ENGINEERING SERVICE CENTER
Port Hueneme, California 93043-4370

TECHNICAL REPORT
TR-2234-ENV

ASSESSMENT OF ENVIRONMENTAL EFFECTS OF ORDNANCE COMPOUNDS AND THEIR TRANSFORMATION PRODUCTS IN COASTAL ECOSYSTEMS

Prepared by

R. Scott Carr, PhD
Columbia Environmental Research Center
Marion Nipper PhD
Texas A&M University-Corpus Christi

Prepared for
Naval Facilities Engineering Service Center

December 2003

Approved for public release; distribution is unlimited.

Printed on recycled paper

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0811

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information, it if does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From – To)*
December 2003	Final	

4. TITLE AND SUBTITLE

ASSESSMENT OF ENVIRONMENTAL EFFECTS OF ORDNANCE COMPOUNDS AND THEIR TRANSFORMATION PRODUCTS IN COASTAL ECOSYSTEMS

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

R. Scott Carr, PhD
Columbia Environmental Research Center
Marion Nipper PhD
Texas A&M University-Corpus Christi

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESSES

Commanding Officer
Naval Facilities Engineering Service Center
1100 23rd Avenue
Port Hueneme, CA 93043-4370

8. PERFORMING ORGANIZATION REPORT NUMBER

TR-2234-ENV

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITORS ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study was undertaken to help understand the fate and effects of two munitions and explosives of concern, 2,6-dinitrotoluene (2,6-DNT) and 2,4,6-trinitrophenol (picric acid) in marine environments. Specifically, biotransformation and degradation in marine sediments, and photo-transformation in seawater were assessed. Studies were conducted with sediments spiked in the laboratory with the two chemicals of concern for the biotransformation assessments, and by spiking filtered seawater with these chemicals for the photo-transformation assessments.

15. SUBJECT TERMS

Ordnance; photo-transformation; biotransformation; degradation; marine sediments

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	U	164	

Final Report

on

ASSESSMENT OF ENVIRONMENTAL EFFECTS OF ORDNANCE COMPOUNDS AND THEIR TRANSFORMATION PRODUCTS IN COASTAL ECOSYSTEMS

Prepared by

R. Scott Carr, Ph.D.
Project Director, Principal Investigator
U.S. Geological Survey
Columbia Environmental Research Center
Marine Ecotoxicology Research Station

Marion Nipper, Ph.D.
Principal Investigator
Texas A&M University-Corpus Christi
Center for Coastal Studies

Submitted to

Naval Facilities Engineering Service Center
Port Hueneme, CA

April 30, 2003
revised
June 26, 2003

Center for Coastal Studies Report No.
TAMU-CC-0303-CCS

COASTAL CENTER STUDIES TAMU-CC

Texas A&M University-Corpus Christi
The Island University

USGS
science for a changing world

Final Report

on

ASSESSMENT OF ENVIRONMENTAL EFFECTS OF ORDNANCE COMPOUNDS AND THEIR TRANSFORMATION PRODUCTS IN COASTAL ECOSYSTEMS

PRINCIPAL INVESTIGATORS

R. Scott Carr, Ph.D., Project Director, Principal Investigator
US Geological Survey
Biological Resources Discipline
Columbia Environmental Research Center
Marine Ecotoxicology Research Station
Texas A&M University-Corpus Christi
Center for Coastal Studies, NRC Suite 3200
6300 Ocean Drive, Corpus Christi, TX 78412

Marion Nipper, Ph.D., Principal Investigator
Marine Ecotoxicology Research Station
Texas A&M University-Corpus Christi
Center for Coastal Studies, NRC Suite 3200
6300 Ocean Drive, Corpus Christi, TX 78412

submitted to

Naval Facilities Engineering Service Center
1100 23rd Avenue
Port Hueneme, CA 93043

April 30, 2003

revised

June 26, 2003

ACKNOWLEDGEMENTS

This study was supported by contracts with the Naval Facilities Engineering and Service Center. This study would not have been possible without the participation of numerous individuals. Karen Miller was the project officer and was instrumental in coordinating the participation of the Washington Department of Ecology, which provided the sediment from Carr Inlet, Puget Sound, WA, to be used in this study. We are indebted to the expert technical assistance of Jim Biedenbach, Russell Hooten and Linda May who helped spiking the sediments, extracting pore waters and conducting the toxicity tests. A starter culture of the copepod used in toxicity tests, *Schizopera knabeni*, was kindly donated by Dr. Gui Lotufo, with US Army Engineering Research & Development Center (ERDC). Sediment microbial analyses were conducted by Dr. Tom Johnson at the USGS Columbia Environmental Research Center. Initial chemical characterization of each sediment and GC/MS analyses for the identification of transformation products were performed by Yaorong Qian, under the direction of Dr. Terry Wade, at GERG, Texas A & M University – College Station. Dr. Tracy Villareal, at University of Texas Marine Sciences Institute, performed nitrite analyses in some select porewater samples. Sodium picramate was kindly donated by Dr. Tom Jenkins, US Army Corps of Engineers, Cold Regions Research Engineering Lab.

DISCLAIMER

The contents, views and opinions expressed in this document are those of the authors and do not necessarily represent the policies or positions of the USGS, the United States Government, or other organizations named in this report. Additionally, the mention of trade names for products or software does not constitute their endorsement.

TABLE OF CONTENTS **Page**

EXECUTIVE SUMMARY 1

INTRODUCTION 4

MATERIAL AND METHODS 4

PART 1: Bio- and Photo-transformation of 2,6-DNT and Picric Acid in Spiked Sediments 4
Sediment Collection, Processing and Sterilization 4
Preparation and Chemical Analyses of Stock Solutions for Sediment Spiking 5
Sediment Spiking and Porewater Extraction 6
Chemical Analyses of Pore Waters and Sediments 7
Sediment Microbiological Assessment 8
Total Coliforms and Heterotrophic Bacteria 8
Microbial Community Analysis 9
Enzyme Induction Experiments 10
Photo-transformation of 2,6-DNT and Picric Acid in Seawater 11

PART 2: Toxicity Assessments 13
Porewater Extraction, Storage and Water Quality Measurements 13
Chemical Analyses 14
Toxicity Tests 14
Reference Toxicant Tests 15
Statistical Analyses 16

RESULTS AND DISCUSSION 17

PART 1: Bio- and photo-transformation of 2,6-DNT and Picric Acid in Spiked Sediments 17
Pore Water and Sediment Chemical Assessment 17
2,6-Dinitrotoluene 17
Picric Acid 21
Dissolved Organic Carbon (DOC) 25
Sediment Microbiological Assessment 25
Total Coliforms 25
Heterotrophic Bacteria 28
Microbial Community Analysis and Enzyme Induction Experiments 28
Photo-transformation of 2,6-DNT and Picric Acid in Spiked Sediments 33

PART 2: Toxicity Assessments 37
Water Quality Measurements 37
Tests with Single Chemicals and Photo-transformed 2,6-DNT 37
Tests with Pore Waters 37

Toxicity Assessment of Ordnance Compounds and Biotransformation Products in Seawater 37
Porewater Toxicity Assessment 40
2,6-Dinitrotoluene 40
Picric Acid 43
Toxicity Assessment with Photo-transformed 2,6-DNT 47
Photo-induced Toxicity Assessments 47
Reference Toxicant Tests 49

SUMMARY AND CONCLUSIONS 49

REFERENCE LIST 50

TABLES

Table 1. Irradiance measurements taken at the surface of the exposure vessel for the photo-transformation and photo-activation experiments 13

FIGURES

Figure 1. Syringes with filtration devices for porewater extraction by vacuum: left - stainless steel mesh filtering medium; right - ground silica air stone filtering medium 7

Figure 2. Schematic diagram of the Biolog EcoPlate® showing the position of the different substrates 11

Figure 3. Spectral comparison of mid-day sun (red) in December in Corpus Christi, Texas versus the solar simulator (blue) 12

Figure 4. HPLC chromatograms of: A) 2,6-DNT stock solution in methanol; B) Sandy sediment immediately after spiking with 2,6-DNT stock solution; C) Fine-grained sediment immediately after spiking with 2,6-DNT stock solution 19

Figure 5. Concentration of 2,6-DNT and its biotransformation product, 2-A-6-NT in sediments at different storage times and temperatures: A) Sandy sediment at 10oC; B) Sandy sediment at 20°C; C) Fine-grained sediment at 10°C; D) Fine-grained sediment at 20°C 22

Figure 6. Concentration of picric acid in sediments at different storage times and temperatures: A) Sandy sediment at 10°C; B) Sandy sediment at 20°C; C) Fine-grained sediment at 10°C; D) Fine-grained sediment at 20°C; E) Vials with pore water extracted prior to storage (day 0 – yellow), and after 56-day storage of sandy (red) and fine-grained (purple) sediment 22

Figure 7. HPLC chromatograms of: A) Picric acid stock solution in filtered seawater; B) Sandy sediment immediately after spiking with picric acid stock solution; C) Fine-grained sediment immediately after spiking with picric acid stock solution 23

Figure 8. Dissolved organic carbon (DOC) concentration in pore waters used in toxicity tests, from sediments spiked with 2,6-DNT and picric acid, and respective blanks, at different storage times 26

Figure 9. Total coliforms (MPN) data comparing the ordnance-spiked (blue) and appropriate reference sediment (maroon, with MeOH for 2,6-DNT and MFS for picric acid) 27

Figure 10. Total heterotrophic bacteria in sandy and fine-grained sediments spiked with 2,6-DNT and corresponding methanol blanks, at several storage times. Data are expressed as most probable number (MPN) of bacterial cells/mg sediment wet weight 29

Figure 11. Total heterotrophic bacteria in sandy and fine-grained sediments spiked with picric acid and corresponding seawater blanks, at several storage times. Data are expressed as most probable number (MPN) of bacterial cells/mg sediment wet weight 30

Figure 12. Comparison of total heterotroph MPN data (white) and EcoPlate® data for control (blue) and 2, 6- DNT-spiked sediments (red) 31

Figure 13. Comparison of total heterotroph MPN data (white) and EcoPlate® data for control (blue) and picric acid-spiked sediments (red) 32

Figure 14. Ecoplate® patterns for five common sediment bacteria Enterobacter aerogenes, Pseudomonas fluorescens, Escherichia coli, Klebsiella sp. and Aeromonas sp. before and after exposure to picric acid 34

Figure 15. Concentration of 2,6-DNT and picric acid in spiked seawater exposed to simulated solar radiation (SSR) for 72 hours and 47 days, respectively 35

Figure 16. HPLC chromatograms of seawater spiked with 2,6-DNT and exposed to simulated solar radiation (SSR) for: A) 0 hours; B) 48 hours; C) 96 hours. Vials in A and C show the change in color of the 2,6-DNT solution from clear to orange with SSR exposure 36

Figure 17. Survival and reproduction effective concentrations in copepod tests with single chemicals 39

Figure 18. Effective concentrations in Ulva fasciata zoospore tests with single chemicals 39

Figure 19. Effect of 2,6-DNT-spiked sediment storage time on the toxicity of pore waters to copepod reproduction, expressed as number of nauplii produced per female, in sandy (A) and fine-grained (B) sediment. Asterisks indicate significant difference from controls (not shown) 41

Figure 20. Effect of 2,6-DNT-spiked sediment storage time on the toxicity of pore waters to Ulva fasciata zoospore germination (A, B), germling growth in length (C, D) and in cell number (E, F) in sandy and fine-grained sediments 42

Figure 21. Effect of picric acid-spiked sediment storage time on the toxicity of pore waters to copepod survival (A, B) and reproduction (C, D, expressed as number of nauplii produced per female), in sandy and fine-grained sediments. Asterisks indicate significant difference from the controls (not shown) 44

Figure 22. HPLC chromatograms of pore waters from sediments spiked with picric acid, after several storage times: A- D = Storage times 0, 28, 56 and 180 days, respectively, in sandy sediment; E- H = Storage times 0, 7, 56 and 180 days, respectively, in fine-grained sediment 45

Figure 23. Effect of picric acid-spiked sediment storage time on the toxicity of pore waters to Ulva fasciata zoospore germination (A, B), germling growth in length (C, D) and in cell number (E, F) in sandy and fine-grained sediments 46

Figure 24. Toxicity of 2,6-DNT and photo-degraded 2,6-DNT. EC50s for photo-degraded 2,6-DNT are based upon concentrations of 2,6-DNT prior to SSR irradiation 48

Figure 25. Effect of simulated solar radiation (SSR) on the toxicity of ordnance compounds: A) S. knabeni exposed to ~75% of the 96-h LC50 of 2,6-DNT and picric acid prior to SSR exposure. SSR at 280-320 nm = 15.6 FW/cm2 = 4% UVB of summer sun; B) D. gyrociliatus exposed to ~50% of the 96-h LC50 of 2,6-DNT prior to SSR exposure. SSR at (280-320 nm = 18.7 FW/cm2) 48

APPENDICES

Appendix A. Measured concentrations of 2,6-DNT, picric acid and some transformation products in samples exposed to bio- or photo-transformation and in samples used for toxicity testing.

Appendix A1. Concentration of 2,6-DNT and its major initial transformation product, 2-A-6-NT in sterilized and non-sterilized samples spiked with 2,6-DNT and stored at 10 or 20°C for up to 180 days. Sediments were analyzed wet and dried at room temperature.

Appendix A2. Percent loss of 2,6-DNT over time, in different sediments and at different temperatures.

Appendix A3. Concentration of picric acid in spiked sediment and corresponding pore waters during different storage times at 10 and 20°C.

Appendix A4. Percent loss of picric acid over time, in different sediments and at different temperatures.

Appendix A5. Percent loss of 2,6-DNT and picric acid with time, under simulated solar radiation.

Appendix A6. Measured concentrations of picric acid and some of its major biotransformation products in pore waters from sediments stored at 10°C and used for toxicity testing.

Appendix B. Water quality measurements in porewater and spiked seawater samples used in toxicity tests.

Appendix B1. Water quality data for porewater samples used in toxicity tests with copepods.

Appendix B2. Water quality data for porewater samples used in toxicity tests with algae.

Appendix B3. Water quality data for toxicity tests with single chemicals using *Schizopera knabeni.*

Appendix B4. Water quality data for toxicity tests with single chemicals using *Ulva fasciata*.

Appendix B5. DOC concentration in ordnance-spiked pore waters from sandy and fine-grained sediment over time at different temperatures.

Appendix C. Microbial analyses of sediments spiked with 2,6-DNT and picric acid.

Appendix C1. Total heterotrophic bacteria counts in ordnance-spiked sediments and respective blanks.

Appendix C2. Ecoplate® data for the degradation experiments conducted with the sandy sediment for control samples (MFS=Millipore filtered seawater, and MeOH = methanol) and picric acid and 2,6-DNT-spiked sediments at two different temperatures.

Appendix C3. Ecoplate® data for the degradation experiments conducted with the fine-grained sediment for control samples (MFS=Millipore filtered seawater, and MeOH = methanol) and picric acid and 2,6-DNT-spiked sediments at two different temperatures.

Appendix D. Results of toxicity tests with single chemicals (ordnance compounds and major transformation products) spiked in seawater.

Appendix D1. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to copepod, *Schizopera knabeni*, gravid female survival.

Appendix D2. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to copepod, *Schizopera knabeni*, embryo and nauplii survival.

Appendix D3. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to algae, *Ulva fasciata*, zoospore germination.

Appendix D4. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to algae, *Ulva fasciata*, germling length.

Appendix D5. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to algae, *Ulva fasciata*, germling cell number.

Appendix D6. Results of macroalgae toxicity tests with sodium dodecyl sulfate.

Appendix D7. Results of copepod toxicity tests with the reference toxicant, sodium dodecyl sulfate.

Appendix E. Results of toxicity tests with pore waters from sediments spiked with 2,6-DNT and picric acid.

Appendix E1. Toxicity of pore waters from sediments spiked with 2,6-DNT to copepod, *Schizopera knabeni*, survival.

Appendix E2. Toxicity of pore waters from sediments spiked with 2,6-DNT to copepod, *Schizopera knabeni*, embryo and nauplii survival.

Appendix E3. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata*, zoospore germination.

Appendix E4. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata*, germling length.

Appendix E5. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata*, germling cell number.

Appendix E6. Toxicity of pore waters from sediments spiked with picric acid to copepod, *Schizopera knabeni*, female survival.

Appendix E7. Toxicity of pore waters from sediments spiked with picric acid to copepod, *Schizopera knabeni*, embryo and nauplii survival.

Appendix E8. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata*, zoospore germination.

Appendix E9. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata*, germling length.

Appendix E10. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata*, germling cell number.

ATTACHMENTS

Attachment 1. (SOP 10.24) Extraction and storage of porewater samples using *in situ* devices.

Attachment 2. (SOP 10. 23) Algal zoospore germination and germling growth toxicity test.

Attachment 3. (SOP 10.25) Culture and toxicity testing methods for *schizopera knabeni* (Copepoda, Harpacticoida, Diosaccidae).

Attachment 4. (SOP 10.10) *Dinophilus gyrociliatus* toxicity test.

EXECUTIVE SUMMARY

This study was undertaken to help understand the fate and effects of two munitions and explosives of concern, 2,6-dinitrotoluene (2,6-DNT) and 2,4,6-trinitrophenol (picric acid) in marine environments. Specifically, biotransformation and degradation in marine sediments, and photo-transformation in seawater were assessed. Studies were conducted with sediments spiked in the laboratory with the two chemicals of concern for the biotransformation assessments, and by spiking filtered seawater with these chemicals for the photo-transformation assessments.

For the biotransformation study, two different natural non-contaminated sediments were used: one sandy (79.9% sand, 10.4% silt and 9.7% clay) and 0.25% total organic carbon (TOC) content, and one fine-grained (15.9% sand, 68.8% silt and 15.3% clay) and 1.1% TOC. Sediments subsamples were spiked with 2,6-DNT or picric acid and subdivided in two lots, one stored at 10°C and one at 20°C, for 6 months, and biotransformation along time was assessed by chemical, microbiological and porewater toxicological analyses at selected time intervals. Sterilized sediments were used as biotransformation controls and were chemically analyzed at the same time intervals as the non-sterilized sediments.

Sediments and pore water were analyzed by HPLC, and some selected samples were analyzed by GC/MS for identification of biotransformation products. Biotransformation started taking place during the spiking procedure, and proceeded for several months, generating a variety of breakdown products during its course. Biotransformation was faster at 20°C and in the fine-grained sediment, than at 10°C and in the sandy sediment, but seemed to follow the same paths in all conditions, although at different rates. The major biotransformation product of 2,6-DNT was 2-amino-6-nitrotoluene (2-A-6-NT). 2-nitrotoluene (2-NT) was also identified as a biotransformation product, and minor components found in 2,6-DNT-spiked samples during the storage time were N,N-dimethyl-3-nitroaniline, benzene nitrile, methylamino-2-nitrosophenol and diaminophenol. After more prolonged storage these chemicals tended to be replaced with larger molecular weight polymers, possibly derived from 2,6-DNT. Several breakdown products of picric acid were identified by GC/MS, and their nature changed with storage time. Breakdown compounds identified at earlier stages of biotransformation (0 to 56 days) were 2,4-dinitrophenol, amino dinitrophenols (including 2-amino-4,6-dinitrophenol, a.k.a. picramic acid), 3,4-diamino phenol, amino nitrophenol, nitro diaminophenol, and peaks possibly corresponding to aminonitrotoluene isomers, diaminotoluenes, dinitrotoluenes and nitrotoluenes were present but this identification is somewhat dubious, i.e., the identity of the peaks was not confirmed.

Analyses of total heterotrophic bacteria were performed and had a similar behavior in all ordnance-spiked samples: there was a strong initial increase in bacterial abundance relative to the respective blanks, followed by a depression that took bacterial abundance to similar levels of the blanks and lasted a few days, then followed by a remarkable increase in the spiked samples, but not in the blanks. The results suggest that the bacteria were using the nitroaromatic compounds as sources of nutrients and causing their transformation into some measured and other unidentified breakdown products. Microbial community analyses suggested a shift in composition, in addition to the observed changes in total heterotrophs.

Toxicity tests were performed using the copepod, *Schizopera knabeni*, and the macroalga, *Ulva fasciata*. Copepod test endpoints were female survival, and nauplii hatching and survival. The latter was the most sensitive endpoint. Macroalga test endpoints were zoospore germination and germling growth.

Some of the major biotransformation products of 2,6-DNT and picric acid were purchased in purified form and analyzed for toxicity in filtered seawater. The main 2,6-DNT biotransformation product, 2-A-6-NT, was more toxic than its parent compound in copepod, *Schizopera knabeni*, embryo and nauplii survival tests, but was less toxic in the macroalga, *Ulva fasciata*, zoospore germination test. The opposite was observed with 2-NT, which was less toxic than the parent compound to copepods and slightly more toxic to macroalgae zoospore germination, but not to germling growth. Some of the main identified breakdown products of picric acid, 2,4-DNP and picramic acid, were more toxic to copepods and macroalgae zoospores than their parent compound, with toxicity ranking in the following order: 2,4-DNP > picramic acid > picric acid.

Porewater test EC_{50} values for samples spiked with 2,6-DNT were calculated as the sum of measured initial concentration of 2,6-DNT and 2-A-6-NT. Similarly to results of tests with purified 2,6-DNT and 2-A-6-NT, porewater samples in which 2,6-DNT was completely transformed and only 2-A-6-NT could be measured (7-day storage) were more toxic to copepod nauplii survival than those where a high amount of 2,6-DNT was measured (prior to storage). After 180 days of storage no 2-A-6-NT was detectable in either sediment sample, and toxicity to copepod reproduction decreased in the fine-grained sediment, but it increased somewhat in the sandy sediment, relative to the sample stored for 7 days. The data suggests that in the fine-grained sediment after 6 months the 2,6-DNT breakdown products had been further metabolized to less toxic forms, whereas in the sandy sediment they had not broken down to the same stage. It is expected that further biotransformation would occur in the sandy sediment, in which breakdown processes typically took longer to occur. Unlike with copepod nauplii, in the tests with *U. fasciata* zoospores porewater toxicity decreased with sediment storage time for both sediments, indicating that 2,6-DNT biotransformation products were less toxic than the parent compounds to the macroalgae early life stages, thus showing the importance of using more than one species and endpoints for toxicity assessments.

