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TOXICITY OF MARINE SEDIMENTS AND PORE WATERS SPIKED WITH ORDNANCE COMPOUNDS

An Investigation Conducted by

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This study was undertaken to help quantify the potential effects of sediment contamination with ordnance compounds to the benthic biota. This objective was achieved by conducting toxicity tests with sediments spiked with ordnance compounds, using whole sediment tests with a benthic amphipod and porewater tests with different life stages of three marine organisms. The selection of ordnance compounds and porewater test species for this study was based on the results of previous studies, where a database for toxicity of eight different ordnance compounds to six marine species was generated. The ordnance compounds selected for the present study were 2,6-dinitrotoluene (2,6-DNT), tetryl and picric acid. Two kinds of sediments were spiked with each ordnance compound. Spiked sediments were allowed to equilibrate for approximately 1 week prior to porewater extraction and to toxicity testing of the solid-phase. Solid-phase tests were conducted using the 10-day acute test with the benthic amphipod, *Ampelisca abdita*. Porewater toxicity tests were conducted using the 48-hour embryological development test with the sea urchin, *Arbacia punctulata*, the 96-hour zoospore germination and germling growth test with the macro-alga, *Ulva fasciata*, and the 7-day survival and reproduction test with the burrowing polychaete, *Dinophilus gyrociliatus*. These species and endpoints were identified previously as being among the most sensitive for these ordnance compounds.

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EXECUTIVE SUMMARY

This study was undertaken to help quantify the potential effects of sediment contamination with ordnance compounds to the benthic biota. This objective was achieved by conducting toxicity tests with sediments spiked with ordnance compounds, using whole sediment tests with a benthic amphipod and porewater tests with different life stages of three marine organisms. The selection of ordnance compounds and porewater test species for this study was based on the results of previous studies, where a database for toxicity of eight different ordnance compounds to 6 marine species was generated (USGS, 1999). The ordnance compounds selected for the present study were 2,6-dinitrotoluene (2,6-DNT), tetryl and picric acid. Two kinds of sediments were spiked with each ordnance compound: one from Carr Inlet, Puget Sound, WA, predominately silt/clay and containing 1.1% total organic carbon (TOC), and one from Redfish Bay, Texas, predominately sand, with 0.1% TOC. Spiked sediments were allowed to equilibrate for approximately a week prior to porewater extraction and to toxicity testing of the solid-phase. Solid-phase tests were conducted using the 10-day acute test with the benthic amphipod, *Ampelisca abdita*. Porewater toxicity tests were conducted using the 48-hour embryological development test with the sea urchin, *Arbacia punctulata*, the 96-hour zoospore germination and germling growth test with the macro-alga, *Ulva fasciata*, and the 7-day survival and reproduction test with the burrowing polychaete, *Dinophilus gyrociliatus*. These species and endpoints were identified previously as being among the most sensitive for these ordnance compounds (USGS, 1999).

An initial experiment analyzing the spiking procedure and minimum equilibration period prior to initiating toxicity tests led to the decision of allowing a one-week equilibration, after which amphipod tests with the whole sediment were started, and pore water for further tests was extracted. Very low concentrations of 2,6-DNT and tetryl were recovered form the muddy sediment from Puget Sound even immediately after spiking, suggesting that either biodegradation in this organically rich sediment or irreversible binding had occurred.

Neither of the two sediments was toxic when spiked with the highest possible concentration of 2,6-DNT, and the Puget Sound sediment spiked with tetryl was also not acutely toxic to amphipods, although the tetryl-spiked sandy sediment from Texas was significantly toxic in the highest concentration, with an LOEC of 3.6 mg/ kg sediment dry weight and an EC_{50} of 3.2 mg/kg dry weight. Picric acid was toxic in the sandy sediment, with NOEC and LOEC values of 73 and 162 mg/kg, respectively, and an LC_{50} of 144 mg/kg dry weight. Toxicity in the picric acid test in Puget Sound sediment peaked in intermediate concentrations, being reduced in the lowest and highest test concentrations. An anomalous behavior, which resulted in the amphipods not leaving their tubes to molt, is suggested as the mechanism that caused this unusual pattern of toxicity.

In the porewater toxicity tests with different marine species and life-stages, the sensitivity varied not only with the tested ordnance compound but also with the sedimentary origin of the spiked pore water. The sensitivity of the different test methods and endpoints did not differ more than one order of magnitude, with the sea urchin embryological development test tending to be the least sensitive overall, except for tetryl in the Puget Sound pore water. Tetryl was also the most toxic chemical in all tests with pore water extracted from both kinds of sediments, and picric acid was the least toxic.

HPLC analyses of the 2,6-DNT spiked porewater samples at test start showed a relatively large peak of a degradation product, which was identified by GC-MS as 2-methyl-3-nitroaniline. Peaks of possible degradation products were also observed in the HPLC chromatograms from some of the tetryl and picric acid spiked samples, but the respective chemicals were not identified. It is suggested that degradation products may have played a significant role in the toxicity of the analyzed samples.

In a previous survey, porewater toxicity tests were conducted with samples from areas suspected of ordnance compound contamination in Puget Sound (USGS, 1999). Toxicity identification evaluation (TIE) procedures indicated that ordnance compounds were not responsible for measured toxicity, but phase II of the TIE study, which aimed to the identification of the compound(s) responsible for toxicity, had not been completed when the report for the initial survey was written. The results of the phase II TIE did not identify a specific contaminant that was responsible for the observed toxic effects (Appendix A). The data demonstrate that none of the chemicals included in the standard comprehensive analysis (PAHs, metals, butyltins, organochlorines, H_2S, or ammonia) were present at a sufficient concentration to account for the toxicity observed.

INTRODUCTION

Contamination of sediments with explosives and related compounds is seen as a potential problem in the vicinity of Naval facilities and harbors throughout the USA. There is a paucity of data in the scientific literature regarding the toxicity of ordnance compounds adsorbed to marine sediments and no Sediment Quality Standards (SQS) currently exist for these substances or their degradation products. The only toxicity study available for an individual ordnance compound in spiked marine sediment reports the toxicity of 2,4,6-trinitrotoluene (2,4,6-TNT) to an amphipod and a polychaete (Green *et al.*, 1999).

In a previous survey, a marine toxicity database was developed for ordnance compounds in aqueous solutions and toxicity tests were conducted with pore waters from areas suspected of ordnance compound contamination in Puget Sound (USGS, 1999). The porewater toxicity tests and subsequent toxicity identification evaluation (TIE) procedures indicated that ordnance compounds were not responsible for the measured toxicity, but phase II of the TIE study, which aimed to the identification of the compound(s) responsible for toxicity, was still underway when the report for the initial survey was written. The data obtained for the phase II TIE are presented as an appendix to the current report (Appendix A).

Based on the toxicity database developed for ordnance compounds in seawater, three compounds, 2,6-dinitrotoluene (2,6-DNT), tetryl and picric acid, were selected for further analyses of toxicity in spiked marine sediments and pore water, with views to identify the concentrations of these compounds that should be expected to cause adverse biological effects when associated with different kinds of sediments.

The current study was undertaken with the following primary objectives:

1) The generation of toxicity data (10-day survival test with the amphipod, *Ampelisca abdita*) for the selected ordnance compounds spiked onto two marine sediments with different organic carbon concentrations (0.1 and 1.1%).

2) The analysis of the toxicity of pore water obtained from the spiked sediments using three different toxicity tests: sea urchin (*Arbacia punctulata*) embryo development test, macro-algae (*Ulva fasciata*) zoospore germination test, and polychaete (*Dinophilus gyrociliatus*) life-cycle test.

3) Chemical characterization of sediments and pore waters used in all tests.

The specific objectives of this study were to:

1) Generate toxicity information for three ordnance compounds associated with marine sediments with different characteristics, by:

   - Spiking two non-contaminated sediments containing 0.1 and 1.1% organic carbon with each of the ordnance compounds and analyzing acute mortality effects to the amphipod, *Ampelisca abdita*.

- Calculating LC_{50} (Lethal Concentration to 50% of the test organisms) values and No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for each ordnance compound and sediment, based on the concentration in sediment and in the pore water at test initiation.

2) Generate toxicity information for three ordnance compounds contained in the pore water from the spiked sediments, by:

- Extracting pore water from the spiked sediment at the time of amphipod tests initiation, and conducting short-term chronic toxicity tests with sea urchin embryos, macro-algae zoospores, and polychaetes.

- Calculating EC_{50} (Effective Concentration to 50% of the test organisms) values and NOEC and LOEC for each compound in the pore water from each sediment based on chemical measurements of ordnance compound concentrations in the pore water at test initiation.

3) Conduct a storage effects and mass balance experiment by:

- Spiking sub-samples of each sediment (with 0.1 and 1.1% organic carbon) with each ordnance compound and conducting periodical chemical analyses of the sediment and pore water over an 8-week time frame.

MATERIALS AND METHODS

PART 1: *Spiked Sediment Storage and Simulated Amphipod Test Experiments*

Sediments to be used in this study were collected by grab sampling or coring in two relatively pristine sites, one on the Northwest coast of the USA and one in the Gulf of Mexico. The sediment from the Northwest coast was collected at Carr Inlet, Puget Sound, WA, and was selected for its fine grain size (76.5% silt, 16.0% clay and 7.5% sand) and 1.1% total organic carbon (TOC) content. A second batch of this sediment had to be collected to repeat one of the solid-phase toxicity tests (with picric acid), and it had a slightly higher TOC content of 1.5%. The sediment from the Gulf of Mexico was collected at Redfish Bay, Texas, and was selected for its sandy characteristics (99.4% sand) and 0.1% TOC. Upon arrival at the laboratory, each sediment was sieved through a 500 μm mesh for removal of the indigenous fauna, and then dewatered, either by pneumatic porewater extraction or by centrifugation. Sediments were then kept in the refrigerator until needed for use in each experiment.

For quality assurance purposes, both sediments were characterized for an extensive list of chemicals (metals, polycyclic aromatic hydrocarbons, organochlorinated hydrocarbons, ordnance compounds, organotins). All chemicals were in very low or non-detectable levels. Concentrations of metals were slightly higher in the Puget Sound sediment than in the Texas sample, but well below expected toxic levels based on sediment quality guidelines (Long *et al.*,

1995).

The sediment spiking method, stability of chemicals in sediments during different storage periods, and changes of chemical concentrations in a simulated amphipod test were analyzed prior to performing toxicity tests.

Sediments were spiked with stock solutions prepared with pure ordnance compounds (≥99% purity), acquired from the following sources: picric acid and 2,6-DNT were purchased from Chem Service (660 Tower Lane, West Chester, PA 19381-9941), and tetryl was re-crystallized and kindly donated by the Naval Surface Warfare Center, Indian Head Division, MD.

Stock solutions of ordnance compounds for the storage and mass balance experiments were prepared by dissolution of the highest possible concentration of each ordnance compound in standard dilution water (0.45 µm Millipore® Filtered Seawater - MFS) (see Attachment 1), with salinity adjusted to 30 $^{o}/_{oo}$. Chiou & Shoup (1985) suggested that the addition of non-aqueous solvents during soil spiking seems to modify sorption/desorption processes when compared to field conditions where water is the solvent. Therefore, no non-aqueous solvent carriers were used to prepare stock solutions, and water was the only solvent used for sediment spiking procedures. This was done with the intention of being as realistic as possible, rather than generating higher sediment concentrations of ordnance compounds than those that could occur under natural field conditions.

Stock solutions were prepared by adding the desired amount of chemical to dilution water and vigorously stirring on a magnetic stirrer for 48 hours. Tetryl solutions were stirred on a heated plate (50 ± 5°C) for the first 24 hours to accelerate initial dissolution in seawater, followed by cooling off to room temperature for the remaining 24 hours of the procedure, so that excess dissolved tetryl would re-crystallize and a realistic concentration would be achieved. Picric acid and 2,6-DNT were stirred at room temperature for 48 hours. Each stock solution was filtered through a 0.45 µm Millipore® filter and the concentration was measured prior to sediment spiking. Chemical measurements were performed by HPLC following USEPA method 8330 (USEPA, 1994).

Picric acid was the most water-soluble of the three chemicals and a concentration of 22.2 g/L was achieved. Tetryl and 2,6-DNT were less soluble, and the highest concentrations reached were 39.5 and 127.8 mg/L, respectively.

The spiking procedure involved the addition of equal volumes of dewatered sediment and stock solutions into glass jars, and stirring on a magnetic stirrer for 4 hours. Sediments were then kept at room temperature (20 ± 1°C) for 24 hours prior to siphoning off the overlying water (stock solution excess).

A sub sample of the spiked sediments was placed in small jars and kept in the refrigerator for 8 weeks. These sediments were thoroughly stirred and sub samples taken periodically for chemical analyses. Chemical measurements were conducted periodically following USEPA Method 8330 (USEPA, 1994). The rest of the sediment was used immediately after spiking to conduct a small-scale simulated amphipod experiment without test organisms. For this experiment, sub samples of each spiked sediment were added to 250 ml jars in a 1:4 proportion, i.e., with 50 ml of sediment and 150 ml of seawater at 30 $^{o}/_{oo}$ salinity, simulating the sediment/water proportion used in an amphipod toxicity test. These jars were placed in an incubator at 20 ± 1°C for 10 days, simulating the test conditions. Three replicates were prepared for each sample, and chemical measurements of the sediment, pore water and overlying water were conducted on days 0, 5 and 10 of the experiment. The overlying water was exchanged 24

hours after initial addition to the jars, and the initial overlying water chemical measurements were conducted prior to overlying water exchange, to assess the initial loss of ordnance compounds from the sediment. For both the simulated amphipod experiment and the sediment storage experiment the pore water was extracted by centrifugation and the sediment chemical measurements were performed with the dewatered sediment after porewater extraction.

PART 2: *Amphipod* (Ampelisca abdita) *Toxicity Tests with Spiked Samples*

Range Finding Test

The range finding tests were conducted to determine the ordnance compounds concentrations to be used in the definitive test. They consisted of typical 10-day solid-phase toxicity tests with *Ampelisca abdita*, conducted according to SOP F10.15 (Attachment 2), with the difference that only two replicates were prepared for each treatment.

Stock solutions were prepared and sediments were spiked following the procedures described in Part 1, but the picric acid stock solution was prepared so that a lower concentration would be achieved. The concentrations of picric acid, 2,6-DNT and tetryl in the stock solutions were 2.205 g/L, and 120.0 and 29.4 mg/L, respectively. A series of two 10-fold dilutions was prepared with each stock solution and used for sediment spiking. In addition to the stock solutions, sediments were also spiked with 0.45µm Millipore® Filtered Seawater (MFS), representing a blank treatment, i.e., sediment handled in the same manner as the spiked sediments but without the addition of ordnance compounds. These blank treatments were used as test controls, in addition to the control sediment from the amphipod collection site, which was press sieved through a 500 µm screen to remove resident amphipods and predators.

A 96-hour toxicity test with the reference toxicant, sodium dodecyl sulfate (SDS), in aqueous solution was conducted concurrently to the range finding test to ensure that the amphipods used in different experiments had similar sensitivity.

Definitive Test

Stock and Test Solution Preparation and Sediment Spiking Procedure

The concentrations of the stock solutions to be used in the definitive test were selected based on the results of the range finding test, and consisted of 1,067 mg/L for picric acid, 110 mg/L for 2,6-DNT, and 43 mg/L for tetryl. Stock solutions were prepared and sediments were spiked following the general procedures described in Part 1, including the preparation of blank treatments. The picric acid stock solution was kept at room temperature and 2,6-DNT and tetryl stock solutions were kept at 15°C until used for sediment spiking, which was performed as soon as possible after stock solution preparation (less than 6 days). The stock solutions were serially diluted by 50% to prepare four additional concentrations for sediment spiking. Based on the results of the range finding test, lower concentrations of picric acid were used in the test with Puget Sound sediment, with 900 mg/L as the highest concentration and four 50% serial dilutions thereafter. Spiked sediments and blanks were kept in the refrigerator for an equilibration period of 10 days for picric acid, 9 days for 2,6-DNT and 5 days for tetryl, prior to test initiation.

Toxicity Tests

Amphipods (*Ampelisca abdita*) for the toxicity test were purchased from Brezina and Associates, Dillon Beach, CA. The organisms were shipped overnight and kept in the laboratory for 24 hours prior to use in the toxicity tests, which were conducted according to SOP F10.15 (Attachment 2). In summary, spiked sediments were added to test jars 24 hours prior to test initiation, placed in controlled temperature chambers at 20 ± 1°C, with mild aeration in each jar. Six replicates were prepared for each treatment: five for toxicity assessment and one for chemical measurements at test start and end. Each replicate contained 200 ml sediment and 700 ml seawater at 30 $^{o}/_{oo}$ salinity. Test jars were kept in the dark for the initial 24 hours. The overlying water was replaced on the day of test initiation, and 20 juvenile amphipods were randomly selected from the holding tanks and inserted into each jar, including the replicates for chemistry. Constant lighting was kept throughout the duration of the test. The five replicates of each treatment to be analyzed for toxicity were inspected for dead amphipods and for organisms floating on the surface film or emerged to the sediment surface on experimental days 1, 2, 4, 6 and 8. The replicates for chemical analyses were inspected on days 3 and 8. Dead organisms were removed and those floating on the surface film were gently pushed down into the water column with a glass rod. Aeration was inspected daily to ensure constant airflow into each jar. The test was terminated on day 10.

The first test with Puget Sound sediment spiked with picric acid failed to cause more than 50% mortality in the highest concentration, and was therefore repeated with higher concentrations. The second test is the one reported herein.

A 96-hour test with picric acid in aqueous solution was also conducted, with the highest concentration of 50 mg/L and four 50% serial dilutions thereafter, in addition to a control using dilution water. A reference toxicant test with SDS was conducted concurrently to each amphipod test. These tests were conducted in complete darkness to avoid excessive stress to the amphipods, at 20 ± 1°C, and seawater salinity of 30 $^{o}/_{oo}$. No food was provided.

Water Quality Measurements

Water quality measurements, consisting of dissolved oxygen, pH, salinity and ammonia, were made in every replicate of the sediment experiment immediately before test termination. Pore water quality was measured in the chemistry replicates, and included sulfide concentration in addition to the parameters mentioned above. In the water only tests with picric acid and SDS, water quality was measured in one replicate of each treatment, since a more stable environment is expected in this kind of test. Dissolved oxygen (DO) was measured with an YSI® meter, model 59; pH, ammonia and sulfide were measured with an Orion® meter, model 290A, and the respective probes; salinity was measured with a Reichert® refractometer. Un-ionized ammonia (expressed as nitrogen) concentrations (NH_3) were calculated for each sample using the respective salinity, temperature, pH, and total ammonia (NH_4) measurements.

Chemical Analyses

Chemical concentrations of ordnance compounds in the spiked sediments and pore waters, including the test blanks and the control sediment, were measured at test initiation and

termination, and in the overlying water at test termination, following USEPA Method 8330 (USEPA, 1994). Samples for chemical analyses were taken from the appropriate jars on days 0 and 10. On day zero a 4 ml sediment sub sample was taken for measurement of ordnance concentrations in the sediment and a 12 ml sub sample was taken for porewater extraction. The sediment sub samples were weighed for wet weight, dried in a chemical hood at room temperature for three to four days, gently ground and prepared for HPLC analyses as recommended in USEPA Method 8330 (USEPA, 1994). The pore water was extracted from the additional sub samples by centrifuging at 1500 *g* for 30 minutes and frozen until chemical analyses could be conducted.

Ordnance compounds were measured against calibration curves prepared using the standards recommended in Method 8330. This method was modified for the measurement of picric acid, for which an isocratic mixture of 65% 0.1M sodium acetate buffer with pH adjusted to 4.8, and 35% methanol, was used as solvent for the HPLC analysis. Picric acid standards in acetonitrile, at 1,000 µg/ml, were purchased from Chem Service and used for the method calibration.

The mass balance for the sediment-bound and porewater-dissolved fractions of each ordnance compound was calculated by the following formulas, respectively:

$$S_b = S_t - (PW_t \times \%M)/100$$

and

$$PW_b = (PW_t \times \%M)/100$$

where:

S_b = Sediment-bound ordnance compound
S_t = Total ordnance compound measured in the sediment
PW_t = Total ordnance compound measured in the pore water
PW_b = Porewater-bound ordnance compound
%M = Percent moisture in the sediment

Data Analyses

The LC_{50}, NOEC and LOEC values were calculated using the concentrations of the tested chemicals in the sediment measured at the start of each experiment. Prior to statistical analysis, the transformed data sets were screened for outliers (SAS, 1992). Outliers were assessed by comparing the studentized residuals to a critical value from a *t*-distribution chosen using a Bonferroni-type adjustment. The adjustment is based on the number of observations, *n*, so that the overall probability of a type I error is at most 5%. The critical value, *cv*, is given by the following equation: $cv = t(df_{Error}, .05/(2 \times n))$.

After omitting outliers but prior to further analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). Statistical comparisons among treatments for the assessment of NOEC and LOEC values were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989).

The Trimmed Spearman-Karber method (Hamilton *et al.*, 1977) with Abbott's correction (Morgan, 1992) was used to calculate LC_{50} values. The data only allowed LC_{50} calculations for some of the Texas samples and the Texas blank data was used for the Abbott's correction.

PART 3: *Urchin Embryo* (Arbacia punctulata), *Polychaete* (Dinophilus gyrociliatus) *and Macro-algae* (Ulva fasciata) *Toxicity Tests with Pore Water from Spiked Sediments*

Pore Water Preparation

Pore water to be used in toxicity tests and chemical analyses was extracted from the spiked sediments on the same day that sediments were added to the amphipod test jars, i.e., after 10, 9 and 5 days of equilibration for picric acid, 2,6-DNT and tetryl, respectively. Pore water was obtained by centrifuging the sediments at 1200 *g* for 20 minutes. The supernatant was removed and centrifuged once more to remove excess particles. Each sample was subdivided into several sub samples with volumes between 60 and 120 ml, stored in amber jars with Teflon lids and screw cap tops and frozen at -20°C until use in toxicity tests.

Two days prior to toxicity tests the pore water was moved from the freezer to a refrigerator at 4°C. One day prior to testing, samples were thawed in a tepid (20°C) water bath. Temperature of the samples was maintained at 20 ± 1°C, and water quality measurements (dissolved oxygen, pH, ammonia, and salinity) were made as described in Part 2.

Following water quality measurements and adjustments, the samples were stored overnight at 4°C, but returned to 20 ± 1°C before the start of the toxicity tests. Some of the pore water samples obtained from sediment from Carr Inlet, Puget Sound had a precipitate form after the freezing process, and test solutions for the toxicity tests were prepared by pipetting off the supernatant, avoiding the precipitate.

At initiation of the toxicity tests, sub-samples of each pore water used in the tests were stored in scintillation vials and frozen for future chemical analyses. A sub-sample of the pore water from the two blank treatments (Texas and Puget Sound) was filtered through a 0.45 µm nylon syringe filter and stored frozen for dissolved organic carbon (DOC) analysis. Samples for DOC analysis were filtered but not acidified prior to freezing to prevent loss of volatile organic carbon.

Test Organisms

Arbacia punctulata urchins used in this study were obtained from Gulf Specimen Company, Inc., Panacea, FL. Polychaetes, *D. gyrociliatus*, have been in culture in our laboratory for over four years. Original organisms were isolated from material obtained from Long Beach Harbor, CA. Fronds of the macro-alga *U. fasciata* were collected during a low tide on Port Aransas, TX, jetties.

Toxicity Tests

All toxicity tests were conducted following standardized methodologies, with one modification: tests were conducted in complete darkness to minimize photo-degradation of the

ordnance compounds during the exposure period. The salinity of the test solutions was 30 ± 1 $^{o}/_{oo}$ and test temperature was 20 ± 1°C.

The sea urchin (*A. punctulata*) 48-hour embryological development test, the polychaete (*D. gyrociliatus*) 7-day survival and reproduction test, and the alga (*U. fasciata*) 96-hour zoospore germination and germling growth test were conducted following SOPs F10.7, F10.10 and F10.23, respectively (Attachments 3-5).

Test treatments were prepared by 50% serial dilutions of each pore water sample, including some dilutions of the blank treatments. MFS was used as the diluent, and a control series was also prepared with MFS.

Reference Toxicant Test

A reference toxicant (SDS) test was conducted concurrently with each test series.

For the sea urchin test, the EC_{50} values obtained in the reference toxicant tests were compared to a control chart prepared using the results of the 20 most recent tests conducted in our laboratory (Environment Canada, 1990). According to the control charts, the EC_{50} values for the embryological development test should be between 1.6 and 6.9 mg/L.

The SDS EC_{50} value obtained with the macro-algae, *U. fasciata*, test was compared to a control chart prepared with the results of 13 previous tests. Based on the control chart, the EC_{50} of a SDS test with *U. fasciata* zoospores should be between 1.2 and 5.6 mg/L.

A control chart with SDS data was prepared for *D. gyrociliatus* using the five last tests conducted in our laboratory. According the this control chart, the LC_{50} of an SDS test should be between 2.3 to 7.1 mg/L, and the reproductive EC_{50}, between 1.7 to 4.9 mg/L.

Water Quality Measurements

Initial water quality measurements, consisting of dissolved oxygen, pH and ammonia, were made for the dilution water, blank treatments and the highest spiked pore water concentration extracted from each sample, following the procedures described in Part 2. Since high ammonia in some of the Puget Sound samples might have been a confounding factor in the sea urchin test, pH and ammonia levels were measured in the whole concentration series. Water quality measurements were also conducted at test termination for the *D. gyrociliatus* test, because its long exposure period could possibly result in DO depletion and ammonia accumulation.

Chemical Analyses

Initial and final concentrations of the three ordnance compounds in pore waters were measured in each treatment of each toxicity test, following USEPA method 8330 (USEPA, 1994), with modifications for picric acid (see Part 2). In order to assess if the loss of some compounds during the test period was enhanced by the presence of organisms and possibly reflected adsorption to the test organisms or food provided, chemical measurements at test termination were also made in replicates subject to the same test conditions but kept without organisms.

Samples for DOC analyses were thawed and run in triplicate. DOC was measured using an O I Analytical Model 1010 Wet Oxidation Total Organic Carbon Analyzer following the model

1010 operators' manual (OI Analytical, 1998). One-milliliter volumes were syringe injected into the reaction vessel. Samples were analyzed in the TOC mode with 400 µl of acid and 4000 µl of oxidant. Total inorganic carbon react and detect times were 2:00 (min:sec) and 1:35 (min:sec), respectively. Total organic carbon react and detect times were 8:30 (min:sec) and 2:00 (min:sec), respectively. At least one blank and one laboratory control was run with each batch of samples. Analysis was repeated if the percent recovery of the laboratory control failed to meet the 90-110% level.

Data Analyses

The EC_{50} and/or LC_{50}, NOEC and LOEC values were calculated using the concentrations of the tested chemicals measured at the start of each experiment. Prior to statistical analysis, the transformed data sets were screened for outliers (SAS, 1992) in the sea urchin and macro-alga tests. Outliers were assessed as described in the data analyses section of Part 2. Data from the polychaete tests were not tested for outliers due to the natural variability of this test's results.

After omitting outliers but prior to further analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). Statistical comparisons among treatments for the assessment of NOEC and LOEC values were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989), except for the results of the polychaete and sea urchin tests in the Puget Sound pore water. These data were compared back to the equivalent blank dilutions by t-tests to assess significance of the results, because of the significant difference between some of the blank results and the dilution water control.

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

The Trimmed Spearman-Karber method (Hamilton *et al.*, 1977) with Abbott's correction (Morgan, 1992) was used to calculate EC_{50} or LC_{50} values. The Texas and Puget Sound blank samples were used as controls for the application of Abbott's correction. For endpoints with continuous values, such as polychaete reproduction (number of laid eggs/adult female), and algae germling length and cell number, the test result was converted into percent of the control, and EC_{50} values were calculated using these percentage data. Some data sets did not meet the requirements for the application of the trimmed Spearman Karber method, e.g., all concentrations had effect above or below 50%, or there was an all-or-none effect, i.e., 100% effect in one concentration and no significant effect in the next. In these cases, other statistical methods were applied, such as Probit or nonlinear interpolation.

RESULTS AND DISCUSSION

PART 1: *Spiked Sediment Storage and Simulated Amphipod Test Experiments*

Spiked Sediment Storage

The distribution of ordnance compounds in sediment and pore water throughout 56 days varied with each ordnance compound and sediment type (Table 1).

The concentration of 2,6-DNT in the sandy Texas sample remained stable in the sediment but suffered a gradual loss from the porewater, reaching nearly 50% after 56 days. Porewater concentrations were one order of magnitude higher than in the sediment. In the fine grain Puget Sound sample 35% of the 2,6-DNT was lost from the sediment in the first week and 65% were lost over 8 weeks, whereas nearly none remained in the pore water already after 7 days. This suggests a high rate of degradation, possibly due to microbiological activity in this sediment.

