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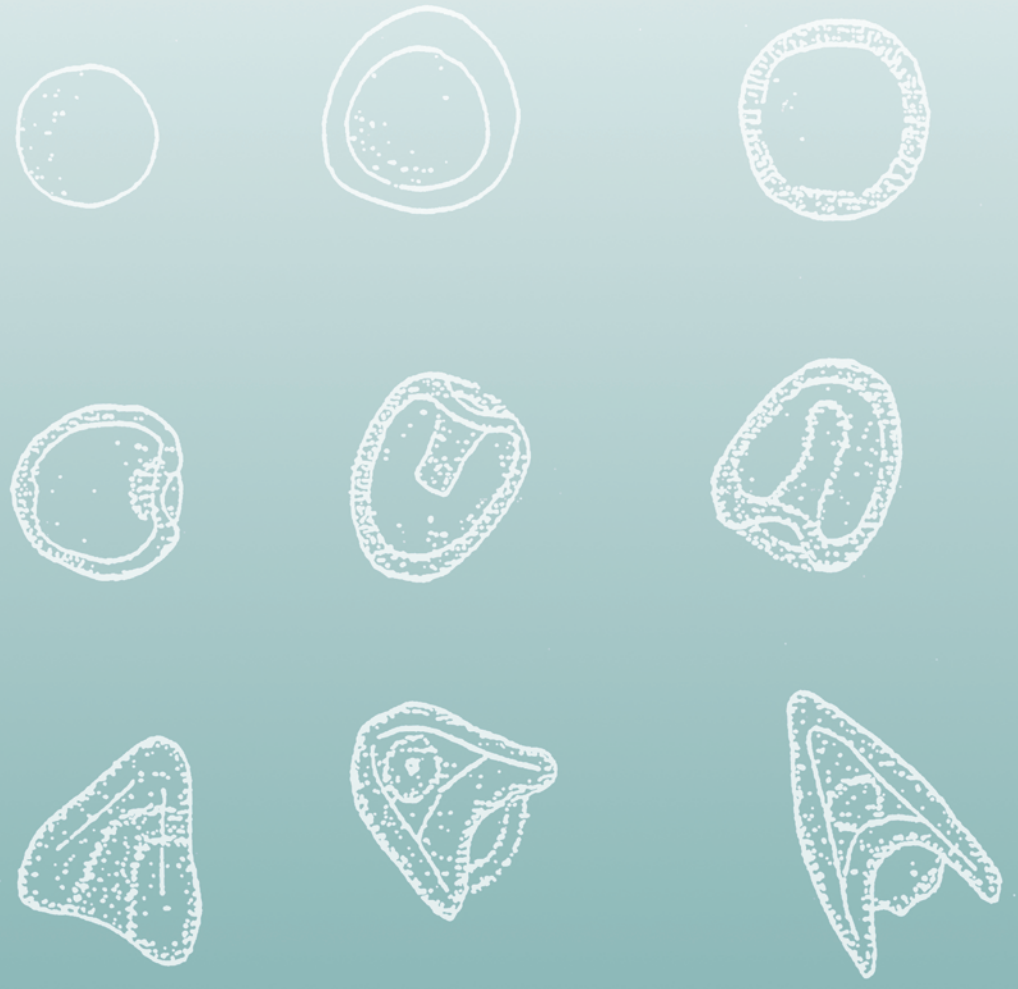
Sediment Pore-Water Toxicity Test Results and Preliminary Toxicity Identification of Post-Landfall Pore-Water Samples Collected Following the Deepwater Horizon Oil Release, Gulf of Mexico, 2010



Open-File Report 2011–1078

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

Cover. Artist's sketches of stages in development of sea urchin, from unfertilized egg to pluteus larvae.

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By James M. Biedenbach and Robert S. Carr

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U.S. Geological Survey
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U.S. Geological Survey, Reston, Virginia: 2011

Suggested citation:
Biedenbach, J.M., Carr, R.S., 2011, Sediment pore-water toxicity test results and preliminary toxicity identification of post-landfall pore-water samples collected following the Deepwater Horizon oil release, Gulf of Mexico, 2010: U.S. Geological Survey Open-File Report 2011–1078, 79 p.

Acknowledgments

The authors thank Dr. Marion Nipper, Dr. Kim Withers, and Misti Grohman of the Texas A&M University–Corpus Christi Center for Coastal Studies, for their assistance in completing the toxicity testing and water-quality analysis.

The authors also thank Suzanne Roberts of the U.S. Geological Survey for her assistance in creating the figures.

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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)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
milliliters (mL)	0.3382	ounce, fluid (fl. oz)
microliters (µL)	0.00003382	ounce, fluid (fl. oz)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
milligrams (mg)	0.00003527	ounce, avoirdupois (oz)
micrograms (µg)	0.00000003527	ounce, avoirdupois (oz)
	Length	
micrometer (µm)	0.00003937	inches (in)

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

$$°F=(1.8\times°C)+32$$

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).

Sediment Pore-Water Toxicity Test Results and Preliminary Toxicity Identification of Post-Landfall Pore-Water Samples Collected Following the Deepwater Horizon Oil Release, Gulf of Mexico, 2010

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Abstract

Pore water from coastal beach and marsh sediments from the northern Gulf of Mexico, pre- and post-landfall of the Deepwater Horizon oil release, were collected and evaluated for toxicity with the sea urchin fertilization and embryological development assays. There were 17 pre-landfall samples and 49 post-landfall samples tested using both assays. Toxicity was determined in four pre-landfall sites and in seven post-landfall sites in one or both assays as compared to a known reference sediment pore-water sample collected in Aransas Bay, Texas. Further analysis and testing of five of the post-landfall toxic samples utilizing Toxicity Identification Evaluation techniques indicated that ammonia, and to a lesser extent metals, contributed to most, if not all, of the observed toxicity in four of the five samples. Results of one sample (MS–39) indicated evidence that ammonia, metals, and non-ionic organics were contributing to the observed toxicity.

Introduction

On April 20, 2010, the Deepwater Horizon oil rig exploded and collapsed to the sea floor resulting in the Nation's largest documented oil release to the marine environment (Crone and Tolstoy, 2010). The U.S. Geological Survey (USGS) was commissioned by the U.S. Coast Guard Unified Area Command to assess any onshore effects related to the release and the subsequent cleanup. Sediment and water samples from beaches and wetlands were collected along the coast from Florida to Texas with a focus on those areas that were anticipated to be affected or were affected by oil coming to shore (fig. 1, at the back of the report). USGS scientists were tasked with identifying and fingerprinting oil in the sediment and dispersants in the water applied in the response effort, and determining the severity of the toxicity of surficial sediment collected from the study area (Rosenbauer and others, 2010). Toxicity was assessed using sediment pore water in the sea urchin (*Arbacia punctulata*) fertilization and embryological development tests. A secondary effort also was initiated to characterize the observed toxicity indicated in the post-landfall samples. Sediment samples were collected by USGS scientists in each of the five affected States in May and June, 2010, before the oil making landfall, and in October 2010, following landfall and subsequent cleanup in some areas. Sediment samples were shipped to the USGS in Corpus Christi, Texas for toxicity testing (fig. 1).

The specific objectives of this study were to:

- Extract sediment pore water from sediment samples collected pre- and post-landfall from areas of possible ecological effects in Florida, Alabama, Mississippi, Louisiana and Texas (fig. 1).
- Measure water-quality parameters (salinity, dissolved oxygen, pH, sulfide, and ammonia) of pore-water samples before toxicity testing.
- Conduct the fertilization and embryological development toxicity tests with pore water using sea urchin (*A. punctulata*) gametes.
- Perform preliminary phase I Toxicity Identification Evaluation (TIE) toxicity testing with toxic post-landfall samples to determine the class of contaminant(s) contributing to the toxicity.

Materials and Methods

Sediment Sample Receipt and Tracking

Surficial sediment samples were collected from 70 sites along the Florida, Alabama, Mississippi, Louisiana, and upper Texas coasts in May and June 2010 before the Deepwater Horizon oil making landfall. Of the 86 sediment samples

received at USGS, 83 had sufficient sediment volume and moisture to be extracted for pore water. Extended holding times and unacceptable temperatures during shipment resulted in 17 of those samples (representing 15 sites) meeting the criteria for quality control; therefore, only those 17 samples were tested in the pre-landfall sampling round (table 1, at the back of the report). The limited volume of some samples (2 of 17) did not allow for a complete dilution series; therefore, a reduced number of replicates were tested in these samples.

In October 2010, surficial sediment samples were resampled at 49 sites along the Gulf Coast. These 49 samples were collected using more rigorous protocol developed during summer 2010. A single site was collected in August 2010, to test the implementation of the new protocol and was included in the pore-water extraction and post-landfall testing. Despite variations in the sampling protocols between pre- and post-landfall, toxicity results are comparable as all tested samples were collected with inert materials and met the quality control criteria upon arrival at USGS. Samples were placed in pre-cleaned 1 liter glass containers, chilled, and shipped overnight by FedEx® in insulated coolers with bagged ice to USGS. Shipments were accompanied by chain of custody sheets, were recorded into a log book, and incoming temperatures were recorded for each container with a Fisher-Scientific Traceable® Ultra™ waterproof thermometer with probe/cable. All post-landfall pore-water samples were extracted within 4 days from the time of field collection of sediment, and within 12 hours of arrival at the USGS laboratory (table 2, at the back of the report).

Sediment Pore-Water Extraction Procedure

Pore-water volumes ranging from 40 to 450 milliliters were extracted from each sediment sample using a pneumatic extraction device (Carr and Chapman, 1992; 1995; Carr and others, 1996a, 1996b; U.S. Geological Survey, 2007). The extractor is made of polyvinyl chloride (PVC) and uses a 5-micrometer (μm) polyester filter mesh. The apparatus and extraction procedures are detailed in CERC SOP P.649 (appendix 1). After extraction, the pore-water samples were centrifuged in polycarbonate bottles at 1,200 times gravity for 20 minutes to remove any suspended particulate material; the supernatant was collected, and stored in precleaned samples bottles (from one to three depending on the volume collected), and frozen at negative 11 plus or minus (±) 1 degree Celsius (°C).

Toxicity Testing

Pore-Water Quality Measurements and Salinity Adjustment

Two days before conducting a toxicity test, pore-water samples were allowed to partially thaw at room temperature for 4 hours before being moved to a refrigerator kept at 4 ºC. One day before testing, samples were brought to room temperature in a tepid (20 ± 2 ºC) water bath for 1 hour. Sample salinity was measured and adjusted to 30 ± 1 parts per thousand (‰), if necessary using Milli-Q® purified de-ionized water or concentrated brine (102 ‰). Following any salinity adjustments, the samples were stored overnight at 4 ºC, but were returned to 20 ± 1 ºC (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 measured for water-quality parameters (dissolved oxygen, pH, and ammonia). Additional subsamples were preserved with Sulfide Anti-oxidant Buffer (SAOB II®) reagent for sulfide measurements (Thermo Fisher Scientific Inc., 2007) and refrigerated to 4 ºC until the following day, when they were brought to room temperature and measured. Dissolved oxygen (DO) concentrations were measured with a Yellow Springs Instruments Incorporated (YSI®) model 59 dissolved oxygen meter with a YSI® model 5905 Biological Oxygen Demand (BOD) probe. Salinity was measured with a Reichert® temperature compensated refractometer. Sulfide (as S^{-2}), pH, and total ammonia (expressed as total ammonia 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 Fisher-Scientific Traceable® Ultra™ Waterproof Thermometer with probe/cable calibrated to National Institute of Standards and Technology (NIST) standards. Un-ionized ammonia (expressed as un-ionized ammonia nitrogen; UAN) concentrations were calculated for each sample using the respective salinity, temperature, pH, and TAN values (Bowers and Bidwell, 1978). No samples had dissolved oxygen levels below 80 percent saturation; therefore, aeration was not conducted.

Pre-Landfall and Post-Landfall Toxicity Testing with Sea Urchins

Sea urchins (*A. punctulata*) were obtained from Gulf Specimens Marine Laboratories, Inc., Panacea, Florida (fig. 1). For fertilization and embryological development tests, each pore-water sample with adequate volume was tested in a dilution series at 100, 50, and 25 percent of the sample (after salinity adjustment) with five replicates per dilution treatment (CERC SOP P.647, appendix 2, and CERC SOP P.648, appendix 3). A reduced number of replicates or removal of dilutions was implemented in cases where sample volume was limited.

A pretest was conducted to determine the optimum sperm dilution that would give acceptable fertilization rates in the reference pore water, dilution water, and reference toxicant dilutions. All pretests and fertilization tests were conducted at 20 ºC in a temperature controlled chamber, and an exposure time of 30 minutes each for the sperm and the sperm plus eggs. At the end of the exposure, the tests were terminated by

the addition of buffered formalin. The presence or absence of a fertilization membrane was determined by examining 100 eggs/replicate using a compound microscope. The percent fertilization was determined using the following formula:

$$\frac{Total\ Number\ of\ Eggs - Number\ of\ Eggs\ Unfertilized}{Total\ Number\ of\ Eggs} \times 100 = Percent\ Eggs\ Fertilized$$

The embryological development test was performed in a similar manner to the fertilization test, except that the eggs were fertilized before being added to the sample vials. The test was terminated after 48 hours by the addition of buffered formalin. The percent normally developed pluteus stage larvae was determined by microscopic examination.

Dilutions were made with 0.45-µm Millipore® filtered seawater (MFS). A reference pore-water sample was collected from Aransas Bay, Texas, (fig. 1) using a PVC corer, refrigerated (4 °C), and extracted identically to the test samples, as a negative toxicity control. This reference sediment was collected on October 20, 2010, and held refrigerated and processed for pore water on October 21, 2010. This site is far removed from any known sources of contamination and has been used previously as a reference site (U.S. Geological Survey, 2007). In addition, a dilution water blank of filtered seawater was included in each test and a brine blank (control pore water diluted to 6 ‰ with Milli-Q® purified water and subsequently increased with brine) was included in those tests that contained samples that were adjusted with brine or Milli-Q® water. A rinsate blank also was included in each initial post-landfall test, which consisted of MFS put through all the extraction equipment, and centrifuged just as a sediment sample had been processed to determine if there was contamination from any cleaning procedure. Finally, a reference toxicity test (positive control sample) was conducted in each assay with a dilution series of sodium dodecyl sulfate (SDS) to evaluate overall test sensitivity.

Toxicity Identification Evaluation Toxicity Testing with Sea Urchins

Sites determined to be toxic in the sea urchin fertilization or embryological development test were analyzed further using TIE procedures to attempt to characterize the type of contaminant(s) contributing to the observed toxicity. The procedures used to perform the TIE are described by the United States Environmental Protection Agency (U.S. Environmental Protection Agency, 1996). Because of the limited sample volume remaining, four sample manipulations were selected to characterize the toxicity of each sample, and no dilutions or pH manipulations were performed. The sample manipulations included the addition of Disodium Ethylenediamine Tetraacetate (EDTA) at a concentration of 60 mg/L to chelate metals; vigorous aeration to drive off volatile organics and hydrogen sulfide; C_{18} solid phase extraction (SPE) to remove non-ionic organic compounds; and exposure to the algae *Ulva lactuca* (0.083 grams per milliliter (g/mL) to adsorb ammonia (U.S. Environmental Protection Agency, 1996).

EDTA was added to a subsample of each pore-water sample by first preparing a 25 grams per liter (g/L) EDTA stock solution and adding 12 microliters (µl) per 5 milliliters (mL) of sample volume contained in a beaker, and, incubating at 20 °C for 3 hours before testing. Following incubation, samples were pipetted into vials for testing.

Six mL Bakerbond™ SPE Octadecyl (C_{18}) disposable extraction columns containing 1,000 mg of resin were used to remove active non-ionic organic contaminants. A separate column was used for each sample, and each was activated with 10 mL of methanol and rinsed with MFS before receiving the sample. Samples were pushed through the columns with a glass syringe into glass beakers, and subsequently pipetted into test vials.