The toxicity of pore waters from picric acid-spiked sediments was variable over time. In the sandy sediment, toxicity to copepod nauplii survival was at its highest after a 28-day storage, and it only decreased significantly after a 180-day storage, although still toxic at a 25% porewater dilution. Similarly, toxicity in the *U. fasciata* zoospore test tended to decrease with storage time of the sandy sediments up to 56 days, with a slight increase in toxicity to the percent germination endpoint with samples from 180-day storage. Picramic acid and 2,4-DNP are likely to have caused or contributed to the observed toxicity in samples from up to 56-day storage, but they were not measured after a 180-day storage. Further biotransformation products are expected to have caused the milder observed effects after the 6-month storage. Possible products related to picric acid found in this sample were products containing amino, nitro and hydroxyl groups, in addition to nitrophenol isocyanate. The toxicity of the latter to the aquatic organisms has not been established. Samples from the fine-grained sediment were the most toxic at the 56-day storage point, with reduced effect after 180-day storage. However, some toxicity still occurred, suggesting that toxic chemicals resulting from the picric acid biotransformation were still present after 6 months, although they could not be quantified by GC/MS analyses, which only indicated the presence of a very small amount of nitrodiaminophenol. Major aromatic compounds in this sample were benzoic acids, benzene acetic acids, the source of which is not clear. However, as for 2,6-DNT spiked samples, toxicity in the fine-grained sediment after 180-day storage was lower than in the sandy samples, suggesting that further metabolization of picric acid breakdown products had occurred, generating less toxic products.

Photo-transformation of 2,6-DNT and picric acid was assessed in seawater, under simulated solar radiation (SSR) in a constant temperature chamber kept at 10°C. A comparison between the spectra of natural sunlight and the solar simulator indicated that the experiments were conducted at approximately 58% of the summertime solar maximum at a latitude of approximately 39°N.

No significant photolysis of picric acid was observed in the current study in up to a 47-day exposure to SSR at irradiance levels of 196 and 1023 $\mu W/cm^2$ UVB and UVA, respectively. Photo-transformation of 2,6-DNT in seawater under SSR (292 and 1398 $\mu W/cm^2$ UVB and UVA, respectively) began soon after the initial exposure, with 10% loss in the first 2 hours, 89% photo-transformed in 24 hours, and none left after 72 hours. A thin peak appeared in the HPLC chromatograms at an elution time of 1.7 minutes and the color of the stock solution changed from clear to orange, but the final photo-transformation product could not be identified by GC/MS, although GC/MS and LC/MS analyses showed high molecular weight chemicals with mass spectra ranging from molecular weight (MW) 200 to 500 compared to the MW 182 for DNT. Complexity of the mass spectra and mass differences among fragments suggest that multiple compounds were possibly co-eluting. The photolysis of 2,6-DNT might have promoted polymerization which formed these high MW compounds.

Samples of photo-transformed 2,6-DNT were assessed for toxicity concurrently to tests with dilution water spiked with 2,6-DNT. The original compound, 2,6-DNT, was more toxic than its breakdown product to *U. fasciata* zoospores, copepod female survival, and to the polychaete, *Dinophilus gyrociliatus*, reproduction, but not to copepod nauplii survival, where slightly higher toxicity was exhibited by the photo-irradiated solution.

In addition to photo-transformation studies, photo-induced toxicity was assessed at the termination of toxicity tests with 2,6-DNT and picric acid in seawater using copepods and polychaetes. Photo-induced toxicity was not exhibited by either chemical, although some adverse effects, including sluggishness of the animals, were observed at the end of the SSR exposure. These effects also occurred in the controls and, therefore, results were inconclusive.

In conclusion, biotransformation of 2,6-DNT and picric acid in marine sediments is temperature- and sediment type-dependent, but tends to proceed relatively rapidly and follow the same chemical paths under all tested conditions. Whereas some of the major initial biotransformation products of both 2,6-DNT and picric acid were more toxic than the parent compounds in some toxicity assessments, further transformation tended to lead to reduction in toxicity. However, it would seem prudent to include some of the major identified degradation products in the list of analytes of concern in field assessments of sediments suspected of contamination by nitroaromatics, as these compounds are not identified in the standard HPLC analysis for ordnance compounds.

INTRODUCTION

Ordnance compounds in marine environments are a matter of concern due to several factors including past use, storage, improper disposal, and incineration of these compounds at Navy facilities, or the presence of unexploded ordnance in some areas off the US coast. As a result of previous studies, which suggested that the degradation/transformation of some ordnance compounds enhanced their toxicity to marine organisms (Carr and Nipper 2000, Nipper *et al.* 2002), a study was conducted to help identifying hazardous ordnance transformation products, causes and pathways of transformation and degradation, and stability of such products prior to complete mineralization. Ordnance compounds considered of priority for this study were 2,6-dinitrotoluene (2,6-DNT) and 2,4,6-trinitrophenol (picric acid), due to observed transformation in previous studies.

The specific objectives of this study were:

1) To determine the effect of sediment type (grain size distribution and organic carbon content) on the microbial transformation/degradation of 2,6-DNT and picric acid in marine sediments;

2) To evaluate the effect of photo-transformation and photo-induced toxicity on 2,6-DNT and picric acid in marine waters, by exposure of contaminated waters and organisms to simulated solar radiation (SSR);

3) To determine if bio- and photo-transformation products of 2,6-DNT and picric acid differ in their nature;

4) To assess whether the degradation of 2,6-DNT and picric acid will proceed through mineralization given sufficient time;

5) To determine if the transformation products of 2,6-DNT and picric acid are more or less toxic to marine organisms than the parent compounds.

MATERIALS AND METHODS

PART 1: *Bio- and Photo-transformation of 2,6-DNT and Picric Acid in Spiked Sediments*

Sediment Collection, Processing and Sterilization

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 US 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 (15.9% sand, 68.8% silt and 15.3% clay) and 1.1% total organic carbon (TOC) content. The sediment from the Gulf of Mexico was collected at Redfish Bay,

TX, and was selected for its sandy characteristics (79.9% sand, 10.4% silt and 9.7% clay) and 0.25% TOC.

Upon arrival at the laboratory each sediment was press-sieved through 1mm mesh for removal of the indigenous fauna and stored at 4°C for no more than 6 weeks, when it was spiked with ordnance compounds. Each sediment was fully characterized for concentration of metals, PAHs and organochlorinated compounds prior to use in the experiments. Very low levels were exhibited by all measured chemicals, although slightly higher in the fine-grained sediment from Puget Sound than in the sandy sample from Texas, but generally one or two orders of magnitude below expected toxic levels based on sediment quality guidelines (Long *et al.* 1995; MacDonald *et al.* 1996).

Prior to spiking with ordnance compounds a sub-sample of each sediment was sterilized by autoclaving. The sterilized sediments were used as chemical controls, to help distinguish between biotransformation and other forms of product reduction and breakdown (e.g., hydrolysis, redox potential) (Haderlein and Schwarzenbach 1995).

Preparation and Chemical Analyses of Stock Solutions for Sediment Spiking

Picric acid and 2,6-DNT (≥98% purity) were purchased from ChemService (West Chester, PA, USA). Although the ordnance compounds could not be sterilized, stock solutions were prepared under sterile conditions, in a laminar flow hood using autoclaved glassware. Desired concentrations of stock solutions were calculated in mg/L, but data are reported in μmoles/L or μmoles/kg dry weight, for pore waters and sediments, respectively. Molecular weights of 2,6-DNT and picric acid are 182.14 and 229.11, respectively, which were used for the calculation of molar concentrations.

All chemical measurements of stock solutions, pore waters and sediments were performed using U.S. EPA Method 8330 (USEPA, 1994) with some modifications: an ODS column was used; the injection volume for nitrotoluenes and derivatives was 10 μl and for picric acid and derivatives it was 2.5 μl; the mobile phase for measurements of picric acid and derivatives consisted of an isocratic mixture of 35% methanol and 65% 0.1 M sodium acetate with pH adjusted to 4.8 and the absorbance was measured at a wavelength of 250 nm. Standards of the different analytes in acetonitrile or methanol were used for method calibration. Picric acid and 2,6-DNT standards were purchased from Restek (Bellefonte, PA).

A 2,6-DNT stock solution was prepared by addition of 5 g of the chemical to 250 ml of HPLC-grade methanol and stirring on magnetic stirrer for 30 minutes, until no more crystals were visible in the solution. This solution was used to spike the sandy sediment, and kept at 4°C for 24 hours prior to spiking the fine-grained sediment. Due to the extremely high concentration of 2,6-DNT in this stock solution, a 100-fold dilution of the stock solution was prepared for HPLC measurement. The measured concentration of 2,6-DNT in the 100-fold dilution was 157.5 mg/L (864.7 μmoles/L), i.e., the final concentration of 2,6-DNT in the stock solution was 15.75 g/L (86.47 mmoles/L). A new measurement was performed prior to spiking the fine-grained sediment. The measured concentration was 208.5 mg/L (1144.7 μmoles/L) in the 100-fold dilution, i.e., a final concentration in the stock solution of 20.85 g/L (114.47 mmoles/L). This increase in the concentration of the stock solution may have been caused by further dissolution of 2,6-DNT micro-crystals, not visible to the naked eye, into the methanol during the overnight storage.

A picric acid stock solution was prepared by addition of 12 g of this chemical (with ~30% moisture) to 900 ml of sterilized seawater at 30 ppt salinity. Sterilization was achieved by filtering the seawater through an autoclaved Millipore® apparatus with a 0.45 μm filter inside a laminar flow hood. The solution was stirred on magnetic stirrer for 5 hours, and then a 100-fold dilution was prepared for HPLC measurement. The measured concentration of picric acid in the 100-fold dilution was 80.40 mg/L (350.9 μmoles/L), i.e., the final concentration in the stock solution was 8.04 g/L (35.1 mmoles/L).

HPLC control measurements were also conducted on the dilution water (0.45 μm filtered seawater), acetonitrile, methanol, and a methanol sample pushed through a 0.45 μm Teflon filter of the type used to filter extracted samples. None of the blanks showed any peaks, as expected, except for the solvent front peak.

Sediment Spiking and Porewater Extraction

The rolling jar method was used for sediment spiking (Ditsworth *et al.* 1990), and the general methodology described by Murdoch (Murdoch *et al.* 1997), with the use of a solvent carrier, was followed with 2,6-DNT. Glass jars of different sizes, depending on the final volume desired, with Teflon®-lined lids were used for the spiking procedure. In order to maximize spiking efficiency, a maximum of 76% of the total volume of a jar was filled with sediment. Several jars with each type of sediment had to be spiked to achieve desired final volumes spiked with each chemical (2,6-DNT and picric acid).

For sediment spiking with 2,6-DNT, a volume of 8.3 ml of the stock solution in methanol was added for each liter of sediment to be placed in the glass jars to be used for spiking. The rolling table to be used for sediment spiking was put into a fume hood and jars containing the stock solution were rolled with open lids until the methanol evaporated (approximately 1.5 hour) and 2,6-DNT crystals could be seen coating the walls of the jars. The desired type of sediment was then added to each gallon jar. For the sandy sediment, the addition of dilution water in a volume of 3% of the total sediment was added to each jar and contents were thoroughly shaken for initial homogenization. This was necessary to achieve the appropriate sediment consistency for efficient rolling and, consequently, spiking. Jars with walls coated with 2,6-DNT and containing sediments were rolled for 6 hours at a speed of 1.1 rpm.

For the picric acid spiking procedure, desired amounts of sediment were added to the spiking jars and the stock solution was added directly to the sediment. For the sandy sediment, the volume of stock solution added was equivalent to 3% of the sediment volume (30 ml stock solution/L sediment), and for the fine-grained sediment it was 2.1% (21 ml stock solution/L sediment). This difference was due to the fact that calculations for the spiking were done on a dry weight basis. The moisture content was 46.2% for the fine-grained sediment and 29.6% for the sandy. After addition of the stock solution the jars were tightly closed, vigorously shaken, and then rolled at 1.1 rpm for 6 hours.

Sediment blanks (controls) for the 2,6-DNT spiking procedure were prepared in the same manner, but only methanol with no DNT was added to the jars and evaporated prior to sediment addition. Blanks for the picric acid spiking procedure were also prepared in the same manner, but with addition of dilution water only instead of the picric acid stock solution. All blanks were treated in identical manner to the sediments spiked with ordnance compounds.

Sterilized sediments were spiked in the same manner with both compounds and blanks, but in smaller amounts since they were only used as chemical and microbiological controls, but not

for toxicity testing. All steps leading to the spiking of sterilized sediments and processing of them thereafter were done in a sterile environment using sterilized materials.

At the end of the rolling procedure the contents of all jars with one type of sediment and chemical were homogenized into one batch by use of a stainless steel electric paint mixer and then subdivided for storage in the dark at 10 and 20°C. Sediment subsamples were taken for porewater extraction prior to storage, for HPLC and microbiological analyses.

Sediment subsamples were harvested at pre-determined time intervals (based on the results of a preliminary experiment – not reported) and subdivided for porewater extraction, and chemical and microbiological analyses.

Porewater extractions were performed by vacuum, using disposable syringes attached to filtering media (Fig. 1). Extracted pore water was centrifuged for 20 minutes at 1200 *g* for removal of all visible particles (see Attachment 1 – SOP F10.24). Extracted pore water was subdivided into samples for HPLC, GC/MS and dissolved organic carbon (DOC) analyses, and toxicity testing. Whereas the HPLC analyses were conducted immediately, the samples for all other kinds of assessments were frozen. Porewater samples for DOC analyses were filtered through a 0.45µm nylon syringe filter prior to freezing. HPLC analyses were repeated with the samples used for toxicity testing after they were thawed, to verify if any ordnance losses or identifiable chemical changes had occurred due to freezing and thawing.

Figure 1. Syringes with filtration devices for porewater extraction by vacuum: left – stainless steel filtering medium; right – ground silica air stone filtering medium.

Chemical Analyses of Pore Waters and Sediments

All porewater samples were analyzed by HPLC following USEPA Method 8330 (USEPA, 1994) with some modifications as described in a previous section (Preparation and chemical analyses of stock solutions for sediment spiking). Prior to the HPLC analysis the pore waters were mixed with HPLC-grade methanol on a volume/volume basis, since extractions with methanol produced higher recovery rates than those performed with acetonitrile. Samples were then filtered through a 0.45 µm Teflon® filter, as required by Method 8330, and analyzed.

Sediment samples were extracted by two procedures:

a) As recommended by Method 8330, i.e., dried at room temperature to a constant weight, finely ground, vortex mixed with acetonitrile (10 ml acetonitrile for 2 g dry sediment) and put in an ultra-sonicator for 18 hours, followed by removal of 5 ml of the supernatant and addition of 5 ml $CaCl_2$ solution, filtration and measurement by HPLC.

b) Following the same procedure, but without pre-drying the sediment, i.e., wet sediment with weight equivalent to 2 g dry weight (based on moisture content) was mixed with 10 ml acetonitrile, ultra-sonicated, and processed and measured as in the first procedure.

This comparison was done in order to assess the suspected loss of ordnance compound during the drying period, either by degradation or irreversible binding, as was hypothesized in a previous study (Carr and Nipper 2000; Nipper *et al.* 2002).

Selected porewater samples showing peaks suspected of being transformation products were analyzed by GC/MS. Analysis of degradation products in the water samples was experimental in nature and the procedure is variable. Aliquots of water samples were dried with sodium sulfate and the residues dissolved in methanol were analyzed by GC/MS in full scan mode. Aliquots of water samples were also extracted with methylene chloride under both acidic and basic conditions. The combined methylene chloride extracts were analyzed with GC/MS. A portion of the extract was also derivatized with BSTFA/pyridine (bis(trimethylsilyl)trifluoroacetamide) and analyzed by GC/MS. Samples were all evaporated to dryness under a stream of N_2 after derivatization and the residue was redissolved in 200 µl of methylene chloride for instrumental analysis. For further identification of ordnance transformation products, an aliquot of the porewater samples was dried over anhydrous sodium sulfate. Organic compounds were washed into a flask with acetone followed by ethyl acetate and dichloromethane. Solvents were evaporated to near dryness using a rotary evaporator and split into three portions after dissolving in methanol. One portion of the sample was analyzed with GC/MS in full scan mode, another portion of the sample was derivatized with BSTFA/pyridine (bis(trimethylsilyl)trifluoroacetamide) and analyzed by GC/MS, and the third portion of the sample was derivatized with diazomethane and analyzed by GC/MS. BSTFA reacts with –OH, NH2 and –COOH groups. Diazomethane methylates –OH and –COOH groups. Comparison of BSTFA and diazomethane derivatized compounds helped in the identification of amino-containing compounds. Samples were all evaporated to dryness under a stream of N_2 after derivatization and the residue was redissolved in 200 µl of methylene chloride for instrumental analysis.

Porewater samples extracted from picric acid-spiked sediments after a 56-day storage were also analyzed for nitrite, due to elevated toxicity and absence of picric acid or any other compounds measurable by HPLC using the modified method 8330 (USEPA 1994). Analyses were conducted using the standard nitrite colorimetric method (Parsons *et al*. 1984). A Lachat Quikchem 8000 flow injection ion analyzer was used. The nitrite (reduced nitrate plus original nitrite) was determined by diazotization with sulfanilamide under acidic conditions to form a diazonium ion. The resulting diazonium ion was then coupled with N-(l-naphthyl) ethylenediamine dihydrochloride. The resulting pink dye absorbed at 520 nm.

Sediment Microbiological Assessment

Assessments of the sediment microbial community in non-sterilized and sterilized spiked sediments and controls were conducted on every sampling occasion. The procedures described below were applied for these assessments.

Total Coliforms and Heterotrophic Bacteria

The detection of total coliforms is based on IDEXX's Defined Substrate Technology and was conducted using the method described in Clesceri *et al.* (1998, Method 9223). Nutrient indicators that produce a yellow color when metabolized by total coliform bacteria are used to count bacteria present in sediments. Heterotrophic bacteria at 1 colony forming unit (CFU)/100 ml can be detected within 24 h at 35 ± 0.5°C.

Aseptic technique and good laboratory practices were followed for this procedure:

1. IDEXX Coliform media was hydrated by adding a Snap Pack® into a sterile disposable bottle containing 100 ml sterile diluent, capped, and shaken to dissolve.

2. 1 ml of sample sediment suspension was inoculated into the above solution and mixed thoroughly.
3. This mixture was poured into an IDEXX Quanti-tray® with 90 micro-wells and the tray was immediately heat-sealed.
4. The tray was incubated at 35°± 0.5°C for 24 hr in the dark.
5. The wells of the tray were checked for yellow color. If the yellow color was greater than the comparator (control), the presence of total coliforms was confirmed. The yellow wells were counted and the Most Probable Number (MPN) table was used to determine the MPN of total coliforms present in the sample.
6. A standard culture of *Enterobacter aerogenes* was used as a positive control. A tray with media and without sediment sample was used as a negative control.

Heterotrophic bacteria obtain energy, carbon, and nitrogen from organic substances, degrade industrial pollutants, and play an integral role in biogenic cycles in all ecosystems. Heterotrophic bacterial counts give an indication of the general "health" of the sediment as well as an indication of the availability of organic nutrients in the ecosystem. A count of total heterotrophic populations from sediment over time is desirable for environmental risk assessments and resource management. Two enzyme-specific methods were used to enumerate heterotrophic populations in this study: SimPlate and Total Coliforms.

The SimPlate® for enumerating waterborne heterotrophic bacteria is based on the Multiple Enzyme Technology® developed by IDEXX Laboratories (Westbrook, MA). The SimPlate® test contains a substrate-based sterile medium and SimPlates® (Stillings *et al*. 1998) in which substrates are hydrolyzed by microbial enzymes, causing a release of 4-methylumbelliferone, which fluoresces (after 48 h incubation at 35°C) when exposed to long wavelength ultraviolet light (365 nm). Viable heterotrophic bacteria in sediment are detected by testing for the presence of key enzymes known to be present in these bacteria. The SimPlate® uses multiple enzyme substrates that fluoresce blue when metabolized by waterborne bacteria. The sediment sample and media are added to a SimPlate® plate, incubated and examined. The number of fluorescing wells corresponds to a Most Probable Number (MPN) of total heterotrophic bacteria in the sample. The MPN value generated by the SimPlate® correlates with the Pour Plate method using Total Heterotroph Plate Count Agar (Clesceri *et al.* 1998, Method 9215).

Aseptic technique and good laboratory practices were followed for this procedure:

1. IDEXX Heterotroph media was hydrated by filling the vessel to the 100 ml mark with sterile diluent, capped and shaken to dissolve.
2. 1.0 ml sample suspension and 9 ml of hydrated media were pipetted into the center of the SimPlate® base.
3. The plate was covered with the lid and gently swirled to distribute the sample into all the wells.
4. The plate was tipped 90°-120° to drain an excess sample into the absorbent pad.
5. The plate was inverted and incubated for 48 h at 35 ± 0.5°C.
6. The UV light was faced toward the sample. The number of wells that fluoresced by holding a 6-watt, 365 nm, UV light 5 inches above the plate was counted.
7. The MPN table provided by IDEXX was used to determine the Most Probable Number of heterotrophic bacteria in the sample.
8. The laboratory's (Columbia Environmental Research Center) pond sediment was used as a positive control. A SimPlate® with media and without sediment sample was used as a negative control.