Tetryl remained stable for 21 days in the Texas sediment but suffered a 75% loss thereafter, whereas the opposite occurred in the pore water, with gradual reduction throughout the first 21 days, achieving stability at a low level thereafter. Tetryl concentrations in the Puget Sound pore water were mostly below detection, indicating high affinity for the sedimentary phase. The concentration of tetryl in the sediment fluctuated with time, with a sharp decrease after the first week, accompanied by a slight increase in the pore water. The reason for the fluctuation of the tetryl concentration in the sediment is unknown, and not enough of the spiked sample was available to continue measurements past the first 21 days.

Both types of sediments were spiked and treated in exactly the same manner. Therefore, the fact that initial concentrations of 2,6-DNT and tetryl were much lower in the Puget Sound than in the Texas samples suggests that either very rapid degradation or irreversible binding (Caton *et al.*, 1994; Thorne & Leggett, 1997; Achtnich *et al.*, 1999) occurred in the muddy Puget Sound samples, even prior to the initial measurements, which were conducted immediately after the end of the spiking procedure and sediment settling.

Picric acid was very stable in both sediments and pore waters. Picric acid concentrations were slightly higher in the muddy sediment from Puget Sound than in the sandy sediment from Texas, whereas porewater concentrations were slightly lower in the Puget Sound sample relative to Texas. However, in both samples the concentration of picric acid was higher in the pore water than in the sediment. This suggests low affinity of this chemical to the sediment, which corroborates adsorption studies conducted by Goodfellow *et al.* (1983) indicating that picric acid was not readily adsorbed to estuarine sediments. However, Goodfellow et al. (1983) analyzed sediments spiked with ^{14}C-labeled picric acid and it is impossible to assess if they were measuring picric acid or degradation compounds.

Simulated Amphipod Test Experiment

The different chemicals had variable behavior in the two types of sediment (Table 2) during a simulated amphipod experiment, conducted without organisms. The concentration of 2,6-DNT was drastically reduced in the sediment, pore water and overlying water from the sandy Texas sample, suggesting degradation. The porewater concentration of 2,6-DNT in the Puget Sound sample was very low from the beginning of the experiment, and no more 2,6-DNT was released

Table 1. Measured concentrations of ordnance compounds in spiked sediments (mg/kg dry weight) from Texas (TX) and Puget Sound, WA (PS), and respective pore water (mg/L), over 56-day storage in the dark at 4°C.

			Concentration						
			Day						
Matrix	Sample	Chemical	0	4	7	14	21	28	56
Sediment	TX	2,6-DNT	3.334	4.110	3.495	3.923	3.446	3.646	3.656
Pore water	TX	2,6-DNT	87.104	81.840	74.436	61.250	67.711	66.542	45.066
Sediment	TX	Tetryl	0.210	0.268	0.210	0.217	0.255	0.133	0.063
Pore water	TX	Tetryl	2.548	0.286	0.149	0.044	0.026	0.019	0.020
Sediment	TX	Picric Acid	653.4	851.9	374.2	810.1	588.3	683.4	758.2
Pore water	TX	Picric Acid	1707.9	1668.9	1653.0	1741.8	1740.0	1702.7	1900.0
Sediment	PS	2,6-DNT	1.006	0.736	0.656	0.549	0.308	0.356	0.354
Pore water	PS	2,6-DNT	9.088	0.023	0.004	BDL[1]	0.026	0.003	0.001
Sediment	PS	Tetryl	0.066	0.150	0.004	0.165	0.049	NM	NM
Pore water	PS	Tetryl	BDL	BDL	0.008	BDL	BDL	NM[2]	NM
Sediment	PS	Picric Acid	1031.1	1074.7	1021.4	976.8	1075.4	1073.5	1274.3
Pore water	PS	Picric Acid	1411.2	1337.7	1312.5	1311.2	1261.5	1301.4	1517.7

[1] BDL = Below detection limit;

[2] NM = Not measured.

Table 2. Measured concentrations of ordnance compounds in sediment (mg/kg dry weight) from Texas (TX) and Puget Sound, WA (PS), and respective overlying water (mg/L) and pore water (mg/L) on days 0, 5 and 10 of a simulated amphipod experiment without organisms. Overlying water was taken after 24-hour equilibration before water exchange.

Matrix	Sample	Chemical	Concentration		
			Day 0	Day 5	Day 10
Sediment	TX	2,6-DNT	1.472	0.332	0.187
Pore water	TX	2,6-DNT	35.780	2.626	1.024
Overlying water	TX	2,6-DNT	11.392	4.168	2.476
Sediment	TX	Tetryl	0.129	BDL	BDL
Pore water	TX	Tetryl	0.132	BDL[1]	BDL
Overlying water	TX	Tetryl	0.145	0.002	0.013
Sediment	TX	Picric Acid	351.755	62.849	40.654
Pore water	TX	Picric Acid	909.962	227.542	137.147
Overlying water	TX	Picric Acid	175.721	150.863	148.624
Sediment	PS	2,6-DNT	0.719	0.102	0.123
Pore water	PS	2,6-DNT	0.001	BDL	0.002
Overlying water	PS	2,6-DNT	0.809	BDL	BDL
Sediment	PS	Tetryl	0.013	0.020	BDL
Pore water	PS	Tetryl	0.039	0.008	BDL
Overlying water	PS	Tetryl	BDL	0.001	BDL
Sediment	PS	Picric Acid	604.789	220.316	121.939
Pore water	PS	Picric Acid	672.818	296.382	145.185
Overlying water	PS	Picric Acid	58.725	231.107	204.047

[1] BDL = Below detection limit

into the overlying water after the first 24 hours, indicating high adsorption of this chemical to this sediment with higher TOC. However, the loss of 2,6-DNT to the overlying water in the first 24 hours was relatively high in both sediments.

Tetryl was totally lost from both sediments throughout the experiment, with concentrations below the detection limit in the pore water and sediment at day 10, and high loss of tetryl into the overlying water of the Texas sample in the first 24 hours.

Tetryl and 2,6-DNT were in higher total concentrations in the Texas samples than in the Puget Sound samples at the beginning of the experiment, although both were spiked with the same ordnance stock solutions. This suggests either higher biodegradation or irreversible binding in the Puget Sound sediment (Caton *et al.*, 1994; Thorne & Leggett, 1997; Achtnich *et al.*, 1999).

Concentrations of picric acid in the overlying water of both sediment types were relatively similar throughout the experiment. However, in the muddy sample picric acid seemed to be in equilibrium between the sediment and the porewater fractions, whereas in the sandy sample 3 to 4-fold more picric acid was measured in the pore water than in the sediment.

PART 2: *Amphipod* (Ampelisca abdita) *Toxicity Tests with Spiked Samples*

Range Finding Test

In the range finding test, the highest concentration of 2,6-DNT in Puget Sound (PS) and Texas (TX) sediments caused only 30 and 55% amphipod mortality, respectively. Similarly, only 15 and 55% amphipod mortality occurred in the highest concentration of tetryl in the muddy (PS) and sandy (TX) sediments, respectively. Picric acid promoted 100% amphipod mortality in a 492 mg/kg concentration in the sandy sediment and 0.402 mg/kg concentration in the muddy sediment. The reason for this acute difference between the sandy and muddy sediment is not known, but it was observed that picric acid in the muddy sediment had a very strange and unique effect, generating a U-shaped curve, as will be discussed in the definitive tests results.

Definitive Test

Toxicity and Chemistry

Measured concentrations of 2,6-DNT and tetryl were typically higher in the spiked sandy sediment from Texas than in the spiked muddy sediment from Puget Sound (Appendices B1 and B2), although both sediments were spiked with the same stock solutions. The reasons for this are unknown, since large amounts of neither chemical could be detected in the pore water or overlying water of the muddy sediment, indicating that a leaching process would not have been responsible for the loss of chemicals. The low concentrations in the Puget Sound sediment may have been caused by enhanced biodegradation in this organically richer sediment or by unavailability of the chemicals due to irreversible binding or to processes that deem the compounds nonsolvent-extractable, which has been reported to happen with TNT and its metabolites in soils and freshwater sediments, (Caton *et al.*, 1994; Thorne & Leggett, 1997; Achtnich *et al.*, 1999) and, therefore, could be expected to occur with other nitroaromatic

compounds in marine sediments. A relatively large peak was seen at an elution time of 4.5-4.7 minutes in all 2,6-DNT chromatograms from the amphipod test sediments, pore waters, and overlying water at test end (Figure 1), suggesting the presence of a degradation product, since the elution time of 2,6-DNT is approximately 9.3 minutes. A GC-MS analysis identified this peak as 2-methyl-3-nitroaniline. Similarly, relatively large peaks were observed in the chromatograms of the amphipod test sediment (but not in the pore water) from Texas sediment spiked with tetryl, with elution times of approximately 4.6 and 7.4-7.6 minutes (Figure 2a), whereas the elution time of tetryl is about 6.1 minutes. Although tetryl was described as a very stable compound, withstanding up to 20 years of storage time at normal temperatures (Yinon, 1990), it seems to be highly degradable when in contact with water and/or sediment as observed in the current and a previous (USGS, 1999) study. Not enough sample was available for the identification of the unknown peaks observed in the HPLC chromatograms, but since picric acid is a primary hydrolysis product of tetryl (Oak Ridge National Laboratories, 1996)), particularly if dissolved in seawater (Hoffsommer & Rosen, 1973), the sediment and porewater samples spiked with tetryl were also analyzed for picric acid. Not only picric acid was identified, but also numerous other peaks were observed at test start and end in the Texas samples spiked with tetryl, when analyzed for picric acid using HPLC (Figure 2b, c). Only some broad peaks were observed in the Puget Sound sediment at test start and pore water test end, for the sample spiked with tetryl (Figure 3). The peaks seen between 2 and 4 minutes of elution time are likely to be non-toxic chemicals naturally contained in the sediments, since they also appeared in the chromatograms from the control and blank samples. A distinct peak was also identified at 4.4 minutes elution time, at test start, in the highest porewater concentrations of Puget Sound samples spiked with picric acid (Figure 4), but no similar peak was observed in any other picric acid spiked samples with either sediment type. The picric acid elution time was 10.7 minutes.

The data presented above suggest relatively high degradability of 2,6-DNT and tetryl, as well as some potential for the degradation of picric acid in marine sediments and pore waters. The persistence and toxicity of such degradation products is not known at this time and would require further research.

Mass balance calculations indicated that picric acid was in equilibrium between the sediment and pore water in both kinds of sediments at test start (Appendix B1), but it tended be released from the sediment into the pore water in the Texas sample, with a 2.5 to 12-fold higher amount in the pore water than bound to the sediment at test end (Appendix B2). Due to its yellow color, a concentration gradient of picric acid could also be seen in the overlying water (Figure 5). In the sandy Texas sample the 2,6-DNT also tended to be released from the sediment, particularly at the highest ordnance compound concentrations. Negative amounts of 2,6-DNT at test start in the Texas sample (Appendix B1) may indicate either degradation of the chemical or occurrence of irreversible binding during the drying and extraction procedures. In the organically richer Puget Sound sample all the 2,6-DNT was bound to the sediment, whereas some tetryl was measured in the pore water. In the sandy Texas sediment most of the tetryl tended to be sediment-bound with some measured in the pore water, particularly in the highest concentrations at test start. Measurements at test end indicate that relatively high amounts of picric acid leached into the overlying water of both kinds of sediments, as did 2,6-DNT in the Texas samples, but not in the Puget Sound sample (Appendix B2). Some tetryl was measured in the overlying water of both kinds of sediments at test end.

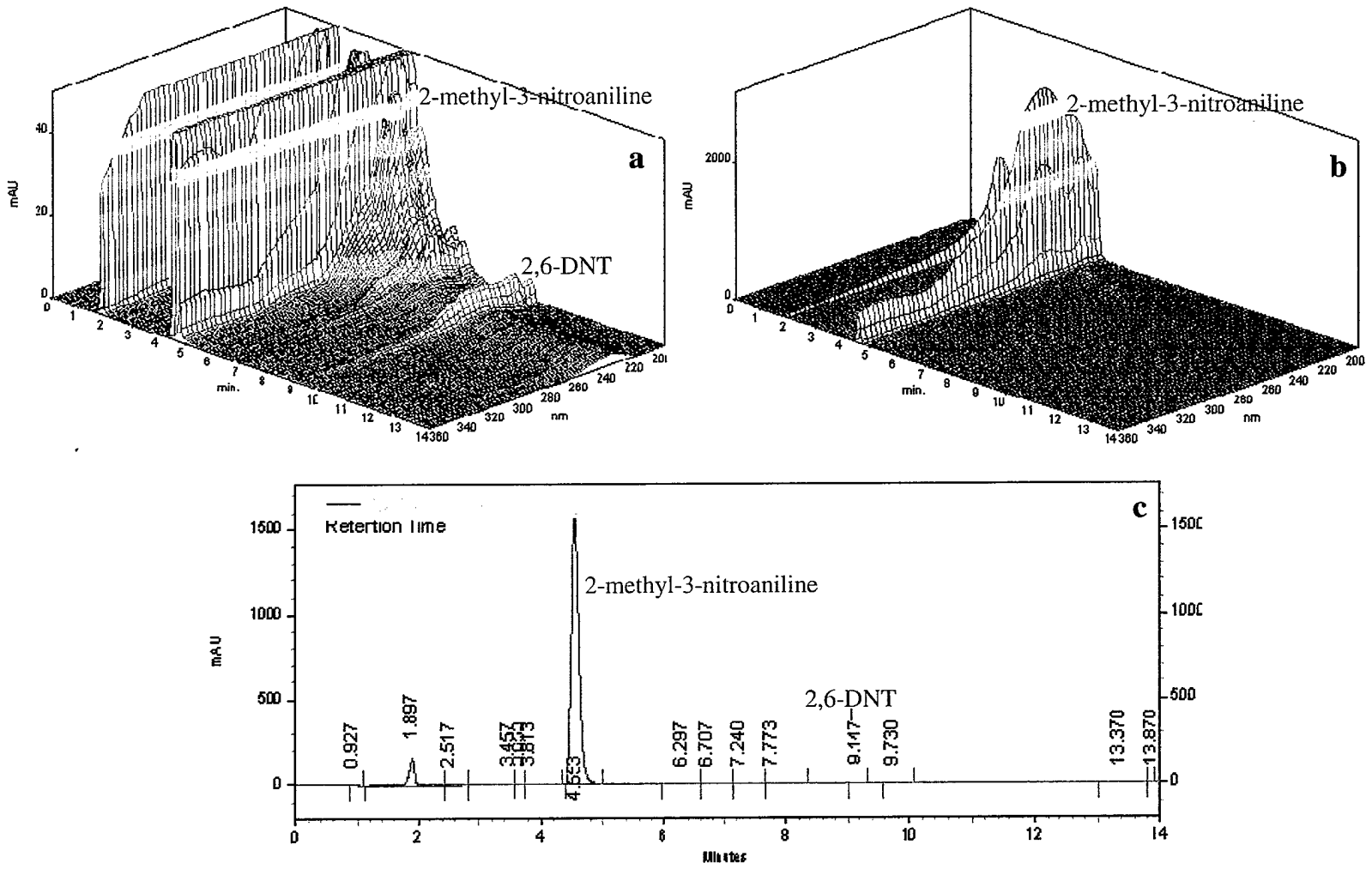


Figure 1. Views of chromatogram of Puget Sound pore water spiked with 2,6-DNT, showing: a) 3-D view with shortened Y axis to allow observation of smaller peaks; b) 3-D view with expanded Y axis for observation of entire degradation product peak; c) 2-D view showing large degradation product peak and 2,6-DNT peak barely visible.

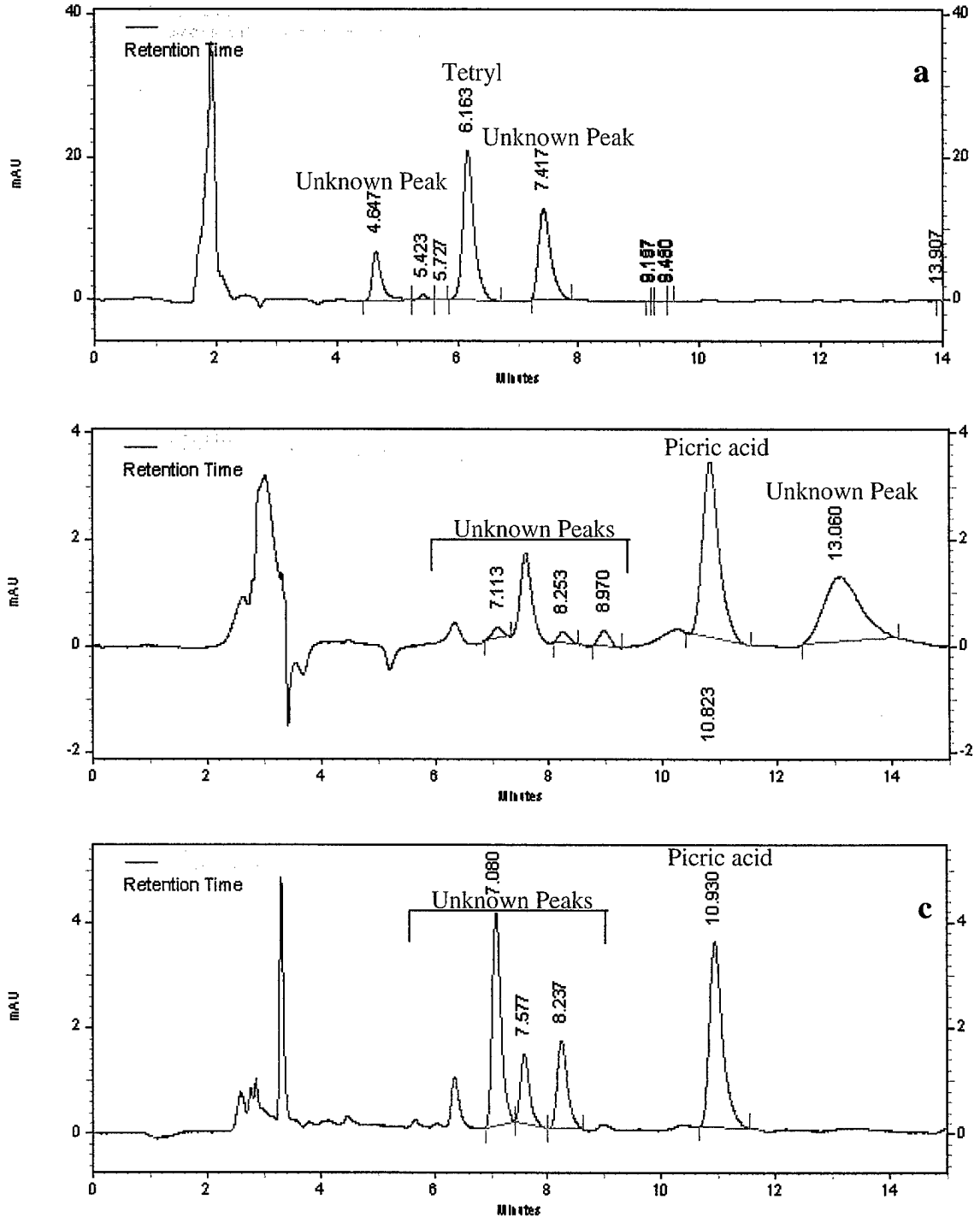
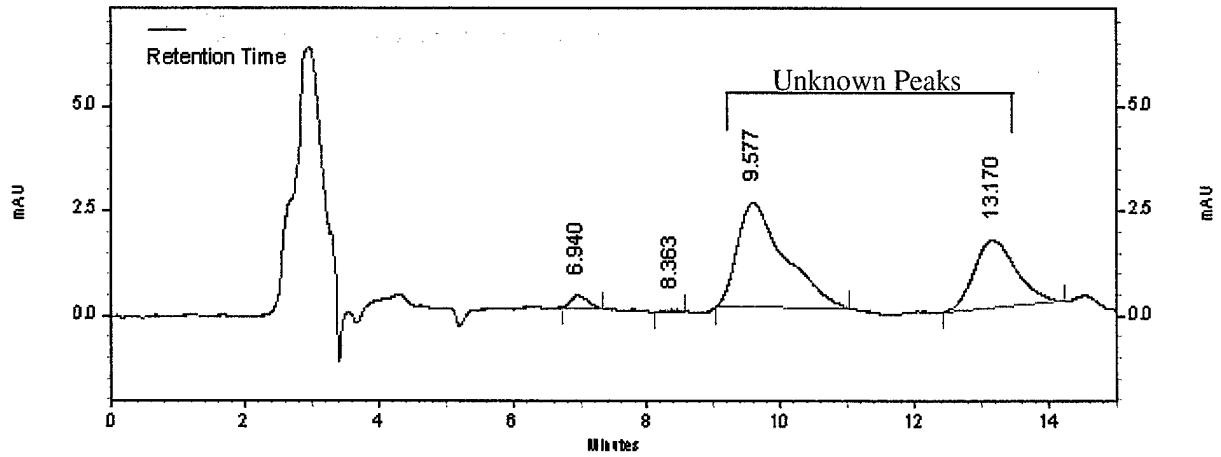


Figure 2. Chromatograms of Texas samples spiked with tetryl, showing unknown peaks, possibly of degradation products: a) Sediment at amphipod toxicity test start, measured for tetryl (Method 8330); b) Same as a, but measured using picric acid method; c) Pore water at amphipod toxicity test start measured for picric acid.



b

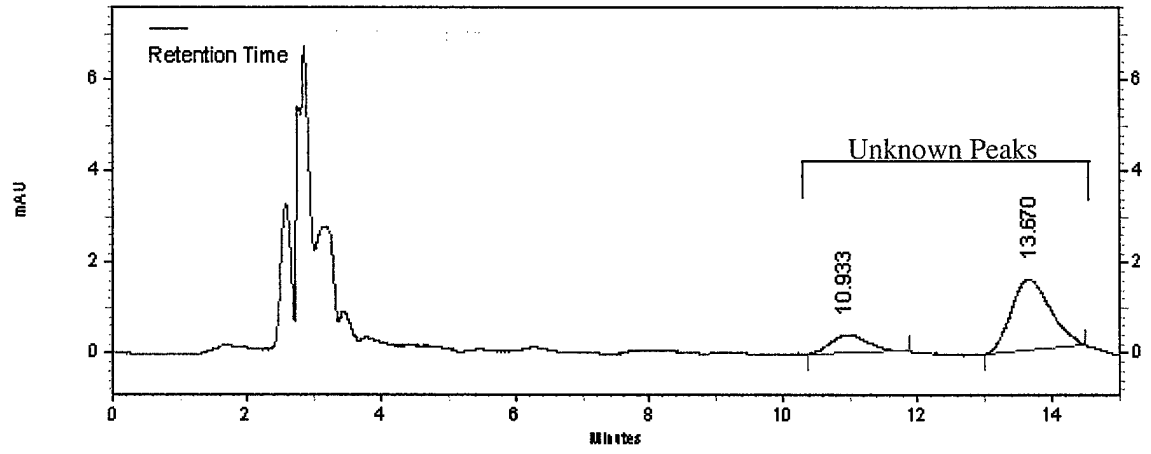


Figure 3. Chromatograms of Puget Sound samples spiked with tetryl and measured using the picric acid method, showing unknown peaks, possibly of degradation products: a) Sediment at amphipod toxicity test start; b) Pore water at amphipod test end.

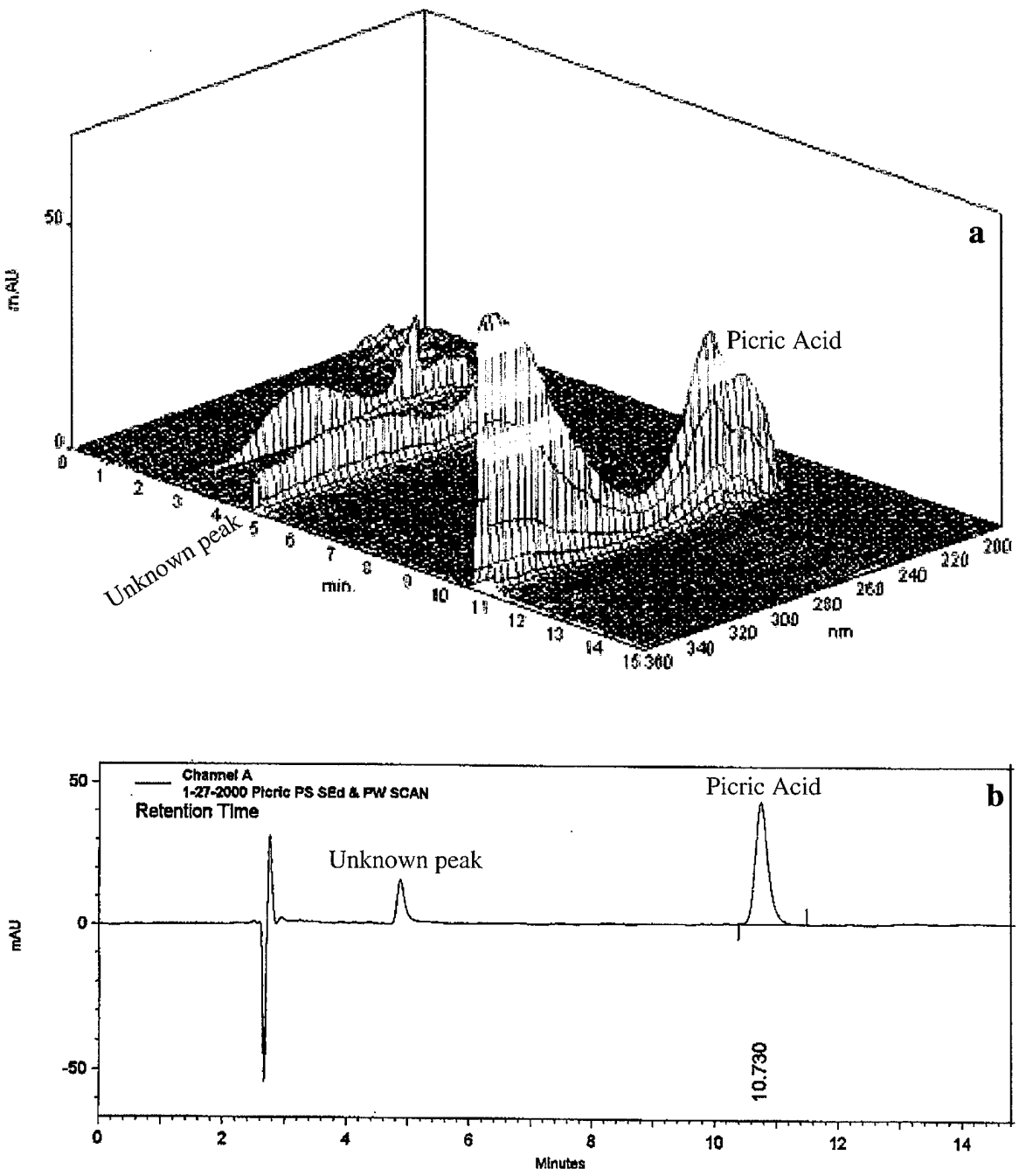


Figure 4. 3-D (a) and 2-D (b) views of chromatogram of Puget Sound pore water spiked with picric acid, showing an unknown peak, possibly of a degradation product.

