soak in standard solution for 5 minutes before use.

2. The silver/sulfide probe should always be stored dry. The rubber cap should be replaced over the element during periods of long storage to protect it.

3. The reference electrode may be stored dry or in internal reference filling solution for short periods of time (less than a week). For longer storage times, the solutions in the probes should be emptied and the chambers rinsed with DI to prevent crystallization.

4.0 TRAINING

Personnel who will perform this task should first read this Standard Operating Procedure (SOP) and operate the probe the first time under supervision.

5.0 SAFETY

SAOB II® is a caustic solution. Lead Perchlorate and Sodium Sulfide are irritants and toxic. Care should be taken and proper safety gear worn to avoid chemical contact with skin or clothing, or prolonged breathing of vapors. Should contact occur, flush the affected area with water and seek medical attention if warranted. An eye flushing station is present in the corner of the lab nearest the door, should it be needed.

6.0 REFERENCES

Thermo Orion® Inc., 1991, Portable pH/ISE Meters Instruction Manual (Models 230A, 250A, and 290A): Beverly, MA.,Thermo Electron Corporation, 60 p.

Thermo Orion® Inc., 2001, Model 94-16 Silver/Sulfide Electrodes Instruction Manual: Beverly, MA., Thermo Electron Corporation, 67 p.

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Appendix 7

Date Prepared : September 19, 2006

Date Revised: May 10, 2007

<div align="center">

SEA URCHIN FERTILIZATION TOXICITY TEST
WITH *STRONGYLOCENTROTUS PURPURATUS*

</div>

1.0 OBJECTIVE

The purpose of the fertilization toxicity test with the sea urchin, *Strongylocentrotus purpuratus*, is to determine if seawater, pore water, sea surface microlayer, or other sample reduces fertilization of exposed gametes relative to that of gametes exposed to a control 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 significantly reduced fertilization or as concentration of test substance which effectively reduces fertilization by 50 percent (EC_{50}).

2.0 TEST PREPARATION

2.1 Test Animals

Gametes from the sea urchin, *S. purpuratus* are used in the sea urchin fertilization toxicity test. Animals can be collected in the field or obtained from a commercial supplier. *S. purpuratus* is a species of sea urchin found along the entire west coast of the United States and are characterized by a dark purple test with short light and dark purple spines. This species also has numerous tube feet between the rows of spines on both the dorsal and ventral surfaces of the test and often is found with algae, rocks and debris being held to the body with the tube feet. Due to the sensitivity of this species to the pH of Gulf Coast waters it is recommended to receive urchins for use in testing on the day of or the day before the actual test is to take place. If urchins are to be held overnight, they should be acclimated slowly to the temperature controlled aquarium water. Temperature of the cultures will prolong the useful life of the urchins and should be maintained at 12 ± 1 °C when gametes are not required. Photoperiod is maintained at 16 hours of light per day. Water quality parameters should be monitored weekly and salinity maintained at 30 ± 3 parts per thousand (‰). Males and females should be kept in separate tanks if the sex can be determined before use in testing.

2.2 Dilution Water

Milli-Q® grade purified water or concentrated seawater brine is used to adjust samples to 30 ‰ as described in Water Quality Adjustment of Samples (CERC SOP P.651). Concentrated seawater brine (90–110 ‰) 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 refrigeration. On the day of the test, pH, ammonia, sulfide and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 micrometer (μm)) seawater adjusted to 30 ‰ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater. Whenever possible, temperatures of wash, dilution and test waters should be maintained at test temperatures.

2.3 Test System: Equipment

When testing samples for potential toxicity, five replicates per treatment are recommended. One replicate is a 5 milliliters (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. For larger tests, (greater than 20 samples) it may be desirable to include control samples including dilutions at the beginning and at the end of the test to account for any variability due to decreased sperm viability during the pipetting process and provide for more statistical robustness for comparisons with a large number of samples.

2.3.1 Equipment

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

2.3.2 Solutions

0.5 molar (M) Potassium Chloride (KCl) solution:

1. 3.73 g KCl crystals
2. 100 mL Milli-Q® reagent grade water

10 percent Buffered Formalin:

1. 1,620 mL MFS
2. 620 mL formaldehyde
3. 6.48 grams (g) Sodium Phosphate Monobasic (NaH_2PO_4) or Potassium Phosphate Monobasic (KH_2PO_4)
4. 10.5 grams (g) Sodium Phosphate Dibasic (Na_2HPO_4) or Potassium Phosphate Dibasic (K_2HPO_4)
5. 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

1. Take two or three urchins and place inverted individually in shallow empty Carolina bowls. Shake free as much water as possible before placing the urchins in each bowl.