Microbial Community Analysis

Subsamples of sediment spiked with 2,6-DNT or picric acid from biodegradation studies underwent microbial community analysis using Biolog EcoPlates®. The Biolog Ecoplate® assay, a sole-carbon test for sediment analysis, identifies microbial isolates based on their substrate utilization profile or metabolic fingerprint (Garland 1997; Gamo and Shoji 1999). Garland and Mills (1991) found that mixed cultures of bacteria inoculated on EcoPlates® developed a distinctive "metabolic fingerprint" that could be measured over time. The characteristic pattern (metabolic fingerprint) developed by communities of bacteria grown on EcoPlates® rapidly and easily provides a large amount of information about the metabolic activity of a complex ecomicrobial community under changing environmental conditions and stresses. This approach of community-level physiological profiling has been demonstrated to be an effective tool to distinguish spatial and temporal changes in microbial communities (Garland 1997).

The Biolog EcoPlate® contains 31 diverse carbon sources (C sources) replicated three times per plate to give strong statistical data for sediment community analysis (Fig. 2). Each bacterial species has different enzymes that metabolize different carbon sources and produce a fingerprint. These community reaction patterns were monitored at one, three and five days. Changes in patterns were compared and analyzed using Principle Components Analysis (PCA) of average well-color development data (Hackett and Griffiths 1997). Changes observed in the metabolic fingerprint patterns provided key data about the microbial populations over time and under changing environmental stressors. In the present carbon source configuration for Ecoplate®, only Gram-negative bacteria are detected from sediments (Lisa Staraci-Schibler, Biolog® Inc., Hayward, CA). Gram-negative bacteria represent the significant majority of decomposers in C and N cycles (Atlas and Barth 1981).

EcoPlates® to be used in the experiments were received as sterile units with 31 carbon sources in a 96-well microplate configuration. The procedure was conducted under standard sterile conditions, as follows:

1) Microplates were inoculated with sediment samples as suspensions using a multi-tip electronic pipette.
2) The plates were incubated at 20°C in the dark for five days.
3) The community-level physiological profile was assessed for key characteristics:
   a) Pattern development (similarity)
   b) Rate of color change in each well
   c) Richness of well response (diversity)

Formation of a purple color (positive reaction) occurs when bacteria utilize the specific C source. Cellular respiration of the C source reduces a tetrazolium dye that is included with each specific source. The metabolic respiration patterns, the oxidative utilization of different C sources by different bacterial groups, were analyzed with a microplate reader (OD_{590}). Statistical analyses of data sets were done using a PCA software package.

Enzyme Induction Experiment

The presence of inducible enzymes in sediment microflora after exposure to picric acid was investigated with pure culture bacteria using five different genera: *Pseudomonas fluorescens*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella* sp. and *Aeromonas* sp. are known important decomposers in sediments and agricultural soils (Atlas and Barth 1981).

Thirty mg of picric acid (aqueous) were added directly onto the surface of sterile nutrient agar plates and immediately spread evenly over the plate surface with a sterile cotton applicator. The plates were permitted to dry for about 30 min before pure cultures of one of the five bacteria (CERC Standard Collection, 2003) in log phase were introduced onto the picric acid-agar plates. The bacteria, grown overnight in Delong flasks as a broth culture in a water-bath shaker at 35 ± 2°C, were introduced and distributed evenly over the agar surface with rapid strokes from a sterile cotton applicator saturated with the broth inoculum. The inoculated petri plates in replicates were immediately inverted and incubated in the dark at 35 ± 2°C for 48 h. Plate colonies that developed on the agar surface were analyzed for metabolic activity with the Ecoplate® protocol previously described.

	1	2	3	4
A	Water	b-Methyl Glucoside	D-Galactonic acid llactone	L-Arginine
B	Pyruvic acid methyl ester	D-xylose	D-galacturonic acid	L-Asparagine
C	Tween 40	L-erythritol	2-hydroxy benzoic acid	L-Phenyl-alanine
D	Tween 80	D-Mannitol	4-hydroxy benzoic acid	L-Serine
E	a-Cylcodextrin	N-acetyl-D-Glucoamine	l-Hydroxybutyric acid	L-Threonine
F	Glycogen	D-Gluco-amine acid	Itaconic Acid	Glyclyl-L-Glutamic Acid
G	D-Cellobiose	Glucose-1-PO_4	a-Ketobutyric acid	Phenylethyl-amine
H	a-D-Lactose	D,L,a-Glycer-ol PO_4	D-Malic Acid	Putrescine

Figure 2. Schematic diagram of the Biolog EcoPlate® showing the position of the different substrates.

Photo-transformation of 2,6-DNT and Picric Acid in Seawater

Dilution water was spiked separately with 2,6-DNT and picric acid and exposed to simulated solar radiation (SSR) for the analysis of photo-transformation under UV exposure. Exposures were done in a constant temperature chamber with the necessary adjustment to keep the temperature inside the liquid samples at 20 ± 1°C. Our solar simulator was not intended to accurately mimic the full spectrum of natural sunlight but to provide irradiance in the UVA,

UVB and visible spectra. A comparison between the spectra of natural sunlight and the solar simulator are shown in Figure 3. The SSR intensity for different wavelength ranges for the different experiments is shown in Table 1. For comparison purposes, the summertime solar maximum UVB irradiance in Columbia, MO was measured to be 427 $\mu W/cm^2$ (Little and Fabacher 1996). Therefore, in the DNT photo-transformation experiment the UVB irradiance was measured to be 249 $\mu W/cm^2$, which is approximately 58% of the summertime solar maximum.

Subsamples were measured by HPLC for the original compounds and breakdown products periodically, over a maximum period of 47 days. GC/MS analyses were also performed in an attempt to identify the nature of the breakdown product(s). After the timeframe when complete photo-transformation was observed (based on HPLC measurements), samples containing photo-transformed 2,6-DNT were assessed for toxicity using the methods detailed below (Toxicity Tests).

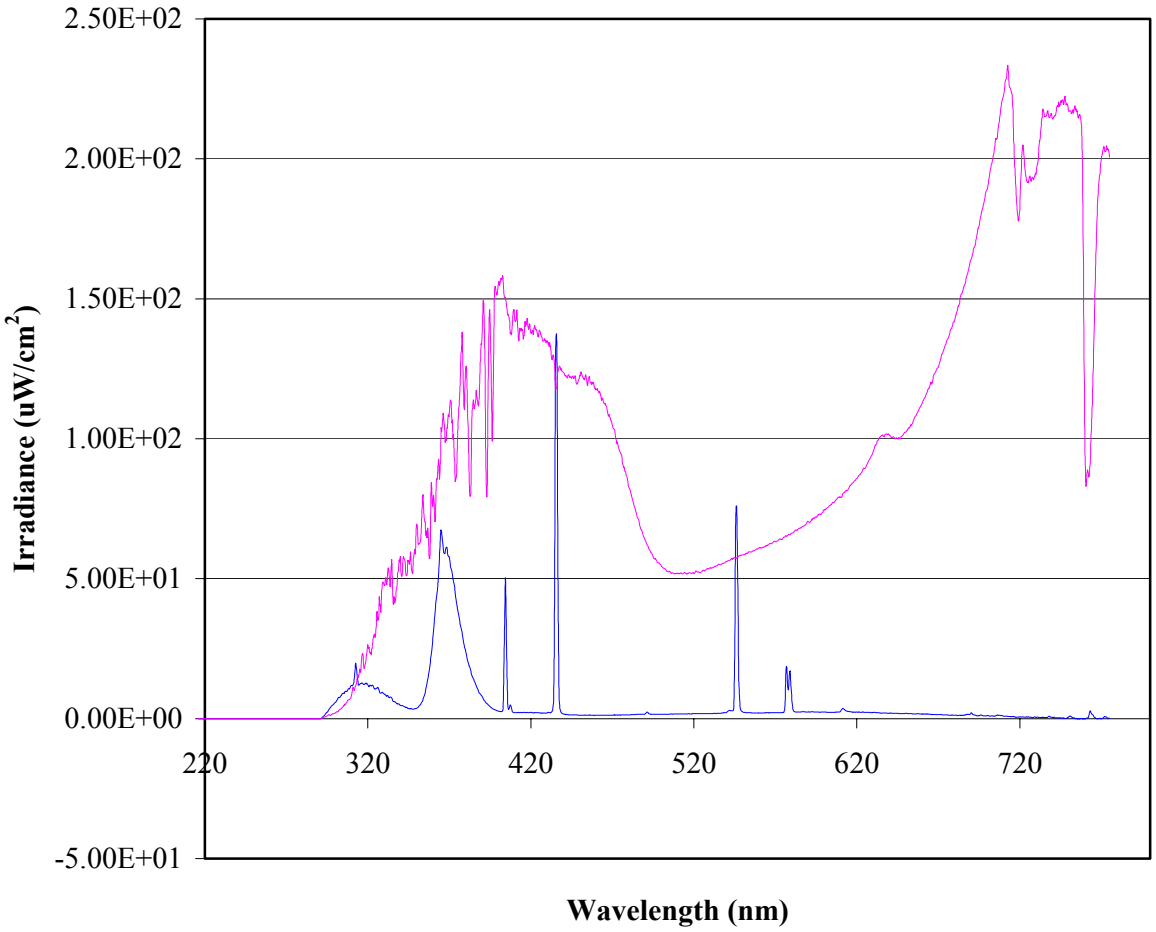

Figure 3. Spectral comparison of mid-day sun (red) in December in Corpus Christi, Texas versus the solar simulator (blue).

Table 1. Irradiance measurements taken at the surface of the exposure vessel for the photo-transformation and photoactivation experiments.

Test Description	Test Dates	Irradiance ($\mu W/cm^2$)		
		280-320 nm (UVB)	320-400 nm (UVA)	220-800 nm
2,6-DNT photo-transformation experiment	March 18-21, 2002	292	1398	2971
Picric acid photo-transformation experiment	May 29-July 18, 2002	196	1023	2273
DNT photo-transformation for toxicity testing	June 7-11, 2002	249	1321	2525
Dinophilus photoactivation experiment	September 18, 2002	15.6	189	942
Schizopera photo-activation experiment (mean of 5 measurements at center and corners of exposure tray)	November 12, 2002	19.3	186	981

PART 2: Toxicity Assessments

Porewater Extraction, Storage and Water Quality Measurements

The pore water extracted by vacuum from the non-sterilized spiked and control sediments at selected storage intervals and temperatures was used in toxicity tests. Two days prior to the 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 the following water quality measurements were made: 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. Unionized ammonia (expressed as nitrogen) concentrations (NH_3) were calculated for each sample using the respective salinity, temperature, pH and total ammonia (NH_4) measurements.

Following water quality measurements and adjustments, the samples were stored overnight at 4°C, but returned to 20 ± 1°C before the start of the toxicity tests. Porewater treatments for use in the toxicity tests were prepared by 50% serial dilutions of each sample, including some dilutions of the blank treatments. Millipore® (0.45 µm) filtered seawater (MFS) was used as the diluent, and a control series was also prepared with MFS.

Chemical Analyses

Concentrations of ordnance compounds and their identified transformation products in the pore waters used in toxicity tests, as well as in the samples from the photo-transformation experiment and the samples from tests with single chemicals were analyzed by HPLC on the day of test initiation and termination following the procedures described in previous sections (Chemical analyses of pore waters and sediments; Preparation and chemical analyses of stock solutions for sediment spiking). An additional experimental replicate without organisms was prepared for this purpose, in order to assess further degradation of the ordnance compounds under the experimental conditions.

Sub-samples of pore waters used for toxicity tests were stored frozen and later analyzed for DOC. Only samples used for toxicity assessments were analyzed for DOC, since the data were used to help interpret bioavailability of organic contaminants in those samples. DOC was measured using an OI Analytical Model 1010 Wet Oxidation Total Organic Carbon Analyzer with an autosampler following the model 1010 operator's manual (OI Analytical 1998). Previously filtered frozen samples were thawed in a tepid water bath. Duplicate one-milliliter volumes were auto-sampled into the reaction vessel. Samples were analyzed in the TOC mode with 400 µl of acid (5% phosphoric acid) and 4000 µl of oxidant (200 g/L sodium persulfate). Total inorganic carbon react and detect times were 2:00 (min:sec) and 1:35 (min:sec), respectively. Total organic carbon react and detect times were 8:30 (min:sec) and 2:00 (min:sec), respectively. At least one blank and one laboratory control were run with each batch of samples. The analysis was repeated if the percent recovery of the laboratory control failed to meet the 90-110% level.

Toxicity Tests

Porewater toxicity tests were performed using the macro-alga (*Ulva fasciata*) 96-hour zoospore germination and germling growth test and the harpacticoid copepod (*Schizopera knabeni*) 96-hour nauplii hatching and survival test. The tests were conducted following standardized procedures, described in SOPs F10.23 and F10.25, respectively (Attachments 2 and 3), but 5 rather than 10 ml porewater were used in each replicate. One additional modification was introduced in the copepod tests, which were conducted in complete darkness to minimize photo-degradation of the ordnance compounds during the exposure period. A 12h light: 12h darkness photoperiod was used for the macro-algae tests. The salinity of the test solutions was 30 ± 1ppt and test temperature was 20 ± 1°C. A copepod toxicity test was also performed with ammonium chloride dissolved in filtered seawater, to establish levels of ammonia that could contribute to, or be responsible for, toxic effects measured in pore waters.

Fronds of the macro-alga *U. fasciata*, from which zoospores were obtained, were collected during low tide on Port Aransas, TX, jetties. A starter culture of the copepod, *S. knabeni*, currently under culture in our laboratory, was kindly donated by Dr. Guilherme Lotufo (USACE,

ERDC, Vicksburg, MS). Organisms were originally isolated from the surface sediment of intertidal mudflats in a *Spartina alterniflora* salt marsh at Port Fourchon, LA, and have been in laboratory culture since 1993.

Toxicity tests with samples of photo-transformed 2,6-DNT were performed using the *U. fasciata* and the *S. knabeni* tests described above, in addition to the life-cycle test with the polychaete, *Dinophilus gyrociliatus*. This species has been in culture in our laboratory for over 6 years and tests were conducted following SOP F10.10 (Attachment 4). Original organisms were isolated from material obtained from Long Beach Harbor, CA.

Toxicity tests with *U. fasciata* and *S. knabeni* were also performed with ordnance compounds and their major identified biotransformation products spiked into dilution water. Tests were conducted with 2,6-DNT, 2-amino-6-nitrotoluene (2-A-6-NT), picric acid, 2-nitrotoluene (2-NT), 2,4-dinitrophenol (2,4-DNP) and sodium picramate, the sodium salt of picramic acid (2-amino-4,6-dinitrophenol). Highly purified 2-A-6-NT and 2-NT (98% purity) were purchased from ChemService (West Chester, PA) and 2,4-DNP (97% purity) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Highly purified picramic acid was not commercially available. Dr. Tom Jenkins (USACE, Cold Regions Research and Engineering Laboratory, Hanover, NH), was able to kindly donate some sodium picramate (97% purity), formerly available at Sigma (St. Louis, MO). Although the dissociation constant (pKa) of sodium picramate and picramic acid could not be found, it is expected to be considerably lower than seawater pH of 8.2, based on the 0.38 and 3.96 pKa values of its parent compound, picric acid and of 2,4-DNP, respectively (Weast et al. 1986-1987). The pKa of phenols usually decreases with the increase in the number of nitro groups (Buikema *et al.* 1979) and, therefore, its value for picramate is expected to be in range of that of 2,4-DNP. Therefore, the sodium picramate was expected to dissociate with the pH increase by dilution in seawater, resulting in the dissociated form in the stock and test solutions, which would be the same dissociated form exhibited by picramic acid. Thus the toxicity of sodium picramate and picramic acid in seawater is expected to be the same, and it was considered acceptable to conduct experiments with picramate. Standards of picramic acid and 2,4-DNP in methanol for chemical calibration were purchased from Protocol Analytical, LLC (Middlesex, NJ) and Ultra Scientific (North Kingstown, RI), respectively. The desired amount of each chemical was weighed on an analytical balance, added to the appropriate amount of dilution water and stirred on magnetic stirrer for 24 hours prior to use in experiments. Chemical analyses of the stock solutions were performed at the beginning of each toxicity test, and of all treatments at test end, to assess for loss of chemicals in the test vials. An additional replicate of each treatment was prepared for this purpose and treated in an identical manner to the toxicity test treatments, except for the absence of organisms. The photo-induced toxicity at the end of the tests with 2,6-DNT and picric acid with *S. knabeni* was assessed by transferring the surviving females to dilution water and immediately exposing to SSR for 1 hour at the irradiance intensities provided in Table 1. A test for the assessment of photo-induced toxicity of these chemicals was also conducted with *D. gyrociliatus.*

Reference Toxicant Tests

A reference toxicant test using sodium dodecyl sulfate (SDS) was conducted concurrently with each test series. Results of the macro-algae and polychaete tests were compared to a control chart prepared using the results of previous tests conducted in our laboratory (Environment

Canada 1990). According to the control charts, the EC_{50} values for the *U. fasciata* germination test should be between 1.2 and 5.6 mg/L. Results for *D. gyrociliatus* survival should be between 2.3 to 7.1 mg/L, and the reproductive EC_{50}, between 1.7 to 4.9 mg/L. Since the copepod test is new for our laboratory, no control charts were available at this time.

Statistical Analyses

The results of the toxicity tests were used for calculation of the EC_{50} and/or LC_{50}, NOEC and LOEC values. These were calculated using the concentrations of the tested chemicals measured at the beginning of each experiment. In the porewater tests with *U. fasciata* zoospores, the blank treatments corresponding to each spiked treatment were used as controls for statistical analyses. For instance, the fine-grained sediment spiked with seawater only was used as the control for picric acid-spiked fine-grained sediment at each corresponding sampling period (day 0, day 7, day 28, and so forth). However, due to varying ammonia levels and the higher sensitivity of the copepod test to this confounding factor, porewater controls were established based on their unionized ammonia concentrations rather than on sampling period. For instance, pore water collected from a 28-day stored fine-grained sediment spiked with seawater was used as the control for the picric acid-spiked sediment after a 56-day storage. This avoided the identification of false positives, i.e., samples that would have been toxic due to their ammonia concentrations rather than to ordnance compounds or their biotransformation products.

The dilutions for each sample and the corresponding blanks were isolated as an independent dataset. Prior to statistical analysis, the transformed data sets were screened for outliers (SAS 1992) in the copepod and macro-alga tests, and tested to ensure that all statistical assumptions were satisfied. Data from the polychaete tests were not tested for outliers due to the natural variability of this test's results. 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 or $\log_{10}$ 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 EC_{50} or LC_{50} values. The respective blank samples of each test were used as controls for the application of Abbott's correction. For endpoints with continuous values, such as copepod and polychaete reproduction (number hatched nauplii or 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.

RESULTS AND DISCUSSION

PART 1: *Bio- and Photo-transformation of 2,6-DNT and Picric Acid in Spiked Sediments*

Pore Water and Sediment Chemical Assessment

Several studies have been conducted for the assessment of the degradability of nitroaromatic compounds under a variety of conditions, e.g., in sewage effluent maintained under aerobiosis or anaerobiosis (Hallas and Alexander 1983), anaerobic digesters with bacterial monocultures (Boopathy and Kulpa 1993), bacterial cultures from soil, compost or mud from a catalytic cracking plant adapted to media rich in phenol (Chambers 1963), an aerobic fluidized-bed biofilm reactor (Lendenmann *et al.* 1998), or freshwaters from a variety of sources (Bausum *et al.* 1992). Most of these studies had the objective of developing bioremediation procedures for contaminated soils and waters rather than understanding the degradation of energetic materials under natural conditions. Relatively little is known about the natural abiotic and biotic transformation and degradation of explosives, particularly in estuarine and marine environments.

Aromatic nitro groups are reduced enzymatically to amino groups (Parris 1980). Aerobic as well as anaerobic systems were found to reduce the nitro groups. Biogeochemical processes and microbial activity in sediments, however, are intimately coupled and an attempt to separate the reduction of nitroaromatic compounds into strictly abiotic or strictly biological does not seem very meaningful (Haderlein and Schwarzenbach 1995).

2,6-Dinitrotoluene

In a previous study, a transformation product of 2,6-DNT was observed in spiked marine sediments and pore waters (Nipper *et al*. 2002). This product was identified as 2-methyl-3-nitroaniline (a.k.a., 2-A-6-NT) (Carr and Nipper 2000, Nipper *et al*. 2002). A large number of genera of aerobic and anaerobic bacteria capable of metabolizing 2,4,6-trinitrotoluene (TNT) have been identified in a variety of environments (Rosenblatt et al. 1991), but not much is known about microbial processes affecting 2,6-DNT, although some research has been conducted. The scientific literature consistently suggests that in several matrixes (soil, water, sediment) and under a variety of conditions 2,4-DNT is more promptly degraded than 2,6-DNT (Spanggord 1980, *apud* Gorontzy *et al.* 1994; Bausum *et al.* 1992). The reduction of both DNT isomers to the corresponding aromatic amines by several different microorganisms has been demonstrated under aerobic conditions (Gorontzy *et al.* 1994). Spanggord *et al.* (1985) suggested that the reduction of the nitro group is one of the major pathways of biological transformation of nitroaromatics, and it has been reported that the DNT isomers can be reduced to the corresponding aromatic amines by several types of microorganisms under aerobic conditions (Gorontzy *et al.* 1994). In aerated sewage, 2,6-DNT was transformed to aminonitrotoluene but did not seem to transform further, whereas under anaerobiosis it slowly disappeared over several weeks (Hallas and Alexander 1983).