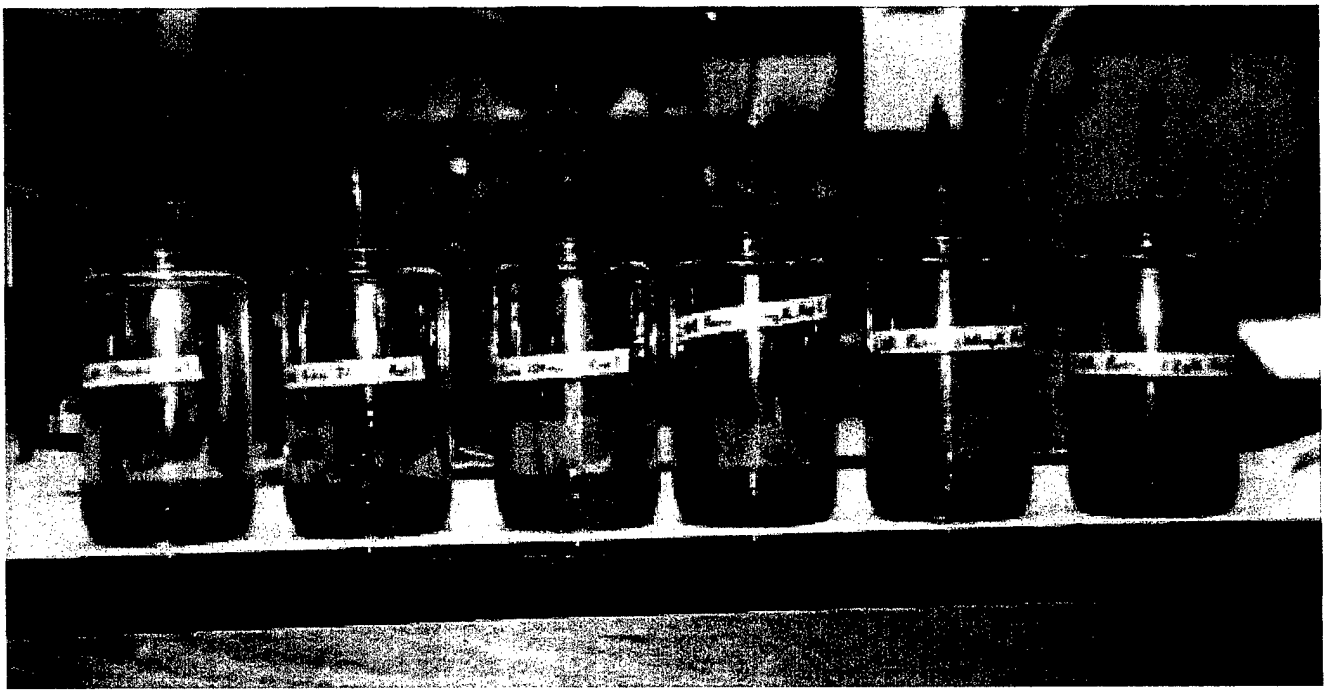


Figure 5: Amphipod test jars with Texas sediment spiked with picric acid, showing color gradient of contaminant released into overlying water. The blank treatment is on the far left.

The data suggests that the chemicals in the highest concentrations reached sediment saturation levels, with release of higher amounts of ordnance compounds into the surrounding water. As concentrations were gradually reduced, more of each chemical remained bound to the sediment (Appendix B2).

Picric acid was more toxic in the muddy than in the sandy sediment in the range finding test, and, therefore, lower concentrations were spiked into Puget Sound sediment for the definitive test (900 mg/L stock solution, compared to the 1200 mg/L stock spiked into the Texas sediment). In spite of this difference in the spiking procedure, the highest measured concentration of picric acid in the sandy and muddy sediment was similar at test start (337 and 304 µg/g sediment dry weight, respectively), but dropped abruptly in the lower concentrations of the muddy sediment relative to the sandy (Appendix B1). At test start, similar amounts of picric acid were measured in the sediment and pore water of both kinds of sediments (Appendix B1). At test end, a 2- to 3-fold higher level of picric acid was measured in the pore water of the sandy sample than in the solid phase, and still higher concentrations were measured in the overlying water (Appendix B2). In the muddy PS sample, approximately 10 times more picric acid was measured in the overlying water than in the sediment or the pore water of the highest concentration at test end, but no picric acid could be measured in any phase of any of the lower concentration treatments. Picric acid could not be detected in the pore water or overlying water of the muddy sediment either, suggesting that it did not simply suffer a leaching process due to low adsorption to sediments (Goodfellow *et al.*, 1983).

Amphipod toxicity tests exhibited consistent results among replicates of each treatment (Appendix B3) and no outliers were detected. Puget Sound sediment spiked with 2,6-DNT and tetryl was not toxic, with survival rates of 80 and 72% (Figs. 6 and 7) in the highest concentrations, respectively, not significantly different from the Puget Sound reference sediment, which had survival of 87 and 88% in two tests. This lack of toxicity could be attributed to both the lower concentrations of 2,6-DNT and tetryl in the Puget Sound sediment relative to the Texas sediment (Appendix B1) and low bioavailability due to the higher TOC, of 1.1%.

High survival also occurred in the sandy Texas sediment spiked with 2,6-DNT, with 63% live amphipods in the highest initial sediment concentration of 4.6 mg/kg sediment dry weight. This was not significantly different from the Texas reference sediment, which had a survival rate of 71% (Appendix B3), suggesting that the amphipods were stressed due to natural sediment features but were not affected by 2,6-DNT in the highest sediment concentrations. The tetryl-spiked sediment from Texas had LOEC and LC_{50} values of 3.6 and 3.2 mg/ kg sediment dry weight, respectively, based on the initial sediment concentration (Appendix B3).

Picric acid was toxic in the sandy sediment, with an LC_{50} of 144 mg/kg dry weight, and NOEC and LOEC values of 73 and 162 mg/kg, respectively (Appendix B3). Picric acid in the muddy sediment seems to have either degraded or been irreversibly bound to the sediment, and chemical measurements could only be made in the two highest concentrations at test start, and at the highest only at test end (Appendices B1 and B2). Curiously, mortality peaked at what would be the intermediate test concentrations, which were below chemical detection limits, decreasing again in the next two higher concentrations (53.4 and 304.2 mg/kg), generating a U-shaped curve (Figure 8). Although the three lowest concentrations on the X-axis of the Puget Sound sector of Figure 8 are below detection limit, they are in increasing order, based on the concentrations of the picric acid solutions used to spike the sediment. It is reported in the literature that toxicity to insects, microcrustaceans, bacteria, fungi and plants increases with the degree of nitration of phenols up to dinitrophenols, but decreases again with picric acid (trinitrophenol) (Simon & Blackman, 1953; Bringmann & Kuhn, 1959). Due to the close phylogenetic relationship of amphipods with other microcrustaceans, this ranking of toxicity of nitrophenols is expected to be true for amphipods also. Picramic acid (2-amino-4,6-dinitrophenol) was also reported as largely more toxic than picric acid to trout and oysters (Goodfellow *et al.*, 1983). Phenols with lower levels of nitration, including dinitrophenols and picramic acid can be produced during the degradation process of picric acid (Goodfellow *et al.*, 1983; Gorontzy *et al.*, 1994; Spain, 1995) and, therefore, could be among the unmeasured chemicals responsible for the toxicity of the Puget Sound samples in the lowest spiked concentrations of picric acid. This would also explain the higher toxicity of the Puget Sound sediments relative to the Texas sediments, since a higher degree of microbial degradation would be expected in the Puget Sound sample, due to its higher TOC content, suggesting the presence of a larger microbial community as well. The higher survival of amphipods in the higher concentrations of picric acid in Puget Sound sediment (Figure 8) would not be related to degradation products but could be due to an anomalous behavior resulting in the amphipods not leaving their tubes to molt. Dead animals and molts were observed on the sediment surface in the second and third lowest concentrations, but not in the two highest concentrations. This suggests that at these higher concentrations the animals stayed inside their tubes and did not molt, therefore being able to survive the harsher conditions. However, in a longer-term test the animals would have to resume normal activities and would eventually be affected by the picric acid present in the sediment and pore water. Therefore, the

concentrations reported here are expected to be deleterious to benthic organisms under natural conditions and should not be considered safe for marine life, in spite of the mortality reduction observed in higher concentrations. Despite this reduction, mortality in all picric acid treatments in Puget Sound sediment was significantly larger than in the control.

A water only test with picric acid resulted in an LC_{50} of 30.2 mg/L and NOEC and LOEC values of 24.8 and 49.7 mg/L, respectively, based on concentrations measured at test start (Appendix B4). Picric acid concentrations suffered minor changes between test beginning and end, indicating low adsorption to surfaces and low degradability in filtered seawater. The sensitivity of amphipods to ordnance compounds was expected to be similar to that of other crustaceans of similar size and life cycle, e.g., mysids such as *Americamysis* (*Mysidopsis*) *bahia*, analyzed in a previous survey. This indeed occurred, with the picric acid LC_{50} to amphipods only twice as high as that to mysids (13 mg/L) and generally an order of magnitude lower than the EC_{50} values of all other previously tested marine organisms (echinoid embryos and gametes, fish embryos, polychaetes and macro-algae zoospores) (USGS, 1999).

The reference toxicant tests with SDS conducted concurrently to all initial amphipod tests and to the picric acid test in Puget Sound sediment had LC_{50} values of 5.99 and 5.08 mg/L, with overlapping 95% confidence intervals, indicating that there was no significant difference between the two LC_{50} values (Appendix B4). These results show that the organisms used in both tests were in the same range of sensitivity to toxicants.

Water Quality Measurements

Water quality measurements conducted with the overlying water at amphipod test termination (Appendix B5) showed that salinity remained stable throughout the experiments, varying from 29 to 32 $^{o}/_{oo}$. The pH ranged from 7.8 to 8.5, and in the water only tests with picric acid and the reference toxicant, SDS, it ranged from 7.4 to 8.1 (Appendix B6). Dissolved oxygen ranged from 87.3 to 97.4% saturation in the overlying water of sediment tests and from 70.4 to 96.4% in the water only tests (Appendices B5 and B6). Un-ionized ammonia was very low in the water only tests, ranging from 0.01 to 1.73 µg/L (Appendix B6), whereas in overlying water of the sediment tests ammonia levels were very variable and tended to increase with increasing concentrations of the ordnance compounds (Appendix B5). The average ammonia concentrations and standard deviation for the overlying water of all replicates from each treatment were calculated and are presented in Appendix B7. The highest ammonia levels occurred in the picric acid treatments, with un-ionized ammonia concentrations ranging from 15 to 449 µg/L in the Puget Sound treatments, and from 0.36 to 7.01 µg/L in the Texas treatments. Higher ammonia levels could be expected in the muddy sediment relative to the sandy, but the concentrations observed in these tests are not explained based on the reference sediments, with un-ionized ammonia concentrations of 0.32 and 0.21 µg/L in the Puget Sound and Texas sediment, respectively. The overlying water in the jars with the control sediment from the amphipod collection site had higher ammonia concentrations, averaging 30.7 µg/L (Appendix B7). The overlying water of the sediments spiked with tetryl and 2,6-DNT presented considerably lower ammonia levels than those with picric acid. Un-ionized ammonia concentrations in the overlying water ranged from 0.3 to 3.4 µg/L, and 0.3 to 2.5, for the tetryl and 2,6-DNT samples with both kinds of sediments, respectively. The un-ionized ammonia

NOEC for overlying water in *A. abdita* tests was established at 400 µg/L (Mueller & Scott, 1995), and, therefore, the only treatment where ammonia could be expected to have slightly influenced toxicity would be the highest picric acid concentration in the Puget Sound sediment, with 449 µg/L of NH_3 (Appendix B7). The ammonia levels in the pore water tended to be considerably higher than in the overlying water, with 243.7 µg/L in the control sediment (Appendix B8), just slightly above the NOEC for *A. abdita* (236 µg/L) (E. Long, NOAA, Seattle, WA, pers. communication). The highest un-ionized ammonia concentration in pore water was for the Puget Sound sample spiked with picric acid, reaching 386.5 µg/L (Appendix B8) in the highest picric acid concentration. This is above the NOEC for *A. abdita*, but far below the LC_{50} of unionized ammonia to *A. abdita* in 96-hour water only tests, of 830 µg/L (Kohn *et al.*, 1994) and therefore is not expected to be solely responsible for the toxicity observed in this sample, particularly if it is considered that the most toxic sample was the intermediate concentration of picric acid, where ammonia levels (167.5 µg/L) were below the NOEC. Water quality measurements in the picric acid test in aqueous phase showed extremely low levels of un-ionized ammonia, ranging from 0.03 to 1.44 µg/L (Appendix B6).

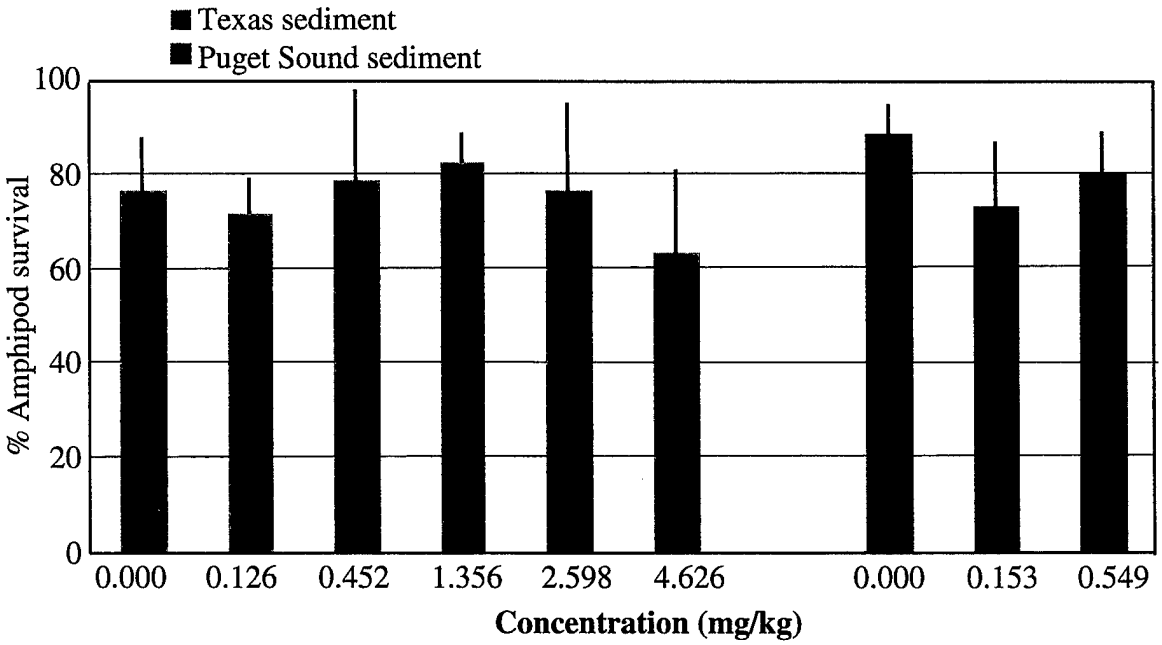


Figure 6: Percent amphipod survival in Texas and Puget Sound sediment spiked with 2,6-DNT. Bars represent standard deviation.

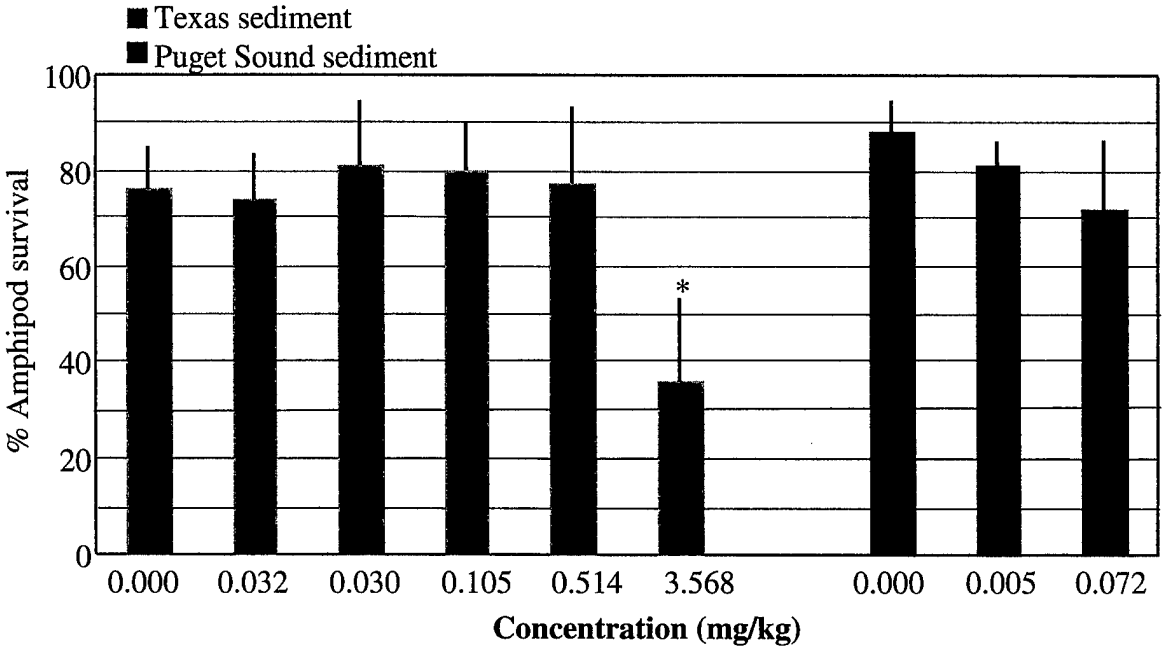


Figure 7: Percent amphipod survival in Texas and Puget Sound sediment spiked with tetryl. Bars represent standard deviation; * indicates significant difference from the control.

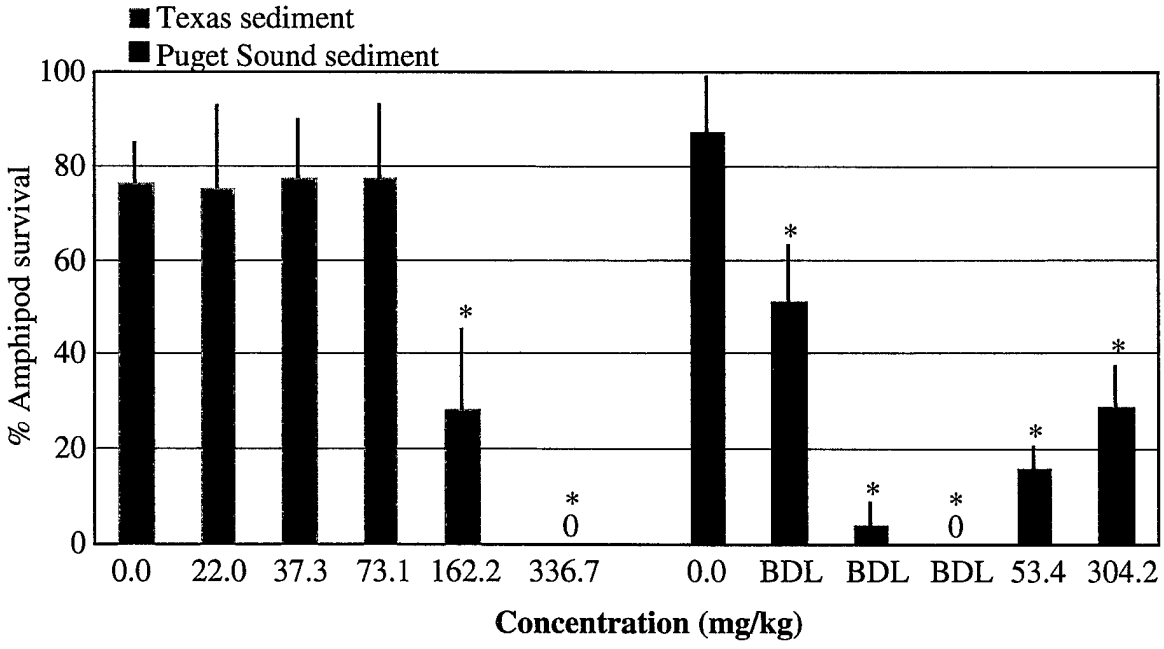


Figure 8: Percent amphipod survival in Texas and Puget Sound sediment spiked with picric acid. Bars represent standard deviation; * indicates significant difference from the control.

PART 3: *Urchin Embryo* (Arbacia punctulata), *Polychaete* (Dinophilus gyrociliatus) *and Macro-algae* (Ulva fasciata) *Toxicity Tests with Pore Water from Spiked Sediments*

Toxicity

Toxicity tests with ordnance compounds in pore water from Puget Sound sediment were more sensitive than the same kinds of tests with ordnance compounds dissolved in filtered seawater (Table 3, Figs. 9 to 11). This suggests that either degradation products of unidentified nature or natural porewater features were responsible for the exhibited toxicity.

Several Puget Sound samples, including the 100 and 50% blanks, were toxic to *A. punctulata* embryos (Appendix C1), probably due to the high levels of un-ionized ammonia in some of those samples (Appendix C2), above the LOEC for *A. punctulata* embryos, of 90 µg/L (Carr *et al.*, 1996). However, the un-ionized ammonia concentration in the Puget Sound porewater samples with 2,6-DNT and picric acid used in the sea urchin test was below toxic levels. The two highest concentrations of the porewater with 2,6-DNT were also very toxic to urchin embryos (Appendix C1), in spite of the very low measured concentrations of this chemical. Dodard et al. (1999) reported that 2,6-DNT was more toxic than some of its degradation products to bacteria and microalgae, but Drzyzga et al. (1995) noted that partial reduction of this compound results in more toxic metabolites, whereas the complete reduction of both nitro groups to amino groups causes detoxification. We suggest that degradation products, including the identified 2-methyl-3-nitroaniline, shown as a large peak at 4.5-minute elution time in the HPLC analysis (Figure 1) and identified by GC-MS, could have caused the toxicity to sea urchin embryos observed in the current study. Smaller peaks also visible on the chromatogram at early elution times (Figure 1) are believed to represent natural non-toxic chemicals present in the seawater, since they routinely appear in filtered seawater and sediment controls.
The two highest concentrations of picric acid in Puget Sound porewater were highly toxic to *A. punctulata* embryos (Appendix C1). Ammonia levels in those samples were below the expected toxic concentration (Appendix C2), but the EC_{50} was still significantly below the picric acid EC_{50} in filtered seawater. Part of this toxic effect could have been caused by unidentified degradation products of picric acid, since a small peak of unknown nature was also observed on the picric acid chromatograms (Figure 4). It is known that phenols with a lower degree of nitration, which can be produced as a result of degradation of picric acid (e.g., 2,4-dinitrophenol) (Rajan *et al.*, 1996; Rieger *et el.*, 1999), are more toxic than the parent compound to a variety of aquatic organisms, including sea urchin embryos (Krahl & Clowes, 1938; Grindley, 1946; Simon & Blackman, 1953; Bringmann & Kuhn, 1959). The PS blank samples were not toxic to any of the *D. gyrociliatus* or *U. fasciata* test endpoints (Appendices D1, E1-E3), indicating that toxicity in the spiked samples could not be attributed exclusively to natural sediment features. However, ammonia concentrations were also high in those samples (Appendix D2, E4) and could have acted as an additional stressor in the polychaete test, as indicated by the results of a toxicity test with ammonia dissolved in filtered seawater (Appendix D1). The EC_{50} values of un-ionized ammonia to *D. gyrociliatus* were 414 (395-434) and 198 (179-219) µg/L for survival and reproduction, respectively, with NOEC and LOEC values of 456.4 and 253.5 µg/L, respectively, for survival, and 253.5 and 131.7 µg/L for the reproduction endpoint (Appendix D1). These values are based on the initial ammonia concentration, since values dropped to approximately half the initial at test termination (Appendix D3). The un-ionized ammonia EC_{50} to *U. fasciata*

zoospore germination is 1650 µg/L and the NOEC is 1530 µg/L (Hooten & Carr, 1998), indicating that stress caused by ammonia should not have been a problem in this test, in spite of the high initial levels in some treatments (Appendix E4).

The macro-alga zoospore toxicity tests with pore water from the Texas sandy sediment spiked with ordnance compounds had similar results to tests with the same compounds in filtered seawater (Table 3). Tetryl and 2,6-DNT in Texas pore water were up to 5-fold less toxic than in filtered seawater in tests with sea urchin embryos and with the polychaete, *D. gyrociliatus* (Table 3). Picric acid in Texas pore water was 2-fold less toxic than in filtered seawater to urchin embryos, but 2-fold more toxic to polychaetes.

Several potential reasons could be pointed out for the mentioned similarities and differences. Tetryl and 2,6-DNT are more hydrophobic than picric acid (solubility in water at 25°C: tetryl = 0.08 g/L; 2,6DNT=0.18 g/L; picric acid=13.1 g/L) (Gorontzy *et al.*, 1994) and, therefore, are expected to have higher affinity and bind more readily to DOC than the latter. This could be responsible for the larger difference in toxicity of 2,6-DNT and tetryl in pore water relative to filtered seawater, since the DOC concentrations in the Texas and Puget Sound pore waters and in MFS were 4.12, 8.12 and 1.54 mg/L, respectively.

An overall assessment shows that tetryl was the most toxic compound to all species in both kinds of pore water, followed by 2,6-DNT and then by picric acid. This corroborates results obtained in tests with filtered seawater (Table 3 – USGS, 1999). The sensitivity ranking of each toxicity test varied not only with the tested ordnance compound but also with the sedimentary origin of the spiked pore water. While the sea urchin embryological development test was the most sensitive only to tetryl in Puget Sound pore water, the polychaete survival and reproduction test was the most sensitive to tetryl and picric acid in Texas pore water, and to 2,6-DNT in Puget Sound pore water. The macro-alga zoospore test was the most sensitive to picric acid in Puget Sound pore water and to 2,6-DNT in Texas pore water. The reasons for this variability are likely to be related to routes of exposure and bioavailability of the ordnance compounds in pore water to each species and life stage. All the EC_{50}, NOEC and LOEC values for the different tests were calculated based on the initial concentration of each chemical in the pore water (Appendix C3, D4, E5), and the duration of the initial exposure, prior to chemical loss by biodegradation and adsorption, would also have influenced the final results and consequent variable sensitivities of each test endpoint. The results of the reference toxicant (SDS) tests conducted concurrently to each toxicity test (Table 3) indicate that the sensitivity of each batch of organisms used in the porewater tests was within the acceptable limits established by our laboratory's control charts.

Water Quality Measurements

Except for ammonia, which was high in a number of porewater samples, as discussed in the previous section, the water quality parameters in all tests were within the acceptable range (Appendices C2, D2 and E4). Sulfide was below detection in all samples of all tests.

The lowest level of dissolved oxygen overall occurred in the sample from Puget Sound spiked with tetryl used in the sea urchin embryological development test, at 73% saturation (Appendix C2). Dissolved oxygen in all other treatments of all tests was above 88% saturation and pH in all porewater samples was in the range of 7.7 ± 0.5.

Table 3. EC_{50} values (mg/L) for all toxicity tests with pore water spiked with ordnance compounds and with the reference toxicant, SDS.

Matrix	Chemical	EC_{50}[1]					
		Urchin	Polychaete		Macro-algae zoospore		
		Embryo	Survival	Eggs/Adult	Germination	Germl. Length	Germl. Cell N°.
TX	2,6-DNT	36.9	21.1	8.16	5.68	3.28	5.14
		(35.3-38.9)	(NR)[2]	(7.43-8.96)	(5.27-6.12)	(2.87-3.74)	(4.63-5.71)
TX	Tetryl	0.27	0.055	0.066	0.82	0.48	0.61
		(0.26-0.27)	(0.05-0.06)	(0.06-0.07)	(0.76-0.88)	(0.44-0.53)	(0.56-0.66)
TX	Picric Acid	592.8	127.5	83.4	574.9	83.1	171.4
		(586-600)	(119-136)	(NR)	(557-592)	(46.2-149.5)	(130-226)
PS	2,6-DNT	>0.029	0.046	0.023	0.092	<0.087	<0.087
		-	(0.045-0.048)	(0.020-0.026)	(NR)	-	-
PS	Tetryl	0.00056	0.0019	0.0019	<0.003	<0.003	<0.003
		(NR)	(NR)	(NR)	-	-	-
PS	Picric Acid	190.2	170.4	64.8	157.2	11.22	47.26
		(185-195)	(164-177)	(54.4-77.2)	(147-168)	(NR)	(NR)
Seawater	SDS	4.07	3.30	3.29	2.66	-	-
		(3.85-4.31)	(3.0-3.6)	(NR)	(2.36-3.01)	-	-
Seawater	SDS[3]	-	3.90	3.47	-	-	-
			(3.7-4.1)	(3.3-3.6)			
Seawater	2,6-DNT	6.7	13	2.1	6.7	2.9	4.2
(from USGS,	Tetryl	0.08	0.06	0.02	0.67	0.34	0.4
1999)	Picric Acid	281	265	155	415	94	118

[1]95% confidence interval in parenthesis; [2] NR = Not reliable; [3] SDS test conducted concurrently to the *D. gyrociliatus* test with PS porewater samples, which were run after the test with Texas samples.