Vigorous aeration was accomplished with an aquarium pump attached to a gang valve manifold to which Pasteur pipettes were attached and placed in beakers containing the samples. Samples were pipetted into test vials after an hour of aeration. Subsamples were collected and preserved for analysis of sulfide reduction.

Ulva lactuca were collected from the jetties in Port Aransas, Texas (fig. 1) the day before testing. Plants were sorted and subsamples weighed (0.083 g/mL) for each treatment sample and placed in large beakers with MFS and gentle aeration. *Ulva lactuca* aliquots were kept incubated in the dark at 15 ºC overnight before the test. On the test day, each algae aliquot was removed from its beaker, carefully dried with a lint free towel, and placed into the beaker containing the pore-water sample. The samples were then incubated at 20 ºC for 5 hours with gentle aeration. At the end of incubation, the pore water was pipetted out of the each beaker into the test vials. Pore-water subsamples were collected and ammonia measurements performed as described previously to determine the extent of ammonia removal.

Each of the five samples was treated with all four manipulations and compared against their untreated counterpart to determine the effect of the manipulation on toxicity. A non-toxic pore-water sample extracted from an Aransas Bay, Texas, sediment served as a blank, and was treated the same as the test samples to determine if any of the treatment manipulations produced toxic artifacts. A brine blank and SDS dilution series also were included to gage overall test sensitivity. Either the fertilization or embryological development test was used for each sample. The test selected was based on the greatest toxic response achieved in the post-landfall testing. An ammonia dilution series (NH_4Cl) in MFS was included in the Toxicity Identification Evaluation testing to determine the sensitivity of this batch of sea urchin embryos to ammonia. The un-ionized ammonia fraction was calculated using the measured TAN, salinity (30 ‰), temperature (20 °C) and the pH of the individual concentrations measured before adding fertilized eggs to the sample vials (Bowers and Bidwell, 1978).

Sea Urchin Toxicity Testing Data Analysis

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). The DSC value for the sea urchin fertilization assay at $\alpha = 0.05$ is 15.5 percent. At $\alpha = 0.01$, the DSC value is 19 percent. The DSC values for the sea urchin embryological development tests are 16.4 percent and 20.6 percent for $\alpha = 0.05$ and 0.01, respectively. 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 $\alpha = 0.05$ for a reference mean of 95 percent would be 80.3 percent [(95-(0.155x95)]. 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 and ammonia dilution series.

For the TIE tests, statistical comparisons between the treated and untreated pore water were made using Analysis of Variance (ANOVA) and Dunnett's one-tailed *t*-test (controls the experiment-wise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989). Replicates were removed only from statistical consideration if there were known errors in the pipetting of the gametes to the vials.

Results

Pre-Landfall Pore-Water-Quality Measurements

Water-quality measurements were conducted on sediment pore water from 15 sites plus 2 duplicates of 1 of the sites (LA–23; table 3, at the back of the report). Salinity adjustment with Milli-Q® purified de-ionized water was required with 4 samples, whereas 12 samples and the reference control required adjustments with brine. Salinities in test samples ranged from nearly fresh water at 0.5 ‰ to greater than full strength seawater at 40 ‰. Initial dissolved oxygen was greater than 80 percent in all the samples. Total ammonia ranged from less than 0.1 to 5.97 mg/L, whereas the un-ionized ammonia (the most toxic fraction) ranged from less than 3.3 to 214.8 µg/L. There were four samples (FL–11 Rep 2, FL–13, FL–14 and FL–26; table 3) that approached or exceeded the EC_{50} for un-ionized ammonia (48.79 µg/L; 95-percent confidence limits 47.53–50.08 µg/L) calculated for the ammonia dilution series included with the TIE testing. This level is slightly lower than what has been previously reported for this species (Winger and others, 2003). Detectable sulfide concentrations were measured in only one pore-water sample (FL–11 Rep 2; table 3) at the detection limit of 0.009 mg/L.

Pre-Landfall Sea Urchin Toxicity Testing

A fertilization test and an embryological development test were conducted with the same sea urchin gametes as part of the sea urchin toxicity testing (tables 4 and 5, at the back of the report). Two of the 17 samples had reduced volume and, therefore, had a reduced number of replicates, dilutions, or both in the tests. Eggs from three females were combined to achieve the necessary quantity required for the tests. Sperm from one male was used in both tests. The EC_{50} value for the SDS positive control for the fertilization test was calculated at 4.18 mg/L. This value is similar to the historical mean for this species for the USGS laboratory of 5.43 mg/L (95-percent confidence limits 3.20 to 7.66 mg/L) and indicates adequate test sensitivity. A significant difference from the reference control in the fertilization test (FL–11 Rep 2, FL–26 and LA–29) was indicated in 3 of the 17 pore-water samples; however, none met the DSC cut-off of 83.4 percent for this test (table 4). The EC_{50} value for the SDS positive control for the embryological development test was calculated at 3.66 mg/L (3.47–3.87 mg/L), which is slightly below the historical mean of 4.24 mg/L (95-percent confidence limits 1.68 to 6.80) for the USGS laboratory, but is within the accepted confidence intervals. Embryological development results from four samples (FL–11 Rep 2, FL–13, FL–14 and FL–26; table 5) were below the DSC cut-off criteria of 71.9 percent for the 100 percent salinity-adjusted pore-water concentration. These four samples also had un-ionized ammonia levels that approached or exceeded the EC_{50} value (48.79 µg/L; 95-percent confidence limits 47.53–50.08 µg/L) calculated for the ammonia series. Sample FL–14 also was below the DSC cut-off values at the 50- and 25-percent pore-water concentrations.

Post-Landfall Pore-Water-Quality Measurements

Water-quality measurements were conducted on sediment pore water from 49 sites, as well as the reference, dilution water, brine, and rinsate blanks (table 6, at the back of the report). Salinity adjustment with Milli-Q® purified de-ionized water was required for 17 pore-water samples, whereas 26 pore-water samples and the reference control required adjustments with brine. Salinities in test samples ranged from nearly fresh water at 1 ‰ to full strength seawater at 35 ‰. Sample LA–26 contained suspended particulates after thawing and water-quality measurement, and required centrifugation before testing to eliminate suspended particles that could interfere with fertilization. Total ammonia ranged from less than 0.1 to 0.793 mg/L, whereas the un-ionized ammonia (the most toxic fraction) ranged from less than 1.9 to 48.9 µg/L. Sulfide concentrations were measured in 14 pore-water samples, and ranged from less than detection at 0.009 mg/L to as much as 0.048 mg/L.

Post–Landfall Sea Urchin Toxicity Testing

On October 26, 2010, three tests were conducted with the same sea urchin gametes; two for the fertilization test, and one for the embryological development test (tables 7–9, at the back of the report). Eggs from four females were combined to achieve the necessary quantity required for the three tests. Sperm from one male was used in all three tests. The EC_{50} value for the SDS positive controls for the fertilization tests were calculated at 5.47 and 5.63 mg/L for tests one and two, respectively. These values are similar to the historical mean for this species for the USGS laboratory of 5.43 mg/L (95-percent confidence limits 3.20 to 7.66 mg/L) and indicate adequate test sensitivity. Only three pore-water samples indicated a significant difference from the reference control in the fertilization tests using the DSC (figs. 2–4, at the back of the report). Fertilization percentages from samples from sites LA–25, LA–29, and LA–36 (fig. 2) were below the DSC cut-off value of 81.9 percent for the 100-percent concentration of the pore water in the first test. Site LA–29 (fig. 2) also was significantly different in the 50- and 25-percent diluted pore-water samples. No sample means were less than the DSC cut-off value of 81.2 percent in the second fertilization test.

Raw data and means from the embryological development test are reported in table 9. The EC_{50} value for the SDS positive control for the embryological development test was calculated at 3.44 mg/L (3.28–3.60 mg/L), which is slightly below the historical mean of 4.24 mg/L (95-percent confidence limits 1.68 to 6.80 mg/L) for the USGS laboratory but was within the accepted 95-percent confidence intervals. Development results from six samples (LA–25, LA–26, LA–31, LA–33, LA–36, and MS–39) were below the DSC cut-off of 78.3 percent for the 100-percent salinity adjusted pore-water concentration (figs. 2–4). No samples were below the DSC at the 50- and 25-percent pore-water concentrations. Notably, samples from sites LA–26 and LA–31 had the greatest ammonia and sulfide concentrations of the samples collected and tested in the post-landfall round of testing.

Toxicity Identification Evaluation Pore-Water-Quality Measurements

After testing of the post-landfall samples, seven samples (LA–25, LA–26, LA–29, LA–31, LA–33, LA–36, and MS–39) were determined to be toxic in either the fertilization or embryological development test based on the exceedance of the DSC. The remaining volume of pore water in frozen storage was sufficient for five samples (all but LA–25 and LA–26) to perform a partial phase I TIE to characterize the toxicity observed. Water-quality measurements were conducted on the five test pore water samples and the reference pore water, as well as the dilution water and a brine blank (table 10, at the back of the report). Initial salinities ranged from 6 to 29 ‰ for the pore-water samples. Total ammonia concentration was greater overall for this set of samples than from the concentration measured during the post-landfall testing conducted in October 2010, indicating a possible problem with the October measurements. Total ammonia concentrations ranged from 0.903 to 2.72 mg/L for the test samples (table 10). Samples LA–31 (94.7 µg/L) and LA–33 (92.0 µg/L) had unionized ammonia values that exceeded the EC_{50} (49.79 µg/L) for toxicity for the sea urchin embryological development test determined in the ammonia series included in this test. Measurable amounts of ammonia were recorded for all the samples initially tested (table 10). Following *Ulva latuca* treatment, a reduction in ammonia levels was observed in every sample to below the detection limit of 0.1 mg/L. Sulfide was measured in two samples (LA–29 and LA–33; table 10) above the detection level of 0.009 mg/L and was comparable to measurements made in the October 2010, post-landfall testing; however, measurements made after aeration indicated little change in sulfide concentration. Sample LA–29 decreased from a reading of 0.04 mg/L to 0.033 mg/L, whereas sample LA–33 virtually was unchanged after aeration. This indicates that the aeration treatment was not effective in removing the sulfide, and may complicate interpretation of results in those samples containing toxic concentrations of sulfide.

Toxicity Identification Evaluation Sea Urchin Toxicity Testing

Sample LA–29 was tested with its various treatment manipulations within the sea urchin fertilization assay (table 11, at the back of the report). The initial untreated pore-water sample exhibited similar toxicity to that documented in the October 2010, post-landfall testing. Of the four treatments, only two had any effect on reducing toxicity to the sample; the addition of EDTA had a small but significant effect raising the mean from 13 percent fertilized to 22.2 percent fertilized. The largest reduction (from 13 percent to 87.3 percent) was observed in the *Ulva lactuca* treatment, as all of the toxicity was removed. This indicates that ammonia with some minor metals contribution is contributing to most of the observed toxicity; however, this is somewhat surprising because the fertilization test is not sensitive to ammonia toxicity when compared to the embryological development assay (Winger and others, 2003), and this sample was not determined to be toxic in the embryological development assay. Sulfides or other related compounds may be playing a role in that they were not removed by the aeration treatment, but may have been taken up by the *Ulva lactuca* treatment. The lack of increased fertilization of the sample treated with the C_{18} column indicates that organics probably are not a primary contributing factor to the toxicity. Treatment blanks with the non-toxic Texas pore water indicated no artifacts imparted to the samples from the various manipulations.

The remaining four samples with their respective treatment manipulations were tested with the sea urchin embryological development test (table 12, at the back of the report). Untreated sample LA–31 exhibited toxicity similar to that

previously measured in the post-landfall assessment. Only the *Ulva lactuca* treatment was successful in reducing toxicity to the level of nontoxic. This indicates ammonia was the primary contaminant and, indeed, the un-ionized ammonia measurement was nearly double the EC_{50} for toxicity determined in the ammonia series included in this test. No other sample manipulation was significant in changing the toxicity.

Untreated sample LA–33 also exhibited toxicity similar to that previously measured in the October 2010 post-landfall assessment. As with sample LA–29, *Ulva latuca* treatment reduced the toxicity completely, whereas EDTA addition also reduced the toxicity level to a lesser but significant extent. C_{18} treatment also seemed to reduce the toxicity level slightly, but was not determined to be significant. This indicates that ammonia and metals contributed to the observed toxicity. The un-ionized ammonia level in this sample (92 µg/L) also was well above the EC_{50} for toxicity determined for this test, indicating that it may have been the primary stressor in sample LA–33.

Sample LA–36 exhibited a reduced toxic response in the untreated sample compared to previous testing in the post-landfall assessment. Although still toxic, the lower response makes it more difficult to discern the effects of the sample manipulations on the level of toxicity; however, two treatments did make an improvement to the level of toxicity. The *Ulva lactuca* treatment and the EDTA addition reduced the toxicity to zero indicating that metals and ammonia are contributing to the observed toxicity. The aeration treatment significantly increased the toxicity of the sample indicating that the sample was evaporating and concentrating the contaminants.

Sample MS–39 exhibited slightly reduced toxicity in the untreated sample compared to the initial testing in the October post-landfall assessment but still had a fairly strong signal. As with the other samples, *Ulva lactuca* treatment removed all of the toxicity and EDTA addition also removed a significant part of it. This sample differed from the others in that the C_{18} treatment also removed nearly all of the toxicity from the sample. Although the measured amount of un-ionized ammonia was not great enough to cause toxicity, the *Ulva lactuca* treatment may have been taking up non-ionic toxicants as well as ammonia. *Ulva lactuca* is known to selectively take up some non-ionic organic toxicants as well as ammonia (Ho and others, 1999). C_{18} is not known to uptake ammonia, but it will chelate some metals such as copper (U.S. Environmental Protection Agency, 1996). It is possible that ammonia, metals, and non-ionic organics are contributing to the observed toxicity of this sample.

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Figure 1. Sample sites in Texas, Louisiana, Mississppi, Alabama and Florida and locations of laboratories.

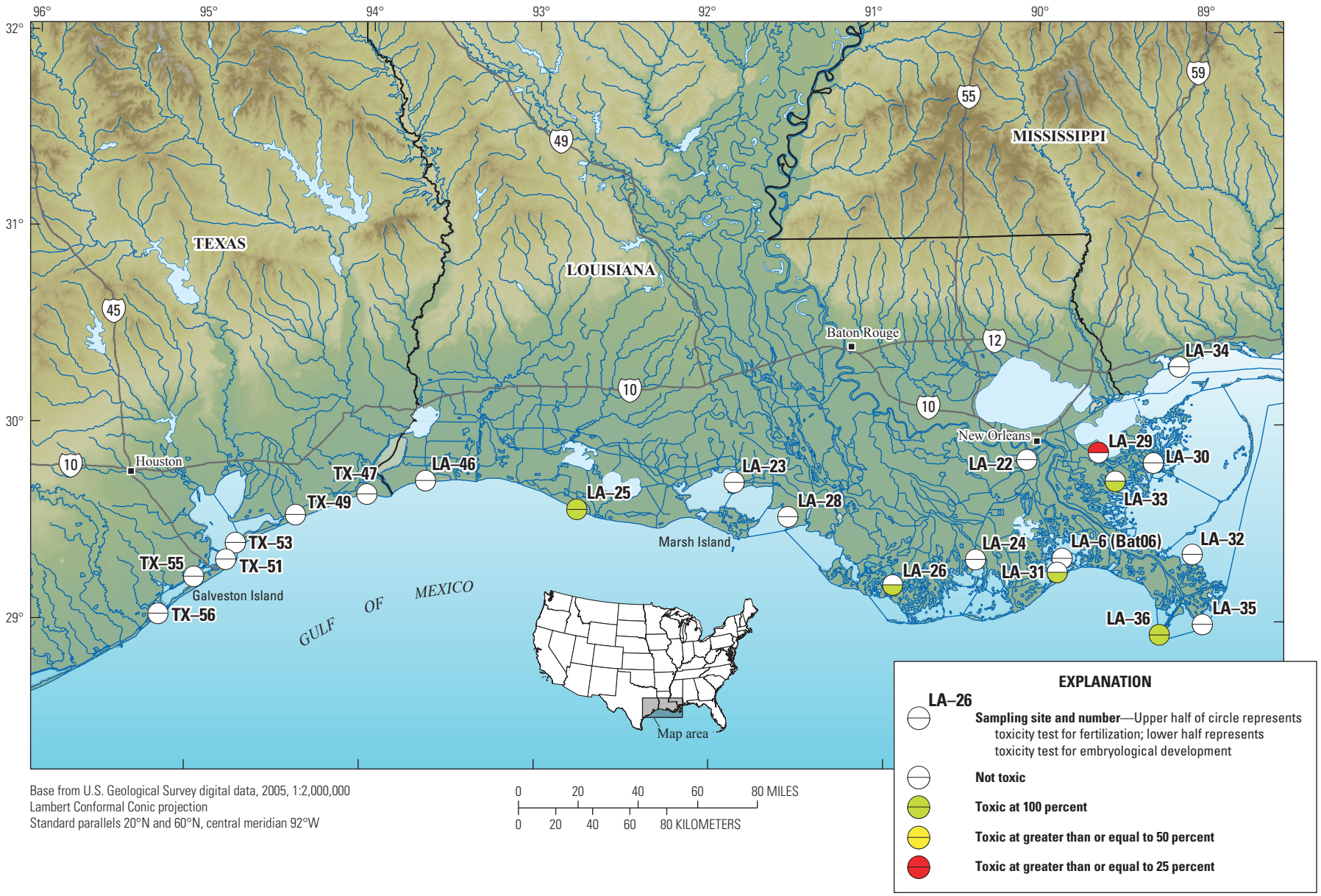

Figure 2. Sample sites in Texas and Louisiana and toxicity test results of the sea urchin fertilization and embryological development post-landfall tests of 100-, 50- and 25-percent salinity adjusted pore water. Color differentiation of circles indicates those sites that met the detectable significance criteria compared to the reference pore water.