2. To stimulate release of gametes from gonopores, inject each urchin with 0.5 to 1.0 mL of a 0.5 molar (M) solution of KCl in several injections surrounding the soft membrane of the mouth opening. Agitate each urchin slightly after injecting. Gametes should appear from the gonopores within a few minutes. If not, inject with another 0.5 to 1 mL aliquot of KCL solution. Replace urchins that shed no gametes after two series of injections.

3. Sperm are white to off white in color and eggs are tan to orange in color. If eggs appear, fill the Carolina dish half full with MFS and allow the urchin to shed eggs into the dish while inverted. Collect a few eggs from the bottom of the dish using a 10 mL disposable syringe with a large gauge blunt-tipped needle attached. Place 2 to 5 drops of eggs onto a scintillation vial containing 10 mL of filtered seawater. Rinse syringe and repeat for each female. Additional injections of KCl may be required to collect a full complement of eggs.

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

5. If sperm is shed from the urchins, place males upright on a pad made up of several layers of paper towels. Dab each male with additional paper towels to remove as much water as possible from the surface of the urchins. It is important to collect the sperm without any water coming in contact with it.

6. If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a Pasteur 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 20−50 microliters (µL) of sperm in 10 mL of filtered seawater. If the proportion of eggs fertilized is high (95−100 percent), 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 B).

2.4.2 Obtain Eggs

1. Place selected female inverted into a small Carolina dish and add enough seawater to cover one half of the urchin's test. Stimulate release of eggs as described above with KCl injections.

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, give one or two additional injections of KCL and agitate the urchin and replace inverted into the dish.

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.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 ‰ 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 percent.

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 greater than 220 use the following formula to calculate the amount of water to add:

   (i.) $\left[\left(Egg\ count - 200\right) \div\ 200\right] \times\ Current\ volume\ of\ eggs = Volume\ of\ seawater\ to\ add\ to\ stock\ (mL)$

   If egg count is less than 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 KCl injected males on a pad made up of several layers of paper towels dab each male with additional paper towels to remove as much water as possible from the

surface of the urchins. Once sperm has contacted water it becomes activated and has a limited life span. This species has a tendency to expel water from the anus during gamete collection so it is important to watch when this happens and discard the sperm on the surface of the urchin at that time, dab with a paper towel to remove water and wetted sperm and continue collecting only dry sperm. It may be necessary to repeatedly invert the urchin for short periods of time or provide additional injections of KCl to facilitate additional shedding of sperm. Collect about one mL of unwetted sperm from between spines using a pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Do not allow the sperm to freeze. To prevent this, wrap each microcentrifuge tube in a small amount of paper towel before placing it on ice. 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 60−95 percent. 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 95 percent and good dose responses may be difficult to obtain with less than 60 percent 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 affect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Due to the sensitivity of *S. purpuratus* sperm to pHs greater than 8.1, a higher concentration of sperm is generally required in tests utilizing Gulf coast sea waters and pore waters. This effectively reduces the overall sensitivity of the test. Generally, a dilution of between 1:2500 and 1:750 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:2500 is required (as determined from the pretest), add 20 μL sperm to 5 mL MFS. Mix thoroughly, and add 1 mL of this solution to 9 mL of MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 10 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 the time of sperm addition. Sperm should be used within 10 minutes of wetting.

2. Incubate all test vials at 12 ±1°C for 20 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 using a clean razor blade to prevent crushing the eggs during pipetting.   Record the time of egg addition.

4. Incubate for 20 minutes at 12 ± 1 °C. The timer may be used again at this point.

5. Add 0.75 mL of 10 percent buffered formalin to each sample with a re-pipette bottle.

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.

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; however, *S. purpuratus* fertilization membranes are typically very easily discernable.

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:

$$(ii.) \quad \frac{\left(\textit{Total number of eggs - Number of eggs unfertilized}\right)}{\textit{Total number of eggs}} \times 100 = \textit{Percent of eggs fertilized}$$

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments C to G). 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 and others, 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 reference pore water, filtered sea water, and/or 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 percent 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 can be 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 A. Equipment List for Fertilization Toxicity Test
Attachment B. Pretest to Insure Selection of Quality Gametes
Attachment C. Water Quality Adjustment Data Form
Attachment D. Sea Urchin Pretest Data Sheet
Attachment E. Sea Urchin Pretest Continuation Data Sheet
Attachment F. Sea Urchin Fertilization/Embryological Development Toxicity Test Gamete
            Data Sheet
Attachment G. Sea Urchin Fertilization Toxicity Test Fertilization Data Sheet

10.0 REFERENCES

Day, R.W., and Quinn, G.P., 1989, Comparisons of treatments after an analysis of variance in ecology: Ecology Monograph, v. 59, p. 433–463.