In the current study, gas chromatography-mass spectrometry (GC/MS) analyses confirmed 2-A-6-NT as the major biotransformation product of 2,6-DNT, and 2-nitrotoluene (2-NT) was also identified as a biotransformation product, possibly as a result of the loss of the amino group from the amino nitrotoluene. Minor components also found in 2,6-DNT-spiked samples along storage time were N,N-dimethyl-3-nitroaniline, benzene nitrile, methylamino-2-nitrosophenol and diaminophenol. After more prolonged storage these chemicals tended to be replaced with

larger molecular weight polymers, which were possibly derived from DNT. It has been reported that several abiotic reactions in oxic environments are believed to cause the combination of aryl amino radicals with each other forming coupling products, including some compounds of environmental concern such as substituted azobenzenes or phenazines (Haderlein and Schwarzenbach 1995). Aminonitrotoluenes, azoxytoluenes and acetamidonitrotoluene were formed when 2,4-DNT was incubated with a fungal monoculture under aerobic conditions (McCormick *et al.* 1978). Parris (1980), however, suggested that the observation of these compounds may be an artifact caused by the high levels of nitroaromatic chemicals used under experimental conditions, and that at more environmentally realistic levels and in the presence of humic acids and other potential reaction partners, such products might be of minor importance. Laboratory experiments using concentration series of ordnance compounds of concern in the marine environment, such as 2,6-DNT and picric acid, would be necessary to establish if Parris and collaborators' assumption is correct.

Natural organic matter in sediments also seems to be important in promoting the covalent binding of amines, followed by irreversible polymerization and rearrangement reactions (Haderlein and Schwarzenbach 1995). Little is known about rates of biotransformation of nitroaromatics such as DNT and trinitrobenzene (TNB) except that they persist in soils and wastewater lagoon sediments for many years (Rosenblatt *et al.* 1991). This is in agreement with findings in marine sediments and animal tissues from the vicinity of Naval facilities in Puget Sound (URS Consultants 1995; EA Engineering, Science, and Technology, Inc. 1996), but seems to be contradicted by the fast rates of biotransformation of 2,6-DNT and picric acid (see next section) observed in the marine sediments studied in the current project. Some light on the subject might come from the finding by Bausum *et al*. (1992) that 2,6-DNT concentrations <1 mg/L in freshwaters failed to stimulate the development of a 2,6-DNT-degrading microbial population. The question that arises is if a minimum level of 2,6-DNT would be the limiting factor for the survival of an established 2,6-DNT-degrading population, which would perish when the 2,6-DNT concentration fell below this level, thus leaving some of the DNT intact. Further research is necessary to better understand this aspect and to assess whether it would also be applicable to picric acid.

Although the possibility of mineralization of 2,6-DNT is suggested in scientific literature (Chambers *et al.* 1963), no conclusive evidence could be drawn from the current study. Although no attempt was made to induce anaerobiosis in the experimental sediments, it is likely that they became anoxic after the initial experimental phase, when they were left in closed jars for several weeks or months. There is evidence in the literature that under anaerobic conditions the amines formed by the reduction of DNTs can be completely deaminated, but the ring molecule was not further metabolized (Gorontzy *et al.* 1994). Boopathy and Kulpa (1993) reported that an anaerobic bacterium, *Desulfovibrio* sp, used nitroaromatic compounds such as DNTs and DNPs as sole source of nitrogen for growth and as electron acceptors, and >60% of the nitroaromatics were transformed within 6 days of incubation in an anaerobic digester. Nitro groups were reduced and deaminated to ammonia but the aromatic ring was not cleaved. Processes like this would explain the higher concentrations of ammonia in the ordnance-spiked sediments than in the blank controls after a few weeks storage (Appendices B1 and B2).

Peaks of 2-A-6-NT were seen in the chromatograms from non-sterilized sediment and porewater measurements performed immediately after the sediment spiking procedure (day 0), but not in the stock solution (Fig. 4), indicating that microbial 2,6-DNT transformation started shortly after its initial contact with these marine sediments (Appendix A1). 2-A-6-NT was also

observed in the sterilized sediments, but to a lesser extent, suggesting that hydrolysis of other breakdown processes might contribute to the transformation of 2,6-DNT into 2-A-6-NT in marine sediments. The percentage of 2,6-DNT transformed into 2-A-6-NT in the sterile samples was only a fraction of that in the non-sterilized samples (Appendix A2), indicating that microbial activity is the major factor responsible for 2,6-DNT breakdown. This is particularly true for the fine-grained sediment, where loss in the non-sterilized sediment was considerably faster and of larger magnitude than in the sterile sediment, and where a larger and more diverse initial microbial community was measured (see section on Sediment Microbiological Assessment, below).

Biotransformation of 2,6-DNT in both sediments was enhanced with temperature increase, but it was considerably faster in the fine-grained sediment than in the sand at both temperatures (Appendix A2). Both results were expected based on the natural increase of microbial activity with temperature and on the larger and more diverse initial microbial community observed in the fine-grained sediment (see respective section below). In the sandy sediment stored at 10°C nearly all 2,6-DNT was broken down on day 28 (Fig. 5A), whereas at 20°C this had already happened after 7 days of storage (Fig. 5B). A similar pattern was observed in the fine-grained sediment, but with most 2,6-DNT already degraded on days 7 and 3 for storage at 10 and 20°C, respectively (Fig. 5C and D). The concentration of 2-A-6-NT also dropped considerably in both sediments with time, with higher losses at the higher temperature and in the fine-grained sediment (Fig. 5). This indicates that the 2-A-6-NT was further broken down into other unidentified products, polymerized, or mineralized. Large molecular weight compounds, possibly derived from DNT, were observed in pore waters from sediment spiked with 2,6-DNT after a 6-month storage. However, mineralization of at least part of the DNT is not ruled out, since cleavage of the aromatic ring and mineralization promoted by natural-water microorganisms have been observed in studies with other nitroaromatic ^{14}C-ring-labelled compounds such as 2,4-DNT and 1,3-dinitrobenzene (Spanggord *et al*. 1985). These authors suggest that the reduction of the nitro group would be a simple process in the presence of additional organic nutrients, which would be the case for both sediments used in this study, but particularly for the fine-grained sediment, which has a higher TOC. The ability of microorganisms to mineralize nitroaromatics, however, might require extended acclimation periods.

Some of the data in Appendix A2 have an asterisk indicating microbial contamination of the sterilized samples. However, if contamination had occurred initially or if the sediments had not been properly sterilized, microbial activity would have been observed in all samples of a certain kind, or at least at all sampling periods after the initial bacterial contamination. Due to the erratic distribution of those samples it is believed that the measured microbial contamination would have happened after transfer from the sterile storage vials containing the spiked sediments and, therefore, would not have compromised the stored spiked sediments as controls for biotransformation. The loss of 2,6-DNT and formation of 2-A-6-NT in the sterilized samples is likely to have been caused by abiotic reactions. Several electron donors capable of reducing nitroaromatic compounds are present in sediments (Haderlein and Schwarzenbach 1995). The higher loss of 2,6-DNT in the sterilized sandy sediment than in the muddy was likely a result of different mineralogical characteristics of the 2 sediments. According to Haderlein and Schwarzenbach (1995), abiotic reduction rates between different environments can differ by orders of magnitude. Whereas some compounds (e.g., TNT) can be rapidly reduced by abiotic processes, the reduction rates of less reactive compounds (e.g., diaminonitrotoluenes, which are

intermediates of TNT reduction) are primarily controlled by microbial activity. Similarly, the reduction of 2,6-DNT by abiotic reactions in the present study may have been higher than that of 2-A-6-NT, leading to a larger accumulation of the latter in the sterilized sediments as the 2,6-DNT was reduced.

Fungal transformation of TNT and 2,4-DNT was achieved by some authors under laboratory conditions using fungi mycelia cultures. TNT transformation was induced by 183 species, out of 190 tested which represented 98 genera, but only 5 of those were able to metabolize 2,4-DNT (Parrish 1977). Reduction of nitro groups from TNT by fungi were only observed at position 4 and, therefore, it is not clear if 2,6-DNT metabolization by fungi would occur. The use of white rot and litter decaying fungi, e.g., *Phanerochaete chrysosporium,* for bioremediation of environments contaminated with nitrotoluenes and their aminated derivatives has generated considerable interest in the last decade, but experimental efforts mostly included liquid cultures or extracellular fungal enzyme preparations (e.g., Fernando and Aust 1991; Spiker *et al.* 1992; Van Aken *et al.* 2000; Bayman and Radkar 1997; Scheibner and Hofrichter 1998). Some lignolitic fungi were found to metabolize TNT into azoxy and aminated derivatives (Bayman and Radkar 1997), while others effectively mineralized TNT and aminated metabolites, provided conditions were appropriate for optimum fungal survival and performance (Fernando *et al.* 1990; Spiker *et al.* 1992; Scheibner and Hofrichter 1998). It was suggested that they may be useful for the bioremediation of sites containing multiple contaminants, due to the versatility of the enzymatic system necessary for degradation of lignin, their natural substrate (Stahl and Aust 1995). Soils amended with the white rot fungus *P. chrysosporium* promoted the mineralization of a small percentage of the TNT within 30 days (Fernando *et al.* 1990), and under ligninolytic conditions the same fungus was able to mineralize 2,4-dinitrotoluene (Valli *et al.* 1992). Most of the studies above, however, were performed in nitrogen-limited media, where mineralization of nitroaromatics was more efficient than with nitrogen-sufficient substrates. Fungi of the white rot type have been identified in the marine environment (Leightley and Eaton 1979), particularly in mangrove and seagrass areas, and were suggested for the bioremediation of samples containing dyes and other aromatic contaminants (Raghukumar *et al.* 1994, 1999; Raghukumar 2000, Raghukumar and Rivonkar 2001). Fungi communities were not analyzed in the current study, and it is not clear if fungi would be capable of metabolizing energetic materials in natural, nitrogen-sufficient, marine environments, but further investigation of this potential means of nitroaromatic degradation in marine environments is recommended.

The measured concentration of both 2,6-DNT and 2-A-6-NT was lower in the dried sediments than in those extracted wet, on nearly all occasions (Appendix A1). This suggests that biodegradation was still occurring during the 3 days that the sediments were allowed to dry at room temperature, following USEPA Method 8330 (USEPA 1994), suggesting that this method is not appropriate for every ordnance compound, and that modifications to the standardized method should be considered.

Picric Acid

The sediments spiked with picric acid behaved similarly to those spiked with 2,6-DNT, i.e., faster biotransformation occurred at the higher temperature (20°C) and in the fine-grained sediment (Appendix A3, Fig. 6). A peak corresponding to a transformation product, 2-amino-4,6-dinitrophenol (picramic acid), was observed in both sediments on day 0, i.e., immediately after the spiking procedure, but no such peak occurred in the stock solution (Fig. 7), again indicating that biotransformation occurred shortly after the picric acid was put in contact with the

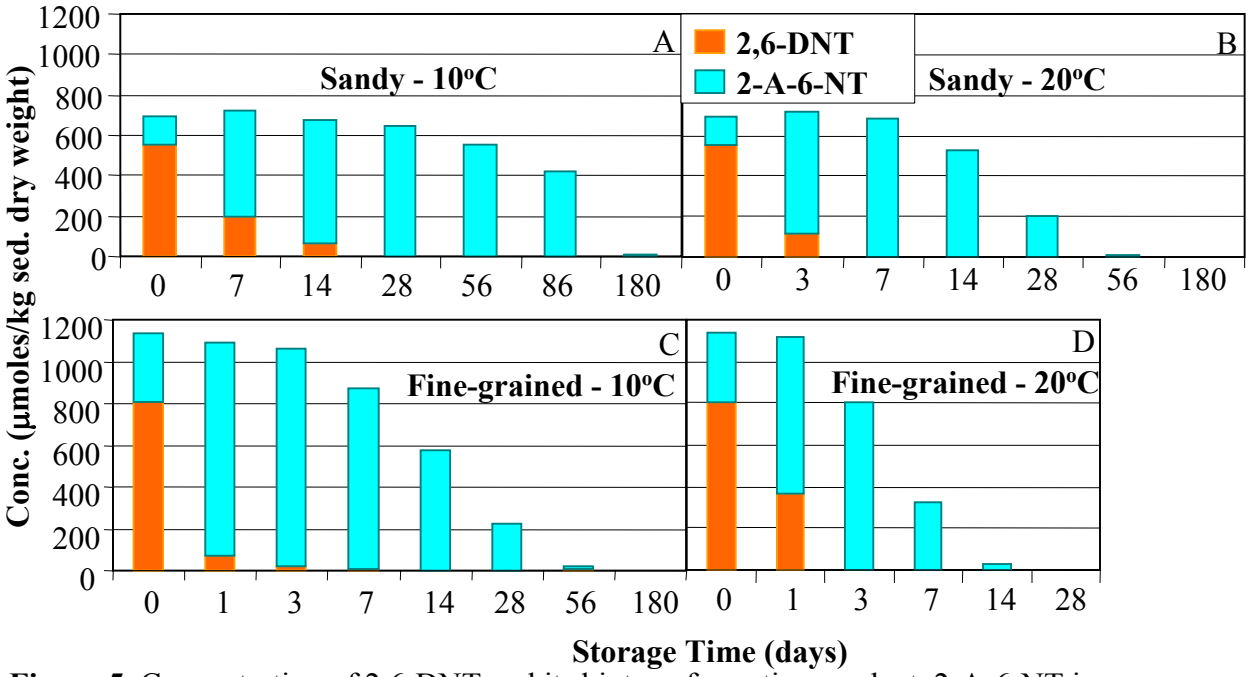

Figure 5. Concentration of 2,6-DNT and its biotransformation product, 2-A-6-NT in sediments at different storage times and temperatures: A) Sandy sediment at 10°C; B) Sandy sediment at 20°C; C) Fine-grained sediment at 10°C; D) Fine-grained sediment at 20°C.

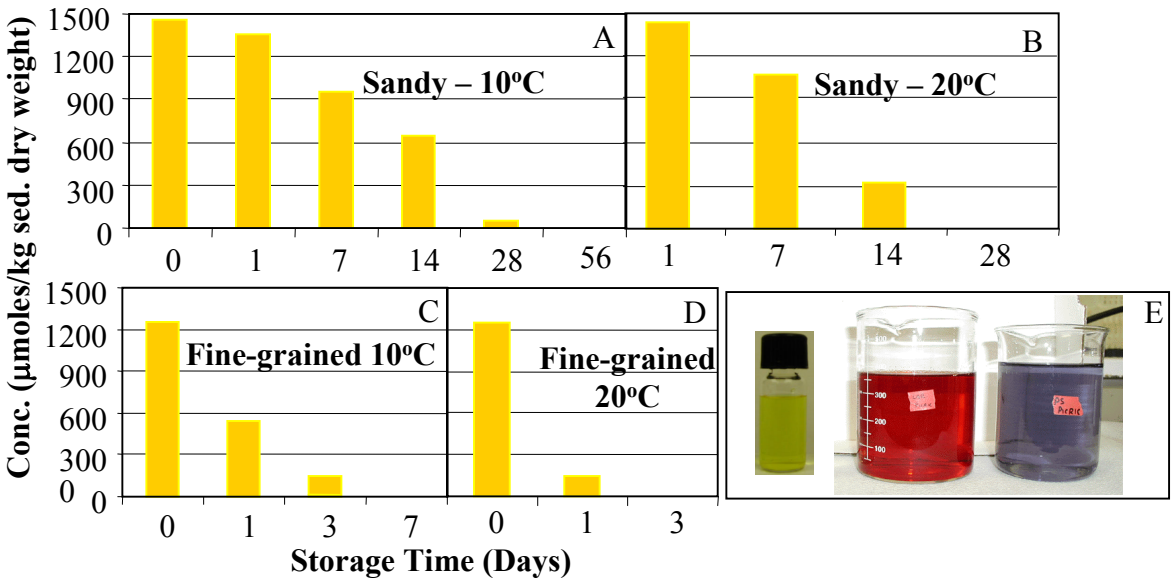

Figure 6. Concentration of picric acid in sediments at different storage times (days) and temperatures: A) Sandy sediment at 10°C; B) Sandy sediment at 20°C; C) Fine-grained sediment at 10°C; D) Fine-grained sediment at 20°C; E) Vials with pore water extracted prior to storage (day 0 – yellow), and after 56-day storage of sandy (red) and fine-grained (purple) sediment.

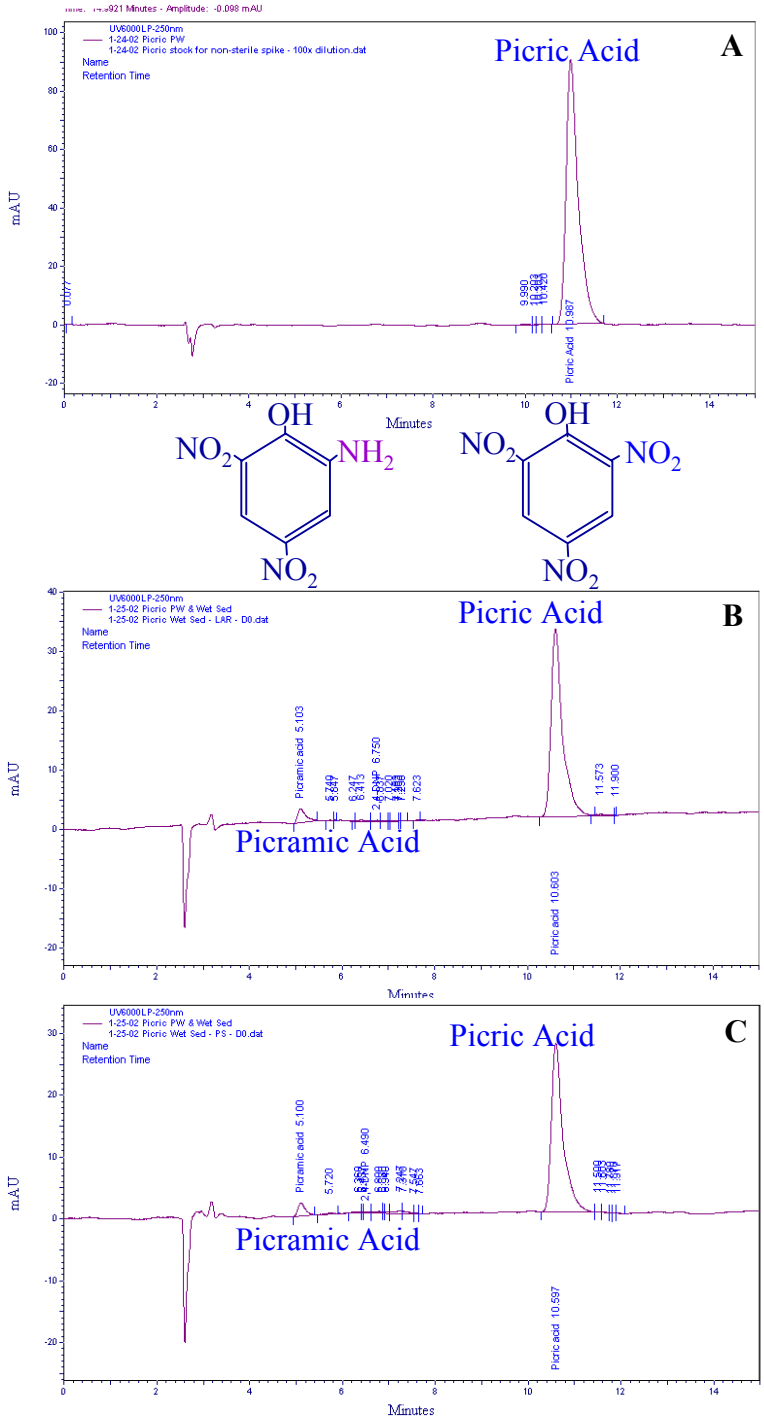

Figure 7. HPLC chromatograms of: A) Picric acid stock solution in filtered seawater; B) Sandy sediment immediately after spiking with picric acid stock solution; C) Fine-grained sediment immediately after spiking with picric acid stock solution.

marine sediments. Picramic acid has been previously reported as an aerobic biotransformation product of picric acid (Wyman *et al.* 1979). After 8 weeks of storage, pore water from sediments spiked with picric acid suffered a dramatic change in color. The sandy sediments produced red pore water whereas the pore water from the fine-grained sample turned to a blue-lavender color (Fig. 5E). The scientific literature reports the use of picric acid as a carbon or nitrogen source by a variety of bacteria, with the transient formation of 2,4-DNP by the liberation of a nitrite (Rajan *et al.* 1996), or the formation of an orange-red metabolite identified as a hydride-Meisenheimer complex (Lenke and Knackmuss 1992). These authors reported that the hydride-Meisenheimer complex further metabolized into 2,4,6-trinitrocyclohexanone as a dead-end product, whereas Rieger *et al.* (1999) described it as a key intermediate for the denitration of picric acid, leading to the formation of 2,4-DNP. Mineralization of picric acid was achieved by Rajan *et al.* (1996) in monospecific bacterial cultures under laboratory conditions.

Several breakdown products of picric acid were identified by GC/MS in the current study, and their nature changed with storage time. Breakdown compounds identified at earlier stages of biotransformation (0 to 56 days) were <u>2,4-dinitrophenol</u>, <u>amino dinitrophenols</u> (including picramic acid), 3,4-diamino phenol, amino nitrophenol, nitro diaminophenol, and peaks possibly corresponding to aminonitrotoluene isomers, diaminotoluenes, dinitrotoluenes and nitrotoluenes were present but this identification is somewhat dubious. The underlined compounds were identified in relatively significant amounts in some of the samples, and were available as highly purified single chemicals. Therefore, further toxicity tests with these individual compounds were performed.

Picric acid, 2,4-dinitriphenol and p-nitrophenol were decomposed by a gram-positive species, *Corynebacterium simplex* (Jensen and Gundersen 1955; Gundersen and Jensen 1956). These nitro groups are converted to nitrite, which is also a toxic compound, although its toxicity is inhibited in seawater and it tends to be readily converted into nitrate, which is essentially nontoxic (Rand and Petrocelli 1985). Nitrite analyses were performed in pore water from picric acid-spiked samples collected after 56-day storage, due to their extremely elevated toxicity (see Porewater Toxicity Assessment section below) and absence of picric acid or any other compounds measurable by HPLC using the modified method 8330 (USEPA 1994). Nitrite measured concentrations were 2.62 and 87.55 μmoles/L in the pore waters from the fine-grained and sandy sediment, respectively. The blank (seawater-spiked) sample from the fine-grained sediment was also analyzed, resulting in 1.53 μmoles/L nitrite.