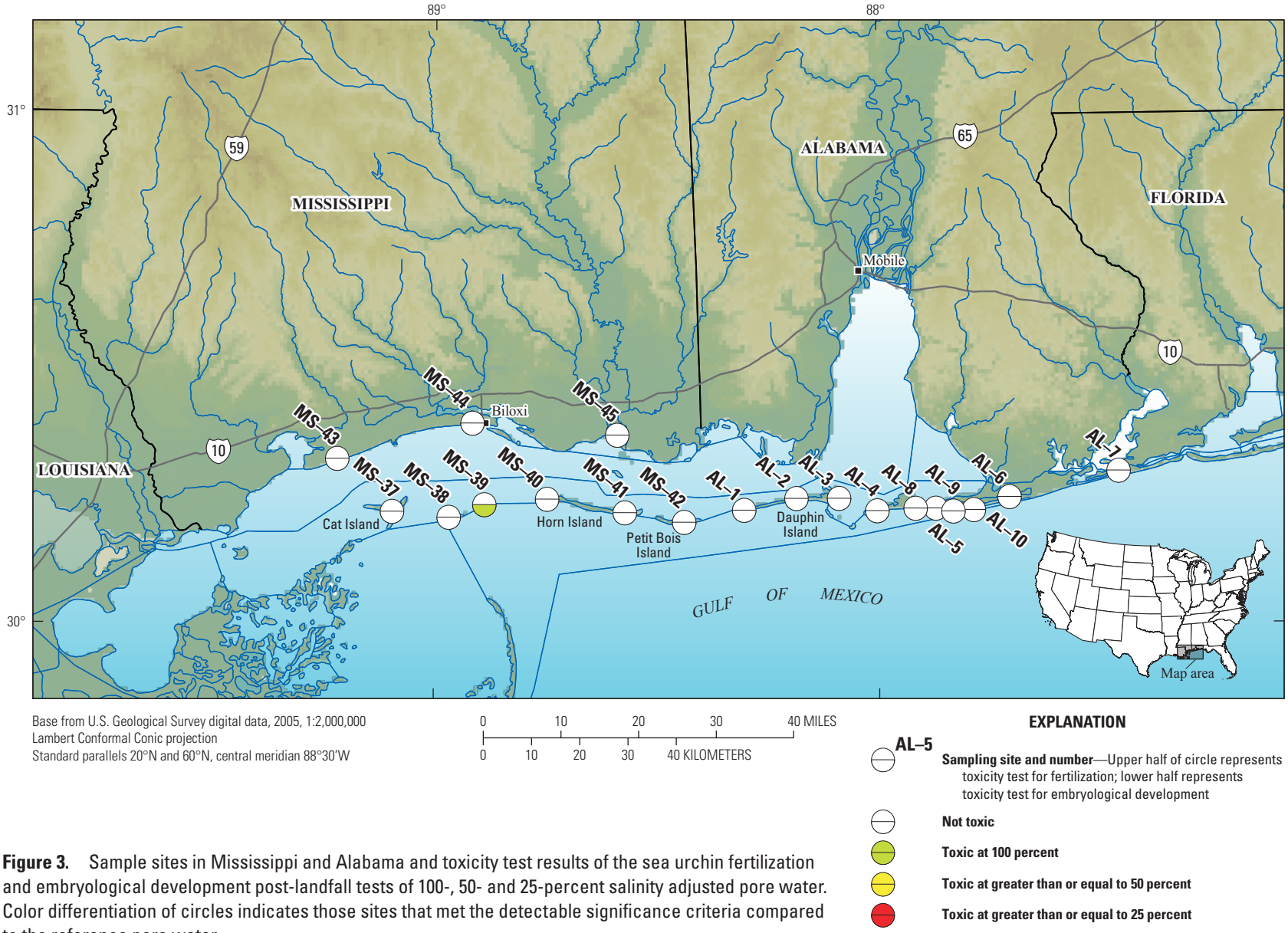

Figure 3. Sample sites in Mississippi and Alabama and toxicity test results of the sea urchin fertilization and embryological development post-landfall tests of 100-, 50- and 25-percent salinity adjusted pore water. Color differentiation of circles indicates those sites that met the detectable significance criteria compared to the reference pore water.

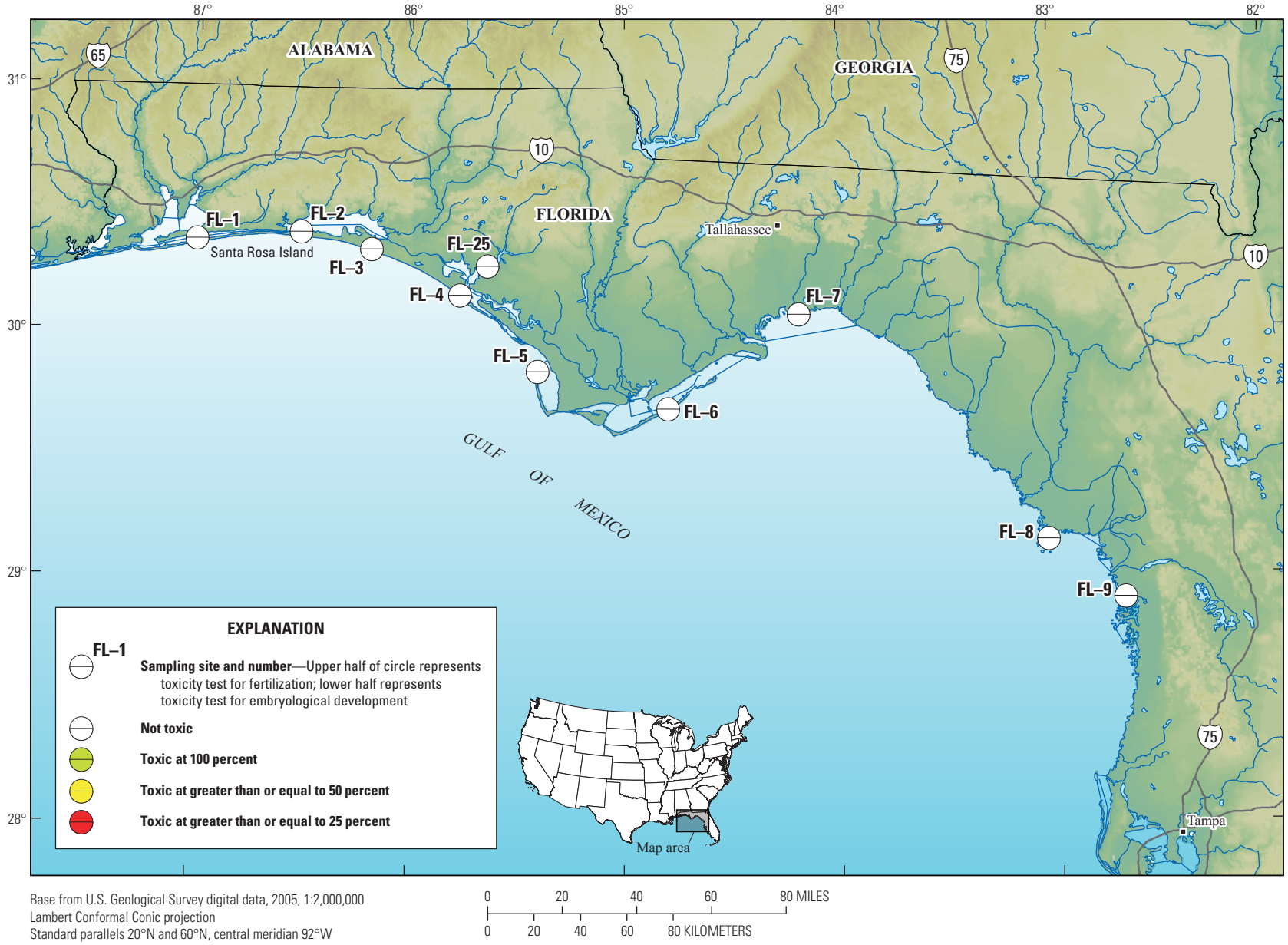

Figure 4. Sample sites in Florida and toxicity test results of the sea urchin fertilization and embryological development post-landfall tests of 100-, 50- and 25-percent salinity adjusted pore water. Color differentiation of circles indicates those sites that met the detectable significance criteria compared to the reference pore water.

Table 1. Relevant sample and extraction dates, times, temperatures and volumes of pore water collected from sediments pre-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; Collection date and time, date and time sample was taken from the field; COC, chain of custody; temps., temperatures, in degrees Celsius; Extraction date, date pore water was collected at Marine Ecotoxicology Research Station Laboratory (MERS); (Rep 2), field replicate two; na, not available; (Rep 1), field replicate one]

Site	Site ID	Collection date	Collection time	Arrival date[1]	Arrival COC log-in time[2]	Incoming sample temps.[3]	Extraction date	Volume extracted[4]
FL–8	290740083031200	5/18/10	17:00	5/21/2010	11:21	7	5/21/2010	110
FL–9	285425082412600	5/19/10	12:00	5/21/2010	11:21	7[5]	5/21/2010	170
FL–11 (Rep 2)	263132082114000	5/20/10	16:30	5/26/2010	11:15	8[5]	5/26/2010	40
FL–13	251329081101100	5/22/10	na	5/26/2010	11:15	8[5]	5/26/2010	190
FL–14	243737082522500	5/20/10	na	5/26/2010	11:15	8	5/26/2010	180
FL–19	265722080045400	6/16/10	13:00	6/18/2010	11:05	3	6/21/2010	230
FL–20	265722080045500	6/16/10	15:00	6/18/2010	11:05	3	6/21/2010	180
FL–24	273605082454900	6/14/10	14:00	6/17/2010	11:00	7.5	6/17/2010	190
FL–25	300223085260800	6/10/10	11:00	6/17/2010	15:30	7.5[5]	6/17/2010	140
FL–26	244325081351500	7/7/10	11:00	7/9/2010	15:30	3	7/12/2010	140
LA–22	294432090083100	5/14/10	12:32	5/21/2010	11:20	6[5]	5/21/2010	100
LA–23	294406091511300	5/13/10	12:30	5/21/2010	11:20	6	5/21/2010	170
LA–23 (Rep 1)	294406091511300	5/13/10	12:31	5/21/2010	11:20	6[5]	5/21/2010	170
LA–23 (Rep 2)	294406091511300	5/13/10	12:32	5/21/2010	11:20	6	5/21/2010	170
LA–24	292046090254500	5/18/10	16:20	5/21/2010	11:21	7[5]	5/21/2010	130
LA–28	293424091321600	5/13/10	9:30	5/21/2010	11:20	6[5]	5/21/2010	170
LA–29	294324089432500	5/18/10	14:00	5/21/2010	11:20	6[5]	5/21/2010	170

[1] Date sample arrived by FedEx® into the USGS Marine Ecotoxicology Research Station Laboratory.

[2] Time chain of custody form was signed at the USGS Marine Ecotoxicology Research Station Laboratory.

[3] Incoming sample temperatures for individual jar of sediment for that site. Temperatures in degrees Celsius.

[4] Volume of pore water extracted in milliliters from the sample and stored frozen.

[5] Temperature estimate based on temperature taken of another sample in the same box.

Table 2. Relevant sample and extraction dates, times, temperatures and volumes of pore water collected from sediments post-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; Collection date and time: date and time sample was taken from the field; COC, chain of custody; temps., temperatures in degrees Celsius; Extraction date: date pore water was collected at Marine Ecotoxicology Research Station Laboratory (MERS)]

Site	Site ID	Collection date	Collection time	Arrival date[1]	Arrival COC log-in time[2]	Incoming sample temps.[3]	Extraction date	Volume extracted[4]
FL–1	302144086581200	10/04/10	14:30	10/06/10	11:25	2.0 and 2.3	10/06/10	285
FL–2	302258086263400	10/05/10	9:30	10/07/10	12:26	4.6 and 4.0	10/07/10	300
FL–3	301926086091800	10/05/10	14:00	10/07/10	12:26	2.5 and 4.6	10/07/10	300
FL–4	300729085440900	10/11/10	13:00	10/13/10	15:00	1.1, 1.1, and 1.1	10/13/10	450
FL–5	294645085243000	10/13/10	10:30	10/15/10	11:52	3.0, 3.5, and 4.2	10/15/10	300
FL–6	294152084460300	10/06/10	11:55	10/08/10	11:55	7.0 and 7.5	10/08/10	300
FL–7	300427084105000	10/07/10	11:30	10/09/10	11:12	1.2 and 0.9	10/09/10	300
FL–25	300223085260800	10/12/10	10:00	10/14/10	11:46	2.2, 3.3, and 2.1	10/14/10	300
AL–1	301338088193500	10/13/10	13:00	10/15/10	10:29	6.0 and 4.3	10/15/10	450
AL–2	301455088110300	10/07/10	12:20	10/08/10	12:40	0.4 and 0.5	10/08/10	230
AL–3	301448088044000	10/06/10	12:30	10/07/10	12:44	0.5 and 1.4	10/07/10	295
AL–4	301329088003000	10/12/10	11:00	10/13/10	14:35	0.1 and 0.1	10/13/10	450
AL–5	301349087541600	10/13/10	13:40	10/14/10	11:30	1.8 and 3.2	10/14/10	300
AL–6	301428087434900	10/14/10	13:30	10/16/10	11:58	3.6, 1.8, and 2.1	10/16/10	300
AL–7	301608087345400	10/14/10	10:00	10/16/10	11:58	3.8, 4.5, and 3.6	10/16/10	300
AL–8	301353087561600	10/13/10	10:45	10/14/10	11:30	2.6 and 3.6	10/14/10	400
AL–9	301343087520200	10/14/10	13:35	10/15/10	11:42	2.9 and 2.4	10/15/10	300
AL–10	301341087495200	10/14/10	10:25	10/15/10	11:42	3.8 and 5.5	10/15/10	300
LA–22	294432090083100	10/13/10	12:30	10/16/10	11:05	2.8 and 2.4	10/16/10	300
LA–23	294406091511300	10/05/10	17:30	10/07/10	12:52	1.3 and 0.9	10/07/10	450
LA–24	292046090254500	10/12/10	10:30	10/13/10	14:45	1.8 and 0.5	10/13/10	170
LA–25	293808092460200	10/07/10	14:00	10/09/10	10:52	0.4 and 0.9	10/09/10	150
LA–26	291507090551800	10/08/10	14:00	10/09/10	11:36	2.8 and 3.7	10/09/10	95
LA–28	293424091321600	10/05/10	12:00	10/07/10	12:52	2.4 and 2.9	10/07/10	300
LA–29	294324089432500	10/13/10	12:00	10/16/10	11:05	2.3 and 3.1	10/16/10	450
LA–30	294108089234500	10/12/10	12:00	10/14/10	11:38	1.8 and 3.1	10/14/10	300
LA–6 (Bat06)	292708089521400	08/23/10	14:30	08/26/10	11:30	9.0 and 10.0	08/26/10	450
LA–31	291537089570100	10/14/10	11:00	10/16/10	11:05	4.5 and 4.1	10/16/10	300
LA–32	291914089105500	10/07/10	11:15	10/09/10	10:52	1.2 and 1.7	10/09/10	450
LA–33	293518089364300	10/14/10	15:00	10/16/10	11:05	3.1 and 3.3	10/16/10	300
LA–34	300907089144500	10/11/10	10:30	10/13/10	14:45	1.2 and 0.7	10/13/10	450
LA–35	285951089085600	10/07/10	14:00	10/09/10	10:52	2.3 and 3.2	10/09/10	150
LA–36	285615089235600	10/14/10	13:30	10/16/10	11:05	3.7 and 3.8	10/16/10	300
LA–46	294456093394801	10/06/10	12:40	10/08/10	12:08	6.0 and 6.1	10/08/10	350