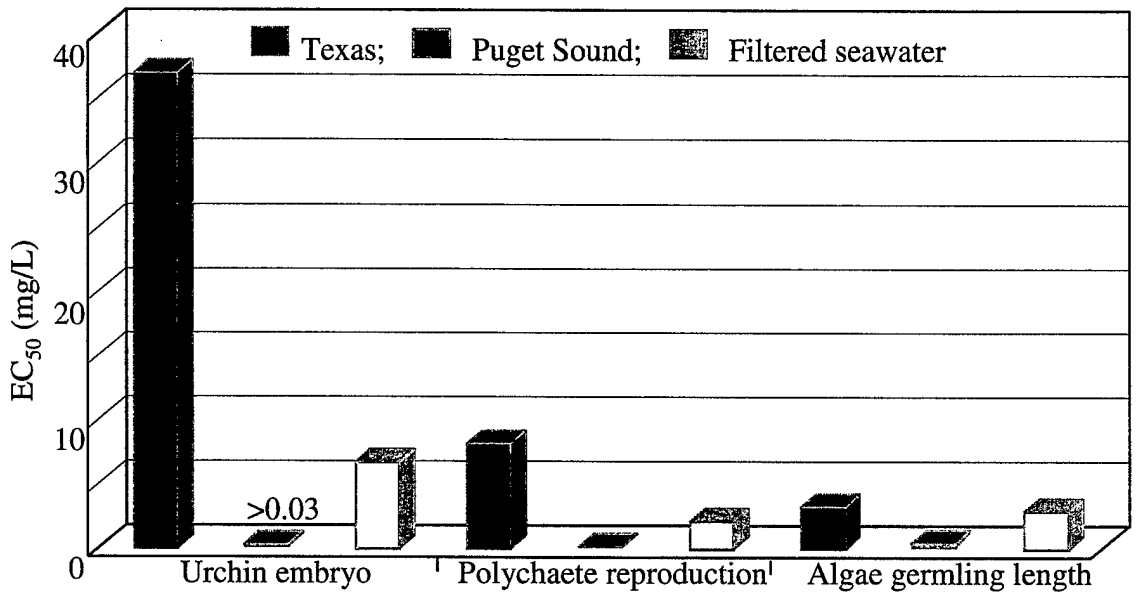


Figure 9: Effects of pore water from sediments spiked with 2,6-DNT on sea urchin *(Arbacia punctulata)* embryological development, polychaete *(Dinophilus gyrociliatus)* egg production, and macro-algae *(Ulva fasciata)* germling growth.

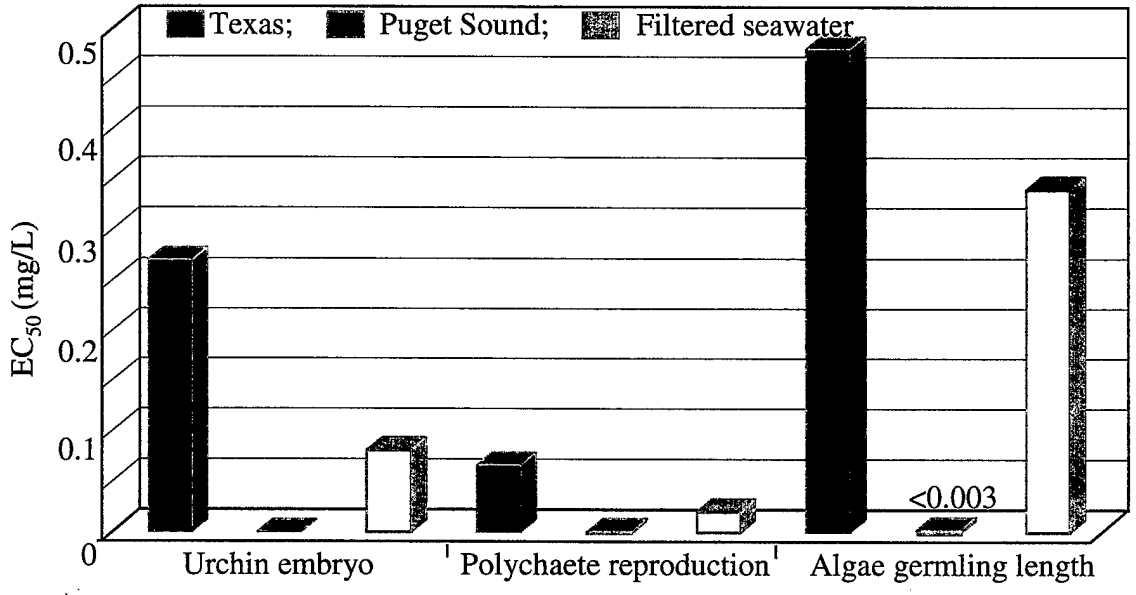


Figure 10: Effects of pore water from sediments spiked with tetryl on sea urchin *(Arbacia punctulata)* embryological development, polychaete *(Dinophilus gyrociliatus)* egg production, and macro-algae *(Ulva fasciata)* germling growth.

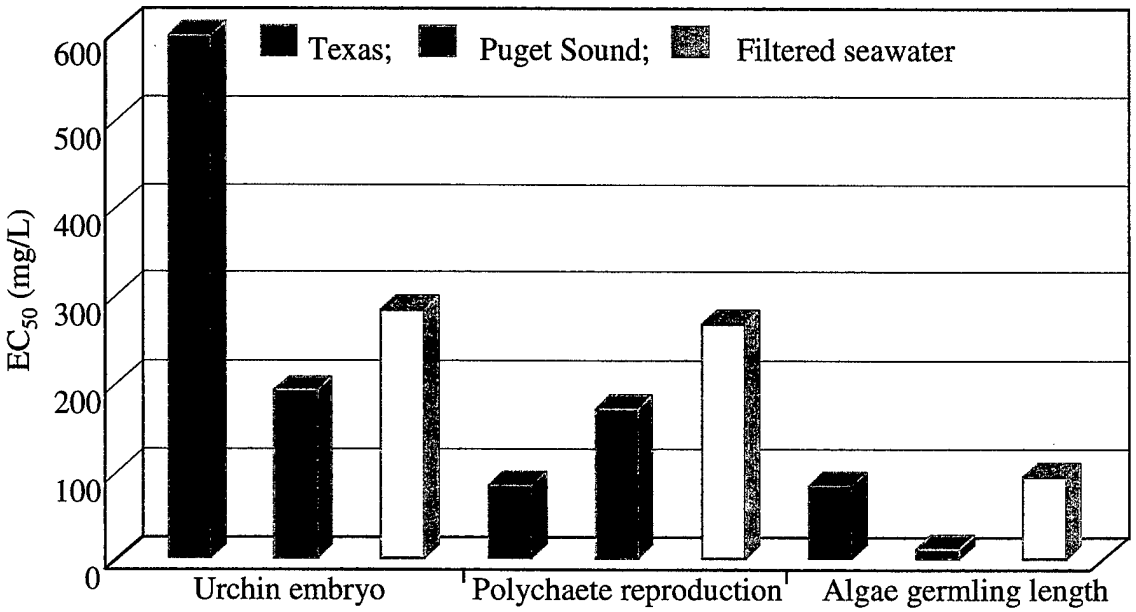


Figure 11: Effects of pore water from sediments spiked with picric acid on sea urchin *(Arbacia punctulata)* embryological development, polychaete (*Dinophilus gyrociliatus*) egg production, and macro-algae (*Ulva fasciata*) germling growth.

Chemical Analyses

Initial chemical measurements conducted with the stock pore water samples with ordnance compounds extracted from Texas and Puget Sound sediments showed that considerably more ordnance compound was released from the sandy Texas sediment into the pore water (Table 4), relative to the fine grain Puget Sound sediment. This was expected based on lower TOC (0.1%) and coarser grain size of the Texas sediment. However, stronger binding would hardly explain the very low concentrations of tetryl and 2,6-DNT in the Puget Sound pore water, suggesting that enhanced biodegradation occurred in the organically rich sediment during the equilibration period. In fact, nearly all 2,6-DNT chromatograms from the urchin, polychaete and algae zoospore test start and end showed a peak of 2-methyl-3-nitroaniline at 4.6 minutes, as also occurred in the amphipod test samples, and this peak was considerably higher in the pore water from Puget Sound samples relative to Texas samples. It also increased with the increase of nominal 2,6-DNT concentrations in the Puget Sound samples, indicating higher amounts of this degradation product. In the tetryl porewater samples from both Puget Sound and Texas, however, no degradation products were identified, either when measured using the standard USEPA 8330 procedure (1994) or the modified picric acid method. In the Puget Sound pore water with picric acid a small peak was identified at 4.6 minutes elution time, in addition to the picric acid peak at 11.1 minutes. No such peak at 4.6 minutes was identified in any of the Texas samples from the sea urchin test.

Table 4. Highest concentration (porewater stock) of 2,6-DNT, tetryl and picric acid in pore water extracted from Puget Sound and Texas spiked sediments, and measured concentrations of ordnance compound stock solutions used to spike the sediments.

Matrix	Chemical	Concentration in Ordnance Stock Spiking Solution (mg/L)	Concentration in Pore Water (mg/L)
TX	2,6-DNT	110	70
TX	Tetryl	43	14
TX	Picric Acid	1,067	825
PS	2,6-DNT	110	0.165
PS	Tetryl	43	0.017
PS	Picric Acid	1,067	511

In spite of the unidentified and one identified peak which are likely to be degradation products, chemical measurements in each test treatment at test start and end indicated that picric acid was stable and 2,6-DNT was also relatively stable in all tests systems and samples, regardless of the presence of organisms (Appendices C3, D4, E5). Of the three ordnance compounds, the highest losses in pore water were observed with tetryl.

In the sea urchin test, all the tetryl was lost from all Puget Sound treatments with and without organisms, whereas in the Texas treatments higher losses occurred in the vials with organisms (Appendix C3). In the polychaete tests all tetryl was lost from the vials with organisms in pore water from both Puget Sound and Texas, but some was left in the vials without organisms (Appendix D4), and similar results were exhibited in the macro-alga zoospore test (Appendix E5).

These data suggest that adsorption to living surfaces is likely to be at least part of the cause of tetryl loss in porewater tests.

CONCLUSIONS

- The three analyzed ordnance compounds, 2,6-DNT, tetryl and picric acid behaved differently in the sandy and muddy sediment with low and high TOC, respectively.
- The three ordnance compounds degraded more rapidly in the fine grain Puget Sound sediment with high organic carbon.
- Nearly all Puget Sound sediment and porewater samples spiked with ordnance compounds were more toxic than the sandy Texas samples and the seawater only containing the same compounds. This could be due to unidentified and one identified (2-methyl-3-nitroaniline) microbial degradation products.

- Nearly all Texas porewater samples were less toxic than filtered seawater spiked with the same ordnance compounds, possibly due to sorption to organic carbon.

- The data suggests that it is not sufficient to look at known and expected ordnance compounds in sediments. Degradation products can play a major role in sediment toxicity and consequent effects to the benthic biota. However, degradation may proceed through mineralization given appropriate conditions and sufficient time, although this aspect was not addressed in the present research. In order to fully evaluate the potential impacts of ordnance compounds to benthic organisms, further studies should be conducted for the assessment of the persistence and biological effects of degradation products of ordnance compounds in marine sediments.

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APPENDICES A-E

Appendix A. Results of Phase II TIE study with contaminated sediments from Puget Sound, WA.

Appendix B. Complete data set for sediment toxicity, chemistry and water quality in tests with the amphipod *Ampelisca abdita*.

Appendix C. Complete data set for porewater toxicity, chemistry and water quality in embryological development tests with the sea urchin *Arbacia punctulata*.

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Appendix A

Results of Phase II TIE study with contaminated sediments from Puget Sound, WA

INTRODUCTION

Previous surveys have shown that sediments in the vicinity of Naval facilities in Puget Sound, Washington were contaminated with ordnance compounds, originating from past use, storage, improper disposal, and incineration of these compounds. It is not possible, however, to predict if sediment samples will be toxic on the basis of analytical chemistry information alone. Toxicity tests are recognized as effective tools to determine the biological significance of contamination found in coastal sediments. The contaminants responsible for any observed toxicity can be determined using toxicity identification evaluation (TIE) procedures. A toxicity survey was used to select sites for comprehensive chemical analyses. The combined toxicity and chemistry data were used to select the sites for the TIE studies.

For the sediment assessment survey, surficial sediments were collected from 52 sites in Puget Sound. This included 25 stations in the vicinity of Jackson Park and 25 in the vicinity of Port Hadlock Naval Facilities, and 2 stations in Sequim Bay (SQ1 and SQ2), which was pre-selected as a reference site (Carr and Nipper, 1999). Sediments were analyzed for porewater toxicity using the sea urchin, *Arbacia punctulata*, fertilization and embryological development tests. The most toxic sediments were characterized chemically. Based on the combined results of the chemical analyses and toxicity tests, three stations adjacent to each other at the Jackson Park site, were selected for a combined TIE study. Sediment from the three sites was sampled and combined prior to the application of TIE procedures. The results of the phase I TIE studies were reported previously (Carr and Nipper, 1999). The results of the phase II TIE studies are reported in this appendix.

MATERIALS AND METHODS

Sediment Porewater Extraction Procedure

Pore water was extracted from the sediments using a pneumatic extraction apparatus (Carr and Chapman, 1995). This extractor is made of polyvinyl chloride (PVC) and uses a 5 µm polyester filter. It is the same device used in previous sediment quality assessment surveys (Carr and Chapman, 1992, 1995; Carr *et al.*, 1996a, 1996b, 1996c, 2000; Carr and Nipper, 1999). After extraction, the porewater samples were centrifuged in polycarbonate bottles at 1200 x *g* for 20 min to remove any suspended particulate material; the supernatant was collected and frozen at -20°C. The pore water was stored frozen until just prior to testing, when water quality parameters were measured and adjusted, if necessary.

Two days before conducting a toxicity test, the samples were moved from the freezer to a refrigerator at 4°C. One day prior to testing, samples were thawed in a tepid (20°C) water bath. Temperature of the samples was maintained at 20 ± 1°C. Sample salinity was measured and adjusted to 30 ± 1 $^{o}/_{oo}$, if necessary, using purified deionized water or concentrated brine at 122$^{o}/_{oo}$ salinity with 10% reference porewater from Redfish Bay, Texas, added. Other water quality measurements (dissolved oxygen, pH, sulfide and ammonia concentrations) were made. Temperature and dissolved oxygen (DO) were measured with YSI® meters; salinity was measured with a Reichert® or American Optical® refractometer; and pH, sulfide (as S^{-2}), and total ammonia (expressed as nitrogen; NH_4) were measured with Orion® meters and their respective

probes. Unionized ammonia concentrations (expressed as nitrogen; NH_3) were calculated for each sample using the respective salinity, temperature, pH, and NH_4 values. Following water quality measurements and adjustments, the samples were stored overnight at 4°C but returned to 20 ± 1°C before the start of the toxicity tests.

Toxicity Tests

Toxicity of the sediment pore water was determined using the sea urchin fertilization and embryological development tests with *A. punctulata*, following the procedures described previously (Carr et al., 1996a, 1996b, Carr and Nipper, 1999). *Arbacia punctulata* urchins were obtained from Gulf Specimen Company, Inc. (Panacea, Florida). Each porewater sample or reconstituted fraction was tested in a dilution series design at 100, 50, and 25% of the water quality adjusted sample with 5 replicates per treatment. Dilutions were made with 0.45 µm Millipore® filtered seawater (MFS). A reference porewater sample collected from Redfish Bay, Texas, which had been handled identically to the test samples, was included with each toxicity test as a negative control. This site is far removed from any known sources of contamination and has been used for more than a decade as a reference site. In addition, dilution blanks of MFS and brine controls (purified deionized water with brine added to reach a 30 $^{o}/_{oo}$ salinity), were also included. The brine control had the objective of identifying any possible adverse effects caused by the brine. A dilution series test with sodium dodecyl sulfate (SDS) was included as a positive control and results were compared to the respective control charts.

Toxicity Identification Evaluation (TIE) Procedures

Phase I

Based on the results of the toxicity tests and of chemical analyses in the initial survey, three of the most toxic stations, which were located in Ostrich Bay and adjacent to each other, were selected for the TIE procedure. Seven gallons of sediment from each station were collected in August 1998, composited, and shipped to the U. S. Geological Survey (USGS) Marine Ecotoxicology Research Station (MERS) in Corpus Christi, Texas. Pore water was extracted from this sediment composite upon arrival to the laboratory, and processed as described below.

The sea urchin fertilization and embryological development tests were used with the TIE procedure. Initially, the toxicity of a frozen and a fresh porewater sample was compared. Since no significant difference in toxicity was detected between the samples, frozen pore water was used for the TIE procedure and treated as described in the previous sections.

Baseline toxicity of the sample was assessed. Phase I TIE treatments were applied following the USEPA protocol (Burgess *et al.*, 1996) and consisted of:

1) Aeration, for the assessment of the contribution of volatile chemicals to the toxicity;
2) Filtration, for the assessment of the contribution of particulate material to the toxicity;
3) C_{18} column, for the assessment of the contribution of organic chemicals to the toxicity;
4) EDTA addition, for the assessment of the contribution of metals to the toxicity;
5) Sodium thiosulfate addition, for the assessment of the contribution of oxidants to the toxicity;
6) pH increase and reduction, for the assessment of the contribution of ammonia to the toxicity.

The results of these phase I studies have been reported elsewhere (Carr and Nipper, 1999).

Phase II

Phase II organic extraction and fractionation was performed on the Ostrich Bay porewater sample utilizing J. T. Baker, Bakerbond Speedisks™ C_{18}, a Speedisk™ remote sample adapter and a vacuum pump attached to a 2 L Erlenmeyer vacuum flask.

Extraction

Two Speedisks™ were utilized to extract five liters of porewater (2.5 liters each). Each Speedisk™ was attached to the vacuum flask using a silicone rubber stopper adapter. The Speedisk™ was preconditioned with two 10 ml aliquots of HPLC grade methanol. The disk was pulled under vacuum at a flow rate of 100-150 ml/minute until 3 to 5 mm of solvent was left covering the disk. Before the porewater sample was added, the disk was rinsed with two 10 ml aliquots of MilliQ® purified water drawn down under vacuum until 3-5 mm of water remained on the disk. The disk was not allowed to dry out between conditioning and sample addition. Sample was added to the disk to bring the level to the top of the reservoir and a (methanol/DI rinsed) remote sample adaptor attached to the top of the reservoir. The Teflon® tubing from the adaptor was clipped to the side of a 4 L beaker containing 2.5 L of the sample pore water. The sample was drawn up the tubing into the reservoir under vacuum through the Speedisk™ at a flow rate of 100-150 ml/min. After the entire volume had been passed through the Speedisk™, the disk was dried under full vacuum for 5 minutes.

Fractionation

Fractions of organics were eluted off the Speedisks™ using the same vacuum system modified to collect the fractions into precleaned 20 ml glass vials. Eight methanol/water fractions were eluted off each Speedisk™. Percent methanol fractions were as follows: 25, 50, 75, 80, 85, 90, 95, and 100%. The non-methanol component of the fractions consisted of 0.45 µm filtered seawater. Elution was performed by adding a 3.5 ml volume of the elution fraction to the disk and allowing a 1 minute soak before collecting the fraction under vacuum into a precleaned glass vial until the Speedisk™ was dry. The procedure was repeated with the same fraction and collected in the same vial for a total volume of 7 ml for each fraction. This procedure was repeated for each methanol/water fraction beginning with 25% methanol and continuing through all the fractions in increasing methanol concentrations. Both Speedisks™ were eluted in the same manner and fractions of the same methanol/water concentration eluted from each disk were combined in a single vial and capped with a Teflon lined cap and refrigerated until toxicity testing. Elution blanks were prepared in the same manner as described above using a separate preconditioned and rinsed Speedisk™.

Phase II Toxicity Testing

Blank and sample fractions were tested for toxicity using the sea urchin fertilization test. Five replicates, each containing 5 ml of dilution water (0.45 µm filtered seawater) injected with

75 µl of each blank or sample fraction were tested. Comparisons were made between sample fractions and blanks of the same methanol/water concentrations using paired t-tests.

Several contaminants were identified in the initial phase I chemical characterization of the composite porewater sample for which no toxicity information was available for the sea urchin tests. Therefore, toxicity tests were conducted for tributyltin (TBT, bis (tributyltin oxide, 96% purity, Sigma-Aldrich Chemical Co.), dibutyltin (DBT, dibutyltin oxide, 98% purity, Sigma-Aldrich Chemical Co.), and arsenic (arsenic (III) oxide, AS_2O_3, 99% purity, Sigma-Aldrich Chemical Co.). The low solubility of these chemicals in seawater required the use of carrier solvents to prepare the stock solutions for the toxicity tests. Methanol was used as the carrier for TBT, acetic acid for DBT, and HCl for arsenic. Appropriate carrier solvent controls were included with each test series which were run in a 50% serial dilution test design.

Chemical Analyses

Chemical analyses of the sediment, porewater, and fractions were performed by Columbia Analytical Services Inc., (CAS), Kelso, Washington. Aliquots of the exposure media for each dilution for the tests with TBT, DBT, and arsenic were also analyzed by CAS by the methods described below. Samples were shipped to CAS on dry ice with chain of custody forms for analyses. Chemical analyses included a suite of ordnance compounds, trace metals, polycyclic aromatic hydrocarbons (PAHs), organochlorinated pesticides, polychlorinated biphenyls (PCBs), and butyltins, as well as particle size distribution. Ordnance compounds were measured by HPLC using Method 8330 (U.S. EPA SW846,1996), and trace metals were measured by ICP/MS using EPA Methods 200.8 (U.S EPA,1993) except for mercury which was measured by CVAA using EPA Method 7471 (U.S. EPA SW846, 1993). PAHs were measured by GC/MS using GC/MS selected ion monitoring as developed by CAS. PCBs were measured by EPA Method 8082 (U.S. EPA SW846,1996) using GC/ECD. Organochlorinated pesticides were measured using method 8081A (U.S. EPA SW846, 1996). Butyltins were measured by GC/FPD using the Columbia Analytical Protocol. Particle size distribution was analyzed by method PS-PSEP, modified (PSEP, 1986).

Data Analysis

The EC_{50} values for dilution series toxicity tests was calculated by the Trimmed Spearman-Karber method (Hamilton *et al.*, 1978) with Abbot's correction (Morgan, 1992). Statistical comparisons between each treatment and the reference pore water were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989). Prior to statistical analysis, the transformed data sets were screened for outliers (SAS, 1992). Outliers were detected by comparing the studentized residuals to a critical value from a *t*-distribution chosen using a Bonferroni-type adjustment. The adjustment is based on the number of observations, *n*, so that the overall probability of a type I error is at most 5%. The critical value, *cv*, is given by the following equation: $cv = t(df_{Error}, .05/(2 \times n))$. After omitting outliers but prior to further analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). Dunnett's one-tailed *t*-test was also used to determine lowest-observed-effect concentrations (LOECs) and no-observed-effect concentrations (NOECs)

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

RESULTS AND DISCUSSION

Toxicity Identification Evaluation - Phase II

Column fractions between 25 to 100% methanol were eluted from the SpeedDisk™ for the Ostrich Bay sample and aliquots of eluant were redissolved in seawater for testing. The 80, 85, 90, and 95% methanol fractions were toxic with the 85 and 90% fractions exhibiting the highest toxicity in the sea urchin fertilization test (Table 1). These toxic fractions were analyzed by CAS for the suite of contaminants analyzed previously in the original porewater sample. Apart from a few PAHs, which were observed in the low µg/L range in the concentrated eluant, the only other substances above the detection limits were phthalates (primarily di-n-butyl phthalate), which most likely leached from the pre-washed plastic syringes used in the TIE procedure. The highest concentration of phthalates was observed in the 75% methanol fraction (2000 µg/L) which was not toxic thereby demonstrating that phthalates were not responsible for the observed toxicity.

Several metals and organotin compounds were detected in the original TIE porewater sample from phase I for which no toxicity data was available for the sea urchin assays from the literature. A summary of the results of tests performed in our laboratory with these contaminants is provided in Table 2. A comparison of these data with the concentration of these chemicals in the porewater sample (Table 3) indicates that they could not have contributed significantly to the observed toxicity. None of the other metals or PAHs measured in the porewater sample were present at concentrations near the known toxic concentrations for the sea urchin assays (Carr et al., 1996). As the toxicity in phase I tests was reduced by the EDTA and C_{18} column treatments, it appears that the observed toxicity in the sea urchin fertilization test, particularly, is due primarily to some unmeasured organic or organo-metallic compounds. In addition to these unmeasured toxicants, ammonia was also a primary contributing factor to the observed toxicity for the embryological development test.

CONCLUSIONS

- No ordnance compounds were detected in the porewater sample used for the TIE study which indicates that explosives of concern in this study were not responsible for the toxicity observed in this composite sample from the most toxic stations.

- The phase I TIE procedures indicated that organic chemicals (PAHs, PCBs, pesticides), and metals to a smaller extent, were the likely causative agents of the toxicity observed in the sea urchin fertilization test.

- The phase I TIE procedures indicated that several classes of chemicals, including organic chemicals (PAHs, PCBs, pesticides), metals and ammonia, were the likely causative agents of the toxic effect in the sea urchin embryological development test.

- Phase II TIE studies indicated that fractions eluted with 80-95% methanol were toxic but no contaminants of concern were identified in these samples.

- The specific contaminants responsible for the observed toxicity were not identified in this phase II study but the compounds in the comprehensive list of analytes which were detected in the porewater sample were not present at high enough concentrations to be implicated.

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Table 1. Toxicity data for sea urchin, *A. punctulata*, fertilization test following phase II TIE procedures.

Treatment	Sample	% Dilution	Mean % Fertilized	Diff.[d]
Baseline	OB[a]	100	22.4	
Baseline	OB	50	40.4	
Baseline	OB	25	74.8	
Baseline	OB	12.5	94	
Baseline	OB	6.25	95.2	
Baseline	REF[b]	100	87.8	
Baseline	REF	50	91.6	
Baseline	REF	25	93	
Baseline	REF	12.5	95.8	
Baseline	REF	6.25	93.8	
Baseline	MFS[c]	100	89.7	
Aeration	OB	100	16.8	
Aeration	OB	50	27.6	
Aeration	OB	25	73.2	
Aeration	MFS	100	83.8	
Filtration	OB	100	30	
Filtration	OB	50	42.6	
Filtration	OB	25	81	
Filtration	MFS	100	83.4	
C18	OB	100	56	**
C18	OB	50	73.8	**
C18	OB	25	89.2	**
C18	MFS	100	84.2	
EDTA	OB	100	44.6	*
EDTA	OB	50	42.6	
EDTA	OB	25	81	
EDTA	MFS	100	93.8	
Na thiosulfate	OB	100	32.8	
Na thiosulfate	OB	50	32	
Na thiosulfate	OB	25	73.8	
Na thiosulfate	MFS	100	91.6	
pH 7.2	OB	100	25	
pH 7.2	OB	50	26	
pH 7.2	OB	25	39.2	**
pH 7.2	REF	100	60.2	
pH 7.2	REF	50	84	
pH 7.2	REF	25	82.4	
pH 7.2	MFS	100	85.8	

Table 1. continued

Treatment	Sample	% Dilution	Mean % Fertilized	Diff.[d]
pH 8.0	OB	100	23	
pH 8.0	OB	50	29.2	
pH 8.0	OB	25	67.2	
pH 8.0	REF	100	38	
pH 8.0	REF	50	84.6	
pH 8.0	REF	25	90.4	
pH 8.0	MFS	100	92.8	
pH 9.0	OB	100	0	**
pH 9.0	OB	50	0	**
pH 9.0	OB	25	13.2	**
pH 9.0	REF	100	11.8	
pH 9.0	REF	50	59.4	
pH 9.0	REF	25	75.6	
pH 9.0	MFS	100	89.4	

[a] Pore water from site selected for TIE, from Ostrich Bay
[b] Reference pore water, from Redfish Bay, Texas
[c] Millipore filtered seawater
[d] Significantly different from Ostrich Bay baseline toxicity, * indicates significant difference at alpha $\leq$ 0.05 and ** indicates significant difference at alpha $\leq$ 0.01.

Table 2. Toxicity test results for sea urchin, *Arbacia punctulata*, fertilization and embryological development tests with tributyltin, dibutyltin, and arsenic.

	Fertilization (µg/L)			Embryological Development (µg/L)		
Chemical	**NOEC**	**LOEC**	**EC_{50}**	**NOEC**	**LOEC**	**EC_{50}**
Tributyltin	1.4	2.7	5.47	1.4	2.7	1.76
Dibutyltin	15	24	>24	24	44	32.3
Arsenic	2110	NC[a]	NC	2110	NC	NC

[a] Not calculable.

Table 3. Summary of chemical measurements in fresh and frozen pore water from Ostrich Bay, WA.

Chemical	Concentration in frozen and fresh pore water (µg/L)	
	Frozen	**Fresh**
Ordnance Compounds	ND[a]	ND[a]
Butyltins	0.118	0.120
Polychlorinated Biphenyls (PCBs)	ND[a]	ND[a]
Polyaromatic Hydrocarbons (PAHs) above detection limit		
Phenol	1.5	1.5
Naphthalene	0.03	0.03
Diethyl Phthalate	0.2	0.2
Phenanthrene	0.05	0.05
Di-n-butyl Phthalate	4.3	4.4
Butyl Benzyl Phthalate	0.06	0.05
Bis(2-ethylhexyl) Phthalate	3	0.2
Indeno(1,2,3-cd)pyrene	0.07	0
Dibenz(a,h)anthracene	0.08	0
Benzo(g,h,i)perylene	0.07	0
Metals		
Arsenic	4.3	8.0
Cadmium	0.04	0.08
Chromium	ND	0.6
Copper	0.2	0.3
Lead	0.06	0.14
Zinc	0.6	1.1

[a] Not detectable.