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, no. 7, p. 714–719; Correction, v. 12, no. 4, p. 417 (1978).

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

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<u>**Attachment A**</u>

EQUIPMENT LIST FOR FERTILIZATION TOXICITY TEST

Small 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)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a
grinding stone)
1 cc syringe with fine tip needle at least 2 cm in length.
0.5 M KCl solution
Paper towels
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 ‰
Data sheets
Milli -Q® reagent grade water
Approximately 100 ‰ concentrated brine
Biological control chamber or incubator adjusted to 12°C

<u>Attachment B</u>

PRETEST TO INSURE SELECTION OF QUALITY GAMETES

1.   Using the procedure in section 2.4.1, select 1 to 4 females and at least 1 male urchin to be
      used in the pretest.

2   Fill pretest vials with 5 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 2 females, 1 male, and 5 pretest sperm concentrations, 20 vials (2 X 2 X 5) would be
     needed. Additional reference solutions may be added to the pretest to aid in selecting the proper
     sperm dilution. Typically, one rep each of three different reference toxicant concentrations (2.5,
     5.0, and 10 mg/L) is added for each sperm concentration to ascertain if the sperm dilution selected
     will fall within the control range for that reference toxicant. Arrange and mark vials accordingly in
     a rack and incubate at 12 °C until the test solutions acclimate to the test temperature.

       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 60–95 percent
          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: 1000 (1 mL of 1:250 and 3 mL MFS)
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)

Sperm must be used within 10 minutes of dilution and should be made up in MFS maintained at
the test temperature. 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 20 minutes at
    approximately 12 °C, and add 200 μL of the egg suspension. Incubate for another 20 minutes, then
    fix with 1 mL of the buffered formalin solution.

Attachment B. Continued

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 C

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____   INITIALS_____

SAMPLE DESIGNATION_____   DATE_____

A. Salinity Adjustment:

    Initial volume (mL)   _____

    Initial salinity (‰)   _____

    Vol. Milli-Q® water added (mL)   _____

    Vol. ___‰ brine added (mL)   _____

    Percent of original sample   _____

    (initial volume/final volume × 100)

B. Character of Sample (after salinity adjustment):

    Volume (mL)   _____

    Salinity (‰)   _____

    pH   _____

    Dissolved oxygen (mg/L)   _____

    DO saturation (percent)   _____

    Total ammonia (mg/L)   _____

    Sulfide (mg/L)   _____

COMMENTS_____

_____

_____

_____

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_____

_____

_____

_____

Attachment D

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:_____

PERCENT FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

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

PERCENT FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

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

<u>**Attachment E**</u>

SEA URCHIN PRETEST CONTINUATION DATA SHEET

STUDY PROTOCOL_____ INITIALS_____
SAMPLE DESIGNATION_____ DATE_____

PERCENT FERTILIZATION   Reference sample designation:_____
   Female #_____   Male #_____

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

PERCENT FERTILIZATION   Reference sample designation:_____
   Female #_____   Male #_____

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

PERCENT FERTILIZATION   Reference sample designation:_____
   Female #_____   Male #_____

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

Attachment F

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 G

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	Unfertilized

COMMENTS_____

Appendix 8.   Sodium dodecyl sulfate reference toxicant control EC50s from toxicity tests with
Strongylocentrotus purpuratus from 1997–2009.

[EC50, Effective concentration 50 percent in milligrams per liter; na, not applicable; °C, degrees Celsius; PS ref tox, Puget Sound
Reference Toxicity Test; PSAMP, Puget Sound Assessment and Monitoring Program; comp, comparison]