After a 6-month storage period, pore waters from the fine-grained sediment kept at 10°C showed barely any aromatic compounds that might be related to picric acid, except for a very small amount of nitro diaminophenol and benzoic acids, and the pore waters were clear, suggesting that nearly complete biodegradation of the parent compound was achieved. In the sandy sediment stored for 6 months at 10°C, GC/MS analyses indicated the presence of the isocyanato group (NCO), in addition to amino, nitro, and hydroxyl groups in the picric acid degradation products, with one of the compounds tentatively identified as nitrophenol isocyanate. A large amount of polar, high molecular weight chemicals, possibly derived from picric acid was identified. It is suggested that they would be polymers of picric acid and of its main breakdown products.

A small peak corresponding to picramic acid was observed in the sterilized sandy sediment, suggesting that abiotic breakdown processes might contribute, to a minor extent, to the transformation of picric acid in some marine sediments. Microbial community analyses suggested that the sterility of the sterilized picric acid-spiked fine-grained sediment was

compromised during the sampling procedure of day 7, after which the loss of picric acid was considerably enhanced (Appendix A4). However, the 100% loss of picric acid in the non-sterilized sediment both at 10 and 20°C on or before day 7 (Fig. 6C and D) shows that microbial activity was, indeed, responsible for the majority of this loss, which did not occur at such an early stage in the samples that were sterile initially. In the sandy sediment, some picric acid could still be measured after 28 days of storage at 10°C, with none left after 56 days (Fig. 6A), whereas at 20°C the breakdown of this chemical was twice as fast, with none measured on day 28 (Fig. 6B). The total loss of picric acid after a 56-day storage, however, did not represent breakdown to mineralization, as shown by the toxicity tests (see Porewater Toxicity Assessments section) or by the cranberry red coloration of the pore water from the sandy sediment and lavender color of the pore water from the fine-grained sediment (Fig. 6E), which indicated the presence of unidentified chemicals. Isomers of azoxy-tetranitrotoluenes were described as having blue-purple to brown-purple color (Bayman and Radkar 1997), and it is possible that chemicals of that nature were present in the sample from the 56-day incubation in fine-grained sediment, although they were not identified by GC/MS analyses.

Unlike with 2,6-DNT, the measured concentration in most samples of sandy sediment spiked with picric acid did not differ all that much between wet and dry extraction (Appendix A3). In the fine-grained sediment, however, the losses of picric acid caused by the drying procedure would have a major impact on data interpretation if the dried sediment results were used to interpret biological effects of picric acid in sediments.

Based on the two sediment extraction methods used in the present study, a change to USEPA's procedure (Method 8330) is recommended and sediment drying at room temperature should be avoided. Sediment moisture content, however, needs to be known for the correct calculation of the concentration of ordnance compound on sediment dry weight basis, in order to allow comparisons of data for sediments of different composition.

Dissolved Organic Carbon (DOC)

DOC concentration tended to decrease in the pore waters with sediment storage time, except for the 2,6-DNT-spiked samples, where an initial increase in DOC occurred, followed by a sharp decrease after a 6-month storage (Appendix B5, Fig. 8). DOC concentrations were always higher in the pore waters from the ordnance-spiked sediments than in their respective blanks. This suggests that the degradation of the ordnance compounds acted as a source of the measured organic carbon.

Sediment Microbiological Assessment

Total Coliforms

The total coliforms most probable numbers (MPN) data for the sediment-spiking experiments are shown in Figure 9. This microbial endpoint did not appear to correspond with changes in the ordnance concentrations or changes in the heterotrophic bacteria discussed in the next section. In some instances the reference sediment tended to have higher total coliforms than the ordnance-spiked treatment (e.g., Figs. 9a, e and h) and in other instances the opposite was observed (e.g., Figs. 9c and g). This endpoint, therefore, was not very useful for estimating changes in the microbial activity during this time course study.

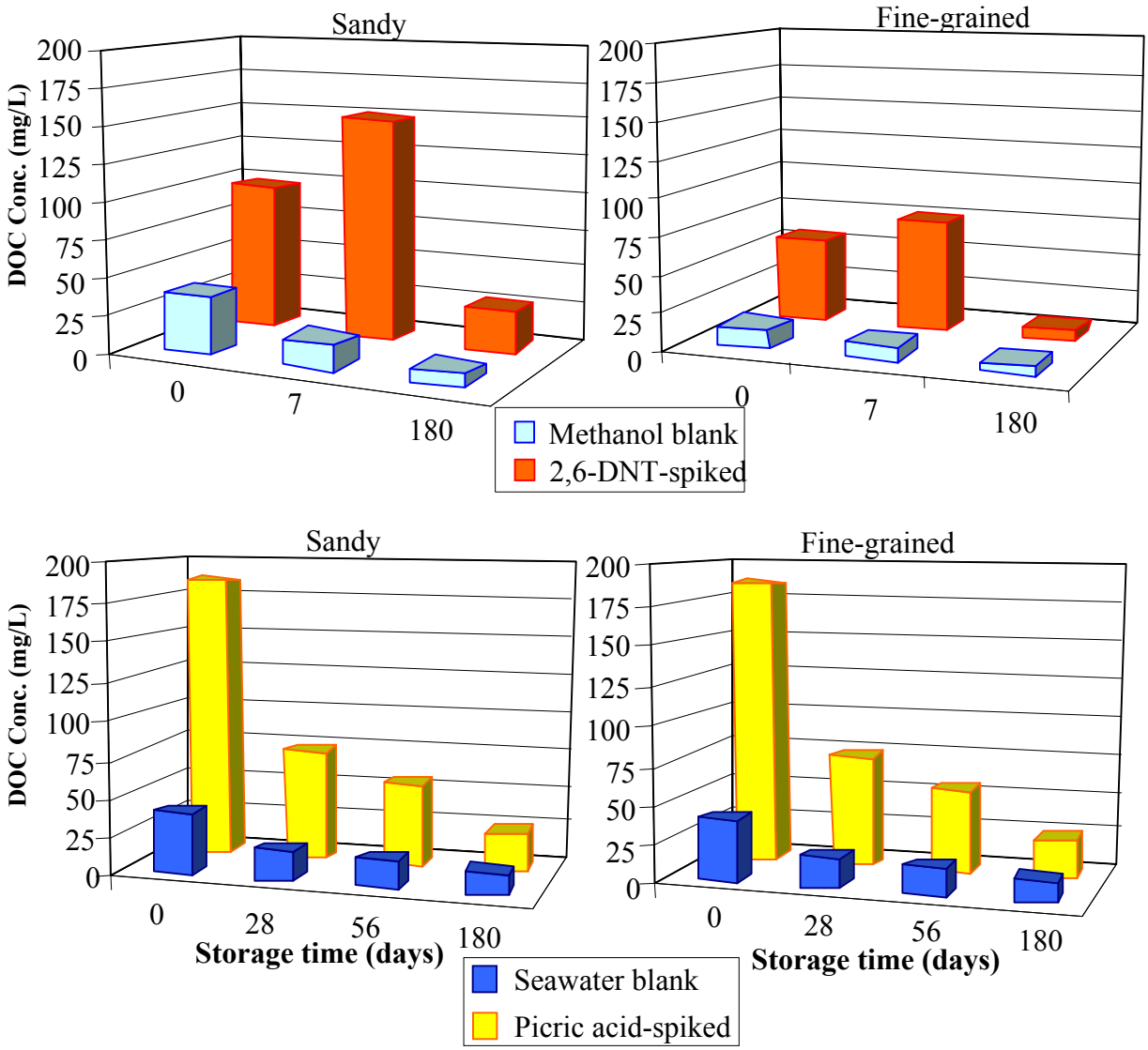

Figure 8. Dissolved organic carbon (DOC) concentration in pore waters used in toxicity tests, from sediments spiked with 2,6-DNT and picric acid, and respective blanks, at different storage times.

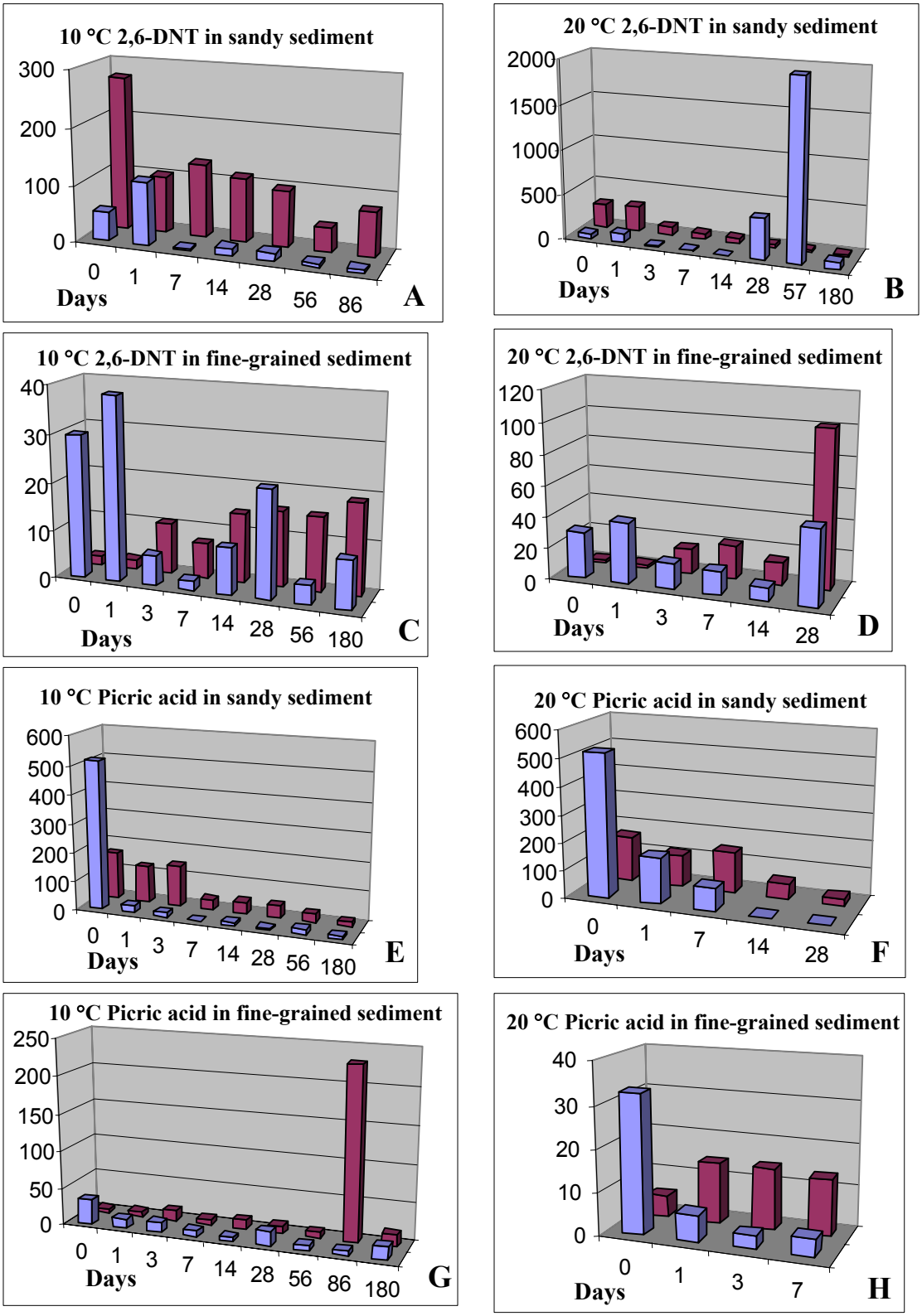

Figure 9. Total coliforms (MPN) data comparing the ordnance-spiked (blue) and appropriate reference sediment (maroon, with MeOH for 2,6-DNT and MFS for picric acid).

Heterotrophic Bacteria

Analyses of total heterotrophic bacteria in sediments had a similar behavior in all ordnance-spiked samples: there was a strong initial increase in bacterial abundance relative to the respective blanks, followed by a depression that took bacterial abundance to similar levels of the blanks and lasted a few days, then followed by a remarkable increase in the spiked samples, but not in the blanks (Appendix C1, Figs 10 and 11), suggesting that the bacteria were using the nitroaromatic compounds as sources of nutrients and causing their transformation into some measured and other unidentified breakdown products. Most probable numbers (MPN) enumeration of 2,6-DNT-utilizing bacteria in natural freshwater rose sharply after incubation with 2,6-DNT at 10 and 20 ppm concentrations (Bausum *et al.* 1992). These authors found clear evidence of concentration-dependent mineralization of 2,6-DNT in freshwater, at concentrations between 1 and 10 ppm, with degradation being undetectable below 1 ppm and being inhibited at 130 ppm. They verified that DNT isomers can be a sole carbon and energy source for bacteria and at least four bacterial types from freshwaters that were active on 2,6-DNT could be distinguished, but pure strains could not be isolated.

The initial (day 0) bacterial community in the sediment blanks (methanol- and seawater-spiked) was about twice as high in the fine-grained samples as in the sandy samples (Appendix C1). However, the spiking procedure with 2,6-DNT and picric acid caused a sharper initial growth of the microbial community in the sandy than in the fine-grained sediment (Figs. 10 and 11 – storage time 0), suggesting that the sandy sediment naturally contained more nitroaromatic-utilizing bacteria which grew once such sources of nutrients were added to their environment. This differs from Bausum *et al.*'s (1992) findings, in which DNT degradation only occurred under laboratory conditions in waters from sites with previous history of pollution by munition compounds, suggesting that prior adaptation of microorganisms had occurred in those waters.

Microbial Community Analysis and Enzyme Induction Experiments

In addition to enumeration of the total heterotrophs, the microbial community was evaluated using the Ecoplate® procedure. Figures 12 and 13 show a comparison of the total heterotroph and Ecoplate® data for the control and ordnance-spiked sediments during the degradation experiment. The Ecoplate® data has been expressed as the number of substrates which were determined to be positive for that sample out of a possible 32 times 100 so that the scales would be similar to the MPN data for the total heterotrophs. The actual patterns for the Ecoplate® results are shown in Appendices C2 and C3. An examination of the data indicates that the patterns between control and ordnance-spiked sediments were often quite dissimilar (e.g., Figs. 12e-f, 12g-h and 13c-d) which suggests that there was a shift in the microbial community composition in addition to the observed changes in total heterotrophs. In the present carbon source configuration for Ecoplate®, only Gram-negative bacteria are detected from sediments (Lisa Staraci-Schibler, Biolog Inc., Hayward, CA, pers. comm.). Only G+C Gram-positive bacteria (e.g., Actinobacteria) have been reported to degrade picric acid (Rajan et al. 1996; Russ *et al*. 2000). Ebert et al. (1999) reported that in *Nocardoides simplex*, the reduction of picric acid involves the unusual redox cofactor deazaflavin F_{420}. This cofactor has so far been described only in archaea, cyanobacteria and high G+C Gram-positive bacteria, but never in Gram-negative bacteria (Russ *et al*. 2000). This could help explain some of the differences between the total heterotroph and EcoPlate® data where the microbes are utilizing the nitroaromatics as a carbon source but are not being detected by the Ecoplate® method.

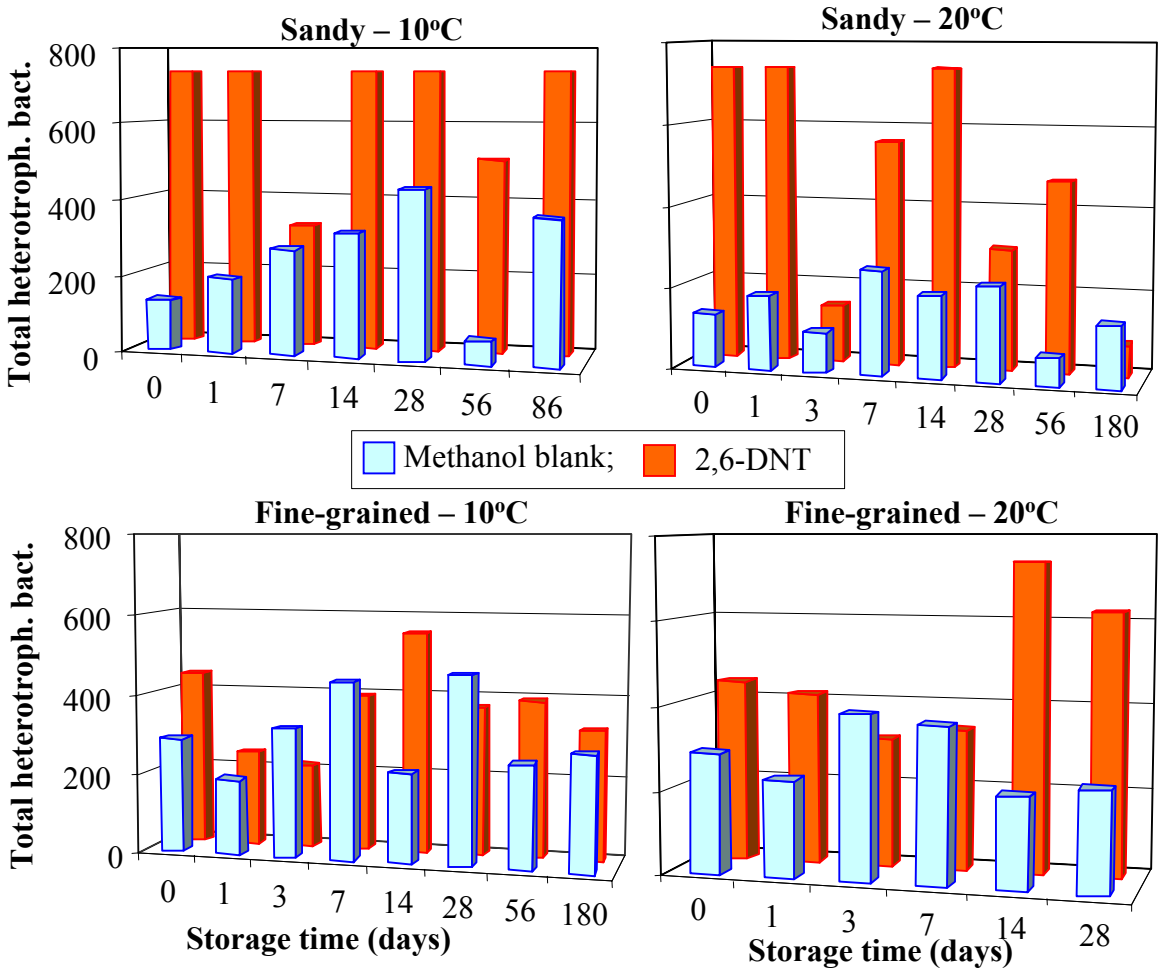

Figure 10. Total heterotrophic bacteria in sandy and fine-grained sediments spiked with 2,6-DNT and corresponding methanol blanks, at several storage times. Data are expressed as most probable number (MPN) of bacterial cells/mg sediment wet weight.

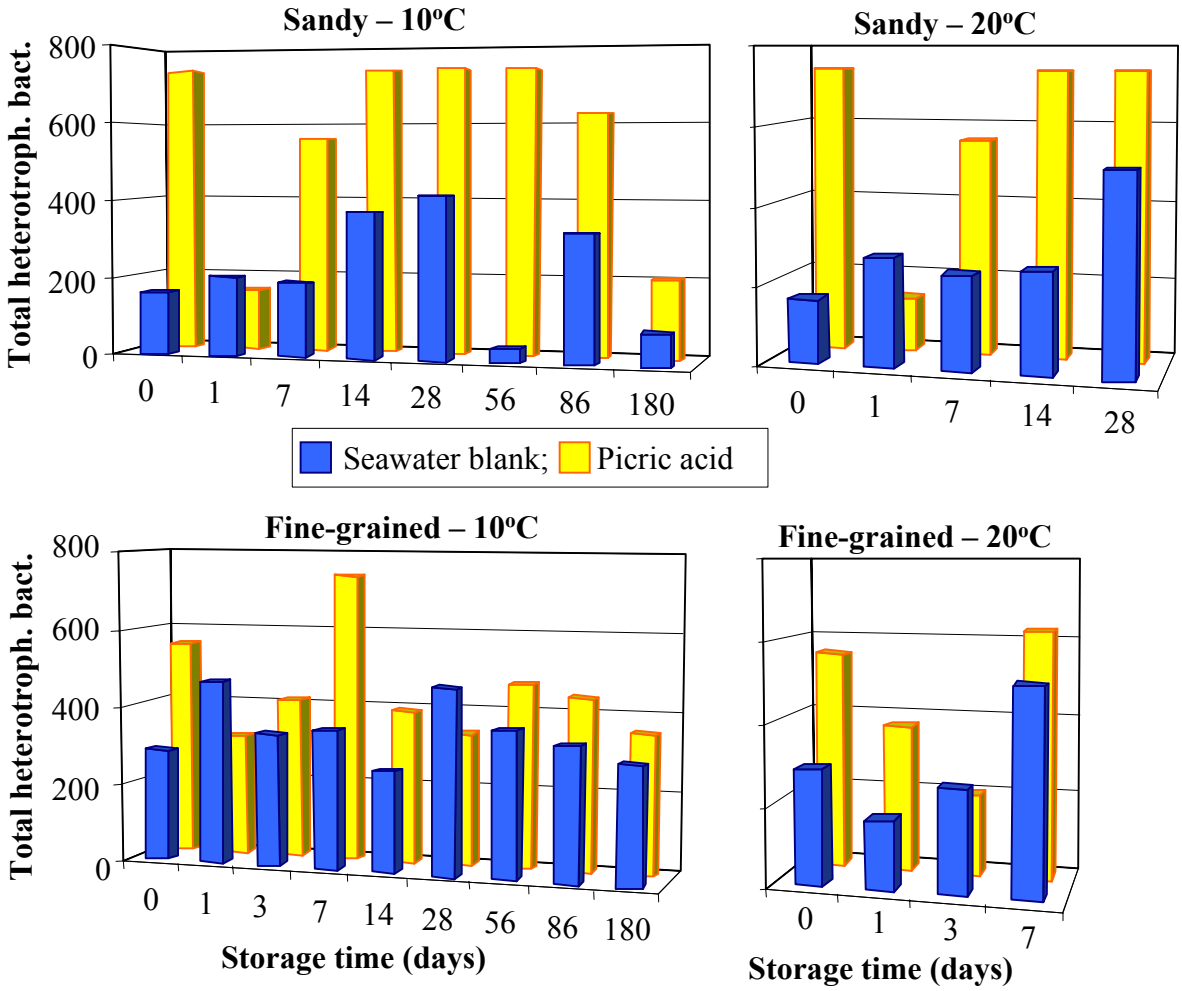

Figure 11. Total heterotrophic bacteria in sandy and fine-grained sediments spiked with picric acid and corresponding seawater blanks, at several storage times. Data are expressed as most probable number (MPN) of bacterial cells/mg sediment wet weight.