Appendix B

Complete data set for sediment toxicity, chemistry and water quality in tests with the amphipod *Ampelisca abdita*

Appendix B1. Mass balance of ordnance compounds based on measured concentrations in the sediment (mg/kg dry weight) and in the porewater (PW) (mg/L), and on sediment moisture content at amphipod test start.

Sediment	Chemical	Conc. #	Moisture (%)	Measured Conc.		Ordnance in 1g dry sed.	
				Sediment	PW	Sediment (mg)	PW Bound (mg)
TX[1]	2,6-DNT	1	27.3	0.126	0.082	0.104	0.022
TX	2,6-DNT	2	26.7	0.452	0.656	0.277	0.175
TX	2,6-DNT	3	27.4	1.356	5.599	-0.181	1.537
TX	2,6-DNT	4	27.9	2.598	19.239	-2.779	5.377
TX	2,6-DNT	5	27.1	4.626	57.464	-10.952	15.578
TX	Tetryl	1	29.2	0.032	0.000 [3]	0.032	0.000
TX	Tetryl	2	27.1	0.030	0.000	0.030	0.000
TX	Tetryl	3	26.9	0.105	0.010	0.102	0.003
TX	Tetryl	4	28.3	0.514	0.176	0.464	0.050
TX	Tetryl	5	28.6	3.568	6.195	1.796	1.772
TX	Picric Acid	1	29.1	22.045	43.163	9.501	12.544
TX	Picric Acid	2	28.5	37.278	89.877	11.658	25.620
TX	Picric Acid	3	29.8	73.065	124.88	35.827	37.238
TX	Picric Acid	4	28.3	162.22	293.46	79.155	83.067
TX	Picric Acid	5	29.7	336.70	595.74	159.65	177.05
PS[2]	2,6-DNT	1	45.3	0.153	0.000	0.153	0.000
PS	2,6-DNT	2	43.2	0.549	0.000	0.549	0.000
PS	Tetryl	1	42.0	0.005	0.000	0.005	0.000
PS	Tetryl	2	42.7	0.072	0.060	0.046	0.026
PS	Picric Acid	1	46.0	0.000	0.000	0.000	0.000
PS	Picric Acid	2	45.7	0.000	0.000	0.000	0.000
PS	Picric Acid	3	45.5	0.000	0.000	0.000	0.000
PS	Picric Acid	4	44.8	53.399	51.445	30.374	23.025
PS	Picric Acid	5	46.8	304.25	245.56	189.42	114.83

[1] TX= Texas sandy sediment; [2] PS = Puget Sound ,WA, muddy sediment;

[3] Zero values = below detection limit, but represented as zero for means of calculations.

Appendix B2. Mass balance of ordnance compounds based on measured concentrations in the sediment (mg/kg dry weight) and in the porewater (PW) (mg/L), and on sediment moisture content at amphipod test end.

Sediment	Chemical	Conc. No.	Moisture (%)	Measured Conc.			Ordnance in 1g dry sed.	
				Sediment	PW	OW[1]	Sediment (mg)	PW Bound (mg)
TX[2]	2,6-DNT	1	26.9	0.011	0.000[4]	0.002	0.011	0.000
TX	2,6-DNT	2	27.5	0.021	0.006	0.047	0.019	0.002
TX	2,6-DNT	3	27.6	0.080	0.048	0.339	0.067	0.013
TX	2,6-DNT	4	24.9	0.300	0.952	1.349	0.063	0.237
TX	2,6-DNT	5	24.5	0.827	8.578	3.894	-1.278	2.105
TX	Tetryl	1	25.1	0.000	0.000	0.000	0.000	0.000
TX	Tetryl	2	25.9	0.000	0.000	0.000	0.000	0.000
TX	Tetryl	3	26.1	0.036	0.000	0.003	0.036	0.000
TX	Tetryl	4	27.7	0.063	0.035	0.008	0.053	0.010
TX	Tetryl	5	28.0	0.163	0.127	0.025	0.127	0.036
TX	Picric Acid	1	24.6	2.022	7.610	3.024	0.153	1.869
TX	Picric Acid	2	29.4	5.065	10.940	6.378	1.846	3.219
TX	Picric Acid	3	24.6	7.053	17.259	8.510	2.815	4.238
TX	Picric Acid	4	26.2	24.946	69.614	26.150	6.682	18.264
TX	Picric Acid	5	27.5	58.130	151.719	55.698	16.457	41.673
PS[3]	2,6-DNT	1	46.3	0.039	0.000	0.000	0.039	0.000
PS	2,6-DNT	2	43.0	0.134	0.000	0.000	0.134	0.000
PS	Tetryl	1	44.3	0.000	0.001	0.002	0.000	0.000
PS	Tetryl	2	45.2	0.000	0.003	0.002	-0.001	0.001
PS	Picric Acid	1	46.1	0.000	0.000	0.000	0.000	0.000
PS	Picric Acid	2	46.5	0.000	0.000	0.000	0.000	0.000
PS	Picric Acid	3	46.5	0.000	0.000	0.000	0.000	0.000
PS	Picric Acid	4	44.0	0.000	0.000	0.000	0.000	0.000
PS	Picric Acid	5	45.5	3.406	3.588	20.596	1.774	1.632

[1] OW = overlying water;

[2] TX= Texas sandy sediment; [3] PS = Puget Sound ,WA, muddy sediment;

[4] Zero values = below detection limit, but represented as zero for means of calculations.

Appendix B3. Amphipod, *Ampelisca abdita*, toxicity test results, with significant differences and LC_{50} values based on initial sediment concentration (95% confidence interval in parenthesis).

Sedim.	Chemical	Initial Sed. Concentr. (mg/kg)	% Amphipod Survival Replicate No. 1	2	3	4	5	Mean % Surv.	St. Dev.	Signif Diff. ($\alpha \leq 0.01$)	LC_{50} (mg/kg dry wt. in sed.)	LC_{50} (mg/L in pore water)
TX[1]	2,6-DNT	0.126	75	75	65	65	75	71	5.5		>4.626	>57.464
TX	2,6-DNT	0.452	65	65	100	75	85	78	14.8			
TX	2,6-DNT	1.356	90	80	80	80	80	82	4.5			
TX	2,6-DNT	2.598	75	55	75	80	95	76	14.3			
TX	2,6-DNT	4.626	50	55	75	55	80	63	13.5			
TX	Tetryl	0.032	85	65	65	80	75	74	8.9		3.2	5.20
TX	Tetryl	0.030	80	80	65	80	100	81	12.4		(2.77-3.79)	(3.90-6.92)
TX	Tetryl	0.105	70	85	70	85	90	80	9.4			
TX	Tetryl	0.514	80	55	85	70	95	77	15.2			
TX	Tetryl	3.568	10	35	50	35	50	36	16.4	**		
TX	Picric Acid	22.04	50	70	90	90	75	75	16.6		144.2	255.20
TX	Picric Acid	37.28	90	65	70	70	90	77	12.0		(140-149)	(247-264)
TX	Picric Acid	73.06	80	60	70	75	100	77	14.8			
TX	Picric Acid	162.22	15	15	20	50	40	28	16.0	**		
TX	Picric Acid	336.70	0	0	0	0	0	0	0.0	**		
PS[2]	2,6-DNT	0.153	75	60	60	80	90	73	13.0		>0.549	-
PS	2,6-DNT	0.549	75	90	85	70	80	80	7.9			
PS	Tetryl	0.005	80	80	75	85	85	81	4.2		>0.072	-
PS	Tetryl	0.072	55	60	80	85	80	72	13.5			
PS	Picric Acid	BDL	55	55	45	35	65	51	11.4	**	<53.4	<51.4
PS	Picric Acid	BDL	0	10	0	5	5	4	4.2	**		
PS	Picric Acid	BDL	0	0	0	0	0	0	0.0	**		
PS	Picric Acid	53.40	15	15	10	20	20	16	4.2	**		
PS	Picric Acid	304.25	20	25	40	35	25	29	8.2	**		
Control	Not spiked	BDL	70	95	75	90	85	83	10.4			
Control[3]	Not spiked	BDL	90	95	80	90	90	89	5.5			
TX	Not spiked	BDL	80	80	85	70	65	76	8.2			
PS	Not spiked	BDL	90	90	95	80	85	88	5.7			
PS[3]	Not spiked	BDL	100	90	85	70	90	87	11.0			

[1] TX= Texas sandy sediment; [2] PS = Puget Sound ,WA, muddy sediment; [3] Control and reference (PS) data for test conducted concurrently with picric acid test in PS sediment.

Appendix B4. Chemistry and toxicity data from *Ampelisca abdita* toxicity tests with picric acid and SDS in aqueous phase. Concentrations of picric acid were measured at test initiation and termination; concentrations of SDS are nominal. SDS test 2 was concurrent to picric acid test with spiked sediment from Puget Sound. SDS test 1 was concurrent to all other tests with *A. abdita*.

Sample	Concentration (mg/L)		% amphipod survival					96-h LC_{50}[3] (mg/l)	Sign. Diff. ($\alpha \leq 0.01$)
	Test Start	Test End	Replicate No. 1	2	3	4	5		
Control (MFS)[1]	0.0	0.0	60	50	60	70	80		
	3.3	3.4	80	70	80	50	60		
Picric	6.2	6.1	70	50	60	70	80	30.16	
Acid	12.5	12.2	50	70	50	70	70	(27.72-32.81)	
	24.8	24.5	40	70	30	70	50		
	49.7	47.9	0	0	0	0	0		**
	1.2	NM[2]	30	60	70	60	80		
	2.5	NM	50	50	60	70	80	5.99	
SDS	5.0	NM	60	30	40	80	20	(5.39-6.65)	
Test 1	10.0	NM	0	10	0	10	0		
	20.0	NM	0	0	0	0	0		
	0.0	0.0	100	90	100	100	80		
	1.2	NM	100	80	90	80	100		
SDS	2.5	NM	90	90	70	90	100	5.08	
Test 2	5.0	NM	70	50	50	60	30	(4.66-5.53)	
	10.0	NM	0	0	0	10	0		
	20.0	NM	0	0	0	0	0		

[1]MFS = Millipore filtered seawater

[2]NM = Not measured

[3]95% confidence interval in parenthesis

Appendix B5. Overlying water quality measurements at amphipod test termination.

Sediment	Chemical	Sediment Conc. at Test Start	Replicate	Salinity (ppt)	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)
TX	2,6-DNT	0.97	1	31	7.12	95.4	7.88	0.008	0.20
			2	30	7.18	95.9	7.89	0.006	0.16
			3	29	6.93	93.1	7.91	0.005	0.12
			4	31	6.86	91.8	7.92	0.010	0.27
			5	30	6.73	90.2	7.92	0.028	0.73
TX	2,6-DNT	3.2	1	30	7.07	94.9	7.87	0.008	0.19
			2	31	6.97	93.6	7.89	0.007	0.17
			3	30	7.03	94.1	7.82	0.015	0.30
			4	30	6.75	91.0	7.91	0.026	0.65
			5	30	6.86	91.9	7.93	0.033	0.88
TX	2,6-DNT	9.8	1	30	7.22	96.5	7.85	0.052	1.17
			2	29	6.97	93.5	7.87	0.038	0.89
			3	30	6.99	94.3	7.85	0.051	1.14
			4	30	7.03	94.0	7.92	0.044	1.15
			5	32	6.86	91.9	7.90	0.047	1.18
TX	2,6-DNT	18.7	1	30	7.12	95.5	8.20	0.072	3.51
			2	31	7.13	95.7	7.87	0.082	1.91
			3	29	6.96	93.5	7.86	0.098	2.24
			4	32	6.69	89.6	7.93	0.063	1.68
			5	30	6.85	92.1	7.94	0.119	3.26
TX	2,6-DNT	34.0	1	31	7.05	94.4	7.89	0.157	3.85
			2	32	7.16	96.1	7.85	0.093	2.08
			3	32	6.82	91.5	7.91	0.063	1.61
			4	30	6.86	92.1	7.90	0.059	1.48
			5	31	6.95	93.1	7.91	0.054	1.37
PS	2,6-DNT	1.2	1	31	7.04	94.5	7.90	0.000	0.01
			2	30	7.02	94.1	7.89	0.004	0.09
			3	30	7.03	93.9	7.87	0.004	0.09
			4	30	6.89	92.9	7.92	0.020	0.53
			5	30	6.99	93.8	7.95	0.021	0.59
PS	2,6-DNT	3.9	1	31	6.80	91.0	7.83	0.155	3.32
			2	31	7.06	94.5	7.83	0.167	3.58
			3	31	6.90	92.4	7.85	0.064	1.44
			4	31	6.85	91.6	7.85	0.083	1.85
			5	31	6.95	93.0	7.87	0.042	0.98

[1] DO = dissolved oxygen

Appendix B5. Continued - Overlying water quality measurements at amphipod test termination

Sediment	Chemical	Conc.	Replicate	Salinity	DO	DO %	pH	Total NH_4	NH_3
TX	Tetryl	0.18	1	30	7.11	95.3	7.86	0.006	0.13
			2	30	7.04	94.0	7.91	0.005	0.13
			3	30	7.15	95.4	7.91	0.002	0.06
			4	32	6.86	92.2	7.90	0.014	0.36
			5	30	6.78	91.3	7.92	0.033	0.87
TX	Tetryl	0.17	1	30	6.96	93.8	7.90	0.004	0.09
			2	31	7.28	97.4	7.87	0.010	0.22
			3	30	7.10	95.2	7.89	0.002	0.06
			4	31	6.84	92.0	7.91	0.017	0.45
			5	30	6.79	91.5	7.91	0.039	1.00
TX	Tetryl	0.44	1	31	7.23	96.8	7.87	0.000	0.00
			2	30	7.16	95.8	7.88	0.100	2.40
			3	30	7.11	95.6	7.86	0.048	1.10
			4	31	6.72	90.5	7.88	0.036	0.85
			5	30	6.73	90.9	7.90	0.039	0.99
TX	Tetryl	1.6	1	30	6.98	94.0	7.85	0.078	1.74
			2	30	7.05	94.4	7.89	0.180	4.41
			3	30	6.91	93.1	7.85	0.072	1.62
			4	31	6.83	91.4	7.90	0.084	2.11
			5	31	6.90	92.5	7.91	0.053	1.35
TX	Tetryl	14.7	1	31	6.94	93.1	7.86	0.243	5.57
			2	32	7.01	93.7	7.95	0.201	5.63
			3	30	6.86	91.7	7.88	0.090	2.16
			4	32	6.88	92.3	7.88	0.093	2.22
			5	30	6.78	91.5	7.86	0.062	1.41
PS	Tetryl	BDL	1	30	7.02	94.3	7.77	0.009	0.16
			2	30	6.91	92.2	7.82	0.011	0.23
			3	30	7.07	94.6	7.80	0.013	0.25
			4	31	6.81	91.3	7.85	0.019	0.42
			5	30	6.85	91.8	7.86	0.019	0.43
PS	Tetryl	0.30	1	32	6.86	92.2	7.86	0.005	0.12
			2	31	6.81	91.7	7.83	0.006	0.13
			3	32	6.70	89.8	7.93	0.006	0.17
			4	30	6.83	91.7	7.92	0.019	0.51
			5	30	6.89	93.2	7.89	0.037	0.91

Appendix B5. Continued - Overlying water quality measurements at amphipod test termination.

Sediment	Chemical	Conc.	Replicate	Salinity	DO	DO %	pH	Total NH_4	NH_3
TX	Picric Acid	22.0	1	31	6.96	93.5	7.87	0.021	0.50
			2	29	7.01	93.7	7.93	0.007	0.18
			3	31	6.73	90.1	7.91	0.006	0.16
			4	31	6.87	92.5	7.89	0.008	0.20
			5	31	6.85	91.9	7.91	0.030	0.76
TX	Picric Acid	37.3	1	29	7.11	94.6	7.88	0.021	0.51
			2	31	7.13	95.2	7.90	0.007	0.18
			3	30	6.92	92.7	7.89	0.024	0.59
			4	31	6.88	92.2	7.89	0.023	0.56
			5	31	6.81	91.3	7.92	0.022	0.59
TX	Picric Acid	73.1	1	30	7.06	93.8	7.88	0.197	4.72
			2	31	6.94	93.2	7.89	0.111	2.72
			3	30	6.86	91.4	7.87	0.077	1.80
			4	31	6.83	91.8	7.88	0.080	1.91
			5	30	6.85	92.3	7.89	0.078	1.90
TX	Picric Acid	162.0	1	30	6.99	93.7	7.88	0.332	7.95
			2	31	7.11	95.1	7.90	0.516	12.93
			3	31	6.94	92.9	7.91	0.132	3.38
			4	30	6.95	93.3	7.88	0.097	2.33
			5	30	6.90	93.1	7.89	0.123	3.01
TX	Picric Acid	336.7	1	30	7.07	94.0	7.87	0.381	8.93
			2	31	7.05	94.4	7.89	0.585	14.33
			3	32	6.97	92.9	7.91	0.186	4.77
			4	32	6.71	89.8	7.91	0.178	4.56
			5	30	6.81	91.9	7.90	0.098	2.47
PS	Not spiked	-	1	31	6.50	87.4	7.93	0.009	0.24
			2	30	7.07	94.9	7.94	0.005	0.14
			3	31	6.86	91.6	7.96	0.009	0.26
			4	31	6.92	92.5	7.91	0.008	0.21
			5	30	6.88	92.9	7.92	0.029	0.77
TX	Not spiked	-	1	30	7.08	94.3	7.87	0.006	0.14
			2	30	7.08	95.0	7.92	0.004	0.11
			3	32	6.86	91.8	7.90	0.012	0.31
			4	31	6.91	92.7	7.91	0.009	0.24
			5	30	6.90	93.0	7.90	0.010	0.25
Control	Not spiked	-	1	32	6.49	87.3	8.15	1.460	63.82
			2	32	6.85	91.9	8.05	0.355	12.44
			3	30	6.66	88.7	8.09	0.930	35.61
			4	32	6.76	90.8	8.22	0.393	20.03
			5	30	6.75	90.9	8.20	0.441	21.51

Appendix B5. Continued - Overlying water quality measurements at amphipod test termination.

Sediment	Chemical	Conc.	Replicate	Salinity	DO	DO %	pH	Total NH_4	NH_3
PS	Picric Acid	BDL-1	1	30	7.00	92.3	8.09	1.010	38.67
			2	31	6.98	92.5	8.07	0.481	17.62
			3	30	6.87	91.1	8.10	0.568	22.23
			4	30	6.97	92.5	8.19	0.003	0.15
			5	30	6.86	91.0	8.10	0.275	10.76
			6	31	6.88	91.3	8.08	0.001	0.03
PS	Picric Acid	BDL-2	1	30	6.96	92.8	8.04	4.260	145.97
			2	31	6.90	91.9	8.05	3.770	132.08
			3	30	6.97	92.6	8.03	4.550	152.48
			4	30	6.91	91.8	8.05	4.290	150.30
			5	30	7.27	95.8	8.08	3.790	141.93
			6	30	7.02	92.8	8.03	4.430	148.46
PS	Picric Acid	BDL-3	1	30	6.90	92.3	8.01	6.180	198.08
			2	31	6.81	91.0	7.98	6.790	203.54
			3	31	6.71	89.4	8.40	6.320	475.09
			4	30	6.78	90.3	8.08	6.770	253.52
			5	30	6.82	90.9	8.02	6.440	211.06
			6	30	6.87	91.1	8.04	6.250	214.16
PS	Picric Acid	53.4	1	31	7.02	93.6	8.01	10.500	336.54
			2	31	6.80	90.3	8.02	12.000	393.29
			3	30	6.78	90.1	8.03	11.300	378.68
			4	30	6.90	91.4	8.02	11.600	380.18
			5	30	6.84	90.9	8.04	11.200	383.77
			6	31	6.78	90.1	8.07	8.930	327.07
PS	Picric Acid	304.3	1	30	7.09	94.8	8.05	12.400	434.44
			2	31	6.93	92.5	8.06	12.400	444.20
			3	31	6.66	88.8	8.11	12.700	508.24
			4	31	6.60	88.1	8.01	12.300	394.24
			5	30	6.70	89.3	8.08	14.000	524.27
			6	30	6.62	88.5	7.99	12.700	389.30
PS[2]	Not spiked	-	1	30	7.19	95.1	8.52	0.000	0.00
			2	30	7.05	93.1	8.27	0.000	0.00
			3	30	6.82	90.2	8.30	0.000	0.00
			4	30	6.97	92.3	8.30	0.000	0.00
			5	30	6.99	92.1	8.51	0.000	0.00
Control[2]	Not spiked	-	1	30	6.99	93.1	8.20	0.005	0.25
			2	30	6.93	91.9	8.20	0.000	0.00
			3	30	6.78	89.8	8.13	0.000	0.00
			4	30	6.98	92.2	8.20	0.000	0.00
			5	30	7.21	95.4	8.31	0.000	0.00

[2] Control and reference sediment data for 2nd test conducted with picric acid in PS sediment.

Appendix B6. Water quality measurements at termination of amphipod tests with picric acid and SDS in aqueous phase.

Chemical	Concentration (mg/L)	Salinity (ppt)	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (mg/L)
SDS[2]	0.0	30	6.90	90.5	7.92	0.090	2.37
SDS	1.2	30	6.76	88.9	7.79	0.060	1.17
SDS	2.5	30	6.52	85.8	7.75	0.039	0.69
SDS	5.0	30	6.64	87.9	7.63	0.002	0.02
SDS	10.0	30	5.35	70.4	7.37	0.001	0.01
SDS	20.0	30	5.65	74.2	7.51	0.001	0.01
SDS[3]	0.0	30	7.22	96.4	8.09	0.046	1.73
SDS	1.2	30	7.17	95.5	7.95	0.043	1.20
SDS	2.5	30	7.08	94.4	7.88	0.031	0.74
SDS	5.0	30	6.68	88.3	7.71	0.014	0.23
SDS	10.0	30	5.84	77.8	7.61	0.051	0.67
SDS	20.0	30	6.20	83.8	7.70	0.018	0.28
Picric Acid	3.3	30	6.80	89.6	7.74	0.066	1.16
Picric Acid	6.2	30	6.86	90.4	7.78	0.075	1.44
Picric Acid	12.5	30	6.62	87.5	7.71	0.066	1.08
Picric Acid	24.8	30	6.78	88.3	7.72	0.041	0.68
Picric Acid	49.7	30	6.79	93.1	7.62	0.003	0.03

[1] DO = Dissolved oxygen;

[2] Reference toxicant test conducted concurrently with all tests but picric acid test in Puget Sound sediment;

[3] Reference toxicant test conducted concurrently with picric acid test in Puget Sound sediment.

Appendix B7. Mean un-ionized ammonia concentration in overlying water of amphipod tests at experiment termination.

Sediment	Chemical	Sediment Conc. at Test Start	Mean NH_3 (µg/L)	Standard Deviation
TX[1]	2,6-DNT	0.97	0.294	0.251
TX	2,6-DNT	3.22	0.440	0.312
TX	2,6-DNT	9.76	1.106	0.124
TX	2,6-DNT	18.70	2.519	0.821
TX	2,6-DNT	34.05	2.079	1.025
TX	Tetryl	0.18	0.310	0.333
TX	Tetryl	0.17	0.365	0.387
TX	Tetryl	0.44	1.068	0.859
TX	Tetryl	1.64	2.246	1.241
TX	Tetryl	14.72	3.398	2.036
TX	Picric	22.05	0.360	0.262
TX	Picric	37.28	0.485	0.174
TX	Picric	73.07	2.610	1.237
TX	Picric	162.22	5.922	4.502
TX	Picric	336.70	7.011	4.717
PS[2]	2,6-DNT	1.15	0.261	0.276
PS	2,6-DNT	3.89	2.232	1.154
PS	Tetryl	BDL	0.299	0.119
PS	Tetryl	0.30	0.367	0.343
PS	Picric	BDL-1	14.91	14.71
PS	Picric	BDL-2	145.20	7.39
PS	Picric	BDL-3	259.24	107.54
PS	Picric	53.40	366.59	27.58
PS	Picric	304.25	449.11	56.52
Control	Not spiked	-	30.681	20.326
Control[3]	Not spiked	-	0.050	0.111
TX	Not spiked	-	0.211	0.083
PS	Not spiked	-	0.322	0.255
PS[3]	Not spiked	-	0.000	0.000

[1]TX= Texas sandy sediment; [2] PS = Puget Sound muddy sediment; [3] Control and reference sediment data for 2_{nd} test conducted with picric acid in PS sediment.

Appendix B8. Pore water quality measurements at amphipod test termination.

Sediment	Chemical	Sediment Conc. at Test Start	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)
TX	2,6-DNT	0.97	7.79	102.7	7.88	0.100	2.40
TX	2,6-DNT	3.22	7.79	100.7	7.89	0.151	3.70
TX	2,6-DNT	9.76	7.10	93.6	7.79	0.308	6.03
TX	2,6-DNT	18.70	7.46	98.6	7.81	1.010	20.67
TX	2,6-DNT	34.05	6.99	89.4	7.75	0.815	14.57
TX	Tetryl	0.18	7.13	94.2	7.85	0.516	11.56
TX	Tetryl	0.17	7.04	93.1	7.86	0.925	21.19
TX	Tetryl	0.44	7.37	97.6	7.92	0.648	16.98
TX	Tetryl	1.64	7.53	99.8	7.86	1.58	36.19
TX	Tetryl	14.72	7.45	98.7	7.82	1.1	23.03
TX	Picric Acid	22.05	7.09	93.8	7.81	0.007	0.15
TX	Picric Acid	37.28	7.16	95.1	7.82	0.115	2.41
TX	Picric Acid	73.07	7.69	100.1	7.79	0.293	5.73
TX	Picric Acid	162.22	7.26	96.5	7.78	1.160	22.19
TX	Picric Acid	336.70	7.37	99.1	7.78	1.460	27.92
PS	2,6-DNT	1.15	6.84	90.4	7.5	1.43	14.48
PS	2,6-DNT	3.89	7.07	93.4	7.29	1.12	7.02
PS	Tetryl	BDL	6.2	82.7	7.34	0.969	6.81
PS	Tetryl	0.30	5.73	76.2	7.3	2.18	13.98
PS	Picric Acid	BDL-1	5.73	75.5	7.47	2.53	23.93
PS	Picric Acid	BDL-2	5.56	72.6	7.56	11.80	137.03
PS	Picric Acid	BDL-3	5.69	73.9	7.54	15.10	167.54
PS	Picric Acid	53.40	5.65	73.2	7.5	19.30	195.50
PS	Picric Acid	304.25	5.56	70.2	7.58	31.80	386.47
Control	Not spiked	-	6.36	85.1	7.63	17.90	243.72
TX	Not spiked	-	7.62	102.3	7.8	0.038	0.77
PS	Not spiked	-	6.8	90.7	7.56	1.470	17.07

[1] DO = dissolved oxygen

Appendix C

Complete data set for porewater toxicity, chemistry and water quality in embryological development tests with the sea urchin *Arbacia punctulata*

Appendix C1. Toxicity data from the sea urchin, *Arbacia punctulata,* embryological development test with pore water spiked with ordnance compounds and in reference toxicant (SDS) test and controls.

Matrix	Chemical	Initial Conc. (mg/L)	% Normal Embryos Replicate No. 1	2	3	4	5	Mean % Normal	St. Dev.	% of Ref.[1]	Sign. Diff.[2]
Seawater	-	-	92	87	85	92	94	91.0	3.16	-	
			90	89	93	94	94				
TX[3]	-	6.2%	94	95	96	97	93	95.0	1.58	-	
TX	-	12.5%	94	93	99	96	93	95.0	2.55	-	
TX	-	25%	95	97	96	97	96	96.2	0.84	-	
TX	-	50%	98	97	97	95	96	96.6	1.14	-	
TX	-	100%	96	94	95	94	94	94.6	0.89	-	
TX	2,6-DNT	8.660	95	97	96	93	94	95.0	1.58	100	
TX	2,6-DNT	17.320	89	93	94	100	93	93.8	3.96	98	
TX	2,6-DNT	33.616	73	49	45	58	66	58.2	11.61	60	**
TX	Tetryl	0.143	99	94	97	94	93	95.4	2.51	105	
TX	Tetryl	0.202	78	65	81	74	71	73.8	6.22	81	**
TX	Tetryl	0.438	0	0	0	0	0	0.0	0.00	0	**
TX	Picric Acid	223.593	91	97	97	98	96	95.8	2.77	100	
TX	Picric Acid	433.897	94	91	95	91	88	91.8	2.77	95	
TX	Picric Acid	858.496	0	0	0	0	0	0.0	0.00	0	**

[1] Represents % of equivalent control: 6.25 to 100% placebo treatment or control filtered seawater depending on sample dilution; [2] ** indicates significant difference at $\alpha \leq 0.01$, and below detectable significance criteria; [3] Pore water from Texas or Puget Sound sediment spiked with filtered seawater as a blank control and subsequent serial 50% dilutions of the pore water with filtered seawater.