Table 2. Relevant sample and extraction dates, times, temperatures and volumes of pore water collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; Collection date and time: date and time sample was taken from the field; COC, chain of custody; temps., temperatures in degrees Celsius; Extraction date: date pore water was collected at Marine Ecotoxicology Research Station Laboratory (MERS)]

Site	Site ID	Collection date	Collection time	Arrival date[1]	Arrival COC log-in time[2]	Incoming sample temps.[3]	Extraction date	Volume extracted[4]
MS–37	301309089044700	10/14/10	11:30	10/16/10	12:30	6.6 and 5.7	10/16/10	300
MS–38	301227088582000	10/14/10	14:30	10/16/10	12:30	3.2 and 4.0	10/16/10	300
MS–39	301358088533300	10/11/10	14:30	10/13/10	15:08	2.6 and 2.4	10/13/10	400
MS–40	301425088440600	10/12/10	16:30	10/14/10	11:56	2.3 and 1.1	10/14/10	450
MS–41	301321088353300	10/12/10	12:30	10/14/10	12:04	4.1 and 4.4	10/14/10	450
MS–42	301208088253600	10/13/10	9:50	10/15/10	11:29	5.5 and 5.6	10/15/10	430
MS–43	301858089141000	10/08/10	10:00	10/09/10	11:28	1.6 and 1.8	10/09/10	400
MS–44	302336088535800	10/07/10	10:30	10/09/10	11:18	1.3 and 1.6	10/09/10	400
MS–45	302034088325200	10/14/10	14:30	10/16/10	12:30	5.9 and 4.5	10/16/10	450
TX–47[5]	294057093572301	10/12/10	11:20	10/16/10	11:37	5.4 and 5.2	10/08/10	300
TX–49	293324094220601	10/07/10	11:20	10/09/10	10:38	5.0 and 4.4	10/09/10	115
TX–51[5]	291815094461001	10/14/10	13:15	10/16/10	11:37	2.8, 5.3, 3.9, and 4.0	10/16/10	300
TX–53	292318094430901	10/07/10	12:43	10/09/10	10:38	6.5 and 7.1	10/09/10	150
TX–55	291251094571401	10/14/10	11:10	10/16/10	12:15	5.2 and 5.7	10/16/10	300
TX–56	290512095063101	10/05/10	12:50	10/07/10	12:35	4.2 and 5.6	10/07/10	150

[1] Date sample arrived by FedEx® into the USGS Marine Ecotoxicology Research Station Laboratory.

[2] Time chain of custody form was signed at the USGS Marine Ecotoxicology Research Station Laboratory.

[3] Incoming sample temperatures for 2 to 4 individual jars of sediment for that site. Temperatures in degrees Celsius.

[4] Volume of pore water extracted from the sample and stored frozen.

[5] Sample was recollected besause of insufficient pore-water extraction from the initial sample.

Table 3. Water-quality measurements after salinity adjustment and original salinity of pore-water samples collected from sediments pre-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; ‰, parts per thousand; %, percent; DO, dissolved oxygen; mg/L, milligrams per liter; TAN, total ammonia as nitrogen; UAN, un-ionized ammonia as nitrogen; µg/L, micrograms per liter; OS, original sample; <, less than; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; ±, plus or minus]

Site	Site ID	Salinity[1] ‰	DO (mg/L)	% DO	pH	TAN (mg/L)	UAN (µg/L)	Sulfide (mg/L)	% OS[2]
FL–8	290740083031200	30	7.24	99.1	8.10	0.667	26.2	<0.009	100
FL–9	285425082412600	12	7.34	100.1	8.03	.1	<3.3	<.009	80
FL–11 (Rep 2)	263132082114000	22.5	7.44	100.6	8.06	5.97	214.8	.009	90.9
FL–13	251329081101100	20	7.31	98.9	8.05	1.54	54.4	<.009	88.0
FL–14	243737082522500	28	7.40	99.7	7.75	4.27	76.8	<.009	97.3
FL–19	265722080045400	37	7.26	97.7	8.08	<.1	<3.7	<.009	81.1
FL–20	265722080045500	33	7.08	95.2	7.97	.178	5.3	<.009	90.9
FL–24	273605082454900	35.5	7.30	97.8	7.91	.809	20.5	<.009	84.5
FL–25	300223085260800	14	7.47	100.8	8.17	<.1	<4.6	<.009	81.9
FL–26	244325081351500	40	7.44	100.1	8.06	1.32	46.8	<.009	75.3
LA–22	294432090083100	2	7.41	99.9	8.31	.288	17.8	<.009	72.0
LA–23	294406091511300	4	7.36	99.3	8.27	<.1	<5.7	<.009	73.6
LA–23 (Rep 1)	294406091511300	4	7.24	97.6	8.25	<.1	<5.5	<.009	73.6
LA–23 (Rep 2)	294406091511300	4	7.10	95.9	8.26	<.1	<5.5	<.009	73.6
LA–24	292046090254500	22	7.51	101.5	8.14	.171	7.3	<.009	90.3
LA–28	293424091321600	0.5	7.36	99.4	8.31	<.1	<6.3	<.009	71.1
LA–29	294324089432500	2	7.07	95.7	8.21	.32	16.0	<.009	72.0
TXREF[3]	None	22.5	7.52	101.8	8.31	.184	11.3	<.009	90.5
MFS[4]	None	32.5	6.34	86.5	8.12	<.1	<4.1	<.009	92.3
Brine blank[5]	None	6	6.14	83.3	8.35	<.1	<6.8	<.009	20.5

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

[2] Percent of original sample after salinity adjustment.

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

[4] Millipore® filtered seawater diluent.

[5] Brine blank of TXREF diluted to 6 ‰ with Milli–Q® purified water and subsequently increased with brine to 30 ‰ (concentrated brine at 102 ‰).

Table 4. Sea urchin fertilization test raw data and means for pore-water samples collected from sediments pre-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
FL–8	290740083031200	100	100	100	100	99	100	99.8	0.4	101.1
		50	98	99	100	99	na	99.0	.8	99.9
		25	98	100	100	98	na	99.0	1.2	99.9
FL–9	285425082412600	100	100	99	100	100	100	99.8	.4	101.1
		50	100	100	99	100	100	99.8	.4	100.7
		25	99	100	100	99	100	99.6	.5	100.5
FL–11 (Rep 2)	263132082114000	100	91	95	94	93	na	93.3	1.7	94.5
FL–13	251329081101100	100	98	100	100	99	100	99.4	.9	100.7
		50	99	100	99	100	100	99.6	.5	100.5
		25	100	100	100	99	100	99.8	.4	100.7
FL–14	243737082522500	100	95	99	99	97	99	97.8	1.8	99.1
		50	100	98	100	97	99	98.8	1.3	99.7
		25	100	99	99	98	97	98.6	1.1	99.5
FL–19	265722080045400	100	100	100	100	100	99	99.8	.4	101.1
		50	99	100	100	100	99	99.6	.5	100.5
		25	99	100	100	100	100	99.8	.4	100.7
FL–20	265722080045500	100	100	100	100	99	100	99.8	.4	101.1
		50	99	98	99	100	99	99.0	.7	99.9
		25	99	100	100	100	100	99.8	.4	100.7
FL–24	273605082454900	100	99	100	99	98	99	99.0	.7	100.3
		50	99	100	98	99	99	99.0	.7	99.9
		25	99	100	100	98	100	99.4	.9	100.3
FL–25	300223085260800	100	99	100	100	99	100	99.6	.5	100.9
		50	100	100	100	98	99	99.4	.9	100.3
		25	100	99	100	100	98	99.4	.9	100.3
FL–26	244325081351500	100	87	88	88	87	84	86.8	1.6	87.9
		50	99	100	99	100	100	99.6	.5	100.5
		25	100	100	100	99	99	99.6	.5	100.5
LA–22	294432090083100	100	100	100	98	99	99	99.2	.8	100.5
		50	100	100	99	100	98	99.4	.9	100.3
		25	100	99	99	100	100	99.6	.5	100.5
LA–23	294406091511300	100	99	99	100	99	99	99.2	.4	100.5
		50	98	100	99	99	100	99.2	.8	100.1
		25	100	99	100	98	100	99.4	.9	100.3
LA–23 (Rep 1)	294406091511300	100	99	98	100	100	100	99.4	.9	100.7
		50	99	99	99	99	97	98.6	.9	99.5
		25	100	100	98	98	99	99.0	1.0	99.9
LA–23 (Rep 2)	294406091511300	100	100	100	100	98	100	99.6	.9	100.9
		50	98	100	98	100	99	99.0	1.0	99.9
		25	99	100	98	99	99	99.0	.7	99.9

Table 4. Sea urchin fertilization test raw data and means for pore-water samples collected from sediments pre-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
LA–24	292046090254500	100	99	98	100	100	100	99.4	0.9	100.7
		50	100	100	99	99	100	99.6	.5	100.5
		25	99	99	100	98	100	99.2	.8	100.1
LA–28	293424091321600	100	98	100	100	100	100	99.6	.9	100.9
		50	100	100	99	99	100	99.6	.5	100.5
		25	100	100	99	100	100	99.8	.4	100.7
LA–29	294324089432500	100	94	97	95	96	94	95.2	1.3	96.5
		50	98	99	99	100	100	99.2	.8	100.1
		25	100	99	98	100	99	99.3	1.0	100.2
TXREF[4]	None	100	98	98	99	100	99	98.7	.9	100.0
			98	100	97	99	99			
		50	98	100	99	99	100	99.1	.7	100.0
			99	100	99	98	99			
		25	99	100	99	100	97	99.1	1.0	100.0
			100	100	99	98	99			
MFS[5]	None	100	99	100	98	99	99	99.1	.7	100.4
			99	98	99	100	100			
Brine blank[6]	None	100	99	100	99	99	99	99.2	.4	100.5
SDS[7]	None	10	0	0	0	0	0	.0	.0	.0
		5	32	36	15	16	21	24.0	9.5	24.3
		2.5	99	99	100	99	99	99.2	.4	100.5
		1.25	99	99	99	100	99	99.2	.4	100.5

[1] Percent of salinity adjusted pore-water sample or concentration of SDS reference toxicant in milligrams per liter.

[2] Percent of TXREF control at the appropriate dilution.

[3] Data point not available because of error in pipetting.

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

[5] Millipore® filtered seawater diluent.

[6] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

[7] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 5. Sea urchin embryological development test raw data and means for pore-water samples collected from sediments pre-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
FL–8	290740083031200	100	91	84	82	83	91	86.2	4.4	100.2
		50	89	89	88	91	na	89.3	1.3	103.4
		25	85	89	90	82	na	86.5	3.7	100.1
FL–9	285425082412600	100	91	87	80	85	90	86.6	4.4	100.7
		50	82	88	84	86	88	85.6	2.6	99.2
		25	86	85	85	87	85	85.6	.9	99.1
FL–11 (Rep 2)	263132082114000	100	0	0	0	na	na	.0	.0	.0
FL–13	251329081101100	100	0	0	0	0	0	.0	.0	.0
		50	88	87	88	85	86	86.8	1.3	100.6
		25	84	87	90	91	91	88.6	3.0	102.5
FL–14	243737082522500	100	0	0	0	0	0	.0	.0	.0
		50	0	0	0	0	0	.0	.0	.0
		25	7	4	2	7	36	11.2	14.0	13.0
FL–19	265722080045400	100	89	85	81	86	82	84.6	3.2	98.4
		50	88	85	86	82	84	85.0	2.2	98.5
		25	83	85	85	89	85	85.4	2.2	98.8
FL–20	265722080045500	100	86	85	80	87	80	83.6	3.4	97.2
		50	86	85	86	94	90	88.2	3.8	102.2
		25	84	84	93	86	88	87.0	3.7	100.7
FL–24	273605082454900	100	80	83	82	81	89	83.0	3.5	96.5
		50	81	85	88	94	84	86.4	4.9	100.1
		25	88	82	91	87	89	87.4	3.4	101.2
FL–25	300223085260800	100	89	83	88	81	84	85.0	3.4	98.8
		50	87	83	84	87	90	86.2	2.8	99.9
		25	87	83	82	78	86	83.2	3.6	96.3
FL–26	244325081351500	100	1	0	0	0	0	.2	.4	.2
		50	81	82	87	79	82	82.2	2.9	95.2
		25	89	86	87	80	90	86.4	3.9	100.0
LA–22	294432090083100	100	86	85	85	87	83	85.2	1.5	99.1
		50	84	85	85	87	85	85.2	1.1	98.7
		25	86	87	84	85	87	85.8	1.3	99.3
LA–23	294406091511300	100	91	88	87	87	82	87.0	3.2	101.2
		50	87	86	86	84	88	86.2	1.5	99.9
		25	82	86	82	86	93	85.8	4.5	99.3

Table 5. Sea urchin embryological development test raw data and means for pore-water samples collected from sediments pre-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
LA–23 (Rep 1)	294406091511300	100	87	88	82	84	85	85.2	2.4	99.1
		50	79	83	89	84	88	84.6	4.0	98.0
		25	89	86	84	80	82	84.2	3.5	97.5
LA–23 (Rep 2)	294406091511300	100	85	86	90	86	85	86.4	2.1	100.5
		50	87	86	83	85	87	85.6	1.7	99.2
		25	87	90	86	84	87	86.8	2.2	100.5
LA–24	292046090254500	100	86	87	91	82	85	86.2	3.3	100.2
		50	87	91	87	88	83	87.2	2.9	101.0
		25	88	91	86	90	89	88.8	1.9	102.8
LA–28	293424091321600	100	82	88	85	84	87	85.2	2.4	99.1
		50	87	84	83	83	86	84.6	1.8	98.0
		25	90	87	85	83	88	86.6	2.7	100.2
LA–29	294324089432500	100	85	87	86	84	86	85.6	1.1	99.5
		50	89	88	82	81	84	84.8	3.6	98.3
		25	86	81	84	82	83	83.8	1.7	96.9
TXREF[4]	None	100	87	88	84	85	88	86.0	2.4	100.0
			87	85	86	89	81			
		50	88	84	86	90	86	86.3	2.9	100.0
			87	89	83	89	81			
		25	92	86	86	86	85	86.4	2.8	100.0
			89	89	84	84	83			
MFS[5]	None	100	88	86	81	83	77	84.7	4.2	98.5
			89	87	88	80	88			
Brine blank[6]	None	100	87	89	90	85	87	87.6	1.9	88.8
SDS[7]	None	10	0	0	0	0	0	.0	.0	.0
		5	9	10	9	7	12	9.4	1.8	10.9
		2.5	81	80	81	81	78	80.2	1.3	93.3
		1.25	89	85	90	84	88	87.2	2.6	101.4

[1] Percent of salinity adjusted pore-water sample or concentration of SDS reference toxicant in milligrams per liter.