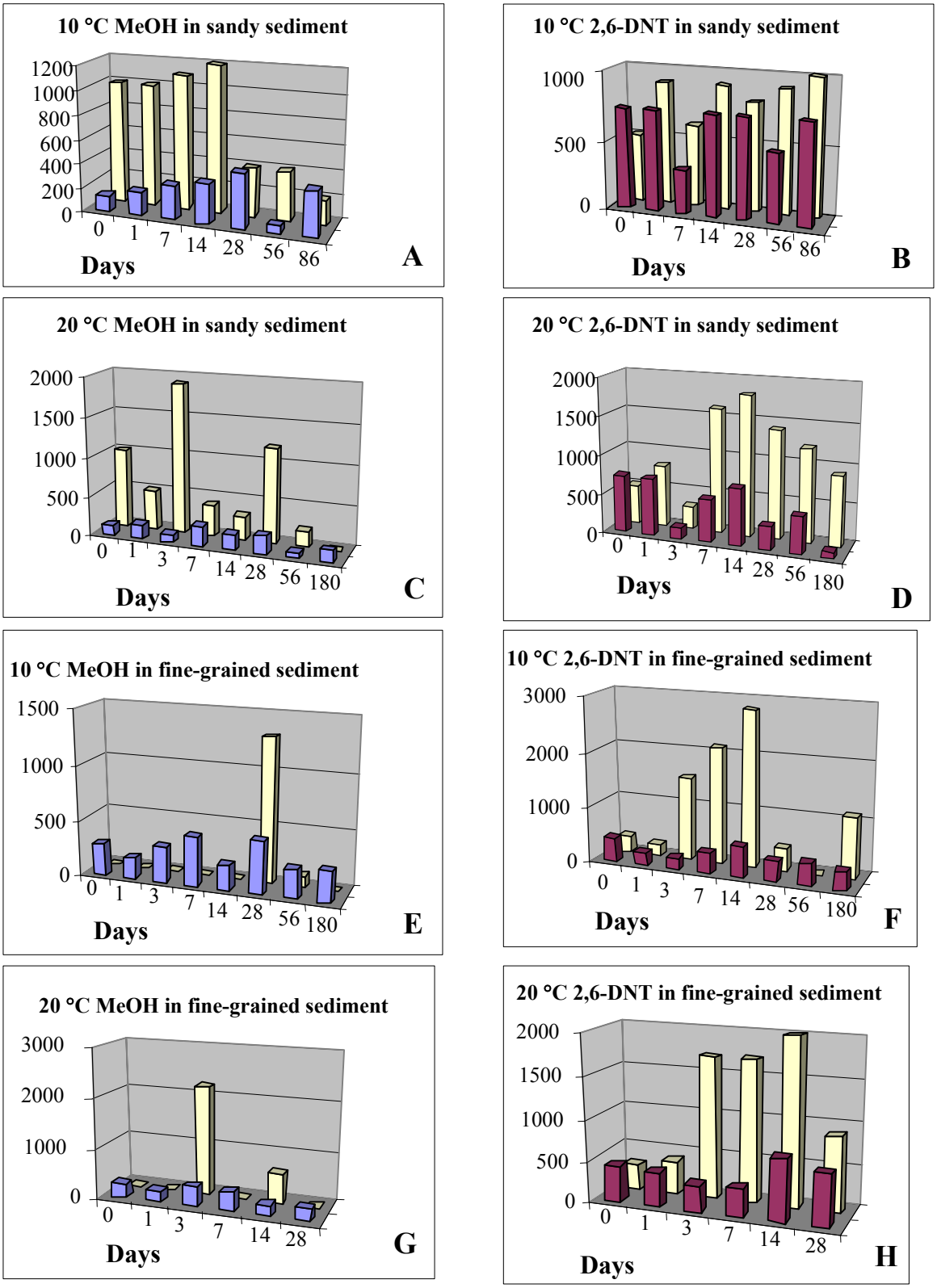

Figure 12. Comparison of total heterotroph MPN data (white) and EcoPlate® data for control (blue) and 2, 6- DNT-spiked sediments (red).

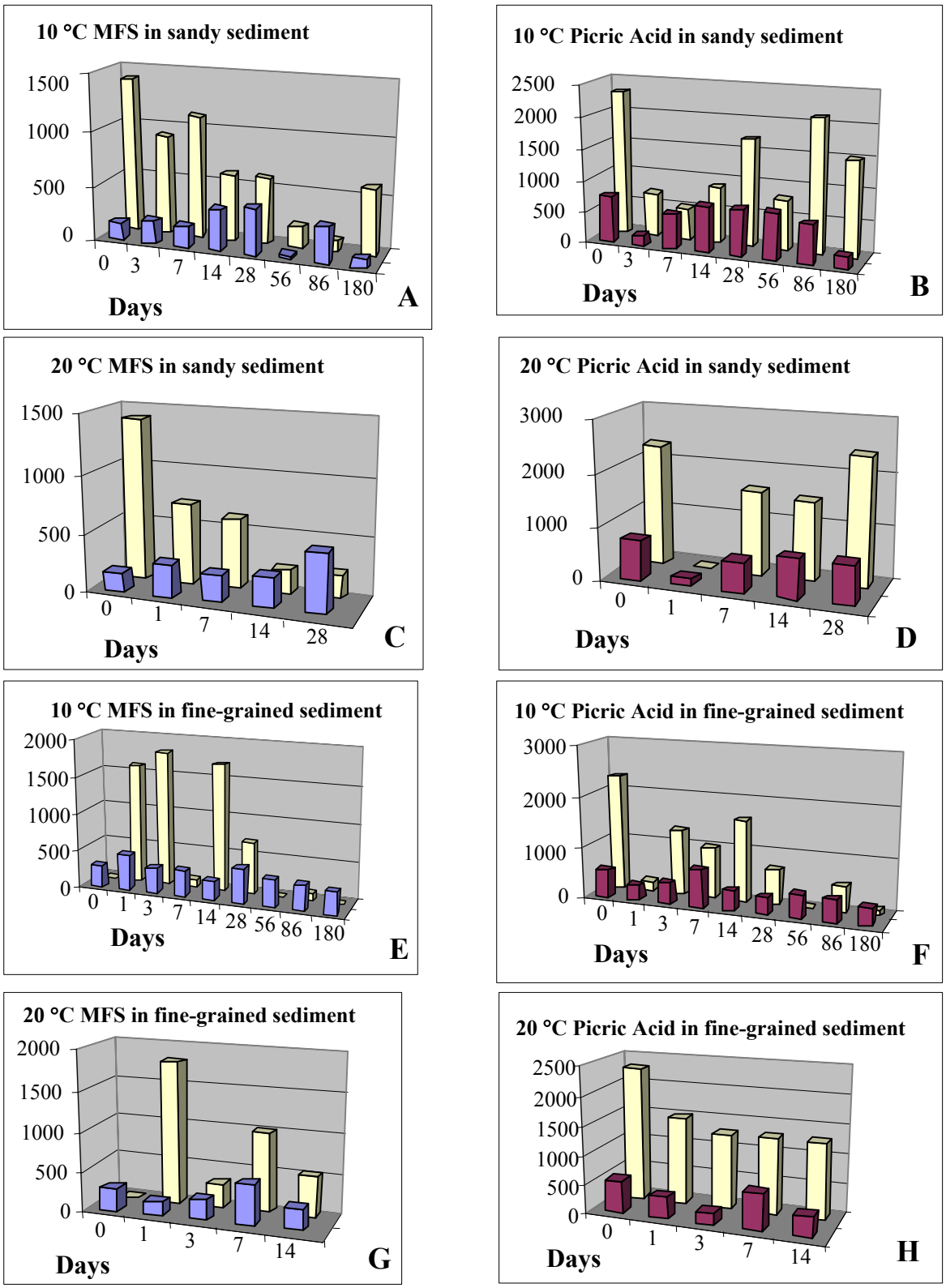

Figure 13. Comparison of total heterotroph MPN data (white) and EcoPlate® data for control (blue) and picric acid-spiked sediments (red).

Some separate experiments were conducted using axenic cultures to aid in the interpretation of the EcoPlate® data. Five common Gram-negative soil forms, *Enterobacter aerogenes*, *Pseudomonas fluorescens*, *Escherichia coli*, *Klebsiella* sp. and *Aeromonas* sp. were measured with the Ecoplate® method before and after being challenged with picric acid (Figure 14). Ecoplate® metabolic fingerprinting pre- and post-exposed to picric acid revealed no significant change in metabolic profiles for four of the five species, with an obvious increase in substrate utilization observed for *Aeromonas* sp. after exposure to picric acid.

Photo-transformation of 2,6-DNT and Picric Acid in Seawater

Photochemical transformations of munitions compounds in waters were considered more important than microbiological transformations because they oftentimes have significantly higher rates (Rosenblatt et al. 1991). The stated importance of photolysis in natural waters seems true for different classes of nitrotoluenes, although this does not necessarily hold for sediments or estuarine environments with turbid waters.

Photolysis is an important mechanism of transformation and can be a dominant fate process of DNTs in natural waters (Spanggord *et al*. 1985; Bausum *et al*. 1992), although these authors did not conduct or report any assessments in seawaters. The photo-transformation of 2,6-DNT in seawater under SSR (292 and 1398 $\mu W/cm^2$ UVB and UVA, respectively) (Table 1) began soon after the initial exposure, with 10% loss in the first 2 hours. Similarly, Kumar and Davis (1997) reported removal of 10 to 13% DNTs after 90 minutes under exposure to a UV light with peak emission at 365 nm. In the present study 89% of the 2,6-DNT was photo-transformed in 24 hours (Appendix A5, Fig. 15) and none was left after 72 hours. During the photo-transformation process, a small peak corresponding to the elution time of aminodinitrotoluenes could be seen in HPLC chromatograms (Fig. 16B), but the final photo-transformation product of 2,6-DNT could not be identified by GC/MS, although a thin peak appeared in the HPLC chromatograms at an elution time of 1.7 minutes and the color of the stock solution changed from clear to orange (Fig. 16C). GC/MS and LC/MS analyses showed high molecular weight chemicals with mass spectra ranging from molecular weight (MW) 200 to 500 compared to the MW 182 for DNT. Complexity of the mass spectra and mass differences among fragments suggest that multiple compounds are possibly co-eluting. The photolysis of 2,6-DNT might have promoted polymerization which formed these high MW compounds. Photolysis of DNTs in freshwaters can produce products such as dinitro- and aminonitrobenzoic acids, and azoxy compounds (Bausum *et al*. 1992). Rosenblatt *et al*. (1991) relate a study in which the photolysis of 2,6-DNT originated unstable mixtures where only some products were tentatively identified, including azoxy- and azobenzenes, in addition to 2,6-dinitrobenzaldehyde and 6-nitroanthranil. Azo compounds are usually strongly coloured as a result of the –N=N- linkage, which brings the aromatic rings into conjugation (Solomons 1997). The color change exhibited by the 2,6-DNT solution under SSR suggests that the compounds resulting from 2,6-DNT photolysis could be chemicals of that nature (Fig. 16C).

It has been reported that the photolysis of a variety of nitrophenols would result in the formation of different hydroxylated aromatic, nitroaromatic and aminoaromatic compounds (Nakagawa and Crosby 1974; Shea *et al*. 1983). However, no significant photolysis of picric acid was observed in the current study in up to a 47-day exposure to SSR at irradiance levels of 196 and 1023 $\mu W/cm^2$ UVB and UVA, respectively (Table 1, Fig. 15).

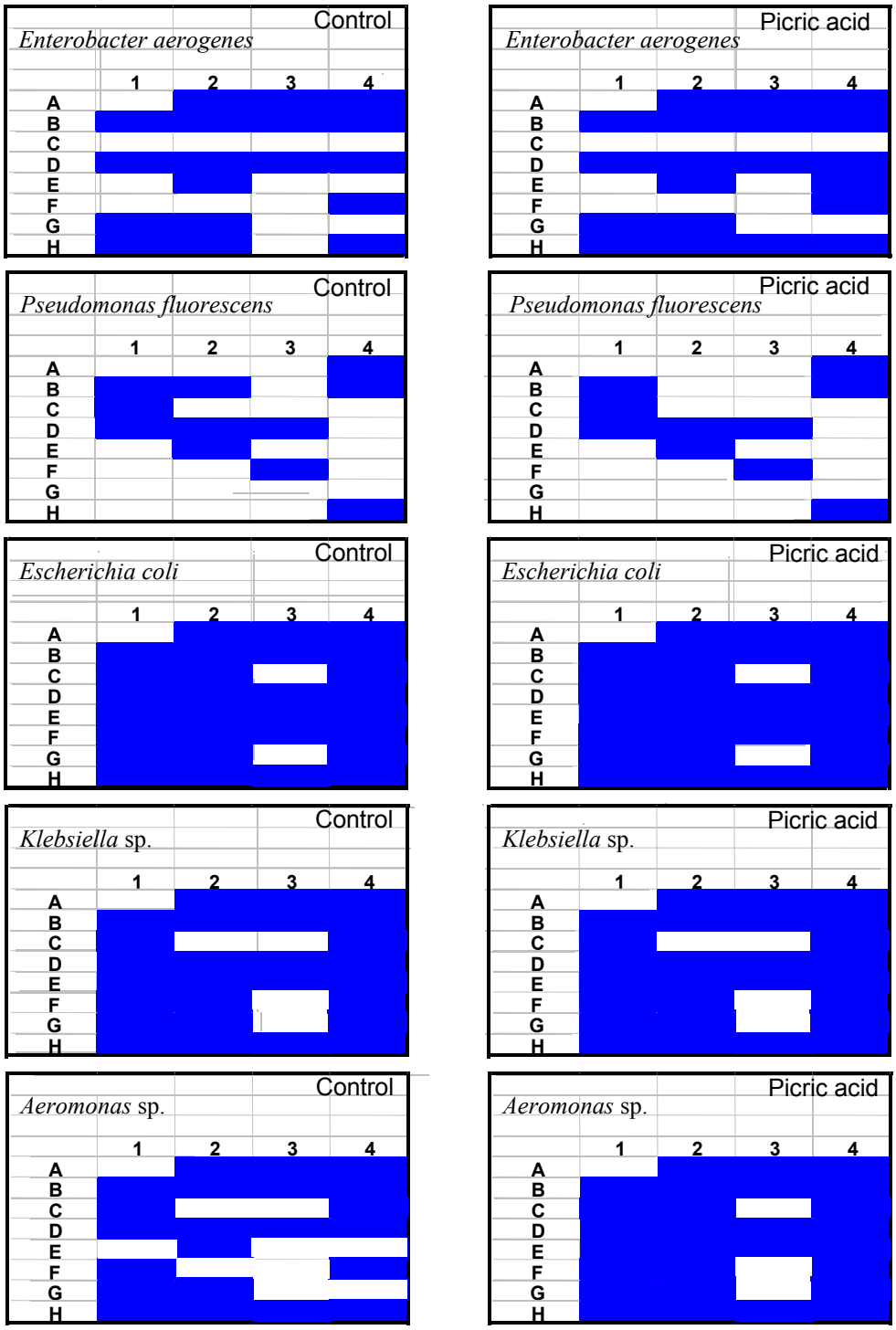

Figure 14. EcoPlate® patterns for five common sediment bacteria *Enterobacter aerogenes, Pseudomonas fluorescens, Escherichia coli, Klebsiella* sp. and *Aeromonas* sp. before and after exposure to picric acid.

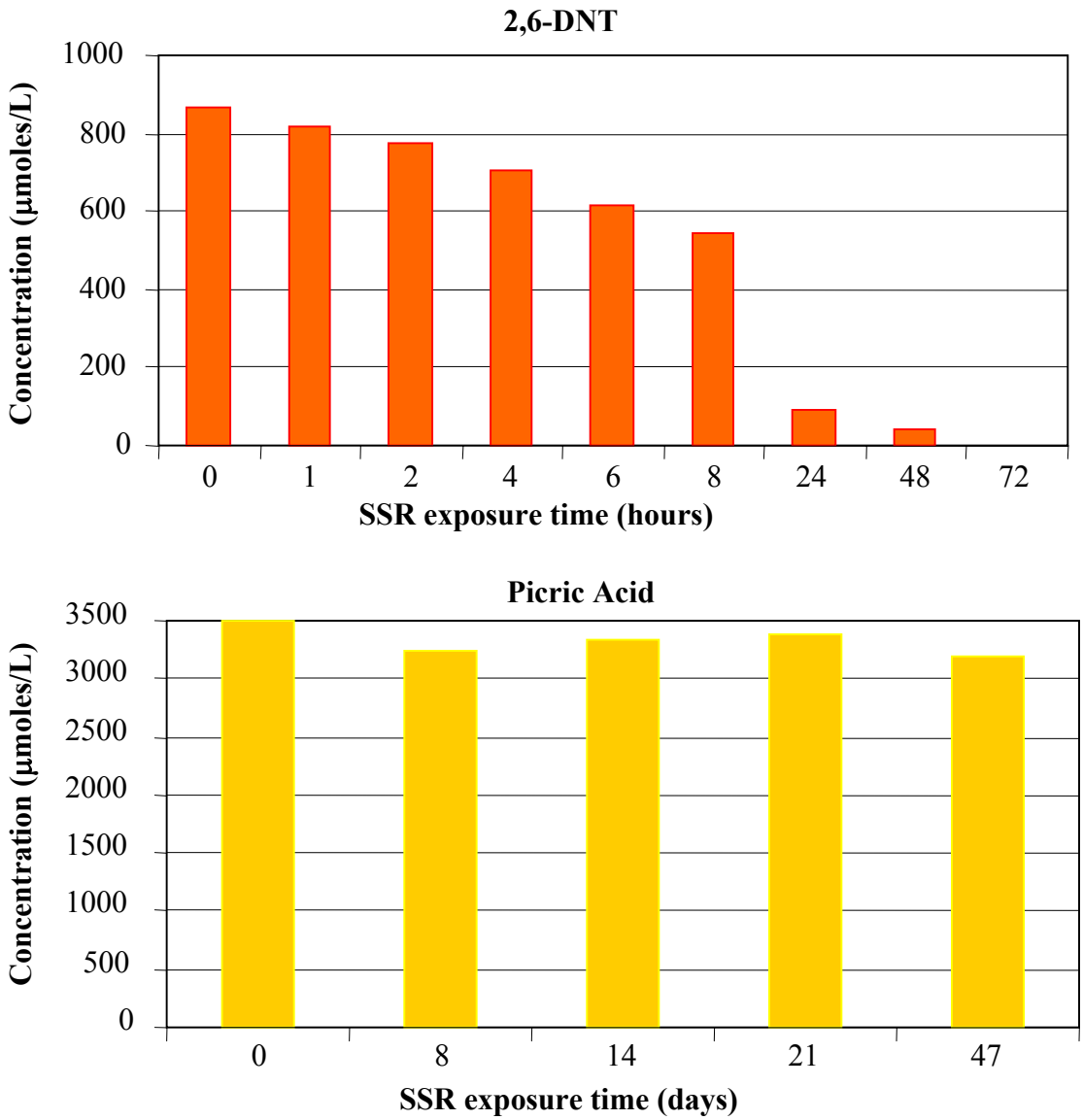

Figure 15. Concentration of 2,6-DNT and picric acid in spiked seawater exposed to simulated solar radiation (SSR) for 72 hours and 47 days, respectively.