Appendix C1. Continued - Toxicity data from the sea urchin test.

Matrix	Chemical	Initial Conc. (mg/L)	% Normal Embryos Replicate No. 1	2	3	4	5	Mean % Normal	St. Dev.	% of Ref.[1]	Sign. Diff.[2]
PS[3]	-	6.2%	90	95	96	96	98	95.0	3.00	-	
PS	-	12.5%	96	92	96	95	96	95.0	1.73	-	
PS	-	25%	90	95	95	96	85	92.2	4.66	-	
PS	-	50%	0	0	0	0	0	0.0	0.00	-	
PS	-	100%	0	0	0	0	0	0.0	0.00	-	
PS	2,6-DNT	0.017	98	98	97	95	91	95.8	2.95	101	
PS	2,6-DNT	0.029	85	89	96	91	86	89.4	4.39	97	
PS	2,6-DNT	0.067	0	0	0	0	0	0.0	0.00	100	NA[5]
PS	2,6-DNT	0.137	0	0	0	0	0	0.0	0.00	100	NA
PS	Tetryl[4]	0.00044	85	83	79	83	82	82.4	2.19	87	
PS	Tetryl[4]	0.00088	0	0	0	0	0	0.0	0.00	0	**
PS	Tetryl[4]	0.00175	0	0	0	0	0	0.0	0.00	0	**
PS	Tetryl[4]	0.00350	0	0	0	0	0	0.0	0.00	100	NA
PS	Tetryl	0.00700	0	0	0	0	0	0.0	0.00	100	NA
PS	Picric Acid	70.754	97	96	95	95	94	95.4	1.14	100	
PS	Picric Acid	134.643	77	86	88	88	71	82.0	7.65	89	
PS	Picric Acid	250.465	5	1	8	10	47	14.2	18.65	100	NA
PS	Picric Acid	465.845	0	0	0	0	0	0.0	0.00	100	NA
Seawater	SDS	2.5	94	93	89	96	91	92.6	2.70	102	
Seawater	SDS	5	22	18	16	19	18	18.6	2.19	20	
Seawater	SDS	10	0	0	0	0	1	0.2	0.45	0	
Seawater	SDS	20	0	0	0	0	0	0.0	0.00	0	

[4] Concentrations below detection limit, therefore were calculated as 50% dilutions of highest measured concentration (0.007 mg/L);

[5] NA = significant differences not applicable because equivalent placebo had 100% effect, i.e., natural features of the pore water did not permit normal embryological development.

Appendix C2. Water quality measurements in the highest concentrations of pore water spiked with ordnance compounds, at the initiation of embryological development toxicity tests with the sea urchin, *Arbacia punctulata*.

Matrix	Chemical	Conc. at Test Start (mg/L)	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)	Sulfide (mg/L)
Seawater	-	-	6.69	89.5	8.16	0.008	0.36	<0.01
TX	Not spiked	-	7.18	95.7	7.55	1.020	11.58	<0.01
TX	2,6-DNT	77	7.59	100.8	7.49	0.581	5.75	<0.01
TX	Tetryl	14	7.74	105.0	7.89	0.657	16.10	<0.01
TX	Picric Acid	737	7.61	101.8	8.13	0.581	24.30	<0.01
PS[2]	Not spiked	6.2%	NM	NM	8.15	1.640	71.70	NM
PS	Not spiked	12.5%	NM	NM	8.10	2.150	84.20	NM
PS	Not spiked	25%	NM	NM	8.03	3.500	117.30	NM
PS	Not spiked	50%	NM	NM	7.89	6.090	149.20	NM
PS	Not spiked	100%	7.13	95.9	7.73	9.850	168.20	<0.01
PS	2,6-DNT	0.137	7.3	97.5	7.75	0.585	10.46	<0.01
PS	Tetryl	0.00044	NM	NM	8.14	1.700	72.70	NM
PS	Tetryl	0.00088	NM	NM	8.12	2.370	97.00	NM
PS	Tetryl	0.00175	NM	NM	8.03	3.940	132.00	NM
PS	Tetryl	0.00350	NM	NM	7.91	6.760	173.20	NM
PS	Tetryl	0.00700	5.48	72.8	7.69	10.000	156.00	<0.01
PS	Picric Acid	466	7.57	101.4	7.21	2.590	13.52	<0.01

[1] DO = dissolved oxygen;

[2] Pore water from sediments spiked with filtered seawater as a blank control, and diluted in 50% serial dilutions with filtered seawater.

Appendix C3. Chemical measurements from porewater samples used in the sea urchin, *Arbacia punctulata*, embryological development toxicity tests.

Sample	Chemical	Measured Concentration (mg/L)		
		With Organisms		No Organisms
		Start	End	End
TX[1]	2,6-DNT	8.660	8.547	9.294
TX	2,6-DNT	17.320	16.721	17.889
TX	2,6-DNT	33.616	31.937	33.804
TX	Tetryl	0.143	BDL[3]	0.045
TX	Tetryl	0.202	BDL	0.148
TX	Tetryl	0.438	0.026	0.398
TX	Picric Acid	216.98	217.11	223.32
TX	Picric Acid	388.94	398.89	408.75
TX	Picric Acid	736.64	716.14	777.04
PS[2]	2,6-DNT	0.017	BDL	0.021
PS	2,6-DNT	0.029	0.029	0.044
PS	2,6-DNT	0.067	0.057	0.086
PS	2,6-DNT	0.137	0.138	0.141
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	0.007	BDL	BDL
PS	Picric Acid	70.75	60.84	68.44
PS	Picric Acid	134.64	110.48	137.57
PS	Picric Acid	250.46	248.13	253.61
PS	Picric Acid	465.84	454.13	467.29

[1] TX= Texas sandy sediment; [2] PS = Puget Sound ,WA, muddy sediment.

[3] BDL = Below Detection Limit.

Appendix D

Complete data set for porewater toxicity, chemistry and water quality in 7-day life cycle tests with the polychaete *Dinophilus gyrociliatus*

Appendix D1. Toxicity data from the polychaete, *Dinophilus gyrociliatus* , life-cycle test with pore water spiked with ordnance compounds and in an ammonia test, as well as reference toxicant (SDS) test and controls.

Matrix	Chemical	Conc. (mg/L)	% Polychaete Survival Replicate No.					Mean % Surv.	St. Dev.	Sign. Diff.[1]	# Eggs/Adult Replicate No.					Mean Eggs/ Adult	St. Dev.	Sign. Diff.[1]
			1	2	3	4	5				1	2	3	4	5			
Seawater	-	-	75	100	100	100	100	95	11.2		2.67	1.75	2.75	3.50	1.75	2.48	0.7	
TX[2]	-	100%PW	100	75	75	100	100	90	13.7		0.00	2.33	0.33	0.25	2.50	1.08	1.2	
TX[3]	-	50%PW	50	100	100	75	75	80	20.9		3.50	4.50	3.25	4.00	1.67	3.38	1.1	
TX	2,6-DNT	0.914	100	75	100	75	100	90	13.7		2.75	2.33	4.00	2.33	1.00	2.48	1.1	
TX	2,6-DNT	1.591	75	75	100	100	75	85	13.7		5.67	3.33	5.00	3.50	3.67	4.23	1.0	
TX	2,6-DNT	3.109	50	75	100	100	100	85	22.4		4.00	1.33	5.50	2.00	2.00	2.97	1.7	
TX	2,6-DNT	7.206	100	75	75	100	100	90	13.7		2.00	1.00	1.00	2.33	1.75	1.62	0.6	
TX	2,6-DNT	14.904	100	100	75	100	100	95	11.2		0.50	0.75	0.33	0.50	0.25	0.47	0.2	**
TX	2,6-DNT	29.904	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0	**
TX	Tetryl	0.008	100	75	50	75	100	80	20.9		2.50	2.67	0.00	2.67	1.25	1.82	1.2	
TX	Tetryl	0.024	50	100	75	100	100	85	22.4		2.50	4.00	0.67	2.25	2.00	2.28	1.2	
TX	Tetryl	0.040	100	75	75	50	75	75	17.7		1.25	2.67	4.00	3.50	2.33	2.75	1.1	
TX	Tetryl	0.077	50	25	25	0	0	20	20.9	**	2.00	2.00	0.00	0.00	0.00	0.80	1.1	
TX	Tetryl	0.123	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0	**
TX	Picric Acid	57.789	100	100	100	100	100	100	0.0		3.50	1.75	4.00	4.25	3.75	3.45	1.0	
TX	Picric Acid	120.438	75	75	50	0	75	55	32.6	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
TX	Picric Acid	227.161	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
Seawater	SDS	1.2	100	75	100	100	100	95	11.2		3.00	0.67	2.00	1.50	2.00	1.83	0.8	
Seawater	SDS	2.5	50	75	75	100	50	70	20.9		2.50	0.33	1.00	3.00	3.50	2.07	1.3	
Seawater	SDS	5.0	0	25	0	50	0	15	22.4		0.00	0.00	0.00	0.00	0.00	0.00	0.0	
Seawater	SDS	10.0	0	0	0	0	0	0	0.0		0.00	0.00	0.00	0.00	0.00	0.00	0	

[1] Significant difference from control or reference sample at $\alpha \leq 0.01$.

[2] Pore water from Texas sediment spiked with filtered sea water as a blank control.

[3] Blank control diluted to 50% with filtered seawater.

Appendix D1. Continued - Toxicity data from the polychaete test.

Matrix	Chemical	Conc.	% Survival - Rep. No.					Mean	St.	Sign.	# Eggs/Adult - Rep. No.					Mean	St.	Sign.
			1	2	3	4	5	Surv.	Dev.	Diff.	1	2	3	4	5	Eggs	Dev.	Diff.
Seawater	-	-	75	100	100	100	100	95	11.2		11.33	10.50	7.60	7.00	8.00	8.89	1.9	
PS[4]	-	100%	100	100	100	75	100	95	11.2		7.50	6.75	3.60	7.00	7.00	6.37	1.6	
PS	-	50%	100	75	100	100	25	80	32.6		10.00	10.33	14.75	8.50	2.00	9.12	4.6	
PS	-	25%	100	100	100	100	100	100	0.0		19.25	9.75	7.75	9.75	10.50	11.40	4.5	
PS	-	12.5%	100	75	100	100	100	95	11.2		3.00	16.67	12.50	6.25	9.00	9.48	5.3	
PS	-	6.2%	100	100	100	100	75	95	11.2		12.25	5.00	14.00	5.50	18.67	11.08	5.8	
PS	2,6-DNT	0.012	100	100	100	100	100	100	0.0		9.25	7.20	4.25	12.50	5.50	7.74	3.3	
PS	2,6-DNT	0.034	100	100	75	100	75	90	13.7		5.50	0.60	2.00	3.25	5.00	3.27	2.0	**
PS	2,6-DNT	0.067	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
PS	Tetryl	0.00175[5]	75	25	25	100	75	60	33.5		9.67	7.00	0.00	11.00	5.00	6.53	4.3	
PS	Tetryl	0.0035[5]	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
PS	Tetryl	0.0070	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
PS	Picric Acid	17.925	100	100	100	100	100	100	0.0		6.25	4.00	4.50	3.00	7.60	5.07	1.8	
PS	Picric Acid	34.341	100	100	100	100	100	100	0.0		10.25	7.00	4.75	8.00	9.00	7.80	2.1	
PS	Picric Acid	67.347	100	100	100	100	100	100	0.0		7.75	0.00	5.25	7.25	4.5	4.95	3.1	
PS	Picric Acid	132.000	75	75	75	100	100	85	13.7		1.33	1.33	0.00	5.00	1.25	1.78	1.9	**
PS	Picric Acid	247.209	0	0	0	0	0	0	0.0	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
Seawater	NH_3	15.2	100	100	100	75	100	95	11.2		11.00	10.75	7.25	10.00	12.00	10.20	1.8	
Seawater	NH_3	60.9	75	100	100	100	100	95	11.2		12.00	14.50	6.25	9.50	5.75	9.60	3.7	
Seawater	NH_3	131.7	100	100	100	75	100	95	11.2		3.00	6.75	9.00	10.67	4.25	6.73	3.2	
Seawater	NH_3	253.5	75	75	100	75	100	85	13.7		5.00	1.67	4.75	5.33	3.00	3.95	1.6	**
Seawater	NH_3	456.4	0	50	25	25	0	20	20.9	**	0.00	0.00	0.00	0.00	0.00	0.00	0.0	**
Seawater	SDS	1.2	100	100	100	50	100	90	22.4		8.75	9.00	10.75	10.50	3.25	8.45	3.0	
Seawater	SDS	2.5	100	100	100	100	100	100	0.0		11.75	4.25	9.00	8.00	8.25	8.25	2.7	
Seawater	SDS	5.0	25	25	0	0	25	15	13.7		0.00	2.00	0.00	0.00	0.00	0.40	0.9	
Seawater	SDS	10.0	0	0	0	0	0	0	0.0		0.00	0.00	0.00	0.00	0.00	0.00	0.0	

[4] Pore water from Puget Sound sediment spiked with filtered sea water as a blank control, and subsequent serial 50% dilutions of the pore water with filtered seawater.

[5] Below detection limit, therefore concentrations are nomimal, calculated based on the highest measured concentration of 0.007 mg/L.

Appendix D2. Water quality measurements at the termination of the polychaete, *Dinophilus gyrociliatus*, tests with pore water spiked with ordnance compounds and in an ammonia test.

Matrix	Chemical	Conc. at Test Start	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)
Sea Water Control TX[2]		-	7.29	99.0	7.89	0.10	2.4
TX[3]	Not spiked	50% PW	7.38	99.6	7.95	0.22	6.0
TX	Not spiked	100% PW	7.37	99.1	8.01	0.32	10.2
TX	2,6-DNT	0.91	7.07	96.6	7.89	0.32	7.9
TX	2,6-DNT	1.59	7.13	97.2	7.87	0.36	8.3
TX	2,6-DNT	3.11	7.00	96.0	7.81	0.36	7.4
TX	2,6-DNT	7.21	7.91	94.3	7.89	0.45	11.0
TX	2,6-DNT	14.90	6.91	94.3	7.91	0.54	13.9
TX	2,6-DNT	29.90	6.92	94.5	7.94	0.82	22.6
TX	Tetryl	0.01	6.92	93.9	7.89	0.25	6.0
TX	Tetryl	0.02	7.02	95.0	7.86	0.20	4.7
TX	Tetryl	0.04	6.98	94.7	7.86	0.21	4.9
TX	Tetryl	0.08	7.05	95.5	7.80	0.22	4.3
TX	Tetryl	0.12	6.94	93.9	7.82	0.23	4.9
TX	Picric Acid	57.79	7.36	98.7	7.92	0.50	13.1
TX	Picric Acid	120.44	7.34	99.1	7.92	0.67	17.5
TX	Picric Acid	227.16	7.21	97.4	7.95	0.65	18.2
Sea Water	SDS[2]	1.3	6.91	94.2	7.88	0.20	4.7
Sea Water	SDS	2.5	6.89	94.0	7.86	0.08	1.9
Sea Water	SDS	5.0	6.92	94.5	7.86	0.01	0.2
Sea Water	SDS	10.0	6.88	94.1	7.86	0.09	2.0
Sea Water	SDS	20.0	6.98	95.0	7.89	0.11	2.7

[1] DO = dissolved oxygen; [2] Sea water control and reference toxicant (SDS) test concurrent with test with pore water from Texas sediment; [3] Pore water from sediments spiked with filtered seawater as a blank control, and diluted in 50% serial dilutions with filtered seawater.

Appendix D2. Continued - Water quality measurements at the polychaete test termination.

Matrix	Chemical	Conc. at Test Start	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)
Sea water	Control PS[4]	-	6.78	91.7	7.93	0.27	7.3
PS	Not spiked	6%	6.92	93.5	7.94	0.47	12.8
PS	Not spiked	13%	6.87	92.7	7.95	0.63	17.7
PS	Not spiked	25%	6.87	92.7	7.98	1.16	34.8
PS	Not spiked	50%	6.66	90.0	8.04	2.39	81.9
PS	Not spiked	100%	6.78	91.5	8.12	3.67	150.1
PS	2,6-DNT	0.01	6.75	91.3	7.96	0.74	21.3
PS	2,6-DNT	0.03	6.65	90.2	7.99	1.11	34.0
PS	2,6-DNT	0.07	6.67	90.2	8.05	2.10	73.6
PS	Tetryl	BDL-1	6.54	88.5	8.00	1.30	40.7
PS	Tetryl	BDL-2	6.60	89.4	8.06	2.00	71.6
PS	Tetryl	0.01	6.70	90.5	8.15	3.69	161.3
PS	Picric Acid	17.93	6.87	92.5	7.93	0.56	14.9
PS	Picric Acid	34.34	6.88	92.3	7.92	0.76	19.9
PS	Picric Acid	67.35	6.82	91.7	7.96	1.16	33.3
PS	Picric Acid	132.00	6.74	90.6	7.97	2.07	60.7
PS	Picric Acid	247.21	6.57	88.4	8.01	3.09	99.0
Sea Water	SDS[4]	1.2	6.76	91.5	7.94	0.26	7.1
Sea Water	SDS	2.5	6.81	92.1	7.95	0.27	7.7
Sea Water	SDS	5.0	6.77	91.6	7.95	0.21	5.9
Sea Water	SDS	10.0	6.65	90.1	7.94	0.17	4.6
Sea Water	SDS	20.0	6.75	91.2	7.98	0.10	3.1

[4] Sea water control and reference toxicant (SDS) test concurrent with test with porewater from Puget Sound sediment.

Appendix D3. Dissolved oxygen and pH measurements at termination of *Dinophilus gyrociliatus* test with ammonium chloride, and ammonia measurements at test initiation and termination.

Matrix	Chemical	DO[1] (mg/L)	DO (% sat.)	pH	Initial NH_4[2] (mg/L)	Final NH_4 (mg/L)	Initial NH_3[3] (µg/L)	Final NH_3 (µg/L)
Seawater	NH_4Cl	6.69	90.9	7.91	1.62	0.67	15.15	17.27
Seawater	NH_4Cl	6.74	91.5	7.90	2.97	1.23	60.92	30.82
Seawater	NH_4Cl	6.63	90.1	7.90	6.42	2.54	131.68	63.65
Seawater	NH_4Cl	6.61	89.7	7.85	12.50	4.43	253.52	99.21
Seawater	NH_4Cl	6.66	90.3	7.86	24.80	9.54	456.44	218.52

[1] DO = dissolved oxygen; [2] NH_4 = total ammonia; [3] NH_3 = un-ionized ammonia

Appendix D4. Chemical measurements from porewater samples used in the polychaete, *Dinophilus gyrociliatus,* toxicity tests.

Sample	Chemical	Measured Concentration (mg/L)		
		With Organisms		No Organisms
		Test Start	End	End
TX[1]	2,6-DNT	0.914	0.794	0.682
TX	2,6-DNT	1.591	1.531	1.308
TX	2,6-DNT	3.109	3.336	3.245
TX	2,6-DNT	7.206	6.566	7.174
TX	2,6-DNT	14.904	13.920	14.866
TX	2,6-DNT	29.904	29.194	29.682
TX	Tetryl	0.008	BDL[3]	BDL
TX	Tetryl	0.024	BDL	BDL
TX	Tetryl	0.040	BDL	0.006
TX	Tetryl	0.077	BDL	0.022
TX	Tetryl	0.123	BDL	0.045
TX	Picric Acid	57.789	61.398	63.666
TX	Picric Acid	120.438	122.618	120.714
TX	Picric Acid	227.161	223.776	231.793
PS[2]	2,6-DNT	0.012	0.016	0.009
PS	2,6-DNT	0.034	0.027	0.037
PS	2,6-DNT	0.067	0.048	0.061
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	0.007	BDL	0.005
PS	Picric Acid	17.925	17.574	18.172
PS	Picric Acid	34.341	32.950	34.728
PS	Picric Acid	67.347	68.071	69.458
PS	Picric Acid	132.000	127.584	134.710
PS	Picric Acid	247.209	254.157	252.372

[1] TX= Texas sandy sediment; [2] PS = Puget Sound ,WA, muddy sediment.

[3] BDL = Below Detection Limit.

Appendix E

Complete data set for porewater toxicity, chemistry and water quality in zoospore germination and germling development tests with the macroalga *Ulva fasciata*

Appendix E1. Toxicity data from the macroalgae, *Ulva fasciata,* zoospore germination test with pore water spiked with ordnance compounds and in reference toxicant (SDS) test and controls: % germination.

Matrix	Chemical	Initial Conc. (mg/L)	% Germination Replicate No. 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control[1]	Sign. Diff.[2]
Seawater	-	-	93	96	88	94	94	93	3.00		
TX[3]	-	50%	91	95	92	91	93	92.4	1.67		
TX[3]	-	100%	84	79	96		81	85	7.62		
TX	2,6-DNT	1.146	96	98	95	92	96	95.4	2.19	103	
TX	2,6-DNT	2.195	94	92	94	93	88	92.2	2.49	99	
TX	2,6-DNT	4.435	62	74	88	67	49	68	14.44	73	**
TX	2,6-DNT	8.382	18	12	12	4	18	12.8	5.76	14	**
TX	2,6-DNT	17.498	0	0	0	0	0	0	0.00	0	**
TX	Tetryl	0.025	92	93	100	100	97	96.4	3.78	104	
TX	Tetryl	0.060	99	97	96	96	96	96.8	1.30	104	
TX	Tetryl	0.149	96	94	95	95	96	95.2	0.84	102	
TX	Tetryl	0.337	84	80	90	92	87	86.6	4.77	93	*
TX	Tetryl	0.736	72	64	72	68	64	68	4.00	73	**
TX	Tetryl	1.375	0	0	0	0	0	0	0.00	0	**
TX	Picric Acid	55.17	87	91	92	89	95	90.8	3.03	98	
TX	Picric Acid	114.73	87	92	91	88	91	89.8	2.17	97	
TX	Picric Acid	214.94	90	96	90	92	92	92	2.45	99	
TX	Picric Acid	409.33	86	83	89	87	84	85.8	2.39	93	
TX	Picric Acid	765.92	11	16	6	10	14	11.4	3.85	13	**
PS[3]	-	50%	99	90		90	96	93.75	4.50		
PS[3]	-	100%	100	98	97	93	94	96.4	2.88		
PS	2,6-DNT	0.087	55	51	62	47	49	52.8	5.93	56	**
PS	2,6-DNT	0.192	0	0	0	0	0	0	0.00	0	**
PS	Tetryl	BDL	0	3	2	0	4	1.8	1.79	2	**
PS	Tetryl	0.006	0	0	0	0	0	0	0.00	0	**
PS	Picric Acid	35.33	85	92	86	91	92	89.2	3.42	96	*
PS	Picric Acid	68.29	82	86	90	88	88	86.8	3.03	93	**
PS	Picric Acid	128.57	85	72	71	87	78	78.6	7.30	85	**
PS	Picric Acid	237.90	0	0	0	0	0	0	0.00	0	**
Seawater	SDS	1.2	81	83	76	80	84	80.8	3.11	87	
Seawater	SDS	2.5	59	48	29	48	37	44	11.52	48	
Seawater	SDS	5.0	35	21	24	17	21	23.6	6.84	25	
Seawater	SDS	10.0	0	0	0	0	0	0	0.00	0	
Seawater	SDS	20.0	0	0	0	0	0	0	0.00	0	

[1] Represents % of equivalent control: 100 or 50% placebo treatment or control filtered seawater depending on sample dilution; [2] * indicates significant difference at $\alpha \leq 0.05$, and ** indicates sign. diff. at $\alpha \leq 0.01$; [3] Pore water from Texas or Puget sound sediment spiked with filtered sea water as a blank control and diluted to 50% with filtered seawater.

Appendix E2. Toxicity data from the macroalgae, *Ulva fasciata,* zoospore germination test with pore water spiked with ordnance compounds and in reference toxicant (SDS) test and controls: germling length.

Matrix	Chemical	Initial Conc. (mg/L)	Germling Length Replicate No. 1	2	3	4	5	Mean Length	St. Dev.	% of Control[1]	Sign. Diff.
Seawater	-	-	56.02	59.34	46.39	64.14	53.74	55.93	6.61		
TX[2]	-	50%	41.57	34.22	39.80	38.53	34.22	37.67	3.33		
TX[2]	-	100%	15.21	15.21	35.74		9.13	18.82	11.64		
TX	2,6-DNT	1.146	32.2	35	36	39	33.7	35.19	2.58	63	**
TX	2,6-DNT	2.195	22.8	19.8	20.3	22.8	23.6	21.85	1.71	39	**
TX	2,6-DNT	4.435	20	17.2	14.5	10.7	15.7	15.62	3.47	28	**
TX	2,6-DNT	8.382	7.35	7.35	8.62	9.38	9.38	8.42	1.02	15	**
TX	2,6-DNT	17.498	0	0	0	0	0	0.00	0.00	0	**
TX	Tetryl	0.025	51.7	42.6	53.7	57.8	46.1	50.40	6.06	90	
TX	Tetryl	0.060	34.7	48.2	44.1	35.7	40.6	40.66	5.65	73	**
TX	Tetryl	0.149	40.3	36	34.5	36	36.5	36.66	2.18	66	**
TX	Tetryl	0.337	29.2	30.9	31.4	28.9	29.4	29.96	1.14	54	**
TX	Tetryl	0.736	14.2	10.4	9.38	10.1	11.9	11.20	1.91	20	**
TX	Tetryl	1.375	0	0	0	0	0	0.00	0.00	0	**
TX	Picric Acid	55.17	19.3	16.2	21.6	26.4	25.1	21.70	4.16	39	**
TX	Picric Acid	114.73	13.2	20.5	17.5	15.7	20	17.39	3.06	31	**
TX	Picric Acid	214.94	14.5	14.5	16.7	18.8	19.3	16.73	2.29	30	**
TX	Picric Acid	409.33	9.63	7.86	8.62	7.61	6.84	8.11	1.06	22	**
TX	Picric Acid	765.92	8.62	8.62	9.38	8.11	8.87	8.72	0.46	46	**
PS[2]	-	50%	53	50.5		44.6	47.2	48.80	3.67		
PS[2]	-	100%	48.7	45.9	45.1	48.7	47.7	47.20	1.63		
PS	2,6-DNT	0.087	10.4	9.63	10.9	9.63	9.63	10.04	0.58	21	**
PS	2,6-DNT	0.192	0	0	0	0	0	0.00	0.00	0	**
PS	Tetryl	BDL	0	11.4	10.1	0	12.7	6.85	6.31	14	**
PS	Tetryl	0.006	0	0	0	0	0	0.00	0.00	0	**
PS	Picric Acid	35.33	10.9	9.13	11.2	10.7	17.8	11.92	3.35	21	**
PS	Picric Acid	68.29	14.7	9.38	10.7	12.7	10.1	11.51	2.16	21	**
PS	Picric Acid	128.57	8.37	8.11	9.89	9.89	10.4	9.33	1.02	17	**
PS	Picric Acid	237.90	0	0	0	0	0	0.00	0.00	0	**
Seawater	SDS	1.2	17.8	22.6	12.7	15.2	18.8	17.39	3.73	31	
Seawater	SDS	2.5	7.61	8.62	8.62	9.38	8.11	8.47	0.66	15	
Seawater	SDS	5.0	7.1	6.08	6.59	7.1	6.08	6.59	0.51	12	
Seawater	SDS	10.0	0	0	0	0	0	0.00	0.00	0	
Seawater	SDS	20.0	0	0	0	0	0	0.00	0.00	0	

[1] Represents % of equivalent control: 100 or 50% placebo treatment or control filtered seawater depending on sample dilution; [2] Pore water from Texas or Puget sound sediment spiked with filtered sea water as a blank control and diluted to 50% with filtered seawater.

Appendix E3. Toxicity data from the macroalgae, *Ulva fasciata,* zoospore germination test with pore water spiked with ordnance compounds and in reference toxicant (SDS) test and controls: germling cell number.