[2] Percent of TXREF control at the appropriate dilution.

[3] Data point not available because of error in pipetting.

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

[5] Millipore® filtered seawater diluent.

[6] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

[7] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 6. Water-quality measurements after salinity adjustment and original salinity of pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; ‰, parts per thousand; %, percent; DO, dissolved oxygen; mg/L, milligrams per liter; TAN, total ammonia as nitrogen; UAN, un-ionized ammonia as nitrogen; µg/L, micrograms per liter; OS, original sample; <, less than; (Rep 1), field replicate one; (Rep 2), field replicate two; MFS, Millipore® filtered seawater; ±, plus or minus]

Site	Site ID	Salinity[1] ‰	DO (mg/L)	% DO	pH	TAN (mg/L)	UAN (µg/L)	Sulfide (mg/L)	% OS[2]
FL–1	302144086581200	34	7.04	91.6	7.97	0.1	2.9	<0.009	88.2
FL–2	302258086263400	30	6.99	90.6	7.81	.139	2.8	<.009	100
FL–3	301926086091800	34	7.05	91.4	8.02	<.1	<3.3	<.009	88.2
FL–4	300729085440900	35	6.97	90	7.98	<.1	<3.0	<.009	85.7
FL–5	294645085243000	34	7.81	101.2	8.00	.102	3.2	<.009	88.2
FL–6	294152084460300	33	7.43	96.3	8.02	.1	<3.2	<.009	90.9
FL–7	300427084105000	24	7.08	91.5	7.99	.134	4.1	.037	92.3
FL–25	300223085260800	20	7.08	91.8	8.02	<.1	<3.3	<.009	88.2
AL–1	301338088193500	32	7.03	91.3	7.99	<.1	<3.1	<.009	93.8
AL–2	301455088110300	32	6.65	86.3	7.96	<.1	<2.8	<.009	93.8
AL–3	301448088044000	25.5	7.04	91.4	8.06	.173	6.2	<.009	94.3
AL–4	301329088003000	32	6.85	88.4	7.90	<.1	<2.5	<.009	93.8
AL–5	301349087541600	34	7.17	92.5	7.99	<.1	<3.0	<.009	88.2
AL–6	301428087434900	34.5	7.14	91.8	7.85	<.1	<2.2	<.009	87.0
AL–7	301608087345400	34.5	6.96	89.4	7.89	<.1	<2.4	<.009	87.0
AL–8	301353087561600	34	7.30	93.9	7.98	<.1	<3.0	<.009	88.2
AL–9	301343087520200	34	7.41	95.7	7.90	<.1	<2.5	<.009	88.2
AL–10	301341087495200	34	7.10	91.4	8.00	<.1	<3.1	<.009	88.2
LA–6 (Bat06)	292708089521400	6	7.71	99.6	8.12	.286	11.6	.021	75.0
LA–22	294432090083100	1.5	7.29	93.7	8.06	.102	3.7	<.009	71.8
LA–23	294406091511300	6	7.19	92.3	8.45	<.1	<8.3	<.009	75.0
LA–24	292046090254500	18.5	7.28	93.1	8.16	.339	15.1	.011	87.6
LA–25	293808092460200	27	7.12	91.1	7.95	.401	11.3	.010	96.2
LA–26	291507090551800	12	7.35	93.5	8.31	.793	48.9	.010	80.0
LA–28	293424091321600	2	7.54	96.4	8.28	<.1	<5.8	<.009	72.1
LA–29	294324089432500	6	7.64	97.7	8.20	.35	17.0	.048	75.0
LA–30	294108089234500	20	7.84	100.4	8.10	<.1	<3.9	<.009	88.2
LA–31	291537089570100	27	7.64	98.2	8.10	.778	30.5	.010	96.2
LA–32	291914089105500	1	7.14	91.5	7.50	.131	1.3	.010	71.4
LA–33	293518089364300	9	7.26	93.2	8.11	.467	18.6	.010	77.7
LA–34	300907089144500	20	7.18	92	8.17	.1	4.6	<.009	88.2
LA–35	285951089085600	19	7.47	96.8	8.34	<.1	<6.5	<.009	86.7
LA–36	285615089235600	18	7.28	94	8.00	.33	10.3	<.009	85.7
LA–46	294456093394801	28	7.51	96.7	8.08	<.1	<3.8	<.009	97.4

Table 6. Water-quality measurements after salinity adjustment and original salinity of pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; ‰, parts per thousand; %, percent; DO, dissolved oxygen; mg/L, milligrams per liter; TAN, total ammonia as nitrogen; UAN, un-ionized ammonia as nitrogen; µg/L, micrograms per liter; OS, original sample; <, less than; (Rep 1), field replicate one; (Rep 2), field replicate two; MFS, Millipore® filtered seawater; ±, plus or minus]

Site	Site ID	Salinity[1] ‰	DO (mg/L)	% DO	pH	TAN (mg/L)	UAN (µg/L)	Sulfide (mg/L)	% OS[2]
MS–37	301309089044700	29	7.24	93.2	7.84	<.1	<2.2	<0.009	100.0
MS–38	301227088582000	31	7.32	94.1	7.77	<.1	<1.9	<.009	100.0
MS–39	301358088533300	29	7.34	94.1	7.96	.237	6.7	<.009	100.0
MS–40	301425088440600	32	7.47	95.7	8.12	<.1	<4.1	<.009	93.8
MS–41	301321088353300	30	7.62	97.6	7.89	<.1	<2.4	<.009	100.0
MS–42	301208088253600	32	7.31	93.7	7.90	<0.1	<2.5	<.009	93.8
MS–43	301858089141000	19	7.54	96.7	8.02	.171	5.6	.017	86.7
MS–44	302336088535800	23	7.55	97	8.04	<.1	<3.4	<.009	91.2
MS–45	302034088325200	28	7.83	100.8	7.87	<.1	<2.4	.010	94.7
TX–47	294057093572301	28	7.75	99.7	8.11	<.1	<4.0	<.009	97.4
TX–49	293324094220601	27	7.49	95.9	8.06	.181	6.5	<.009	96.0
TX–51	291815094461001	32	7.71	99.1	8.05	<.1	<3.5	<.009	93.8
TX–53	292318094430901	26	7.68	98.9	8.32	.108	6.9	.009	94.9
TX–55	291251094571401	27	7.44	96	7.98	<.1	<3.0	.010	96.1
TX–56	290512095063101	30	7.43	95.9	8.11	.157	6.3	<.009	100.0
TXREF[3]	None	23	7.57	98.6	8.32	.24	15.1	.010	90.6
MFS[4]	None	30	6.49	84.3	8.13	<.1	<4.1	<.009	100.0
Brine blank[5]	None	23	6.22	80.9	8.47	<.1	<8.6	<.009	18.8
Rinse blank[6]	None	30	7.08	92.2	8.18	<.1	<4.7	<.009	100.0

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

[2] Percent of original sample after salinity adjustment.

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

[4] Millipore® filtered seawater diluent.

[5] Brine blank of TXREF diluted to 6 ‰ with Milli-Q® purified water and subsequently increased with brine to 30 ‰ (concentrated brine at 102 ‰).

[6] Rinsate blank consisting of filtered seawater run over a complete set of all equipment used in pore-water extraction.

Table 7. Sea urchin fertilization test raw data and means for experiment one with pore-water samples collected from sediments post–landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjustment sample; Rep, replicate; SD, standard deviation; na, not available; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
AL–1	301338088193500	100	96	95	98	96	94	95.8	1.5	98.9
		50	96	95	94	91	96	94.4	2.1	96.4
		25	96	96	91	95	95	94.6	2.1	96.4
AL–2	301455088110300	100	98	98	100	97	98	98.2	1.1	101.3
		50	98	97	97	98	94	96.8	1.6	98.9
		25	98	96	93	93	96	95.2	2.2	97.0
AL–3	301448088044000	100	98	97	97	97	95	96.8	1.1	99.9
		50	99	99	97	97	98	98.0	1.0	100.1
		25	99	99	99	97	96	98.0	1.4	99.9
AL–4	301329088003000	100	100	95	94	98	96	96.6	2.4	99.7
		50	100	93	90	83	95	92.2	6.3	94.2
		25	96	93	92	96	94	94.2	1.8	96.0
AL–5	301349087541600	100	95	98	97	95	97	96.4	1.3	99.5
		50	98	94	96	96	90	94.8	3.0	96.8
		25	94	94	96	93	93	94.0	1.2	95.8
AL–6	301428087434900	100	99	98	99	96	97	97.8	1.3	100.9
		50	95	92	96	92	95	94.0	1.9	96.0
		25	96	97	91	98	91	94.6	3.4	96.4
AL–7	301608087345400	100	99	98	95	98	97	97.4	1.5	100.5
		50	97	96	98	93	98	96.4	2.1	98.5
		25	94	92	94	94	92	93.2	1.1	95.0
AL–8	301353087561600	100	98	100	97	95	96	97.2	1.9	100.3
		50	93	95	93	94	94	93.8	.8	95.8
		25	98	97	96	93	94	95.6	2.1	97.5
AL–9	301343087520200	100	98	96	94	94	97	95.8	1.8	98.9
		50	97	99	96	97	95	96.8	1.5	98.9
		25	96	95	87	92	95	93.0	3.7	94.8
AL–10	301341087495200	100	99	98	93	93	96	95.8	2.8	98.9
		50	97	96	91	95	94	94.6	2.3	96.6
		25	95	98	97	96	95	96.2	1.3	98.1
LA–22	294432090083100	100	99	98	99	98	100	98.8	.8	102.0
		50	96	98	99	96	97	97.2	1.3	99.3
		25	96	99	97	95	95	96.4	1.7	98.3
LA–23	294406091511300	100	98	98	99	98	96	97.8	1.1	100.9
		50	100	97	99	95	97	97.6	1.9	99.7
		25	93	97	91	96	96	94.6	2.5	96.4
LA–24	292046090254500	100	92	80	90	78	80	84.0	6.5	86.7
		50	94	96	95	98	98	96.2	1.8	98.3
		25	99	98	98	98	97	98.0	.7	99.9

Table 7. Sea urchin fertilization test raw data and means for experiment one with pore-water samples collected from sediments post–landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjustment sample; Rep, replicate; SD, standard deviation; na, not available; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
LA–25	293808092460200	100	80	75	71	89	94	81.8	9.6	84.4
		50	98	97	90	97	96	95.6	3.2	97.7
		25	98	98	99	96	96	97.4	1.3	99.3
LA–26	291507090551800	100	96	96	93	88	93	93.2	3.3	96.2
		50	91	96	94	98	99	95.6	3.2	97.7
		25	99	98	97	95	97	97.2	1.5	99.1
LA–28	293424091321600	100	97	94	100	95	99	97.0	2.5	100.1
		50	98	99	96	99	95	97.4	1.8	99.5
		25	91	97	95	98	92	94.6	3.0	96.4
LA–29	294324089432500	100	14	17	14	17	20	16.4	2.5	16.9
		50	27	48	41	50	41	41.4	9.0	42.3
		25	77	na[3]	63	71	76	71.8	6.4	73.1
LA–30	294108089234500	100	99	100	99	97	96	98.2	1.6	101.3
		50	94	97	98	99	100	97.6	2.3	99.7
		25	99	99	97	99	99	98.6	.9	100.5
LA–31	291537089570100	100	97	96	95	91	82	92.2	6.1	95.1
		50	97	95	94	93	96	95.0	1.6	97.0
		25	97	98	98	95	99	97.4	1.5	99.3
LA–32	291914089105500	100	89	92	87	93	77	87.6	6.4	90.4
		50	97	97	97	96	92	95.8	2.2	97.9
		25	96	97	97	98	97	97.0	.7	98.9
LA–33	293518089364300	100	97	92	98	99	96	96.4	2.7	99.5
		50	95	93	93	99	96	95.2	2.5	97.2
		25	98	98	100	99	98	98.6	.9	100.5
LA–34	30090789144500	100	100	99	98	100	99	99.2	.8	102.4
		50	98	98	98	97	98	97.8	.4	99.9
		25	90	98	100	96	97	96.2	3.8	98.1
LA–35	285951089085600	100	99	98	98	100	99	98.8	.8	102.0
		50	98	98	98	98	98	98.0	.0	100.1
		25	95	95	97	96	97	96.0	1.0	97.9
LA–36	285615089235600	100	84	70	26	29	67	55.2	26.1	57.0
		50	99	95	97	98	94	96.6	2.1	98.7
		25	97	97	95	99	98	97.2	1.5	99.1
TXREF[4]	None	100	98	98	93	97	99	96.9	1.8	100.0
			96	98	97	95	98			
		50	99	95	96	99	98	97.9	1.5	100.0
			99	97	100	98	98			
		25	96	96	98	99	97	98.1	1.4	100.0
			99	100	99	99	98			

Table 7. Sea urchin fertilization test raw data and means for experiment one with pore-water samples collected from sediments post–landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjustment sample; Rep, replicate; SD, standard deviation; na, not available; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
MFS[5]	None	100	97	93	95	91	94	95.3	2.3	98.9
			97	99	95	95	97			
Brine blank[6]	None		97	96	93	96	93	95.0	1.9	98.0
Rinse blank[7]	None	100	93	96	94	92	96	94.2	1.8	97.2
SDS[8]	None	20	0	1	0	0	0	.2	.4	.2
		10	0	2	2	5	1	2.0	1.9	2.1
		5	58	52	53	64	61	57.6	5.1	59.4
		2.5	96	95	96	96	94	95.4	.9	98.5
		1.25	98	98	98	95	99	97.6	1.5	100.7

[1] Percent of salinity adjusted pore-water sample or concentration of SDS reference toxicant in milligrams per liter.

[2] Percent of TXREF control at the appropriate dilution.

[3] Data point not available because of error in pipetting.

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

[5] Millipore® filtered seawater diluent.

[6] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

[7] Rinsate blank consisting of filtered seawater run over a complete set of all equipment used in pore-water extraction.