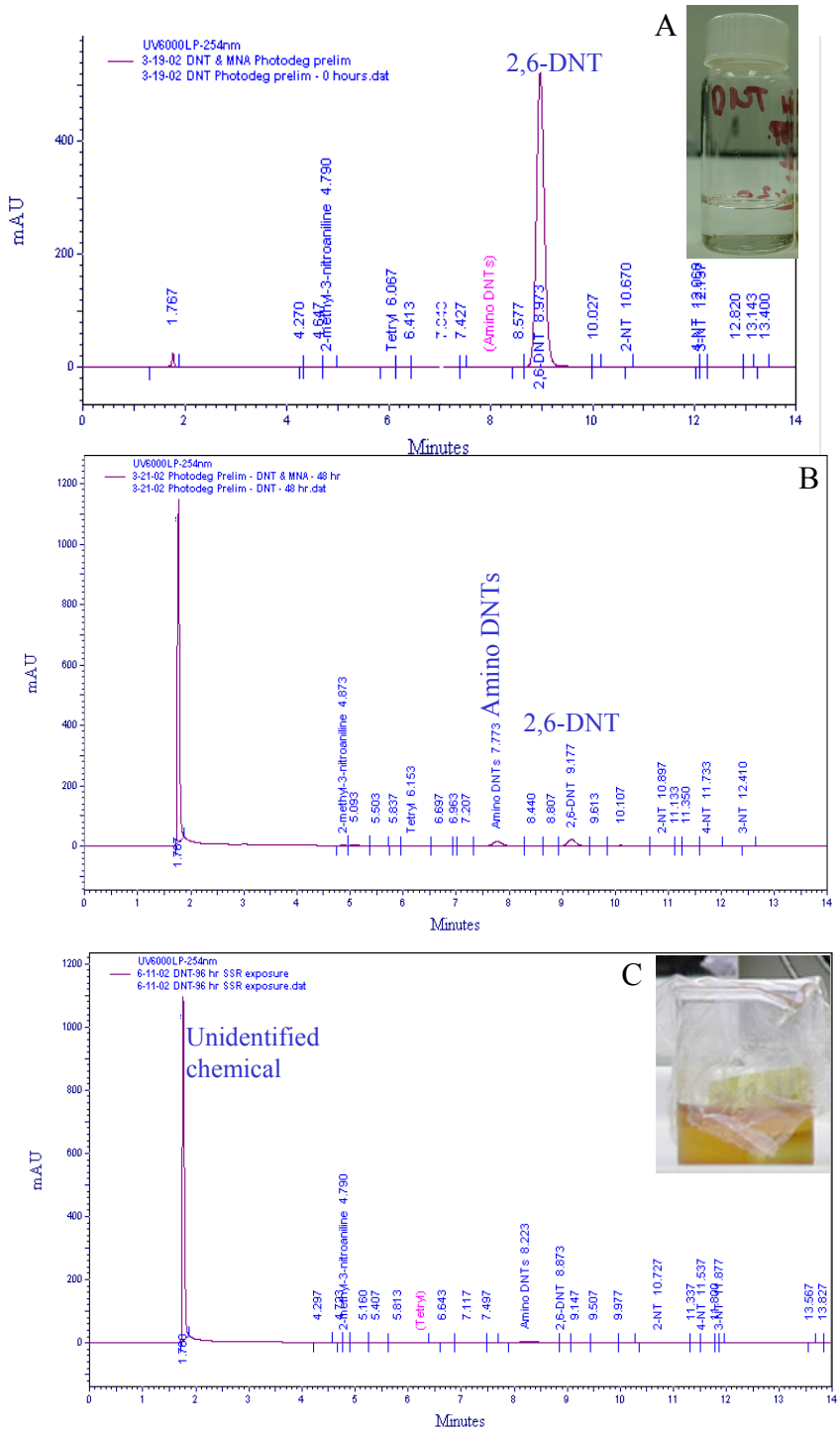

Figure 16. HPLC chromatograms of seawater spiked with 2,6-DNT and exposed to simulated solar radiation (SSR) for: A) 0 hours; B) 48 hours; C) 96 hours. Vials in A and C show the change in color of the 2,6-DNT solution from clear to orange with SSR exposure.

PART 2: Toxicity Assessments

Water Quality Measurements

Water quality measurements indicated that sulfide was below detection (0.01 mg/L) in all toxicity test samples. Therefore, sulfide data are not presented in any of the water quality tables.

Tests with Single Chemicals and Photo-transformed 2,6-DNT

Water quality parameters were within the acceptable range in all samples used for toxicity tests with single chemicals (Appendices B3 and B4). The lowest DO level (in percent saturation) was 90 and 90.1 in the copepod and algae test, respectively, and pH ranged from 7.8 to 8.4 in the copepod test and 7.9 and 8.3 in the algae test. In the copepods tests, with exception of the test performed with ammonium chloride, ammonia was only above detection in the photo-degraded 2,6-DNT sample, with unionized ammonia concentration of 21.7 μg/L, well below the toxic level to this species (NOEC = 692.3 μg/L; see Appendix D1). The highest unionized ammonia concentration measured in single chemicals tests with algae was 16.1 μg/L, also in the photo-degraded 2,6-DNT sample and also well below toxic levels for the test species.

Tests with Pore Waters

Water quality in porewater tests was more variable than in single chemical tests, which was expected due to the more varied matrix (sediment) and conditions to which they were exposed prior to porewater extraction. Oxygen levels were high in all samples, with a minimum of 88% in toxicity tests with both algae and copepods (Appendices B1 and B2). The pH measurements in both tests were a bit more variable, ranging from 7.7 to 9.1 in the copepod test and 7.2 to 8.7 in the algae test. The highest pH values, of 8.8 and 9.1 in the copepod tests, and 8.7 in the algae tests, occurred in the sandy sample spiked with the 2,6-DNT and the respective blank spiked with filtered seawater after a 180-day storage. The pH elevation may have been caused by sedimentary processes taking place during the storage at 20°C. The high pH values drove the levels of unionized ammonia to concentrations that could be toxic to the copepods, but this was compensated with the use of porewater blanks with correspondingly high ammonia as to avoid the detection of false positives due to this confounding factor.

Unionized ammonia concentrations in the pore waters used in copepod tests ranged from 20.9 to 2208 μg/L, with total ammonia ranging from 0.4 to 15.6 mg/L. In the pore waters used in the algae tests, unionized and total ammonia concentrations ranged from 9.9 to 3086.8 μg/L and 0.2 and 36.7 mg/L, respectively. Two extremely high unionized ammonia levels in the algae test (2606 and 3087 μg/L) corresponded to 100% pore water from the fine-grained and sandy sediments spiked with picric acid, after a 56-day storage (Appendix B2). The same levels cannot be seen in the copepod test (Appendix B1) because the highest experimental concentration for those samples was a 25% dilution instead of 100% pore water.

Toxicity Assessment of Ordnance Compounds and Biotransformation Products in Seawater

Some of the major biotransformation products of 2,6-DNT and picric acid were purchased in purified form and analyzed for toxicity in filtered seawater. Neither 2,6-DNT nor its main biotransformation product, 2-A-6-NT, were highly toxic to copepod, *S. knabeni*, adult female

survival, with NOEC values of 277 and 240 μmoles/L, respectively (Appendix D1). The toxicity of the same chemicals to nauplii hatching rate, however, indicated significantly higher toxicity of the biotransformation compound, with EC_{50} values of 52 and 242 μmoles/L for 2-A-6-NT and 2,6-DNT, respectively (Appendix D2, Fig. 17). The opposite happened in the macroalga, *Ulva fasciata*, zoospore germination tests, where the toxicity of the parent compound, 2,6-DNT, was markedly higher than that of 2-A-6-NT. EC_{50} values of 2-A-6-NT were >160, 123 and 137 μmoles/L for germination, germling length and cell number, respectively, whereas the EC_{50} values for 2,6-DNT were 73, 20 and 24 μmoles/L, respectively (Appendices D3-D5). Amino derivatives of TNT have also been found to be less toxic than the parent compound to some organisms but more toxic to others (Amerkhanova and Naumova 1979, *apud* Stahl and Aust 1995). Another biotransformation product of 2,6-DNT, 2-nitrotoluene, was less toxic than the parent compound to copepod females and embryos (Appendices D1 and D2, Fig. 17), whereas it was only slightly more toxic to macroalgae zoospore germination, but not to germling growth (Appendices D3-D5, Fig. 18). However, results of toxicity tests with 2-NT are reported as measured initial concentration of this chemical, since acute losses occurred during the 96-hour tests (see Appendices D1-D5), possibly due to volatilization.

Some of the main identified breakdown products of picric acid were 2,4-DNP and picramic acid. Both biotransformation products were more toxic to copepods and macroalgae zoospores than their parent compound (Figs. 17 and 18, Appendices D1-D5). Toxicity ranking tended to follow this order: 2,4-DNP > picramic acid > picric acid, matching the suggestion by Simon and Blackman (1953) that the toxicity of nitrophenols increases with the first nitration (nitro- to dinitro-phenol) but decreases with the third nitration. A literature review by Buikema *et al.* (1979) also indicates higher toxicity of 2,4-DNP than picric acid to the freshwater cladoceran, *Daphnia magna*, with LC_{50} values of 2,4-DNP ranging from 25 to 103 μmoles/L in 48 and 24-hour tests, respectively. These values are comparable to the 96-hour female and nauplii survival LC_{50} values for the copepod used in the current study, of 77 and 54 μmoles/L, respectively. The toxicity of 2,4-DNP to a variety of fish, as summarized by Buikema *et al.* (1979) and Goodfellow *et al.* (1983), ranged from 1.6 to 157 μmoles/L, depending on species, life stage and exposure time. The high sensitivity of some species suggests that further assessments not only with 2,4-DNP but other picric acid transformation products should be conducted with fish, particularly using early-life stage tests.

Picramic acid was also found to be more toxic than picric acid to American oysters, *Crassostrea virginica*, and rainbow trout, *Salmo gairdneri* (Goodfellow *et al.* 1983). Picramic acid had 2-fold higher toxicity than picric acid in oyster tests, with 144-h LC_{50} values of 251 and 478 μmoles/L, respectively, and about 3-fold higher in trout tests, with 96-h LC_{50} values of 379 and 1112 μmoles/L, respectively. The sensitivity of *S. knabeni* and *U. fasciata* tests to these chemicals fell in this range, with the algae being somewhat less sensitive to picric acid (Appendices D3-D5) and the copepod nauplii survival somewhat more sensitive to picramic acid (Appendix D2). Sublethal effects to oyster shell deposition, however, occurred at lower levels still, with EC_{50} values of 30 and 122 μmoles/L for picramic and picric acid, respectively (Goodfellow *et al*. 1983).

Since some of the porewater samples to be tested for toxicity exhibited elevated levels of ammonia, the toxic concentrations of ammonia to *S. knabeni* survival and nauplii hatching were calculated (Appendices D1 and D2). The EC_{50} of unionized ammonia to copepod survival and reproduction was 1207 and 387 μg/L, respectively. Some of the tested pore water contained ammonia in toxic levels. The identification of false positives due to ammonia was avoided by

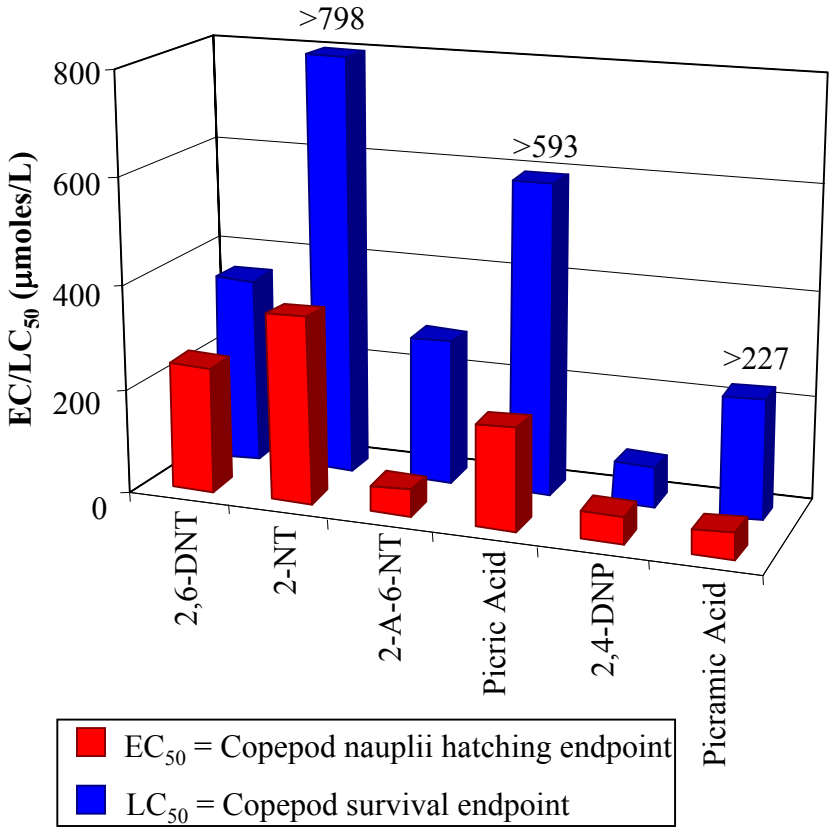

Figure 17. Survival and reproduction effective concentrations in copepod tests with single chemicals.

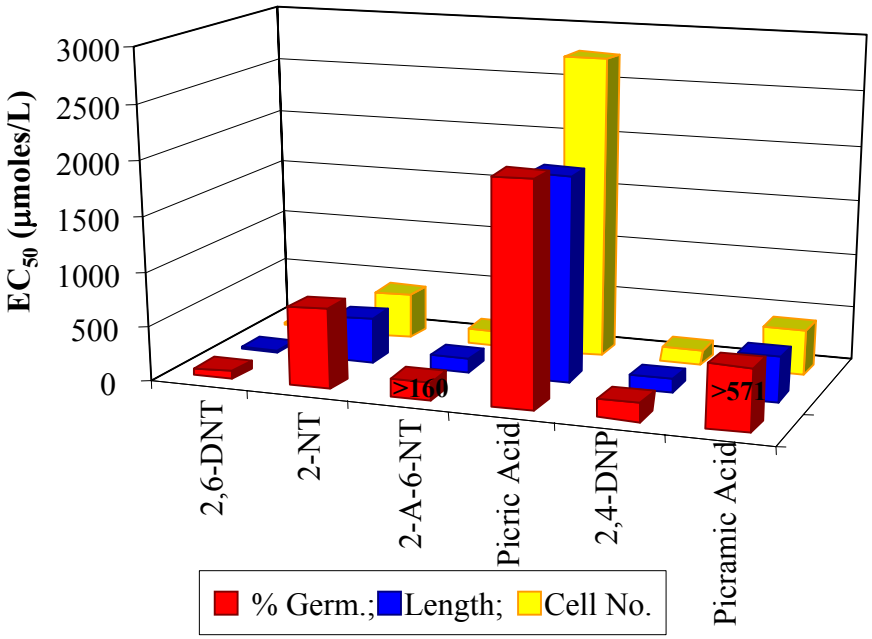

Figure 18. Effective concentrations in *Ulva fasciata* zoospore tests with single chemicals.

using appropriate porewater controls, which had unionized ammonia concentrations similar to those of the samples spiked with ordnance compounds. Therefore, no samples should have been identified as toxic due to ammonia alone. This was not a problem in the *U. fasciata* tests, since these organisms have extremely low sensitivity to ammonia, with a germination EC_{50} of 1650 μg/L unionized ammonia (Hooten and Carr 1998). Only the two sediments spiked with picric acid and stored for 56 days had ammonia concentrations that would be expected to adversely affect the macroalga test endpoints. In both cases, toxic effects were also exhibited in lower porewater concentrations, where ammonia was already below toxic levels, therefore ruling out the possibility of identifying a false positive, i.e., a toxic effect caused by ammonia rather than by ordnance compounds and transformation products.

Porewater Toxicity Assessment

2,6-Dinitrotoluene

The EC_{50} based on the sum of measured initial concentration of 2,6-DNT and 2-A-6-NT was calculated in porewater tests. In the copepod tests, reproduction, expressed as number of nauplii hatched per female added to the test vials, tended to be a more sensitive endpoint than adult female survival (Appendices E1 and E2), with the exception of the sample from sandy sediment on day 0, where survival was more sensitive than reproduction. Calculated EC_{50} values show that the samples in which 2,6-DNT was totally metabolized and only 2-A-6-NT could be measured (7-day storage) were less toxic to copepod survival than those where a high amount of 2,6-DNT was measured (prior to storage) (Appendix E1), whereas the opposite was observed with copepod reproduction (Appendix E2). Similar results were observed in tests with purified DNT and ANT spiked into filtered seawater (Appendices D1 and D2).

After a 180-day storage no 2-A-6-NT was detectable in either sediment sample, and no significant toxicity to copepod survival was measured (Appendix E1, Fig. 19A). Toxicity to copepod reproduction decreased in the fine-grained sediment after a 180-day storage (Fig. 19C and D), but it increased somewhat in the sandy sediment, relative to the initial and 7-day samples (Appendix E2, Fig. 19B). The data suggests that in the fine-grained sediment after 6 months the 2,6-DNT transformation products had been further metabolized to less toxic forms, whereas in the sandy sediment they had not been transformed to the same stage. It is expected that further biotransformation would occur in the sandy sediment, in which such processes typically took longer to occur (see Appendix A1).

Unlike with copepod reproduction, in the tests with *U. fasciata* zoospores porewater toxicity decreased with sediment storage time for both sediments, although increased germling growth, relative to control, occurred in the pore water from the fine-grained sample stored for 180 days (Fig. 20, Appendices E3-E5). This indicates that 2,6-DNT biotransformation products were less toxic than the parent compounds to the macroalgae early life stages, thus showing the importance of using more than one species and endpoints for toxicity assessments.

Tests with filtered seawater spiked with 2,6-DNT and with its identified biotransformation products, 2-A-6-NT and 2-NT, as identified by GC/MS, corroborate the results presented above. The 2,6-DNT EC_{50} values for *U. fasciata* zoospore germination, germling length and germling cell number were 73, 20 and 24 μmoles/L, respectively, whereas for 2-A-6-NT the corresponding EC_{50} values were >160, 123 and 137 μmoles/L, and for 2-NT they were 368 μmoles/L for germination and 207 μmoles/L for both germling length and cell number (Appendices D3-D5).

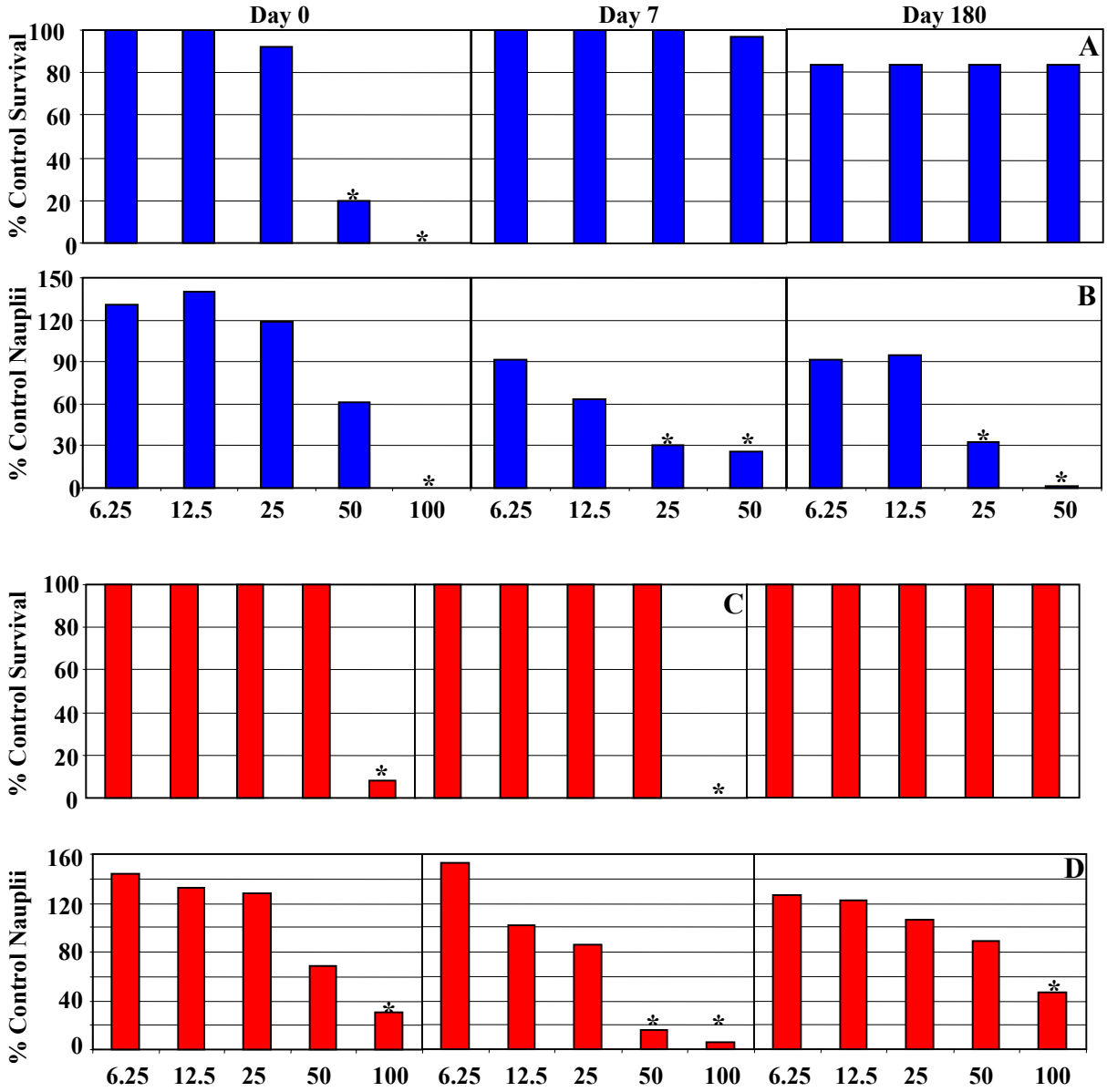

Figure 19. Effect of 2,6-DNT-spiked sediment storage time on the toxicity of pore waters to copepod adult female and nauplii survival in pore waters from sandy (A, B) and fine-grained (C, D) sediment. Asterisks indicate significant difference from the controls (not shown).