Matrix	Chemical	Initial Conc. (mg/L)	Germling Cell No. Replicate No. 1	2	3	4	5	Mean Cell No.	St. Dev.	% of Control[1]	Sign. Diff.
Seawater	-	-	4.7	4.9	4	5.4	4.4	4.68	0.53		
TX[2]	-	100%	1.8	1.7	3.3		1.1	1.98	0.94		
TX[2]	-	50%	4	3.1	3.6	3.3	2.9	3.38	0.43		
TX	2,6-DNT	1.146	3.2	3.9	3.7	3.9	3.6	3.66	0.29	78	**
TX	2,6-DNT	2.195	2.7	2.6	2.8	3	3.1	2.84	0.21	61	**
TX	2,6-DNT	4.435	2.4	2.2	2.5	1.5	1.9	2.10	0.41	45	**
TX	2,6-DNT	8.382	1	1	1	1.2	1	1.04	0.09	22	**
TX	2,6-DNT	17.498	0	0	0	0	0	0.00	0.00	0	**
TX	Tetryl	0.025	4.2	4.1	4.4	4.3	3.9	4.18	0.19	89	
TX	Tetryl	0.060	3.6	4.2	4.1	3.6	3.4	3.78	0.35	81	**
TX	Tetryl	0.149	3.5	3.7	3.3	3.7	3.5	3.54	0.17	76	**
TX	Tetryl	0.337	3.1	3.2	3.2	3.2	3.3	3.20	0.07	68	**
TX	Tetryl	0.736	1.5	1.1	1	1.2	1.4	1.24	0.21	26	**
TX	Tetryl	1.375	0	0	0	0	0	0.00	0.00	0	**
TX	Picric Acid	55.17	2.1	1.7	2.2	3.3	3.2	2.50	0.71	53	**
TX	Picric Acid	114.73	1.4	2.2	1.9	1.6	2	1.82	0.32	39	**
TX	Picric Acid	214.94	1.4	1.6	1.9	2	2	1.78	0.27	38	**
TX	Picric Acid	409.33	1	1	1.1	1	1	1.02	0.04	30	**
TX	Picric Acid	765.92	1	1	1	1	1	1.00	0.00	51	**
PS[2]	-	100%	5.7	5.1	4.7	5.2	5.5	5.24	0.38		
PS[2]	-	50%	6.9	5.4		5	5.4	5.68	0.84		
PS	2,6-DNT	0.087	1	1.1	1	1	1	1.02	0.04	18	**
PS	2,6-DNT	0.192	0	0	0	0	0	0.00	0.00	0	**
PS	Tetryl	BDL	0	1.2	1.3	0	1.4	0.78	0.72	14	**
PS	Tetryl	0.006	0	0	0	0	0	0.00	0.00	0	**
PS	Picric Acid	35.33	1.3	1.1	1.3	1.2	1.9	1.36	0.31	29	**
PS	Picric Acid	68.29	1.8	1.2	1.3	1.4	1.1	1.36	0.27	29	**
PS	Picric Acid	128.57	1	1.3	1.2	1.2	1.4	1.22	0.15	26	**
PS	Picric Acid	237.90	0	0	0	0	0	0.00	0.00	0	**
Seawater	SDS	1.2	2	2	1.8	1.8	1.9	1.90	0.10	41	**
Seawater	SDS	2.5	1	1	1.1	1.1	1	1.04	0.05	22	**
Seawater	SDS	5.0	1	1	1.1	1	1	1.02	0.04	22	**
Seawater	SDS	10.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	**
Seawater	SDS	20.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	**

[1] Represents % of equivalent control: 100 or 50% placebo treatment or control filtered seawater depending on sample dilution; [2] Pore water from Texas or Puget sound sediment spiked with filtered sea water as a blank control and diluted to 50% with filtered seawater.

Appendix E4. Water quality measurements in the highest concentrations of pore water spiked with ordnance compounds, at the initiation of zoospore germination toxicity tests with the macro-alga, *Ulva fasciata*.

Matrix	Chemical	Conc. at Test Start (mg/L)	DO[1] (mg/L)	DO (% sat.)	pH	Total Ammonia (mg/L)	Un-ionized Ammonia (µg/L)	Sulfide (mg/L)
Seawater	-	-	6.89	86.5	8.03	0.005	0.17	<0.01
TX	Not spiked	-	7.00	93.5	7.82	0.366	7.66	<0.01
TX	2,6-DNT	70	7.09	94.6	7.86	0.595	13.63	<0.01
TX	Tetryl	14	7.15	95.5	7.86	0.221	5.06	<0.01
TX	Picric Acid	765	6.94	92.5	7.62	0.952	12.67	<0.01
PS	Not spiked	-	7.24	96.5	7.97	9.820	287.87	<0.01
PS	2,6-DNT	0.192	7.24	96.2	8.06	9.160	328.13	<0.01
PS	Tetryl	0.006	7.08	94.4	8.08	11.600	434.39	<0.01
PS	Picric Acid	511	7.07	94.1	7.50	4.670	47.30	<0.01

[1] DO = dissolved oxygen

Appendix E5. Chemical measurements from porewater samples used in the macroalgae, *Ulva fasciata,* toxicity tests.

Sample	Chemical	Measured Concentration (mg/L)		
		With Organisms		No Organisms
		Test Start	Test End	Test End
TX[1]	2,6-DNT	1.146	1.115	1.085
TX	2,6-DNT	2.195	2.265	2.048
TX	2,6-DNT	4.435	4.013	3.974
TX	2,6-DNT	8.382	7.708	7.496
TX	2,6-DNT	17.498	15.888	15.785
TX	Tetryl	0.025	BDL[3]	BDL
TX	Tetryl	0.060	BDL	BDL
TX	Tetryl	0.149	0.015	BDL
TX	Tetryl	0.337	0.033	0.092
TX	Tetryl	0.736	0.226	0.229
TX	Tetryl	1.375	0.736	0.750
TX	Picric Acid	55.172	56.903	58.416
TX	Picric Acid	114.730	115.195	114.330
TX	Picric Acid	214.943	215.163	217.729
TX	Picric Acid	409.332	407.726	407.096
TX	Picric Acid	765.922	752.796	754.194
PS[2]	2,6-DNT	0.087	0.077	0.040
PS	2,6-DNT	0.192	0.169	0.076
PS	Tetryl	BDL	BDL	BDL
PS	Tetryl	0.006	BDL	BDL
PS	Picric Acid	35.328	34.509	34.798
PS	Picric Acid	68.288	66.558	67.656
PS	Picric Acid	128.570	132.866	132.369
PS	Picric Acid	237.903	250.667	249.615

[1] TX= Texas sandy sediment; [2] PS = Puget Sound ,WA, muddy sediment

[3] BDL = Below Detection Limit.

ATTACHMENTS 1-5

Attachment 1. (SOP F10.14) Preparation of Filtered (0.45 μm) Seawater.

Attachment 2. (SOP F10.15) Amphipod Solid-phase Toxicity Test

Attachment 3. (SOP F10.7) Sea Urchin Embryological Development Toxicity Test.

Attachment 4. (SOP F10.10) *Dinophilus gyrociliatus* Toxicity Test.

Attachment 5. (SOP F10.23) Algal Zoospore Germination and Germling Growth Toxicity Test.

Attachment 1. (SOP F10.14) Preparation of Filtered (0.45 μm) Seawater.

Date Prepared: January 10, 1993

PREPARATION OF FILTERED (0.45 μm) SEAWATER

1.0 APPLICATION

Filtered (0.45 μm) seawater (MFS) is used in most of the toxicity tests conducted at this field station with a variety of marine organisms. The acronym MFS is derived from "Millipore® Filtered Seawater" because the original 0.45 μm filtering apparatus purchased at this lab was manufactured by Millipore company. Filters and apparatus manufactured by other companies are acceptable. MFS is distinct from FS, which indicates seawater of any salinity filtered through a 1 μm cartridge filter. MFS serves an important role in the tests as a nontoxic seawater medium. Among other functions, MFS is used as a control medium, to dilute porewater samples, to wash sea urchin eggs, to dilute sea urchin eggs and sperm, and to overlay sediment in amphipod exposure chambers.

2.0 PREPARATION

2.1 Equipment and Labware

See the Equipment List for Preparation of Filtered (0.45 μm) Seawater (MFS) in Attachment 1.

2.2 Source of Seawater

The seawater to be used in the preparation of MFS is natural and free of contaminants. It is typically pre-filtered using a 1 μm cartridge filter to reduce the quantity of 0.45 μm filters needed. Since the salinity of MFS is 30 $^{o}/_{oo}$, it is preferable to start with seawater of 30 $^{o}/_{oo}$. If necessary, adjust seawater salinity to 30 $^{o}/_{oo}$ as described in Water Quality Adjustment of Samples (SOP F10.12).

3.0 PROCEDURES

1. Set up filtering apparatus (Figure 1). Connect tubing to filtering flask, liquid trap, vacuum pump and valve. Plug in the pump. Secure liquid trap to burette stand with clamp. Place bottom of filtering funnel on filtering flask. Remove one 0.45 μm filter from package with forceps (filters are packaged with a paper liner on

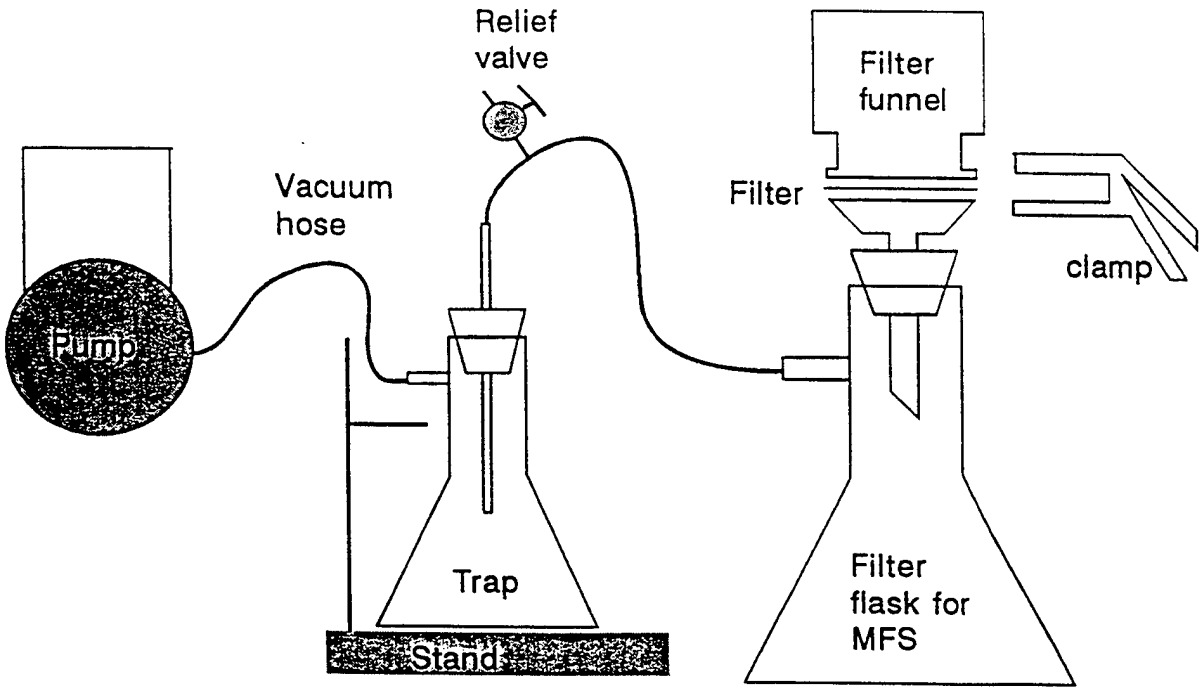

Figure 1. Filtering apparatus.

both sides), wet the filter, and place on the fritted disc of the filtering funnel. Clamp the top of the filtering funnel into place.

2. Add seawater to the filtering funnel. Close the relief valve. Turn on the pump. Add more seawater as the volume in the funnel drops. Continue until the flow slows noticeably or until the filtering flask becomes full.

3. If the flow slows noticeably, replace the filter. Open the relief valve and turn off the pump in that order. Always open the relief valve before turning off the pump. (Doing otherwise leaves a vacuum inside the pump which could damage it.) Remove the filtering funnel and clamp. Remove the used filter and put on a new filter with the forceps. Return the top of the filtering funnel into place and repeat step 2.

4. If the filtering flask becomes full, transfer the MFS to a plastic holding container of appropriate size. Open the valve and turn off the pump. Remove the filtering funnel and flask. Transfer the MFS in the flask to the holding container, using a funnel if necessary.

5. Continue filtering and transfering until sufficient MFS is prepared.

6. After use, disconnect the pump, tubing and glassware. Rinse the glassware with deionized water.

7. Aerate the MFS. Because the filtering process strips oxygen from seawater, the MFS should be aerated to bring the dissolved oxygen (DO) concentration above 80% saturation. Connect airline tubing to an aquarium pump and to a new disposable glass pipette. Place the pipette into the MFS container and aerate until DO concentration is adequate (measure DO with a dissolved oxygen meter).

8. Double-check salinity of the MFS and adjust as needed.

9. Discard MFS approximately one week after preparation, unless the test to be conducted has different requirements.

4.0 TRAINING

Personnel who perform this task will first read this protocol and then operate under supervision during at least his/her first MFS preparation.

5.0 SAFETY

No safety hazards are known to exist.

6.0 ATTACHMENTS

Attachment 1. Equipment List for Preparation of Filtered (0.45 μm) Seawater (MFS)

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Attachment 1

EQUIPMENT LIST
FOR PREPARATION OF FILTERED (0.45 μm) SEAWATER (MFS)

Filters (0.45 μm, preferably gridded, Millipore® or other equivalent brand)
Filter forceps
Filter funnel with clamp
Filtering flask (2 L)
Vacuum trap
Small, plastic valve for vaccuum release
Burette stand with clamp
Standard funnel
Beakers (1 L)
Clear vinyl tubing (eg. Tygon®)
Airline tubing (plastic)
Disposable glass pipettes
Air pump (aquarium type)
Refractometer
Dissolved oxygen meter
Containers for holding MFS (polyethylene, high or low density, are good but should be soaked for at least three days with multiple water changes prior to use)

Attachment 2. (SOP F10.15) Amphipod Solid-phase Toxicity Test

Date Prepared: January 26, 1993

AMPHIPOD SOLID-PHASE TOXICITY TEST

1.0 OBJECTIVE

The purpose of the amphipod solid-phase toxicity test is to determine if test sediment samples reduce survival of exposed animals relative to that of animals exposed to reference sediment. Test results are reported as treatment (station) or a combination of treatments (site) which produces statistically significant reduced survival.

2.0 TEST PREPARATION

2.1 Experimental Design

The amphipod solid-phase toxicity test is a static 10-day experiment conducted in glass exposure chambers (e.g., 1 L glass jars). An equal number of amphipods (normally 20) is stocked in each of the jars which contains a layer of sediment overlayed with filtered (1 μm) seawater. Each test and reference treatment normally consists of five replicate jars. Reference sediment may be either sediment from which the amphipods were collected or another uncontaminated reference sediment. Additionally, a water-only dilution series test with sodium dodecyl sulfate (SDS) may be conducted as a positive control.

2.2 Test Animals

2.2.1 Sources

The solid-phase toxicity test can be conducted with different species of amphipods. Animals can be collected in the field or obtained from a commercial supplier or can be cultured. Generally, the amphipod solid-phase toxicity tests are conducted at this field station with commercially-supplied *Ampelisca abdita*. Test specific requirements such as screen mesh sizes and physical conditions for this species are given in Attachment 1, Specific Requirements for Conducting the Solid-Phase Toxicity Test with *Ampelisca abdita*.

2.2.2 Receipt and acclimation

Amphipods purchased from a commercial supplier are normally delivered 1 to 10 days before use. Generally, amphipods will be shipped in native sediment with

overlaying seawater and enclosed in plastic bags. Depending on the duration of the trip, the bags will be chilled and injected with oxygen. Upon arrival seawater temperature, salinity and dissolved oxygen is measured. The shipping containers are aerated and acclimation to test temperature and salinity begins, if necessary. Filtered seawater of appropriate temperature and salinity is added gradually to the amphipod holding containers to make the adjustments to test conditions. It is recommended that acclimation proceed no faster than 1 °C or 2 $^{o}/_{oo}$ salinity per hour. For temperature control, the amphipod containers may be held in an incubator or water bath. If quality of the amphipods is poor (large percentage emerge, excessive mortality, or otherwise appear unhealthy), the group should be replaced.

2.3 Test and Control Sediments

Sediment samples should be collected and handled using proper methods and chain of custody procedures. Samples should be processed immediately upon collection or receipt or stored at 4 °C until processed. It is recommended that sediment samples be used within 2 weeks of collection. Each sample is press sieved through a 1 mm polyester mesh screen and homogenized before being added to the jars.

2.4 Test System

2.4.1 Equipment

A list of equipment necessary for conducting this test is given in Attachment 2 (Equipment List for Amphipod Solid-Phase Toxicity Test). For clarity, the list is organized by different tasks/phases of the test, so there is some overlap among the different areas.

2.4.2 Solutions

<u>10% Buffered Formalin with Rose Bengal Stain</u>

13.5 L filtered (1 μm) seawater
1.5 L formalin
3/4 cup Borax®
2 tsp Rose Bengal

2.4.3 Overlaying water

Filtered (1 µm) seawater (FS) adjusted to the appropriate test salinity is used to overlay the sediment in each test and control jar.

2.4.4 Test apparatus setup

After sediment is sieved, homogenized and added to the jars, they are capped and held at 4°C until the day before the test. At that time FS is added to each jar to within ~2-3 cm of the top (~700 mL). Disturbance of the sediment should be minimized by introducing the water inside each jar onto a small plastic disk attached to the end of a plastic pipette. The disk is rinsed between treatments. After FS is added, the jars are aerated with small aquarium-type air pumps and maintained at 20°C. Gentle aeration is delivered through disposable glass Pasteur pipettes fitted in a single hole through each lid and liner. To facilitate removing and replacing the individual airlines, which is done daily throughout the test, plastic airline tubing connectors may be fitted to each pipette. This can be done by inserting a connector into a short (~5 cm) piece of airline tubing which has been attached to a pipette using silicon sealant (allowed to dry overnight) and then wrapped with Parafilm® to secure the connection. To ensure that the tips of the pipettes are suspended in the center of the water column (to avoid disturbance of the sediment), label tape may be wrapped around the pipettes at a proper distance from the tip, allowing the pipette to pass only partially through the hole in each lid and liner.

2.4.5 SDS dilution series

A water-only SDS dilution series should be included as a positive control. A range of SDS concentrations (normally 50% dilutions) can be run together with a control containing no SDS. The upper and lower concentrations used in the test should be chosen to bracket the EC_{50}, although this will depend on animal quality as well as the species used. Other concentrations can be tested, but using a range from 1 to 32 mg/L will usually ensure that the EC_{50} is bracketed. After the different dilutions are made, they are transferred to test jars (normally 5 replicates per treatment) and kept at 20°C without aeration until the day of the test.

3.0 TEST PROCEDURES

3.1 Initiation

1. Measure desired water quality parameters in each jar (or in at least one replicate jar from each treatment) and record the information on the Amphipod Solid-Phase Toxicity Test Water Quality Data Sheet (Attachment 3). Take appropriate samples and rinse probes, thermometers, etc. between readings to prevent cross-contamination between jars.

2. Sieve amphipods to remove from native sediment, using seawater having temperature and salinity similar to that to which they have been acclimated. To select an appropriate size range of animals, two screen mesh sizes can be used to eliminate

outsized and undersized specimens. Before sieving, check for and avoid collecting amphipods emerged from the sediment (most easily done by transferring only sediment to the sieve) which may be of inferior quality.

3. Count amphipods, with the aid of a stereomicroscope, into small sample cups containing FS. The amphipods can be collected using a small strip of polyester mesh material (Nitex®, for example). Any outsized and undersized amphipods and molts are avoided. During this step, the amphipods are maintained in aerated seawater of appropriate temperature and salinity.

4. The number of amphipods in each sample cup is verified by another investigator.

5. Amphipods in the sample cups are stocked into the test and control jars.

6. Allow time for the amphipods to burrow. Any that remain emerged should be replaced.

7. Replace the lids, resume aeration and maintain under proper conditions.

3.2 Daily Check and Mortality Count

1. Remove lids from all jars and check each for amphipods emerged on the sediment surface, emerged in the water column, on the surface film, and any dead amphipods. Those caught in the surface film should be gently pushed down into the water with a glass rod or equivalent instrument. Dead amphipods should be removed. Record the information on the form, Amphipod Solid-Phase Toxicity Test Daily Check and Mortality Count (Attachment 4). Any instruments used during this procedure are rinsed between jars to prevent cross-contamination.

2. Repeat step 1 daily thru day 10.

3.3 Termination

1. On day 10, measure water quality as in 3.1 step 1.

2. Sieve contents of each jar, using seawater, and transfer the material to the same jar or a different container. During the sieving procedure, the water flow should be adjusted so the pressure is not too excessive to damage the amphipods or splash material out of the sieve. Transferring the material can be done using a wash bottle containing seawater.

3. For large tests (those that cannot be counted immediately following termination), preserve the sample in ~5% formalin with Rose Bengal by adding a volume of 10%

formalin solution (subsection 2.4.2) approximately equal to the volume of the sieved material plus rinse water. For small tests, preservation of the samples is optional if they can be processed immediately.

4. Check the sieve after rinsing and transferring under a stereomicroscope for missed amphipods. Note the number, if any, on the respective sample jar.

3.4 Procedures for SDS Series

On the day of test initiation, the SDS dilution series jars are water quality checked and stocked with amphipods. Although stocked with a different number of amphipods (normally 10 per jar), they are maintained under the same conditions as the solid-phase test jars and are checked daily for amphipods caught in the surface film. Any on the surface are gently pushed down into the water, however, dead amphipods are not removed as is done with dead amphipods in the solid-phase jars. Also, in contrast to the 10-day run with the solid-phase jars, the SDS treatments are terminated at 96 hr. At that time, water quality parameters may be measured in selected SDS jars. The amphipods may be counted immediately or preserved for later counting.

4.0 SURVIVAL DATA COLLECTION AND TABULATION

4.1 Large Test

Amphipod solid-phase tests commonly consist of too many jars to be counted immediately following termination of the test and have been preserved (subsection 3.3, step 3). Now, the contents of each jar are thoroughly but gently rinsed through a screen to remove formalin and flush excess stain before sorting and counting. Many of the preserved amphipods float on the water surface after being transferred from the sieve to a sorting dish and are easily removed and counted. The remaining amphipods are picked from their tubes (if tube dwellers) or other retained material. Most of the preserved amphipods have retained Rose Bengal stain and are various shades of pink in color. The collected amphipods are transferred to small vials containing 70% ETOH. The number of amphipods counted plus the number of amphipods that remained on the screen on the day of termination is compared with the number of amphipods expected according to the mortality noted during the daily checks. If the number of amphipods unaccounted for exceeds 10% of the total number originally stocked in jar, the sample is rechecked and the number verified by a different investigator. The data are recorded on the standardized data sheet Amphipod Solid-Phase Toxicity Test Termination Survival Data (Attachment 5).

4.2 Small Test

Tests small enough to be processed within one day immediately following termination may not have been previously preserved. The jars are sieved, the material is transferred to a sorting dish, and the amphipods are retrieved and counted. To encourage any live tube-dwelling amphipods to leave their tubes, it may be helpful to add a drop of ethyl alcohol (ETOH) near to one end of the tube. Data are treated as in subsection 4.1.

4.3 SDS Series

Preserved or non-preserved amphipods in the SDS treatments are removed from the jars by pipetting. The number of survivors and percent survival are determined for each jar and data are recorded on the appropriate data sheet (Attachment 5).

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (Attachments 3-5), as previously noted. Under normal circumstances, percent survival in each test treatment is compared to an appropriate reference treatment (native sediment or other reference site sediment collected 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 LC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Sediment samples from other reference sites may be included in addition to the sediment from the area where the amphipods were collected. Initial amphipod counts before stocking are verified by a second investigator. Similarly, final survival counts are verified by a second investigator if 90% of the animals stocked cannot be accounted for.

7.0 TRAINING

The solid-phase amphipod toxicity test consists of many different tasks and, initially, a trainee will follow test procedures under supervision. With time, the trainee will learn how to perform all of the tasks associated with the test and will conduct them independently once he/she has demonstrated his/her ability to accurately reproduce the tasks. Although most steps are test specific activities which cannot be pre-trained, water quality determinations, microscope use, and sieving procedures can be taught in advance. For further information regarding 10-day amphipod solid-phase toxicity testing, trainees may refer to ASTM (1990) and SAIC (1992).

8.0 SAFETY

The amphipod solid-phase toxicity test poses little risk to those performing it, provided a few precautions are taken. Care should be taken when making and dispensing formalin solutions and when sieving preserved samples. A fume hood should be used and the test area should be well ventilated. Protective clothing should be worn when working with formalin solutions and when handling potentially toxic sediment samples.

9.0 ATTACHMENTS

Attachment 1. Specific Requirements for Conducting the Solid-Phase Toxicity Test with *Ampelisca abdita.*
Attachment 2. Equipment List for Amphipod Solid-Phase Toxicity Test
Attachment 3. Amphipod Solid-Phase Toxicity Test Water Quality Data Sheet
Attachment 4. Amphipod Solid-Phase Toxicity Test Daily Check and Mortality Count
Attachment 5. Amphipod Solid-Phase Toxicity Test Termination Survival Data

10.0 REFERENCES

American Society for Testing and Materials (ASTM). 1990. Standard guide for conducting solid phase 10-day static sediment toxicity tests with marine and estuarine infaunal amphipods. ASTM E 1367-90, pp. 1-24.

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Attachment 1

SPECIFIC REQUIREMENTS FOR CONDUCTING THE SOLID-PHASE TOXICITY TEST WITH AMPELISCA ABDITA

1. Stocking density = normally 20 animals per 1 L glass jar

2. Temperature = 20 ± 1 °C

3. Salinity = 30 $^{o}/_{oo}$ (or 28-35 $^{o}/_{oo}$)

4. No feeding during 10-day test.

5. Continuous light.

6. The species is a tube dweller and it is recommended that the sediment layer be 2-3 cm deep.

7. Screen Mesh Sizes:
   Initial sieving of amphipods in native sediment: through 1 mm, retained on 0.5 mm
   Termination sieving of amphipods in test sediment: directly retained on 0.5 mm
   Sieving of preserved amphipods in formalin solution: directly retained on 0.5 mm

8. Collecting *Ampelisca abdita* for initial counting and stocking as in section 3.1 step 3 can be facilitated because the animals will remain on the surface film if the screen is raised above water level and then re-emersed. Once on the surface film, they can be easily collected with the Nytex® strip.

Attachment 2

EQUIPMENT LIST FOR AMPHIPOD SOLID-PHASE TOXICITY TEST

Amphipod Receipt and Acclimation
thermometers
dissolved oxygen meter
refractometer
airpumps
airline tubing
airstones
incubator (or water bath)
filtered (1 μm) seawater

Setup
glass jars (1 L) with lids (Teflon®-lined) or other exposure chambers
sieves
spatulas, spoons
plastic baggies (nontoxic)
incubator (or water bath)
airline tubing
airpumps (aquarium type)
disposable glass Pasteur pipettes
silicon sealant
airline tubing connectors
Parafilm®
label tape
plastic disks
disposable plastic pipettes
min-max or recording thermometers
sodium dodecyl sulfate (SDS)
filtered (1 μm) seawater

Water Quality
dissolved oxygen meter
refractometer
total ammonia probe/meter
pH meter
thermometers
wash bottles
stir bars
small glass sample vials (eg. scintillation vials)
appropriate reagents and solutions

deionized water

Initiation
all water quality equipment
sieves
amphipod holding container
stereomicroscopes
small plastic sample cups
polyester mesh material (eg. Nitex®)
glass rod (or similar instrument)
airpump
airline tubing
airstones

Daily Check
pipettes
glass rod (or similar instrument)
Petri dishes or glass slides
stereomicroscope
deionized water
wash bottles
paper towels

Termination
all water quality equipment
sieves
sample jars
wash bottles
spray nozzles
formalin
Rose Bengal
Borax
stereomicroscope
label tape

Counting
sieves
spray nozzles
wash bottles
glass sorting dishes
fume hood
dissecting instruments
forceps

wide bore pipettes
"formalin only" sieve
formalin waste bin
formalin disposal containers
small glass sample vials (eg. scintillation vials)
ethyl alcohol

Protective Clothing
gloves
lab coat
protective eyeglasses or shield

Attachment 3

AMPHIPOD SOLID-PHASE TOXICITY TEST WATER QUALITY DATA SHEET

TEST ID_____ INITIALS_____STUDY PROTOCOL_____

_____ DATE_____

Trt.	Rep.	Sal.	Temp.	DO	% DO	pH	TAN	UAN	Sulf.