[8] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 8. Sea urchin fertilization test raw data and means for experiment two with pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
FL–1	302144086581200	100	99	99	97	97	98	98.0	1.0	102.0
		50	100	99	100	100	98	99.4	.9	101.5
		25	96	97	98	95	100	97.2	1.9	99.4
FL–2	302258086263400	100	97	94	95	98	99	96.6	2.1	100.5
		50	100	100	99	98	99	99.2	.8	101.3
		25	100	98	99	99	95	98.2	1.9	100.4
FL–3	301926086091800	100	97	99	99	100	98	98.6	1.1	102.6
		50	98	98	99	99	96	98.0	1.2	100.1
		25	99	98	94	98	99	97.6	2.1	99.8
FL–4	300729085440900	100	99	98	98	99	98	98.4	.5	102.4
		50	99	98	100	97	96	98.0	1.6	100.1
		25	99	98	99	100	99	99.0	.7	101.2
FL–5	294645085243000	100	97	97	100	98	97	97.8	1.3	101.8
		50	97	97	96	97	97	96.8	.4	98.9
		25	98	98	100	98	94	97.6	2.2	99.8
FL–6	294152084460300	100	98	99	94	99	98	97.6	2.1	101.6
		50	99	100	97	100	99	99.0	1.2	101.1
		25	99	100	98	97	100	98.8	1.3	101.0
FL–7	300427084105000	100	85	85	93	90	89	88.4	3.4	92.0
		50	99	95	95	91	96	95.2	2.9	97.2
		25	97	96	98	95	99	97.0	1.6	99.2
FL–25	300223085260800	100	99	98	100	96	99	98.4	1.5	102.4
		50	99	98	100	97	100	98.8	1.3	100.9
		25	99	95	98	99	99	98.0	1.7	100.2
LA–6 (Bat06)	292708089521400	100	78	84	86	70	75	78.6	6.5	81.8
		50	93	92	95	99	98	95.4	3.0	97.4
		25	100	98	95	90	99	96.4	4.0	98.6
LA–46	294456093394801	100	98	100	100	100	99	99.4	.9	103.4
		50	na[3]	99	96	99	98	98.0	1.4	100.1
		25	98	98	100	97	100	98.6	1.3	100.8
MS–37	301309089044700	100	98	98	99	95	96	97.2	1.6	101.1
		50	97	98	96	96	98	97.0	1.0	99.1
		25	98	99	99	98	98	98.4	.5	100.6
MS–38	301227088582000	100	99	96	97	98	96	97.2	1.3	101.1
		50	95	97	97	100	97	97.2	1.8	99.3
		25	96	99	98	98	97	97.6	1.1	99.8
MS–39	301358088533300	100	95	97	98	98	99	97.4	1.5	101.4
		50	98	99	98	98	98	98.2	.4	100.3
		25	97	97	98	99	97	97.6	.9	99.8

Table 8. Sea urchin fertilization test raw data and means for experiment two with pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
MS–40	301425088440600	100	98	99	96	99	100	98.4	1.5	102.4
		50	98	98	100	99	100	99.0	1.0	101.1
		25	97	98	98	97	100	98.0	1.2	100.2
MS–41	301321088353300	100	100	99	98	99	96	98.4	1.5	102.4
		50	99	na 3	99	99	93	97.5	3.0	99.6
		25	95	97	99	94	96	96.2	1.9	98.4
MS–42	301208088253600	100	97	99	98	98	99	98.2	0.8	102.2
		50	97	97	98	100	97	97.8	1.3	99.9
		25	95	100	99	100	96	98.0	2.3	100.2
MS–43	301858089141000	100	100	96	95	100	93	96.8	3.1	100.7
		50	100	99	99	99	98	99.0	.7	101.1
		25	98	97	99	100	98	98.4	1.1	100.6
MS–44	302336088535800	100	99	99	98	96	96	97.6	1.5	101.6
		50	99	97	97	98	95	97.2	1.5	99.3
		25	97	100	96	95	96	96.8	1.9	99.0
MS–45	302034088325200	100	98	94	97	97	98	96.8	1.6	100.7
		50	100	98	98	95	98	97.8	1.8	99.9
		25	97	96	99	89	98	95.8	4.6	97.9
TX–47	294057093572301	100	97	99	97	98	99	98.0	1.0	102.0
		50	99	97	100	99	100	99.0	1.2	101.1
		25	99	100	98	99	98	98.8	.8	101.0
TX–49	293324094220601	100	99	100	99	98	99	99.0	.7	103.0
		50	95	97	99	98	98	97.4	1.5	99.5
		25	98	96	98	100	99	98.2	1.5	100.4
TX–51	291815094461001	100	98	98	99	100	97	98.4	1.1	102.4
		50	99	100	99	98	97	98.6	1.1	100.7
		25	100	98	100	97	99	98.8	1.3	101.6
TX–53	292318094430901	100	98	98	98	100	99	98.6	.9	102.6
		50	100	100	99	99	98	99.2	.8	101.3
		25	97	99	100	98	97	98.2	1.3	100.4

Table 8. Sea urchin fertilization test raw data and means for experiment two with pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; na, not available; (Rep 2), field replicate two; (Rep 1), field replicate one; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Fertilized					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
TX–55	291251094571401	100	98	99	99	98	96	98.0	1.2	102.0
		50	99	99	99	98	100	99.0	.7	101.1
		25	99	100	100	99	98	99.2	.8	101.4
TX–56	290512095063101	100	99	98	97	96	99	97.8	1.3	101.8
		50	99	100	98	100	98	99.0	1.0	101.1
		25	98	100	98	100	95	98.2	2.0	100.4
TXREF[4]	None	100	96	97	97	98	97	96.1	1.8	100.0
			96	96	93	93	98			
		50	99	99	96	96	98	97.9	1.5	100.0
			100	97	97	100	97			
		25	98	98	94	98	98	97.8	1.8	100.0
			96	100	100	98	98			
MFS 5	None	100	100	95	98	96	99	97.2	2.0	98.9
			95	97	95	97	100			
Brine blank[6]	None		97	97	100	98	98	98.0	1.2	102.0
Rinse blank[7]	None	100	98	97	96	97	98	97.2	.8	101.1
SDS[8]	None	20	0	0	0	0	0	.0	.0	.0
		10	1	4	4	2	1	2.4	1.5	2.5
		5	na	62	60	60	69	62.8	4.3	65.3
		2.5	96	99	95	97	99	97.2	1.8	101.1
		1.25	100	100	96	99	95	98.0	2.3	102.0

[1] Percent of salinity adjusted pore-water sample or concentration of SDS reference toxicant in milligrams per liter.

[2] Percent of TXREF control at the appropriate dilution.

[3] Data point not available because of error in pipetting.

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

[5] Millipore® filtered seawater diluent.

[6] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

[7] Rinsate blank consisting of filtered seawater run over a complete set of all equipment used in pore-water extraction.

[8] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 9. Sea urchin embryological development raw data and means for pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
AL–1	301338088193500	100	96	93	97	96	91	94.6	2.5	101.0
		50	96	96	96	94	95	95.4	.9	101.2
		25	94	100	96	96	96	96.4	2.2	100.6
AL–2	301455088110300	100	97	98	99	96	94	96.8	1.9	103.3
		50	95	92	95	94	97	94.6	1.8	100.3
		25	95	95	99	95	93	95.4	2.2	99.6
AL–3	301448088044000	100	97	93	93	92	93	93.6	1.9	99.9
		50	90	95	94	98	96	94.6	3.0	100.3
		25	96	94	94	96	94	94.8	1.1	99.0
AL–4	301329088003000	100	92	96	94	97	95	94.8	1.9	101.2
		50	94	93	94	95	96	94.4	1.1	100.1
		25	94	95	96	95	97	95.4	1.1	99.6
AL–5	301349087541600	100	94	97	95	93	97	95.2	1.8	101.6
		50	94	92	96	92	94	93.6	1.7	99.3
		25	92	97	97	90	98	94.8	3.6	99.0
AL–6	301428087434900	100	94	93	95	93	95	94.0	1.0	100.3
		50	96	96	94	97	95	95.6	1.1	101.4
		25	99	92	95	94	96	95.2	2.6	99.4
AL–7	301608087345400	100	92	95	95	95	92	93.8	1.6	100.1
		50	95	95	98	92	95	95.0	2.1	100.7
		25	93	97	94	91	91	93.2	2.5	97.3
AL–8	301353087561600	100	99	97	97	96	95	96.8	1.5	103.3
		50	95	97	97	93	97	95.8	1.8	101.6
		25	96	97	96	93	94	95.2	1.6	99.4
AL–9	301343087520200	100	94	97	94	96	95	95.2	1.3	101.6
		50	93	90	94	97	93	93.4	2.5	99.0
		25	91	95	97	93	96	94.4	2.4	98.5
AL–10	301341087495200	100	98	96	96	96	93	95.8	1.8	102.2
		50	97	95	93	97	95	95.4	1.7	101.2
		25	92	98	91	98	93	94.4	3.4	98.5
LA–6 (Bat06)	292708089521400	100	97	91	93	92	91	92.8	2.5	99.0
		50	94	94	95	95	95	94.6	.5	100.3
		25	95	98	92	96	97	95.6	2.3	99.8
LA–22	294432090083100	100	94	97	97	94	91	94.6	2.5	101.0
		50	92	95	94	96	97	94.8	1.9	100.5
		25	94	94	92	94	92	93.2	1.1	97.3
LA–23	294406091511300	100	90	92	95	99	95	94.2	3.4	100.5
		50	91	90	97	96	94	93.6	3.0	99.3
		25	98	95	97	96	96	96.4	1.1	100.6

Table 9. Sea urchin embryological development raw data and means for pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
LA–24	292046090254500	100	91	88	88	88	87	88.4	1.5	94.3
		50	94	92	95	97	97	95.0	2.1	100.7
		25	95	94	96	95	94	94.8	.8	99.0
LA–25	293808092460200	100	0	4	2	8	0	2.8	3.3	3.0
		50	93	91	94	92	93	92.6	1.1	98.2
		25	97	93	94	95	90	93.8	2.6	97.9
LA–26	291507090551800	100	0	0	0	0	0	0.0	.0	.0
		50	75	86	78	83	89	82.2	5.7	87.2
		25	96	95	93	93	95	94.4	1.3	98.5
LA–28	293424091321600	100	94	95	90	94	96	93.8	2.3	100.1
		50	94	95	95	93	91	93.6	1.7	99.3
		25	94	98	95	94	95	95.2	1.6	99.4
LA–29	294324089432500	100	93	91	91	92	89	91.2	1.5	97.3
		50	93	93	94	91	91	92.4	1.3	98.0
		25	98	95	93	91	95	94.3	3.0	98.4
LA–30	294108089234500	100	92	93	94	97	91	93.4	2.3	99.7
		50	94	93	93	93	94	93.4	.5	99.0
		25	91	94	95	95	97	94.4	2.2	98.5
LA–31	291537089570100	100	0	0	0	0	0	.0	.0	.0
		50	93	92	91	82	86	88.8	4.7	94.2
		25	98	93	96	96	95	95.6	1.8	99.8
LA–32	291914089105500	100	97	95	92	91	97	94.4	2.8	100.7
		50	96	97	95	92	90	94.0	2.9	99.7
		25	95	97	94	97	94	95.4	1.5	99.6
LA–33	293518089364300	100	54	6	8	31	1	20.0	22.2	21.3
		50	97	93	96	92	88	93.2	3.6	98.8
		25	97	97	98	92	98	96.4	2.5	100.6
LA–34	30090789144500	100	94	95	95	96	94	94.8	.8	101.2
		50	87	89	95	98	93	92.4	4.4	98.0
		25	97	95	94	92	93	94.2	1.9	98.3
LA–35	285951089085600	100	93	94	98	92	94	94.2	2.3	100.5
		50	94	95	95	95	93	94.4	.9	100.1
		25	98	96	94	96	97	96.2	1.5	100.4
LA–36	285615089235600	100	6	20	8	11	5	10.0	6.0	10.7
		50	96	94	91	96	92	93.8	2.3	99.5
		25	95	88	96	94	92	93.0	3.2	97.1
LA–46	294456093394801	100	94	97	97	97	98	96.6	1.5	103.1
		50	94	90	95	97	96	94.4	2.7	100.1
		25	98	95	97	89	94	94.6	3.5	98.7

Table 9. Sea urchin embryological development raw data and means for pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
FL–1	302144086581200	100	92	93	91	93	92	92.2	.8	98.4
		50	95	93	99	90	94	94.2	3.3	99.9
		25	98	95	89	92	93	93.4	3.4	97.5
FL–2	302258086263400	100	99	94	96	91	92	94.4	3.2	100.7
		50	94	92	93	94	89	92.4	2.1	98.0
		25	95	92	97	94	94	94.4	1.8	98.5
FL–3	301926086091800	100	92	97	91	95	90	93.0	2.9	99.3
		50	89	94	96	97	92	93.6	3.2	99.3
		25	93	94	91	90	97	93.0	2.7	97.1
FL–4	300729085440900	100	95	92	95	94	91	93.4	1.8	99.7
		50	93	94	96	96	94	94.6	1.3	100.3
		25	93	93	92	93	95	93.2	1.1	97.3
FL–5	294645085243000	100	94	97	92	94	98	95.0	2.4	101.4
		50	99	93	96	93	97	95.6	2.6	101.4
		25	92	97	91	94	98	94.4	3.0	98.5
FL–6	294152084460300	100	91	93	92	95	96	93.4	2.1	99.7
		50	95	93	97	97	95	95.4	1.7	101.2
		25	94	97	95	97	94	95.4	1.5	99.6
FL–7	300427084105000	100	94	94	88	95	92	92.6	2.8	98.8
		50	100	95	93	92	91	94.2	3.6	99.9
		25	96	95	92	98	90	94.2	3.2	98.3
FL–25	300223085260800	100	93	95	95	95	91	93.8	1.8	100.1
		50	91	91	93	94	92	92.2	1.3	97.8
		25	89	99	96	94	97	95.0	3.8	99.2
MS–37	301309089044700	100	97	92	91	95	95	94.0	2.4	100.3
		50	95	98	96	95	96	96.0	1.2	101.8
		25	97	95	99	95	94	96.0	2.0	100.2
MS–38	301227088582000	100	90	91	94	90	94	91.8	2.0	98.0
		50	93	89	96	87	91	91.2	3.5	96.7
		25	95	97	94	97	93	95.2	1.8	99.4
MS–39	301358088533300	100	0	4	1	5	7	3.4	2.9	3.6
		50	92	92	91	87	93	91.0	2.3	96.5
		25	94	96	90	94	96	94.0	2.4	98.1
MS–40	301425088440600	100	94	93	96	94	95	94.4	1.1	100.7
		50	92	92	98	94	96	94.4	2.6	100.1
		25	95	94	96	93	97	95.0	1.6	99.2
MS–41	301321088353300	100	92	95	94	95	92	93.6	1.5	99.9
		50	93	94	93	95	94	93.8	.8	99.5
		25	98	94	95	95	98	96.0	1.9	100.2

Table 9. Sea urchin embryological development raw data and means for pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
MS–42	301208088253600	100	94	98	91	95	96	94.8	2.6	101.2
		50	94	95	98	92	95	94.8	2.2	100.5
		25	96	98	96	92	94	95.2	2.3	99.4
MS–43	301858089141000	100	97	94	98	95	95	95.8	1.6	102.2
		50	96	94	95	92	94	94.2	1.5	99.9
		25	94	99	92	94	94	94.6	2.6	98.7
MS–44	302336088535800	100	92	91	91	91	95	92.0	1.7	98.2
		50	95	94	98	94	96	95.4	1.7	101.2
		25	95	92	94	96	96	94.6	1.7	98.7
MS–45	302034088325200	100	92	95	94	91	90	92.4	2.1	98.6
		50	95	94	98	94	93	94.8	1.9	100.5
		25	94	97	93	98	96	95.3	2.2	99.4
TX–47	294057093572301	100	93	93	94	95	97	94.4	1.7	100.7
		50	93	93	93	93	96	93.6	1.3	99.3
		25	94	94	96	93	89	93.2	2.6	97.3
TX–49	293324094220601	100	94	96	90	87	93	92.0	3.5	98.2
		50	96	93	95	96	96	95.2	1.3	101.0
		25	92	96	94	94	91	93.4	1.9	97.5
TX–51	291815094461001	100	91	92	96	97	96	94.4	2.7	100.7
		50	96	93	96	95	94	94.8	1.3	100.5
		25	93	98	95	94	96	95.2	1.9	99.4
TX–53	292318094430901	100	95	96	97	90	95	94.6	2.7	101.0
		50	96	95	93	97	99	96.0	2.2	101.8
		25	96	98	95	99	94	96.4	2.1	100.6
TX–55	291251094571401	100	92	94	99	93	94	94.4	2.7	100.7
		50	94	93	94	97	98	95.2	2.2	101.0
		25	95	93	94	96	92	94.0	1.6	98.1
TX–56	290512095063101	100	99	94	96	93	93	95.0	2.5	101.4
		50	99	90	96	96	94	95.0	3.3	100.7
		25	97	92	95	93	95	94.4	1.9	98.5
TXREF[3]	None	100	96	94	94	91	94	93.7	1.3	100.0
			93	93	93	95	94			
		50	93	95	92	95	95	94.3	1.4	100.0
			95	97	93	94	94			
		25	94	97	96	98	93	95.8	1.9	100.0
			94	98	94	97	97			
MFS[4]	None	100	96	91	95	91	99	93.2	2.9	99.5
			90	94	93	93	90			
Brine blank[5]	None		93	93	94	93	93	93.2	.4	99.5

Table 9. Sea urchin embryological development raw data and means for pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release.—Continued

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; WQAS, water quality adjusted sample; Rep, replicate; SD, standard deviation; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate]

Site	Site ID	% WQAS[1]	% Normal Pluteus					Mean	SD	% of control[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5			
Rinse blank[6]	None	100	94	96	98	91	94	94.6	2.6	101.0
SDS[7]	None	20	0	0	0	0	0	0.0	0.0	0.0
		10	0	0	0	0	0	.0	.0	.0
		5	5	3	5	4	1	3.6	1.7	3.8
		2.5	91	88	87	79	86	86.2	4.4	92.0
		1.25	96	93	94	95	97	95.0	1.6	101.4

[1] Percent of water quality adjusted pore water sampled or concentration of SDS reference toxicant in milligrams per liter.