Year	Test	EC50	EC50	Method[1]	Stations included
1997	Puget Sound-1	2.41	na	30/30 exposure, 15°C	26–58, 83–100
1997	Puget Sound-2A	3.23	na	30/30 exposure, 15°C	59–82
1997	Puget Sound-4	3.51	na	30/30 exposure, 15°C	1–25
1997	PS ref tox	2.91	na	30/30 exposure, 15°C	None
1998	Puget Sound II-2	2.32	na	30/30 exposure, 15°C	157–205, 114, 115
1998	Puget Sound II-3	5.36	na	30/30 exposure, 15°C	106–132
1998	Puget Sound II-4	4.03	na	30/30 exposure, 15°C	133–156
1999	Puget Sound III-2A	3.69	na	30/30 exposure, 15°C	254–305
1999	Puget Sound III-2B	2.31	na	30/30 exposure, 15°C	206–253
2002	PSAMP 2002	4.77	na	30/30 exposure, 15°C	1–161
2002	PSAMP 2002	5.70	na	30/30 exposure, 15°C	177–2123
2003	PSAMP 2003	na	2.49	20/20 exposure, 12°C	297–523
2003	PSAMP 2003	na	2.76	20/20 exposure, 12°C	527–1387
2005	PSAMP 2004	3.69	na	20/20 exposure, 12°C	8–336
2005	PSAMP 2004	na	3.54	20/20 exposure, 12°C	24–336 (selected)
2005	PSAMP 2003 retest	5.70	na	20/20 exposure, 12°C	297–1387
2006	PSAMP methods comp	na	3.58	20/20 exposure, 12°C	All
2006	PSAMP methods comp	3.49	na	20/20 exposure, 12°C	All
2006	PSAMP test 1	na	3.66	20/20 exposure, 12°C	3–165
2006	PSAMP test 2	na	3.87	20/20 exposure, 12°C	189–667
2007	PSAMP test 1	na	2.62	20/20 exposure, 12°C	5–151
2007	PSAMP test 2	na	3.54	20/20 exposure, 12°C	155–371
2007	Urban waters	na	2.42	20/20 exposure, 12°C	All
2008	PSAMP	na	3.56	20/20 exposure, 12°C	All
2008	Urban waters	na	2.83	20/20 exposure, 12°C	All
2009	PSAMP test 1	na	3.54	20/20 exposure, 12°C	002–234
2009	PSAMP test 2	na	3.54	20/20 exposure, 12°C	239–498
2009	Urban waters	na	3.08	20/20 exposure, 12°C	All
	Mean	3.79	3.22		
	Standard deviation	1.19	.50		

[1] Refers to minutes of exposure of sperm and then egg with sperm at incubated temperature.

Appendix 9.   Aransas Bay negative control 100-percent pore-water and Millipore® filtered seawater (MFS) dilution water means and standard deviations from toxicity tests with *Strongylocentrotus purpuratus* from 1997–2009.

[SD, standard deviation; MFS, Millipore® filtered seawater; na, not applicable; PS ref tox, Puget Sound Reference Toxicity Test; PSAMP, Puget Sound Assessment and Monitoring Program]

Year	Test	Negative control mean	SD	MFS mean	SD	Stations included
1997	Puget Sound-1	na[1]	na[1]	96.2	3.1	26–58, 83–100
1997	Puget Sound-2A	na[1]	na[1]	91.8	5.7	59–82
1997	Puget Sound-4	na[1]	na[1]	92.6	3.9	1–25
1997	PS ref tox	na[1]	na[1]	85.5	4.8	None
1998	Puget Sound II-2	na[1]	na[1]	79.0	4.9	157–205, 114, 115
1998	Puget Sound II-3	na[1]	na[1]	97.3	2.0	106–132
1998	Puget Sound II-4	na[1]	na[1]	98.7	1.7	133–156
1999	Puget Sound III-2A	na[1]	na[1]	96.4	2.4	254–305
1999	Puget Sound III-2B	na[1]	na[1]	96.9	2.1	206–253
2002	PSAMP 2002	98.8	1.3	99.0	1.4	1–161
2002	PSAMP 2002	99.0	1.0	99.5	.8	177–2123
2003	PSAMP 2003	93.6	4.0	92.3	3.2	297–523
2003	PSAMP 2003	94.8	1.5	95.2	2.6	527–1387
2005	PSAMP 2004	94.2	3.2	93.1	6.9	8–336
2005	PSAMP 2004	95.4	1.8	96.9	.8	24–336 (selected)
2005	PSAMP 2003 retest	99.2	.6	98.5	1.2	297–1387
2006	PSAMP test 1	92.4	4.3	98.8	.8	3–165
2006	PSAMP test 2	89.8	3.4	95.2	4.8	189–667
2007	PSAMP test 1	95.3	3.5	85.9	7.6	5–151
2007	PSAMP test 2	92.4	2.7	94.6	2.5	155–371
2007	Urban waters	97.3	2.3	93.3	5.7	All
2008	PSAMP	99.6	.7	99.5	.7	All
2008	Urban waters	98.3	2.1	96.4	2.0	All
2009	PSAMP test 1	99.3	.7	98.5	.5	002–234
2009	PSAMP test 2	99.4	.7	99.0	.9	239–498
2009	Urban waters	82.0	5.3	86.7	9.1	All
	Mean	95.7		94.5		
	Standard deviation	3.1		5.1		

[1] A different control pore water from Redfish Bay was used for these tests.