COMMENTS_____

_____

_____

CALIBRATIONS_____

_____

_____

Attachment 4

AMPHIPOD SOLID-PHASE TOXICITY TEST DAILY CHECK AND MORTALITY COUNT

TEST ID_____ INITIALS_____STUDY PROTOCOL___

_____ DATE_____ DAY___

Treatment	Rep.	# emerged on sediment	# emerged in water column	# on surface film	# dead & removed	Comments

COMMENTS_____

_____

_____

Attachment 5

AMPHIPOD SOLID-PHASE TOXICITY TEST TERMINATION SURVIVAL DATA

TEST ID_____ INITIALS_____STUDY PROTOCOL___

_____ DATE_____

Treatment	Rep.	# Survivors	% Survival	Comments	Initials

COMMENTS_____

_____

Attachment 3. (SOP F10.7) Sea Urchin Embryological Development Toxicity Test.

Date Prepared : April 10, 1990

Date Revised: February 29, 2000

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

1.0 OBJECTIVE

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

2.0 TEST PREPARATION

2.1 Test Animals

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

2.2 Dilution Water

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

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

2.3 Test System: Equipment

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

2.3.1 Equipment

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

2.3.2 Solutions

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

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

0.75 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

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

2.4.1 Selection of Urchins to be Used in Toxicity Test.

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

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

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

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

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

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

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

2.4.2 Obtain Eggs

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

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

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

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

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

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

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

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

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

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

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

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

2.4.4 Obtain Sperm

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

2.4.5 Prepare Appropriate Sperm Dilution

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

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

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

3.0 TEST PROCEDURES

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

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

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

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

4.0 DATA COLLECTION AND TABULATION

1. Transfer approximately 1 mL embryos and water from 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 pleuteus and abnormalities may be lumped together and counted on one key. Attachment 3 has a list of developmental stages and drawings of each.

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

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

5.0 DATA ANALYSIS

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

6.0 QUALITY CONTROL

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

7.0 TRAINING

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

8.0 SAFETY

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

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

9.0 ATTACHMENTS

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

10.0 REFERENCES

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Attachment 1

EQUIPMENT LIST FOR EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

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

Attachment 2
PRETEST TO INSURE SELECTION OF QUALITY GAMETES

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

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

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

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

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

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

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

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

Attachment 3

DEVELOPMENT OF SEA URCHIN EGGS TO PLUTEUS LARVAE

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

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

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

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

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

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

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

Attachment 4

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

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

B. Character of Sample (after salinity adjustment):

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

COMMENTS_____

_____

_____

_____

_____

Attachment 5

SEA URCHIN PRETEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

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

SPERM

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

TEST TIMES

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

SPERM DILUTION _____

COMMENTS _____

_____

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

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

% FERTILIZATION Reference sample designation:_____

Female #_____ Male #_____

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

Attachment 6

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID______________________________ INITIALS______________

STUDY PROTOCOL________________________ DATE________________

% FERTILIZATION Reference sample designation:______________

Female #______________ Male #______________

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

% FERTILIZATION Reference sample designation:______________

Female #______________ Male #______________

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

% FERTILIZATION Reference sample designation:______________

Female #______________ Male #______________

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

% FERTILIZATION Reference sample designation:______________

Female #______________ Male #______________

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

Attachment 7

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT

TOXICITY TEST GAMETE DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

EGGS

Collection time:_____

Initial count/volume:_____

Final count:_____

SPERM

Collection time:_____ Dilution start time:_____

Sperm dilution:_____

Test start temperature:_____

TEST TIMES

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

COMMENTS_____

Attachment 8

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TEST DATA SHEET

TEST ID_____ INITIALS_____

STUDY PROTOCOL_____ DATE_____

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

Comments_____

Attachment 9

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST DATA SHEET

TEST ID________________________________ INITIALS________________

STUDY PROTOCOL________________________________ DATE________________

PERCENT NORMAL PLUTEI

Replicate

Treatment	1	2	3	4	5	Mean±SD	Comments

Attachment 4. (SOP F10.10) *Dinophilus gyrociliatus* Toxicity Test.

Date Prepared : 4-10-90

Date Revised : 7-8-97

DINOPHILUS GYROCILIATUS TOXICITY TEST

1.0 OBJECTIVE

The purpose of the *Dinophilus gyrociliatus* toxicity test is to determine if a sea water, pore water, sea surface microlayer, or other sample reduces survival and/or reproduction in exposed *D. gyrociliatus* polychaetes relative to those exposed to a reference sample. The test may also be used to determine the concentration of a test substance which reduces survival or reproduction. Test results are reported as treatment (or concentration) which produces statistically significant reduced survival or reproduction.

2.0 TEST PREPARATION

2.1 Test Animals

Recently hatched juvenile *D. gyrociliatus* are needed to perform this test. These polychaetes are very easy to culture in the lab. Seed animals for a culture can be collected in the field.

Cultures can be maintained easily in 25-30 $^{o}/_{oo}$ seawater in small widemouth jars or almost any tightly closable container. Cultures are fed a suspension of freeze-dried powdered (<105 µm) spinach every 1-2 weeks. Cultures are generally reestablished every month by transferring a portion of an existing culture into a new culture vessel and adding fresh seawater to make up the difference in the volume. New cultures produce the greatest number of juveniles for use in testing, however cultures may be maintained for several months to provide seed stocks for new cultures. The salinity of cultures should be checked weekly and recorded on standardized data forms (Attachment 1).

2.2 Procurement of Test Organisms From the Cultures

Choose a culture container which has had sufficient time since it was established to produce a sufficient number of juveniles for harvesting (usually about 2-3 weeks). Place a light source such as a fiber optic light at the edge of the jar, near the surface of the water. The newly released juveniles are positively phototactic and will congregate near the light. Using a pasteur pipet and a dissecting microscope, move the animals from the jar into a smaller dish containing fresh filtered seawater. Salinity of the test water should be similar to culture conditions to prevent osmotic shock to the animals.

2.3 Dilution Water

Milli-Q water or concentrated seawater brine is used to adjust samples to the proper salinity (Attachment 2). Concentrated seawater brine (90-110 $^{o}/_{oo}$) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine stock quality remains constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen is also measured. Salinity adjustment and water quality data is recorded on prepared data forms.

2.4 Test System: Equipment

A list of equipment necessary for conducting this test is given in Attachment 3 (Equipment List for *Dinophilus gyrociliatus* Toxicity Test).

3.0 TEST PROCEDURES

3.1 Experimental Design

The tests are conducted in 20-mL stender dishes with ground glass lids with 10 mL of solution per dish. At least four animals are placed into each dish with five dishes per treatment. If brine and Mill-Q water are used as diluents, then both diluted brine and natural seawater controls can be run, as well as an appropriate reference sample. Tests may be conducted as a screening test (one treatment concentration) or as a dilution series test (more than one treatment concentration). The test is run as a static exposure with no water change during the test period.

3.2 Test Initiation

The test is started with one- to two-day-old animals. An experienced investigator can easily differentiate between newly released juveniles and more mature animals due to their rapid growth. The test solutions are first dispensed to the exposure chambers. The animals are taken from the small dish described in Section 2.2 and placed individually into the chambers using a Pasteur pipet with a latex bulb. All observations and manipulations are performed using a dissecting microscope. After the animals have been added, each chamber is reexamined to verify that there are at least four animals per replicate at the start of the test. After the chambers have been reexamined, 50 µL of a 0.5 percent powdered spinach solution is dispensed to each dish.

4.0 DATA COLLECTION

4.1 Record Keeping

All raw data are entered on standardized forms (Attachments 4 and 5). Raw data sheets are kept on file in the lab, and a copy made and kept on file in the care of Project Leader.

4.2 Biological Monitoring

Each chamber is examined at 24 hours (Day 1), 96 hours (Day 4), and at test completion (Day 7). Survival and reproductive data for each chamber are recorded on a standardized data sheet (see Attachment 3). The eggs of *Dinophilus gyrociliatus* are sexually dimorphic with the female eggs being much larger than the males. There are generally 2 to 5 eggs/egg case with the majority of the eggs being female. Because the males die shortly after copulation, which occurs in the egg case, only female eggs are used in the egg production counts. The first eggs are usually laid on Day 4 or 5. New juveniles may begin to emerge by Day 6 or 7. The reproductive data recorded for each chamber are the total number of female eggs, the number of egg cases, the number of eggs still in the coelom, and the number of newly emerged juveniles.

4.3 Environmental Monitoring

The parameters of temperature, salinity, dissolved oxygen, pH, and ammonia concentration will be made on a composite sample of the test solution for each treatment just prior to test initiation and again on Day 7 at the time of test completion. The data will be recorded on the Environmental Conditions Data Form (Attachment 4).

The water quality parameters for the static tests should be maintained within the following ranges:

Parameter	Acceptable Range
Temperature	20°C ± 2°C
Salinity	Test specific ± 2 $^{o}/_{oo}$
Dissolved oxygen	≥ 60% Saturation
pH	7.9 ± 0.4 units

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 1, 2, 4, and 5). Normally, survival and/or reproduction in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea-surface microlayer sample from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's test (Sokal and Rohlf 1981). Since ANOVA assumes that responses are independently and normally distributed with a common variance within treatment levels, a test of the validity of these assumptions is recommended. Bartlett's test or Levine's test may be used to test for homogeneity of variances (Snedecor and Cochran 1980). If the raw data do not satisfy these assumptions, the data may be transformed (for example a natural log or a $\log_{10}$ transformation) to stabilize the variance between treatment levels. If the assumptions for ANOVA cannot be met, a non-parametric Kruskal-Wallis test (Daniel 1978) may be performed.

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

Reconstituted brine, fresh filtered seawater, and reference site controls may be run. A test is unacceptable if more than 20% of control organisms appear stressed or diseased, or die.

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 *Dinophilus gyrociliatus* toxicity test poses little risk to those performing it. Protective gloves and lab coats should be worn when pipetting or dispensing potentially toxic samples.

9.0 ATTACHMENTS

Attachment 1. Culture Maintenance Record
Attachment 2. Water Quality Adjustment Form
Attachment 3. Equipment List for *Dinophilus gyrociliatus* Toxicity Test
Attachment 4. Toxicity Test Environmental Conditions
Attachment 5. Biological Monitoring Data

10.0 REFERENCES

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Attachment 1

CULTURE MAINTENANCE RECORD

Organism: *Dinophilus gyrociliatus* Culture Designation _____

Date Culture Started:_____ Project Number _____

Date Culture Terminated:_____

Date/Time	Salinity (‰)	Temp (°C)	Comments	FED Initials

Attachment 2

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____

SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

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

B. Character of Sample (after salinity adjustment):

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

COMMENTS_____

Attachment 3

EQUIPMENT LIST FOR *DINOPHILUS GYROCILIATUS* TOXICITY TEST

Glass stender dishes with ground glass lids (approximately 20-mL size)
Dissecting microscope with illuminator (fiber optics is suggested)
Pasteur pipets (with latex bulbs)
5-mL Oxford-type pipetter (with tips)
50 µL pipetter
2 to 3 small Carolina type dishes
Filtering apparatus (with 0.45-µm filters)
Vacuum pump
Colored labeling tape
Pens and markers
Data sheets
Manual counter
Kimwipes

For Food Preparation:

Freeze-dried spinach (from frozen grocery bought pack)
150-µm sieve
Mortar and pestle or electric coffee grinder

Attachment 4

DINOPHILUS GYROCILIATUS
TOXICITY TEST ENVIRONMENTAL CONDITIONS

Test Material_____ Test Description_____
Date/Time Test Started_____ Date/Time Test Completed_____
Observation Period_____ Date_____ Time_____

Parameter

Treatment	Temp (oC)	Salinity ()	DO (mg/L)	pH	mV	Ammonia (mg/L) (Mg/L)

Method: _____

Entered by:_____ Date: _____

Observation Period: _____ Date: _____ Time: _____

Parameter

Treatment	Temp (oC)	Salinity ()	DO (mg/L)	pH	mV	Ammonia (mg/L) (Mg/L)

Method: _____

Entered by:_____ Date: _____

Attachment 5

DINOPHILUS GYROCILIATUS

BIOLOGICAL MONITORING DATA

Test Material_____ Test Description_____

Date Test Started_____ Date Test Completed_____

	Survival Data				Reproductive Data				
	No.	No.	No.	Day 4	Day 7				
Treatment/ Replicate	Observed Day 1	Observed Day 4	Observed Day 7	Total No. Eggs	Total No. Eggs	No. Egg Cases	No. Eggs In Coelom	No. Eggs/ Adult	Comments

Organism Source: _____

Data Entered By: _____ Approval: _____

Comments:_____

Attachment 5. (SOP F10.23)
Algal Zoospore Germination and Germling Growth Toxicity Test.

Date Prepared: November 4, 1996

ALGAL ZOOSPORE GERMINATION AND GERMLING GROWTH TOXICITY TEST

1.0 OBJECTIVE

The purpose of the algal germination and germling growth toxicity test using *Ulva fasciata* and *U. lactuca* zoospores is to determine if sea water, pore water, or other aqueous samples inhibit germination and/or suppress growth of exposed algal zoospores and developing germlings relative to the response of zoospores and germlings exposed to a reference sample.

In this procedure, motile, quadriflagellate zoospores are exposed to test solutions for 96 hours, during which time they settle on glass cover slides in the test chambers. Each slide is examined microscopically to determine the percentage of zoospores that failed to germinate. Also, the length and cell number of ten randomly selected germlings are measured and counted, respectively, for each replicate. Test results are reported as the treatment (or concentration) that produces a statistically significant reduction in germination and growth or as the concentration that reduces germination by 50 percent (EC_{50}).

2.0 TEST SYSTEM

2.1 Equipment

A complete list of equipment necessary to conduct an algal zoospore test is provided in Attachment 1.

2.2 Dilution Water

Ultra-pure or concentrated seawater brine is used to adjust samples and filtered sea water to 30‰ as described in Water Quality Adjustment of Samples (SOP 10.12).

Filtered (0.45µm) seawater adjusted to 30‰ is used to rinse algal samples after collection and rewet thalli to initiate the release of reproductive bodies. It is also used to prepare zoospores stock solutions.

Filtered (0.45µm) seawater adjusted to 30‰ and diluted 10-15% with pore water (also adjusted to 30‰) is used as sample dilution water (DPW). The pore water, which is extracted from sediment collected from a site known to be free of contamination,

provides nutrients necessary for normal algal growth. The amount of pore water added to dilute filtered seawater is pre-determined with a pore water dilution test.

2.3 Test Chambers

Porewater samples may be tested in 20 mL glass beakers *(other containers may be suitable e.g., Stender dishes).* For tests with metal toxicants, 25 mL *polyethylene* beakers are preferred, however, glass beakers may be used. Place circular (20 mm diameter), glass cover slides flat on the bottom of the test chambers to provide a settling substrate. Five replicates per treatment are recommended. One treatment consists of 10 mL of test solution in a test chamber. When conducting dilution series tests, fifty percent serial dilutions may be made in the test chambers using DPW as the diluent.

3.0 TEST ORGANISMS

3.1 Life History

The test organisms for this protocol are the zoospores of *Ulva fasciata* Delile and *U. lactuca* Linnaeus, two marine, macrophytic Chlorophytes commonly known as sea lettuce. *Ulva* provides food and habitat to vertebrate and invertebrate species.

Ulva fasciata and *U. lactuca* have an alternation of isomorphic gametophytic and sporophytic generations. Motile gametes and zoospores are the primary dispersal mechanism for *Ulva* and are particularly sensitive stages in the life cycle. Each cell in gametophyte and sporophyte blades has the potential to produce 8 to 16 and 4 to 8 reproductive cells, respectively. Gametes and zoospores are differentiated by the number of flagella they possess. Gametes are biflagellate and zoospores are quadriflagellate. Mature sporophytes (2n) release zoospores which settle, germinate and develop into gametophytes (n). Gametophytes reach maturity within six weeks and release gametes which unite and develop into sporophytes, completing the life cycle (Kapraun 1970).

3.2 Species Identification

Both *Ulva fasciata* and *U. lactuca* occur in the intertidal zone. They are common on jetties, bulkheads and other hard substrates and may be found attached to rocks and shells. The two species may be distinguished by thallus morphology. *Ulva fasciata* thalli are divided into narrow, linear segments usually less than 1.5 cm wide but may range from 0.5-5.0 cm wide. *Ulva lactuca* have simple broad thalli with irregular lobes. Consult Kapraun (1970) for more information on *Ulva* sp. in the vicinity of Port Aransas, TX .

3.3 Collection of Algae

Because *Ulva* sp. gametophytes and sporophytes are isomorphic, it is not possible to distinguish one from the other in the field. Positive identification can be made only after reproductive cells have been released.

1. Collect algae at low tide on the evening before a test is to be conducted. During low tide, *Ulva* is exposed to air and becomes slightly desiccated, which is a necessary stage in the zoospore release process. Collect entire plants including the holdfast. The plants collected should be damp; do not collect dry, brittle algae. Place algae in a plastic bucket for transport to the laboratory.

2. Collect at least 20 individual plants from several locations along the jetty. Collections should be made in areas free of pollution to minimize the possibility of genetic or physiological adaptation to pollutants. Samples are collected from several different areas to increase the probability of having several sporophytes among the samples collected.

3. Only collect algae whose thalli are uniform in color or have slightly darker green margins. Algae whose thalli have clear margins should not be collected. Clear margins indicate that reproductive bodies have been released.

3.4 Storage of Algae

1. After collection, rinse samples with filtered (0.45µm) seawater and gently wipe with cheese cloth to remove debris, epiphytes and other associated organisms. Special attention should be given to cleaning the holdfast. The rinsing process should be done as quickly as possible as over-washing may stimulate the algae to release their reproductive bodies prematurely.

2. Discard any small thalli pieces not attached to a holdfast.

3. Layer washed samples (lasagna style, without overlap) between paper towels dampened with filtered (0.45 µm) seawater, place into a box with a lid and keep in the dark at 20^{0}C overnight. Samples should be used within 18 hours of collection.

3.5 Collection of Zoospores

To induce zoospore/gamete release, thalli must be subjected to mild desiccation in the dark, followed by rewetting and a sudden change in light intensity (Reed *et al.* 1991,

Anderson and Hunt 1993). Test solutions may be prepared while reproductive bodies are being released.

3.5.1 Zoospore Release

1. Remove several (5-10) clean plants from the dark box. If possible, select plants with dark green or olive colored thalli margins.

2. Place thalli from single plants into 150 or 250 -mL beakers (1 plant/beaker) containing approximately 100 mL of filtered (0.45µm) seawater at 20°C and illuminate with ambient room light (cool white fluorescent).

If thalli from a chosen plant have particularly wide, darkened edges, indicating that a large number of reproductive bodies are available for release, then only two or three thalli and not the entire plant are needed for the release procedure. Place the unused portion of the plant between damp paper towels in a labeled box. If that particular plant is identified as a sporophyte and more zoospores are required for a test, the unused portion will be available. Reproductive bodies should not be collected from plants whose thalli margins have turned tan, brown or golden brown.

3.5.2 Zoospore Identification/Motility Check

Either the formation of a green ring at the water-air interface along the inside of the beaker, or a green cloudiness in the water indicates that reproductive bodies have been released.

1. Examine a sample of the released organisms microscopically (200X) to identify them as zoospores or gametes. Preferably, zoospores from three or four plants should be examined.

2. Once zoospores from several plants have been identified, they should be examined to determine motility. If zoospores from a particular plant are inactive immediately after release, they should not be used in a test and spores from a separate plant should be evaluated. If zoospores are active, they may be accepted as potential test organisms.

3.6 Zoospore Concentration

3.6.1 Concentration Determination

1. Remove thalli from release beaker.

2. Thouroughly mix zoospore solution by stirring and pipet 4.5 mL of the solution into a scintillation vial. Add 0.5 mL of buffered formalin to the scintillation vial.

3. Determine the concentration of the zoospore stock solution subsample microscopically with an Improved Neubauer hemacytometer at 100X.

4. Use the formula and worksheet (Attachment 2) modified from Anderson and Hunt (1993) to calculate the zoospore concentration and the volume of stock solution to add to each test chamber to achieve a 12,750 zoospores/ml concentration. To prevent over-dilution of the test solution, the volume of zoospores added to each test chamber should be between 0.05 and 1% of the test solution volume (i.e., 50 to 100 µl).

5. If the zoospore concentration of the release beaker falls within the specified range to produce 12,750 zoospores/mL of sample, then the release beaker may be used to stock test chambers.

3.6.2 Concentration Adjustments

The concentration of the zoospore stock solution may be adjusted if it is too concentrated or diluted to meet the specified volume range that may be introduced into test solutions.

1. If the zoospore stock solution is too concentrated, dilute it with filtered seawater and recalculate the zoospore concentration.

2. If the stock solution is too dilute, allow zoospores to accumulate at the water-air interface in the release beaker and pipet them into a small beaker. If necessary, water from the bottom of the prepared stock solution may be removed after allowing the zoospores to accumulate at the water's surface. Recalculate the zoospore concentration.

4.0 TOXICITY TEST PROCEDURE

4.1 Exposure to Test Solutions

1. Observe a sample of zoospores from the stock solution before adding them to the test chambers to verify that they are swimming.

2. Pipet the calculated volume of zoospore stock solution into each test chamber.

3. Record the time zoospores are introduced into test chambers on the Algal Test Data Form (Attachment 2).

4.2 Incubation

1. Cover stocked test chambers with clear plastic Petri dish halves (50 mm diameter).

2. Incubate test for 96 h on a 12 h light-12 h dark photoperiod at 20°C.

3. Record the time test chambers are placed into incubators on the Algal Test Data Form (Attachment 2). Zoospores begin to germinate within 48 h. The additional 48 hours allows germling length and cell number to be included as sublethal endpoints.

4.3 Data Collection

The test is terminated after 96 hours. The endpoints for this test are percent germination, germling blade length and germling blade cell number. Salinity from at least five test chambers should be measured and recorded to insure it remained constant throughout the test.

4.3.1 Germination

A zoospore is considered germinated if it has divided into at least two cells; one cell being the initial rhizoid cell which produces a uniserate filament or germ tube, and the other being the frond or blade cell which will give rise to the thallus (Kapraun 1970). However, at 96 hours, germinated zoospores have generally developed into germlings with at least a three or four blade cells. Settled zoospores that have not germinated are usually spherical, between 7 and 10 µm in diameter, and appear light green. Germlings 96 h old are easily differentiated from ciliates or other protists which may be in water samples or may be introduced with the algal zoospores. If an object cannot be identified definitively as a germinated or non-germinated zoospore, it should not be counted.

1. Remove the slide from the test solution and hold it vertically for a moment to allow any test solution to drip off.

2. Invert the cover slide and, using a paper wipe, lightly press it onto a standard microscope slide. Care should be taken when pressing the cover slide onto the microscope slide. If it is pressed too hard, germlings may be destroyed to the point that germling length and cell number data may be impossible to obtain.

3. If necessary, blot around the edge of the cover slide to prevent it from sliding on the microscope slide.

4. Observe the slide microscopically (200X) and record the developmental progress of the first 100 settled zoospores encountered. Record all data on a standardized data sheet (Attachment 3).

4.3.2 Growth measurements

Growth of germlings is determined by measuring the length and counting the number of cells in ten randomly selected germling blades per replicate of each treatment.

1. Randomly select germlings (10) by moving the slide to a new field of view without looking through the eyepiece.

2. With the ocular micrometer, measure the germling lying closest to the micrometer in each field of view and count its cell number. Do not include the rhizoid in germling length measurements. Germling length is initially recorded in ocular units and must be converted to micrometers. (For our Zeiss compound microscope using the 20X objective, the conversion factor is 2.57.)

Ocular Units * 2.57 = germling length (µm)

3. If germination is significantly inhibited and fewer than 30% of the zoospores germinate, the first ten germinated zoospores encountered should be measured and counted (Anderson and Hunt, 1993). Record all data on a standardized data sheet (Attachment 3).

4.4 Preservation of Tests

Tests may be preserved by adding 1 mL of 10% buffered formalin to each test chamber. (Preliminary results indicate that there is no significant difference for germling length and cell number between chambers evaluated immediatley after test termination and those preserved with formalin and evalueted one week after test termination. The use of gluteraldehyde will be evaluated in the future)

5.0 DATA ANALYSIS

5.1 Statistical Analysis

Percent germination, germling length and germling cell number for each treatment are compared to an appropriate reference.

5.1.1 Germination Data

Statistical comparisons are made using one-way analysis of variance (ANOVA) and Dunnett's *t*-test on arcsine transformed germination data (SAS Institute, Inc 1989). Prior to analysis, transformed data sets should be screened for outliers (SAS Institute, Inc 1992). After removing outliers, data sets should be tested for normality and homogeneity of variance with Levene's test (SAS Institute, Inc 1992).

The trimmed Spearman-Karber method (Hamilton *et al.* 1977) with Abbott's correction (Morgan 1992) is used on germination data to determine the Median Effective Concentration (EC_{50}).

5.1.2 Growth Data

ANOVA and Dunnett's *t*-test are used to determine significant differences of germling length and cell number between test and control treatments. Data sets should be screened for outliers and tested for normality and homogeneity of variance. Appropriate transformations should be applied to germling length and cell number data when assumptions of equal variance are violated.

6.0 QUALITY CONTROL

Quality control tests may be conducted using both positive and negative controls with multiple replicates. Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is included with each test to evaluate the sensitivity of the zoospores chosen. Negative controls may include a reference pore water, dilution water and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining the zoospore stock solution concentration is a test specific activity. This function can be performed independently after a trainee has demonstrated the ability to accurately reproduce the test.

8.0 SAFETY

The algal zoospore germination and germling growth test poses little risk to those conducting it. Protective gloves may be worn when pipetting potentially toxic samples.

Care should be taken when collecting algae on the jetties. Protective footwear with soles that provide good traction should be worn to protect feet from barnacle cuts and slipping on algal mats. Preferably, collections should not be made alone.

9.0 ATTACHMENTS

Attachment 1. Equipment list for Algal Zoospore Germination and Germling Growth Toxicity Test
Attachment 2. Water Quality Adjustment Data Form
Attachment 3. Zoospore Release Data Form
Attachment 4. Algal Toxicity Test Data Sheet

10.0 REFERENCES

Anderson, B.S. and J.W. Hunt. 1993. Giant kelp germination and growth short-term toxicity test protocol. Procedures Manual for Conducting Toxicity Tests Developed by the Marine Bioassay Project, 90-10WQ.

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Attachment 1

EQUIPMENT LIST FOR ALGAL TOXICITY TEST

20 mL glass beakers or 25 mL plastic beakers for use as test chambers
22 mm diameter circular microspope cover slides and standard microsope slides
50 mm diameter Petri dish halves (or equivalent)
150 or 250 mL glass beakers to conduct zoospore release procedure
1000 mL glass beaker for dilution water preparation
25 mL and 100 mL graduated cylinder
Pasteur pipets and latex bulbs
Improved Neubauer Hemocytometer
Compound microscope with ocular micrometer and 10X and 20X objectives
Thermometer
Refractometer
Writing pens
50-100 µl pipetter
5 mL pipetter
Hand tally counter
Standard, glass microscope slides
Calculator
Plastic bucket to collect algae from the jetties
Filtered sea water (0.45µm), adjusted to 30‰
Filtered sea water (0.45µm), adjusted to 30‰ with pore water added
Concentrated brine
Ultra-pure water
Algae Test Data Form
Test data sheets
Incubator with contolled lighting

Attachment 2

Algae Test Data Form

Date:

Study Identification:

Investigator:

Condition of thalli used: poor fair good

Time blades placed in release beaker:_____

Time spores removed from release beaker:_____

Temperature of spore solution:_____

Spore motility check:_____

Zoospore Concentration Check

Determine concentration with 5 counts:

1. _____
2. _____
3. _____
4. _____
5. _____

Mean:_____ S.D.:_____

Mean *10,000*1.11= _____spores/ml. This is the concentration of the zoospore release.

To determine volume of spores to deliver to test chamber:

12,750 spores/ml x _____ml test solution/chamber = _____spores per test container.

_____spores/chamber ÷ spore concentration_____spores/ml = _____ml/test container

_____μl/test container

Temperature of spore solution: _____

Temperature of test containers: _____

Salinity of test containers (before/after): _____

Time test containers stocked: _____

Incubation start time:_____

Test termination time: _____

Comments:_____

_____

_____

Attachment 3

Algal Toxicity Test Data Sheet

Test Start Date:_____ Test End Date:_____ SDS EC_{50}:

Start Time:_____ End Time:_____ Comments_____

Micrometer conversion factor:_____ _____

Study Identification:_____ _____

Sample ID	Germ	Non Germ	Length Measurements/Cell Number										Mean Length/Cell No.
			L1/C1	L2/C2	L3/C3	L4/C4	L5/C5	L6/C6	L7/C7	L8/C8	L9/C9	L10/C10	

Sample ID	Germ	Non Germ	Length Measurements/Cell Number										Mean Length/Cell No.
			L1/C1	L2/C2	L3/C3	L4/C4	L5/C5	L6/C6	L7/C7	L8/C8	L9/C9	L10/C10	