[2] Percent of TXREF control at the appropriate dilution.

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

[4] Millipore® filtered seawater diluent.

[5] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

[6] Rinsate blank consisting of filtered seawater run over a complete set of all equipment used in pore-water extraction.

[7] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 10. Water-quality measurements after salinity adjustment and original salinity of pore-water samples collected from sediments post-landfall of the Deepwater Horizon oil release and tested in the Toxicity Identification Evaluation (TIE).

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; ‰, parts per thousand; %, percent; DO, dissolved oxygen; mg/L, milligrams per liter; TAN, total ammonia as nitrogen; UAN, un-ionized ammonia as nitrogen; µg/L, micrograms per liter; OS, original sample; <, less than; MFS, Millipore® filtered seawater; ±, plus or minus]

Site	Site ID	Salinity[1] ‰	DO (mg/L)	% DO	pH	TAN (mg/L)	UAN (µg/L)	Sulfide (mg/L)	% OS[2]
LA–29	294324089432500	6	7.67	104.6	8.13	0.906	38.1	0.040	75.0
LA–31	291537089570100	27	7.34	98.5	8.05	2.72	94.7	<.009	96.2
LA–33	293518089364300	9	7.81	105.7	8.33	1.41	92.0	.009	77.7
LA–36	285615089235600	18	7.59	103	7.55	.903	10.3	<.009	85.7
MS–39	301358088533300	29	6.69	90.4	8.05	.742	26.2	<.009	100.0
TXREF[3]	None	25	7.31	98.7	8.30	.587	35.4	<.009	93.6
MFS[4]	None	32	6.87	92.8	8.17	<.1	< 4.6	<.009	93.8
Brine blank[5]	None	25	6.13	84.2	8.25	.183	10.0	<.009	18.1

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

[2] Percent of original sample after salinity adjustment.

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

[4] Millipore® filtered seawater diluent.

[5] Brine blank of TXREF diluted to 6 ‰ with Milli-Q® purified water and subsequently increased with brine to 30 ‰ (concentrated brine at 102 ‰).

Table 11. Toxicity Identification evaluation (TIE) fertilization raw data ans means for pore-water samples identified as toxic in the Deep Water Horizon post-landfall sea urchin fertilization test.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; Rep, replicate; SD, stansard deviation; Sig, significance; na, not applicable; EDTA, Disodium Ethylenediamine Tetraacetate; **, significant reduction in toxicity; ns, not significant; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; mg/mL, milligrams per milliliter]

Site	Site ID	Treatment[1]	% Fertilized					Mean	SD	Sig[2]	% of control[3]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
LA–29	294324089432500	Untreated	15	11	5	20	14	13.0	5.5	na	100.0
		EDTA	24	20	20	26	21	22.2	2.7	**	170.8
		Aeration	18	10	9	13	11	12.2	3.6	ns	93.8
		C_{18}	29	7	6	12	12	13.2	9.3	ns	101.5
		Ulva lactuca	97	84	87	81	84	87.3	6.9	**	671.2
TXREF[4]	None	Untreated	82	79	84	82	84	82.8	2.3	na	100.0
		EDTA	85	86	81	85	80	82.4	3.1	ns	99.5
		Aeration	84	76	83	80	84	82.9	3.4	ns	100.1
		C_{18}	86	80	86	83	87	83.7	4.0	ns	101.1
		Ulva lactuca	85	76	90	83	81	84.0	4.5	ns	101.4
MFS[5]	None	Untreated	89	81	83	90	82	85.0	4.2	na	102.7
Brine blank[6]	None	Untreated	89	89	89	91	81	87.8	3.9	na	106.0
SDS[7]	None	10	0	0	0	0	0	.0	.0	ns	.0
		5	27	38	37	27	39	33.6	6.1	ns	40.6
		2.5	81	83	81	72	85	80.4	5.0	ns	97.1
		1.25	91	89	87	87	87	88.2	1.8	ns	106.5

[1] Manipulations performed on sample to reduce toxicity including: no treatment (untreated); addition of EDTA•$2H_20$ (sodium salt) at 60 mg/L for 3 hours; vigorous aeration for 1 hour; C_{18} solid phase extraction (SPE); ans 5 hour exposure of sample to the algae *Ulva lactuca* at 83.3 mg/mL or concentration in milligrams per liter in the case of the SDS positive control.

[2] Significance in relation to the untreated sample; Dunnetts *t*-test $\alpha < 0.01$.

[3] Percent of the control (in this case) the untreated sample or in the case of MFS, brine blank, ans SDS the untreated TXREF control.

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

[5] Millipore® filtered seawater diluent.

[6] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ ans subsequently increased with brine to 30 ‰ with concentrated brine.

[7] Sodium dodecyl sulfate positive control (in milligrams per liter).

Table 12. Toxicity Identification evaluation (TIE) embryological development raw data and means for pore-water samples identified as toxic in the Deep Water Horizon post-landfall sea urchin embryological development test.

[Site, U.S. Geological Survey (USGS) identifier for site locality; Site ID, USGS identifier number; %, percent; Rep, replicate; SD, standard deviation; Sig, significance; na, not applicable; EDTA, Disodium Ethylenediamine Tetraacetate; ns, not significant; **, no significant reduction in toxicity; ++, significant increase in toxicity; MFS, Millipore® filtered seawater; SDS, sodium dodecyl sulfate; mg/L, milligrams per liter; mg/mL, milligrams per milliliter; µg/L, micrograms per liter]

Site	Site ID	Treatment[1]	% Normal Pluteus					Mean	SD	Sig[2]	% of control[3]
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5				
LA–31	291537089570100	Untreated	0	1	0	2	0	0.6	0.9	na	100.0
		EDTA	4	12	0	8	2	5.2	4.8	ns	866.7
		Aeration	0	0	0	0	0	.0	.0	ns	.0
		C_{18}	0	0	0	0	2	.4	.9	ns	66.7
		Ulva lactuca	87	91	91	91	88	89.3	2.1	**	14,875.0
LA–33	293518089364300	Untreated	0	2	8	0	8	3.6	4.1	na	100.0
		EDTA	29	19	39	45	29	32.2	10.1	**	894.4
		Aeration	0	0	0	0	0	.0	.0	ns	.0
		C_{18}	8	14	50	10	20	20.4	17.2	ns	566.7
		Ulva lactuca	88	94	93	93	86	90.0	3.6	**	2,500.0
LA–36	285615089235600	Untreated	78	78	75	80	77	77.6	1.8	na	100.0
		EDTA	93	87	95	86	94	91.0	4.2	**	117.3
		Aeration	64	58	78	61	70	66.2	7.9	++	85.3
		C_{18}	81	76	85	81	81	80.8	3.2	ns	104.1
		Ulva lactuca	91	92	90	93	90	91.0	1.4	**	117.3
MS–39	301358088533300	Untreated	15	29	28	23	31	25.2	6.4	na	100.0
		EDTA	84	79	58	76	86	76.6	11.1	**	304.0
		Aeration	8	19	2	31	17	15.4	11.1	ns	61.1
		C_{18}	93	90	91	89	94	91.4	2.1	**	362.7
		Ulva lactuca	95	94	96	99	98	97.0	1.8	**	384.9
MFS[4]	None	Untreated	95	96	95	92	95	94.6	1.5	na	99.2
Brine blank[5]	None	Untreated	93	95	100	97	100	97.0	3.1	na	101.7
TXREF[6]	None	Untreated	97	95	95	96	94	95.4	1.4	na	100.0
		EDTA	98	96	93	95	95	95.8	1.6	ns	100.4
		Aeration	96	97	94	96	98	95.7	1.6	ns	100.3
		C_{18}	96	96	94	97	93	94.1	2.7	ns	98.6
		Ulva lactuca	96	96	92	93	88	93.0	3.3	ns	97.5
SDS[7]	None	10	0	0	0	0	0	.0	.0	ns	.0
		5	0	0	0	0	0	.0	.0	ns	.0
		2.5	82	75	85	77	82	80.2	4.1	ns	84.1
		1.25	94	90	96	94	93	93.4	2.2	ns	97.9
NH_3[8]	None	129.8	0	0	0	0	0	.0	.0	ns	.0
		68.1	5	1	2	4	1	2.6	1.8	ns	2.7
		34	93	95	95	96	93	94.4	1.3	ns	99.0
		19.2	95	98	94	93	97	95.4	2.1	ns	100.0

[1] Manipulations performed on sample to reduce toxicity including: no treatment (untreated); addition of EDTA•$2H_20$ (sodium salt) at 60 mg/L for 3 hours; vigorous aeration for one hour; C_{18} solid phase extraction (SPE); and 5 hour exposure of sample to the algae *Ulva lactuca* at 83.3 mg/mL or concentration in mg/L in the case of the SDS positive control or µg/L in the case of NH_3 series.

[2] Significance in relation to the untreated sample; Dunnetts *t*-test $\alpha < 0.01$.

[3] Percent of the control in this case the untreated sample or in the case of MFS, brine blank, and SDS the untreated TXREF control.

[4] Millipore® filtered seawater diluent.

[5] Brine blank consisting of TXREF reference pore water diluted to 6 ‰ and subsequently increased with brine to 30 ‰ with concentrated brine.

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

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

[8] Un-ionized ammonia calculated from measured total ammonia, pH, and temperature (in µg/L).

Appendixes

Date Prepared: May 5, 1990

Date Revised: July 18, 2007

EXTRACTION AND STORAGE OF PORE-WATER SAMPLES

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 et al., 1989; Carr and Chapman, 1992) pore water was extracted from sediments using a device constructed of Teflon®. Since then, the design has been improved (Carr and Chapman, 1994) The polyvinyl chloride (PVC) extractors in current use are less costly to construct and easier to operate. This device has been used in numerous sediment quality assessment surveys (Carr, et al., 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 cm height and 13 cm diameter) with threaded ends and threaded open compression nuts (Figure 1–1). The coupling is fitted with end plates cut from 7/16" thick PVC sheeting that are held in place by the threaded end nuts. The gaskets provided with the coupling are discarded and silicon O-rings are used to seal the top and bottom connections. The top end plate is fitted with a quick-release fitting where the pressurized air is supplied, and a safety pressure relief valve. Like the original Teflon® extractor, the bottom end plate (Figure 1–1) has several interconnected concentric grooves to facilitate flow of the pore water to the central exit port. A 5 μm polyester filter is situated between the bottom end plate and the silicon O-ring. Before a sediment sample is loaded, the bottom end nut is tightened in place by using the stationary bottom wrench (Figure 1–1) and a standard strap wrench.

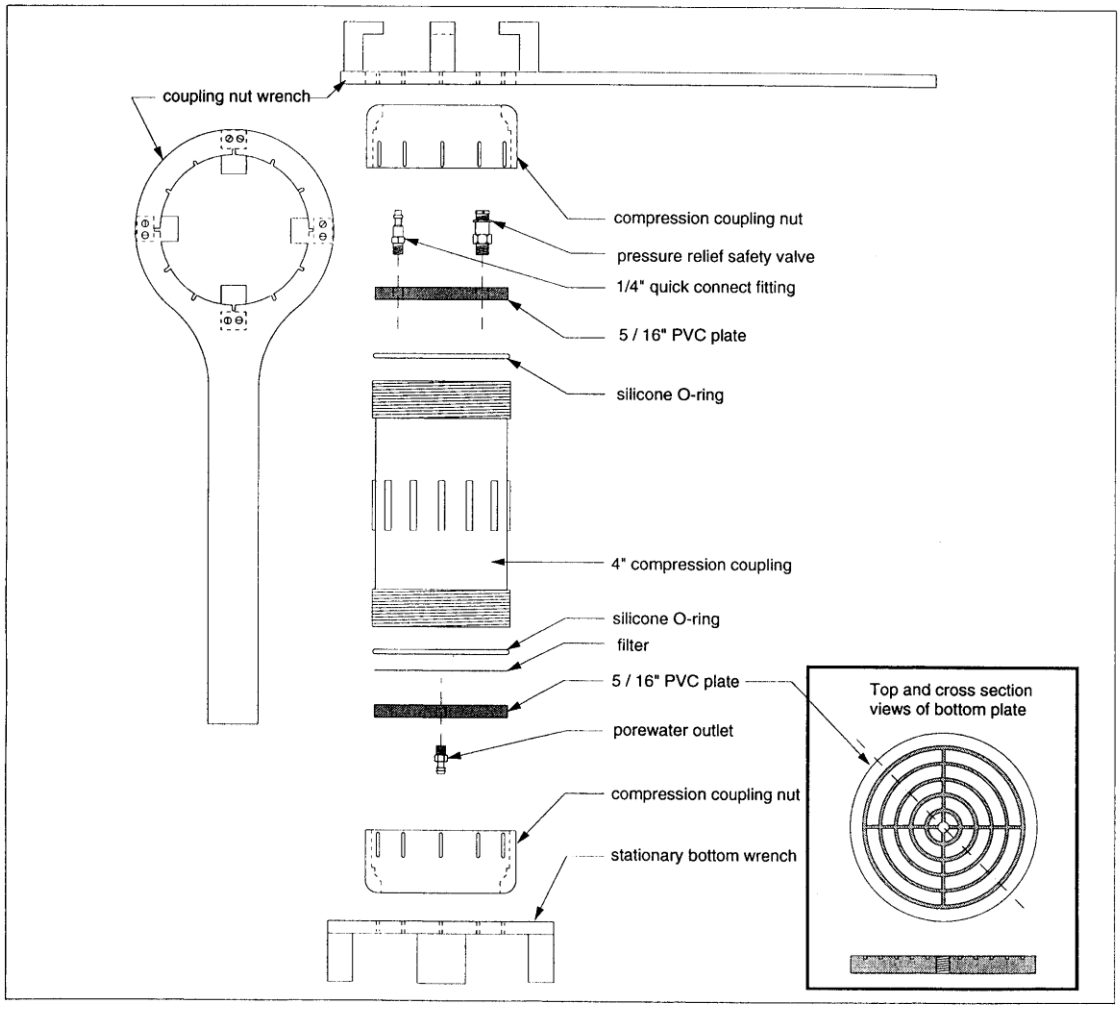

Figure 1–1. Schematic of sediment pore water squeeze extraction device.

The extractors are pressurized with air supplied from a standard SCUBA cylinder via a SCUBA first stage regulator which delivers air to a manifold with a valving system

(Figure 1–2). With this system, multiple cylinders can be pressurized simultaneously, using the same SCUBA cylinder.

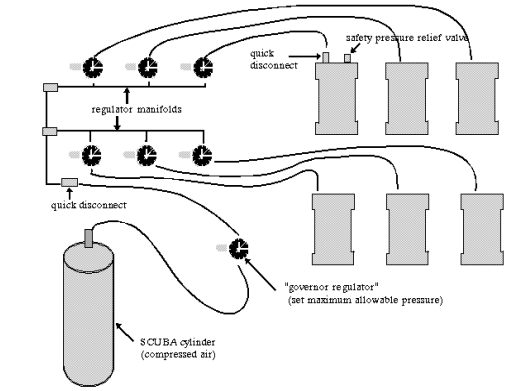

Figure 1–2. Schematic of sediment pore-water pressure extraction system.

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 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 I-Chem® amber 250 mL or 125 mL glass jar cleaned to EPA standards, with Teflon® lid liner) underneath the outlet.

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

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

4. After sediment is loaded, the top end plate within the top compression coupling nut is installed . To tighten the top nut, the strap wrench and the coupling nut wrench (Figure 1–1) 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 (Figure 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 to1,200 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 (I-Chem® amber glass jars) of pore water collected, d) centrifugation information, if performed, e) date frozen and location (freezer no.), and e) date and jar no. thawed and used in which test. The Sample History Forms are kept in a three-ring binder at the same location where the samples are stored.

5.0 TRAINING

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

6.0 SAFETY

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

8.0 REFERENCES

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Attachment A

REQUIRED EQUIPMENT AND MATERIALS

To construct a sediment pore water extraction device:

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

To use a pore water extraction device:

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

Other required supplies/equipment:

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

Attachment B

SAMPLE HISTORY DATA FORM

Sample Designation: _____ Study Protocol: _____ Initials: _____

Date of acquisition: _____ Sample type: _____

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

_____

_____

_____

Initials	Date	Action Taken

Date Prepared : April 10, 1990

Date Revised: July 18, 2007

SEA URCHIN FERTILIZATION TOXICITY TEST

1.0 OBJECTIVE

The purpose of the fertilization toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample reduces fertilization of exposed gametes relative to that of gametes exposed to a reference sample. The test may also be used to determine the concentration of a test substance which reduces fertilization. Test results are reported as treatment (or concentration) which produces statistically significant reduced fertilization or as concentration of test substance which reduces fertilization by 50 percent (EC_{50}). This test can be performed concurrently with Sea Urchin Embryological Development Toxicity Test (CERC SOP P.648) using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

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

2.2 Dilution Water

Milli-Q® 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 no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 µm) seawater adjusted to 30 ‰ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

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

2.3.1 Equipment

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

2.3.2 Solutions

10 percent Buffered Formalin:

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

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

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

2.4.1 Selection of Urchins to be Used in Toxicity Test.

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

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V, 0.15 ampere transformer.

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

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

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

6. Stimulate release of sperm from gonopores by touching test with electrodes from the12V, 0.15 ampere transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

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

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V, 0.15 ampere transformer.

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

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

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 ‰ 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 > 220 use the following formula to calculate the amount of water to add:

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

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

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

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V transformer, and collect about 0.5 mL of unwetted sperm from between spines using a Pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Wrap the microcentrifuge tube in a small piece of paper towel before storing on ice to prevent the sperm from freezing when coming in contact with the 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 60-90 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 90 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 effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:10,000 and 1:2500 will result in desirable fertilization rates, if the animals are in good condition.

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

3.0 TEST PROCEDURES

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

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

3. While gently swirling the egg solution to maintain even mixing of eggs, use a 200 µL pipetter to add 200 µL diluted egg suspension to each vial. Pipette tips are cut back using a clean razor blade to prevent crushing the eggs during pipetting. Record time of egg addition.

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

5. Using the dispenser, add 0.75 mL of 10 percent buffered formalin to each sample.

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

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

4.0 DATA COLLECTION AND TABULATION

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

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

3. Calculate fertilization percentage for each replicate test:

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

5.0 DATA ANALYSIS

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

6.0 QUALITY CONTROL

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

7.0 TRAINING

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

8.0 SAFETY

The sea urchin fertilization toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10 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 are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

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

10.0 REFERENCES

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Attachment 1

EQUIPMENT LIST FOR FERTILIZATION TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL Wheaton scintillation vials (These should be type shipped with caps with polyseal cone liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 µL pipetter
50-200 µL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 µm) seawater, adjusted to 30 ‰
Data sheets
Milli-Q® reagent grade water
Approximately 100 ‰ concentrated brine

Attachment 2

PRETEST TO INSURE SELECTION OF QUALITY GAMETES

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

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

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

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90 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: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

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

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

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

Attachment 3

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

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

B. Character of Sample (after salinity adjustment):

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

COMMENTS_____

Attachment 4

SEA URCHIN PRETEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

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

SPERM

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

TEST TIMES

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

SPERM DILUTION _____

COMMENTS _____

_____

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 5

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID________________________ INITIALS____________
STUDY PROTOCOL____________________ 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

PERCENT FERTILIZATION Reference sample designation:____________

Female #____________ Male #____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4

Attachment 6

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST GAMETE DATA SHEET

TEST ID_____ INITIALS_____
STUDY PROTOCOL_____ DATE_____

EGGS

Collection time:_____

Initial count/volume:_____

Final count:_____

SPERM

Collection time:_____ Dilution start time:_____

Sperm dilution:_____

_____

Test start temperature:_____

TEST TIMES

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

COMMENTS_____

_____

_____

Attachment 7

SEA URCHIN FERTILIZATION TOXICITY TEST
FERTILIZATION DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

PERCENT FERTILIZED

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

COMMENTS _____

Date Prepared : April 10, 1990

Date Revised: July 18, 2007

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

1.0 OBJECTIVE

The purpose of the embryological development toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample affects development of exposed embryos (development arrested at an early stage or a developmental abnormality) relative to that of embryos exposed to a reference sample. The test may also be used to determine the concentration of a test substance which affects development. Test results are reported as treatment (or concentration) which produces statistically significant developmental effect. This test can be performed concurrently with Sea Urchin Fertilization Toxicity Test (CERC SOP P.647) using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

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

2.2 Dilution Water

Milli-Q® 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 no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 µm) seawater adjusted to 30 ‰ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

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

2.3.1 Equipment

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

2.3.2 Solutions

10 percent Buffered Formalin:

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

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

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

2.4.1 Selection of Urchins to be Used in Toxicity Test.

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

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V, 0.15 ampere transformer.

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

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

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

6. Stimulate release of sperm from gonopores by touching test with electrodes from the12V, 0.15 ampere transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

Generally, a gamete check is performed in order to ensure that both the male and the female urchins used in the test have gametes with a high degree of viability. If the gamete check is performed, two to five females (depending on confidence in the proportion of urchins in the holding facility in good reproductive status) and at least two males should be selected using the above procedures. The check is performed by adding 5 to 7 drops of a concentrated dilution of sperm to the eggs in the scintillation vials (collected as described above) and observing the eggs under the microscope after 10 minutes. The concentrated dilution of sperm is usually made by diluting 20-50 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 2).

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V, 0.15 ampere transformer.

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

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

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 ‰ 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 > 220 use the following formula to calculate the amount of water to add:

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

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

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

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V, 0.15 ampere transformer, and collect about 0.5 mL of unwetted sperm from between spines using a Pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Wrap the microcentrifuge tube in a small piece of paper towel before storing on ice to prevent the sperm from freezing when coming in contact with the 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

As in the Sea Urchin Fertilization Test, it is desirable for control fertilization to be 70-90 percent. Although controls outside these bounds do not automatically disqualify a test, particularly if a valuable dose response is generated, the chance of inducing polyspermy is increased with increased concentrations of sperm, and good dose responses may be difficult to obtain with less than 70 percent normal pluteus in controls. Density of sperm in the sperm solution should be determined with this goal in mind. Condition of the animals and length of acclimation to the aquarium may effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:1250 and 1:7500 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:5000 is required (as determined from the pretest), add 20 µL sperm to 10 mL MFS. Mix thoroughly, then add 1 mL of this solution to 9 mL MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 30 minutes before the test has begun, including sperm dilutions used in any pretest, should be discarded and a new dilution made from sperm kept on ice. The quantity of sperm to be added to the egg dilution is calculated by dividing the total volume of eggs by five and adding 50 µL of sperm dilution per that number. Sperm should be allowed to incubate with the eggs for 10 minutes to allow fertilization to take place. After 10 minutes, eggs should be evaluated under 100 X magnification for fertilization membranes. If

70-90 percent of the eggs are fertilized, the embryos can be pipetted into the test vials. If the percentage is lower than 70 percent, additional sperm may be added and/or more time allowed for fertilization. If the fertilization does not increase above 70 percent after 30 minutes, the embryos should be discarded and new gametes selected for use. Embryos should not be allowed to undergo division before pipetting them into the test vials.

3.0 TEST PROCEDURES

1. Just before the embryos are to be used, add 2 mL of a penicillin-G stock solution (5000 units/mL) per 100 mL of eggs in the fertilized embryo suspension. The addition of penicillin to the embryological development test has been shown to be beneficial in evaluation of the stages of development by inhibiting bacterial growth which can cause the embryos to disintegrate before the test is terminated. The penicillin stock solution is prepare by diluting 296 mg of Penicillin-G sodium salt (1690 units/mg) in 100 mL of MFS and mixing until dissolved. The addition of 2 mL/100 mL of eggs will result in a final concentration of 4 units/mL in each replicate. The number of units of penicillin per mg of penicillin-G sodium salt is variable with each lot. Thus, the quantity added to the stock will change in order to keep the final concentration at 4 units/mL.

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

3. Incubate all test vials at 20 ± 1°C for 48 hours.

4. Using the dispenser, add 0.75 mL 10 percent buffered formalin to each vial.

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

4.0 DATA COLLECTION AND TABULATION

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

2. Count 100 embryos/sample using hand counter with multiple keys (such as a blood cell counter), using one key to indicate normally developed pluteus larvae and others to indicate unfertilized eggs, embryos arrested in earlier developmental stages, and other abnormalities or for more efficient data collection, stages other than pluteus and abnormalities may be lumped together and counted on one key. Attachment 3 has a list of developmental stages and drawings of each.
3. Calculate the proportion of normal plutei for each replicate test:

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

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 4-9). Normally, percent normal development (normal plutei) in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea surface microlayer from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's *t*-test (Sokal and Rohlf, 1981) on the arc sine square root transformed data. For multiple comparisons among treatments, Ryan's Q test (Day and Quinn, 1989) with the arc sine square root transformed data is recommended. The trimmed Spearman-Karber method with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al., 1977)

6.0 QUALITY CONTROL

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

7.0 TRAINING

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

8.0 SAFETY

The sea urchin embryological development toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10 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 are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

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

10.0 REFERENCES

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

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Attachment 1

EQUIPMENT LIST FOR EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL Wheaton scintillation vials (These should be type shipped with caps with polycone liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 µL pipetter
50-200 µL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 µm) seawater, adjusted to 30 ‰
Data sheets
Milli-Q® reagent grade water
Approximately 100 ‰ concentrated brine

Attachment 2

PRETEST TO INSURE SELECTION OF QUALITY GAMETES

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

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

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

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90 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: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

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

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

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

Attachment 3

DEVELOPMENT OF SEA URCHIN EGGS TO PLUTEUS LARVAE

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

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

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

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

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

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

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

Attachment 3 Continued

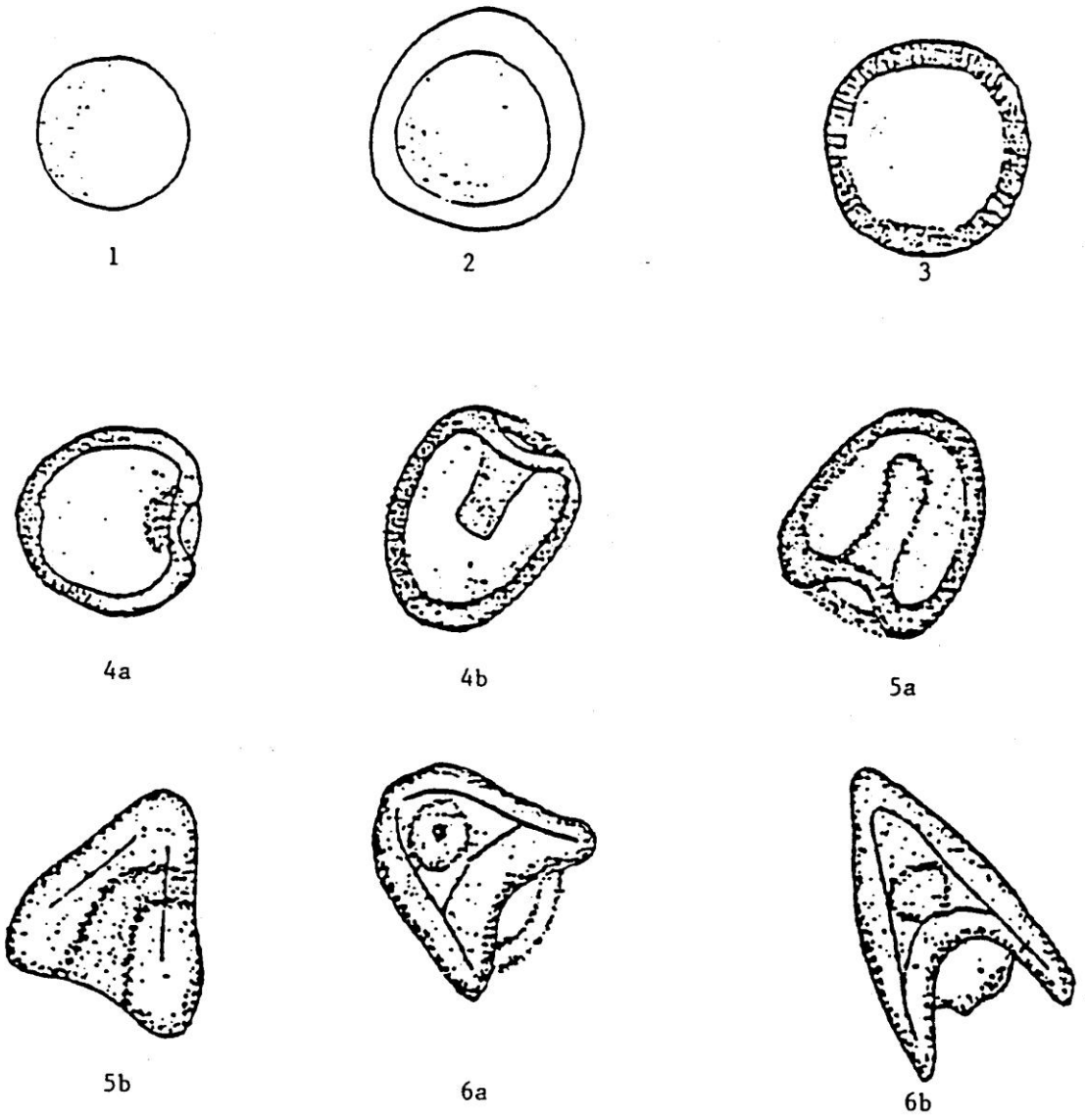

Figure 3–1. Stages in development of sea urchin, from unfertilized egg to pluteus larvae. Numbers relate to descriptions on previous page.

Attachment 4

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

Initial volume (mL) _____

Initial salinity (‰) _____

Vol. Milli-Q® water added (mL) _____

Vol. ___‰ brine added (mL) _____

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

B. Character of Sample (after salinity adjustment):

Volume (mL) _____

Salinity (‰) _____

pH _____

Dissolved oxygen (mg/L) _____

DO saturation (percent) _____

Total ammonia (mg/L) _____

Sulfide (mg/L) _____

COMMENTS_____

Attachment 5

SEA URCHIN PRETEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

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

SPERM

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

TEST TIMES

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

SPERM DILUTION _____

COMMENTS _____

_____

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 6

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID______________________________ INITIALS______________
STUDY PROTOCOL________________________ 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
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

PERCENT FERTILIZATION Reference sample designation:______________

Female #______________ Male #______________

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

Attachment 7

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST GAMETE DATA SHEET

TEST ID_____ INITIALS_____
STUDY PROTOCOL_____ DATE_____

EGGS

Collection time:_____

Initial count/volume:_____

Final count:_____

SPERM

Collection time:_____ Dilution start time:_____

Sperm dilution:_____

_____

Test start temperature:_____

TEST TIMES

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

COMMENTS_____

_____

_____

_____

Attachment 8

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

PERCENT NORMAL PLUTEUS

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

COMMENTS _____

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