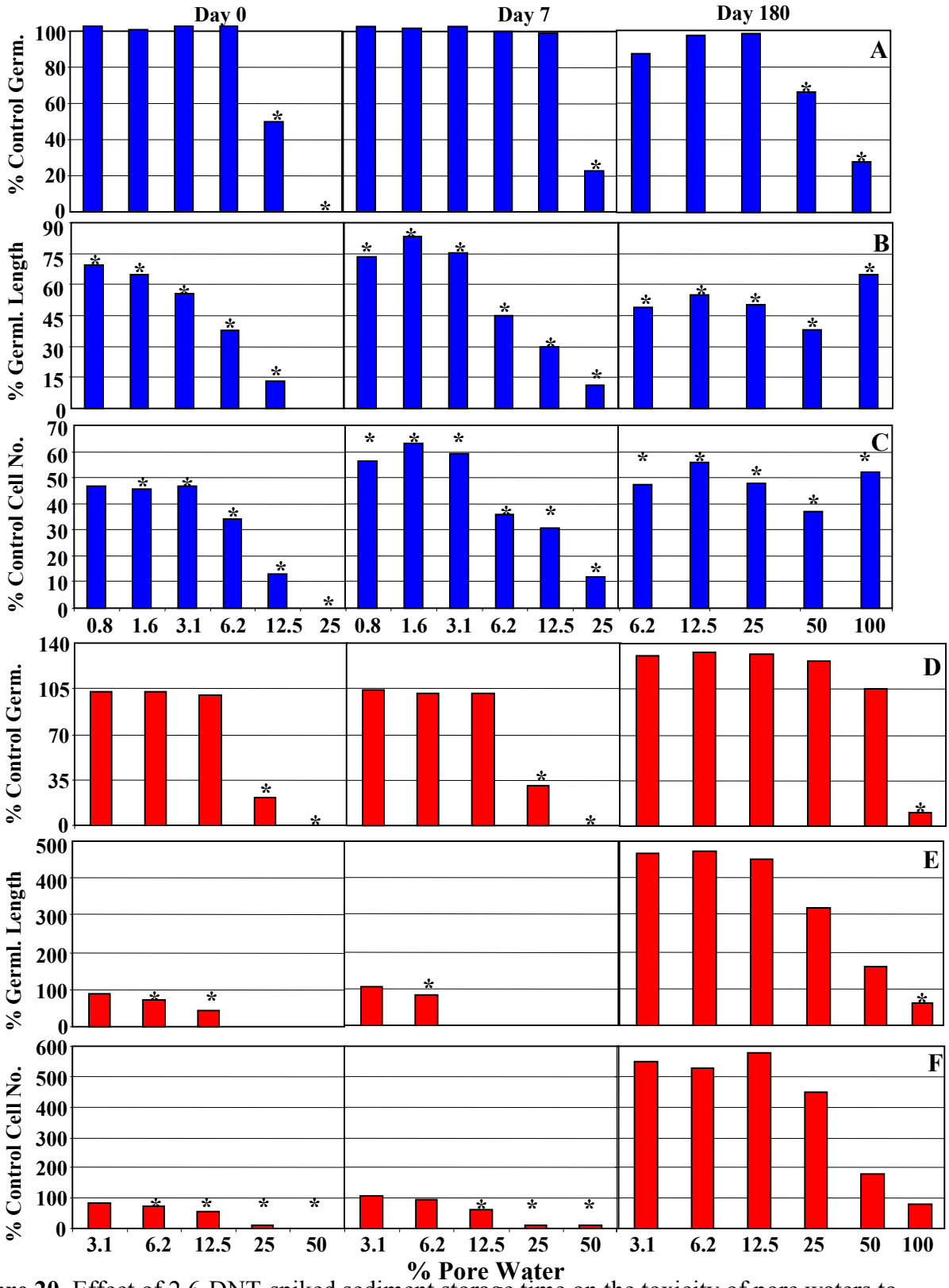

Figure 20. Effect of 2,6-DNT-spiked sediment storage time on the toxicity of pore waters to *Ulva fasciata* zoospore germination (A, D), and germling growth in length (B, E) and in cell number (C, F). A-C: Samples from sandy sediment stored for 0, 28, 56 and 180 days; D-F: Samples from fine-grained sediment stored for 0, 7, 56 and 180 days.

Picric Acid

The toxicity of pore waters from picric acid-spiked sediments was variable over time. In the sandy sediment, toxicity to copepod female survival disappeared after a 28-day storage of the spiked sediment (Appendix E6, Fig. 21A), whereas for the reproductive endpoint (nauplii survival) toxicity was only significantly decreased after a 180-day storage, although still toxic at a 25% porewater dilution (Appendix E7, Fig. 21B). The most toxic sample to copepod nauplii hatching and survival was after 28-day storage, where high toxicity still occurred at a 6.25% dilution of the pore water (Fig. 21B). This sample, at 100% porewater, contained only 32 µmoles picric acid /L, well below the NOEC of 74 µmoles/L (Appendix D2). However, it also contained 148 µmoles 2,4-DNP/L and 15 µmoles picramic acid/L (Fig. 22B), which are likely to have caused or contributed to the observed toxicity. Both biotransformation products were still present after 56-day storage, although in lower concentrations (Fig. 22C), but they could not be measured after a 180-day storage (Fig. 22D). Further biotransformation products are expected to have caused the milder observed effects. Possible products related to picric acid found in this sample were products containing amino, nitro and hydroxyl groups, in addition to nitrophenol isocyanate. The toxicity of the latter to aquatic organisms has not been established.

In samples from the fine-grained sediment the lowest copepod female survival occurred after 56 days of storage (Appendix E6, Fig. 21C), and no effect was observed after 180-day storage. Copepod nauplii hatching and survival followed a similar pattern, with the highest toxicity in the 56-day storage sample (Appendix E7, Fig. 21D). After a 180-day storage, reproduction was still significantly affected in 50 and 100% porewater (Appendix E7), suggesting that toxic chemicals resulting from the picric acid biotransformation were still present, although they could not be quantified by GC/MS analyses, which only indicated the presence of a very small amount of nitrodiaminophenol. Major aromatic compounds in this sample were benzoic acids, benzene acetic acids, the source of which is not clear. However, as for 2,6-DNT spiked samples, toxicity in the fine-grained sediment after 180-day storage was lower than in the sandy samples, suggesting that further metabolization of picric acid breakdown products had occurred, generating less toxic products.

Similarly to the copepod reproductive endpoint, toxicity in the *U. fasciata* zoospore test tended to decrease with storage time of the sandy sediments up to 56 days, with a slight increase in toxicity to the percent germination endpoint with samples from 180-day storage (Fig. 23A-C, Appendices E8-E10). A different response was exhibited by the samples from the fine-grained sediment, where extremely high toxicity, with no germination at all, was exhibited after a 56-day storage of the spiked sediment, but no toxic effect to germination occurred after 180-day storage, with only a mild effect on germling growth (Fig. 23D-F, Appendices E8-E10). The high toxic effects of the fine-grained day 56 sample could neither be explained by any measured ordnance compounds or their metabolites (Fig. 22; see Appendices D3-D5 for toxicity of picramic acid and 2,4-DNP), nor by elevated ammonia or nitrite. Although ammonia could have been at toxic levels to the copepods, blanks with similar levels of ammonia were used for results interpretation. Nitrite concentrations in those samples (2.6 and 87.6 µmoles/L in 100% pore water from sandy and fine-grained sediment, respectively) also do not explain the extremely elevated toxicity of the samples, based on literature data. The highest crustacean sensitivity to nitrite found in the scientific literature was for eggs of the shrimp *Penaeus paulensis*, with a 24-hour LC_{50} of 3.1 mg/L, equivalent to 67.4 µmoles/L (Ostrensky and Poersch 1992-1993). Similarly to the copepod test, porewater toxicity to *U. fasciata* also exhibited a strong decrease after a 180-day storage, particularly in the fine-grained sediment.

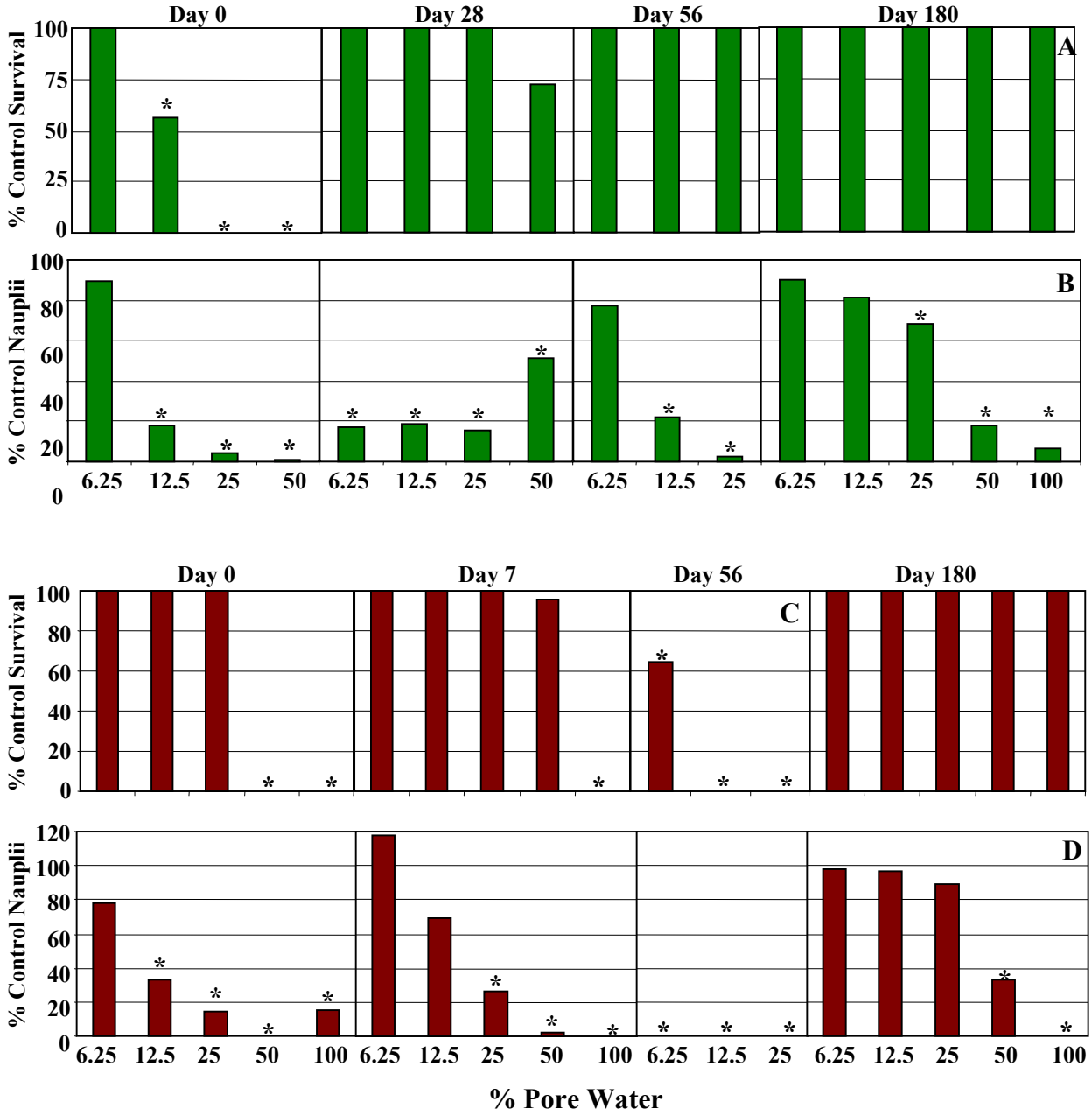

Figure 21. Effect of picric acid-spiked sediment storage time on the toxicity of pore waters to copepod adult female and nauplii survival in pore waters from sandy (A, B) and fine-grained (C, D) sediment. Asterisks indicate significant difference from the controls (not shown).

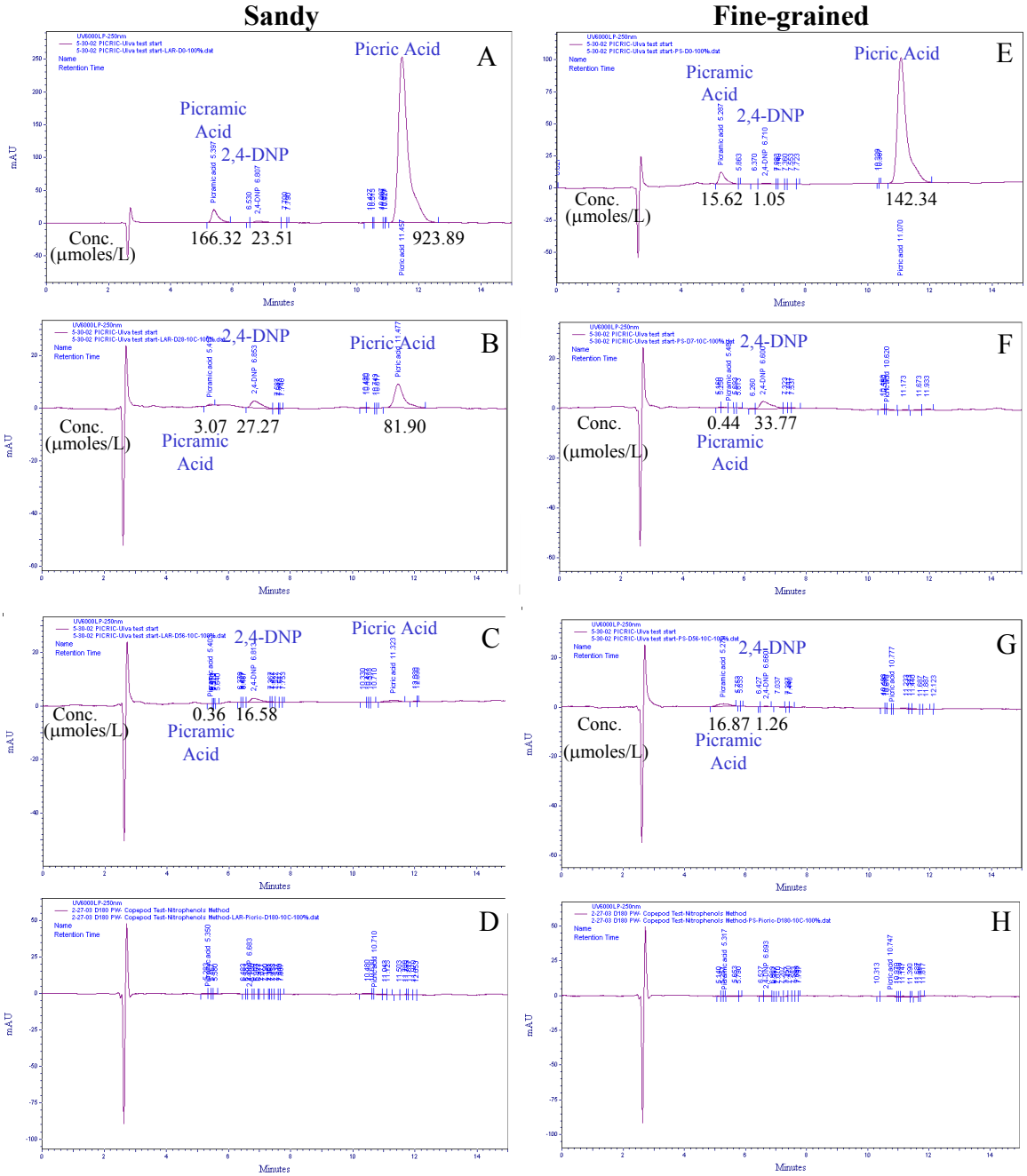

Figure 22. HPLC chromatograms of pore waters from sediments spiked with picric acid, after several storage times: A - D = Storage times 0, 28, 56 and 180 days, respectively, in sandy sediment; E - H = Storage times 0, 7, 56 and 180 days, respectively, in fine-grained sediment.

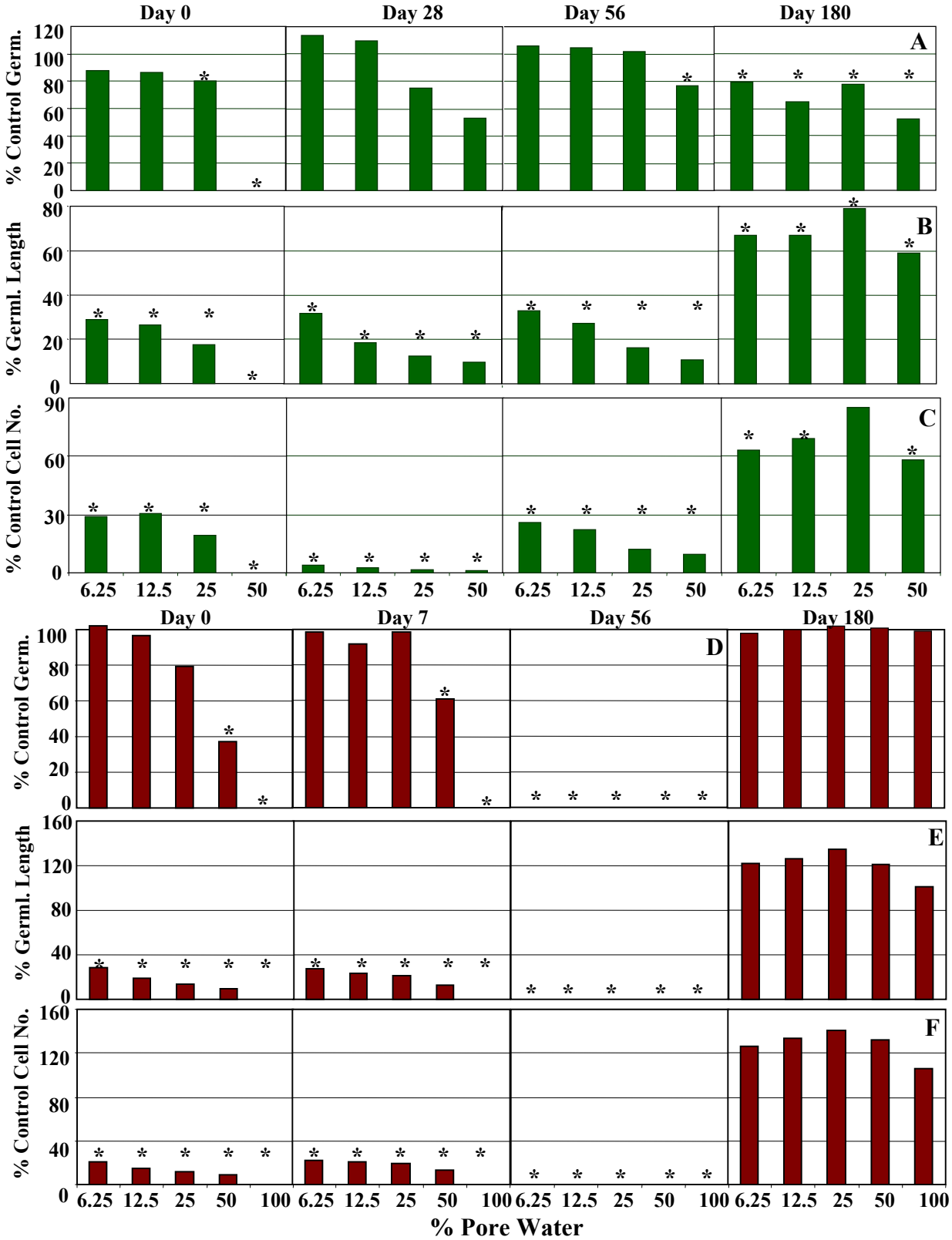

Figure 23. Effect of picric acid-spiked sediment storage time on the toxicity of pore waters to *Ulva fasciata* zoospore germination (A, D), and germling growth in length (B, E) and in cell number (C, F). A-C: Samples from sandy sediment stored for 0, 28, 56 and 180 days; D-F: Samples from fine-grained sediment stored for 0, 7, 56 and 180 days.

Two of the major biotransformation products of picric acid, 2,4-DNP and picramic acid, as identified by GC/MS, had higher toxicity than its parent compound in copepod and macroalgae tests (Appendices D1-D5), with a more marked increase in toxicity to the copepod reproductive endpoint and algae germling growth. The concentrations of 2,4-DNP and picramic acid were measured by HPLC in the pore waters used for toxicity testing (Appendix A6). The EC_{50} of pore waters calculated based on picric acid concentrations tended to present a lower value than the EC_{50} of picric acid spiked into filtered seawater. The measured picric acid biotransformation products, 2,4-DNP and picramic acid, could have contributed to the increased toxicity in the pore waters, but do not fully explain the highly elevated toxic effects of some samples. This suggests that other unidentified breakdown products would be present and causing strong adverse effects to the test organisms. The extremely high toxicity of the pore water from the fine-grained sediment after 56-day storage, particularly, could not at all be due to 2,4-DNP or picramic acid, since only low concentrations of these chemicals could be measured by HPLC in those porewater samples (Fig. 22C and G).

Toxicity Assessment with Photo-transformed 2,6-DNT

Samples of photo-transformed 2,6-DNT were assessed for toxicity concurrently to tests with dilution water spiked with 2,6-DNT. Results of the assay with the photo-transformed product are expressed as percent of original test solution (Appendices D1-D5). The original 2,6-DNT concentration in the 100% solution prior to photo-transformation was 783.7 μmoles/L in the copepod test and 705.3 μmoles/L in the macroalgae test. This difference is due to the addition of 10% reference pore water to the samples used in algae tests, as a source of nutrients.

The EC_{50} values of the 2,6-DNT photo-transformed product(s) were also calculated as μmoles/L original 2,6-DNT in the stock solution, prior to photo-transformation, in order to allow comparison of the toxicity data. Toxicity of the original compound was higher than that of its breakdown product to *U. fasciata* zoospores, copepod survival, and to the polychaete, *D. gyrociliatus*, reproduction, but not to copepod reproduction, where slightly higher toxicity was exhibited by the photo-irradiated solution (Fig. 24). Similarly to what was observed in the current study with 2,6-DNT, TNT photolysis caused toxicity to decrease to several species of freshwater fish, the cladoceran, *Daphnia magna*, and the earthworm, *Lumbriculus variegatus* (Rosenblatt *et al*. 1991). The toxicity of TNT to a freshwater amphipod and midge was not modified by photolysis.

Photo-induced Toxicity Assessments

The assessment of photo-induced toxicity was performed at the termination of toxicity tests with 2,6-DNT and picric acid in dilution water using copepods and polychaetes. Photo-induced toxicity was not exhibited by either chemical (Fig. 25). Some adverse effects, including sluggishness of the animals, were observed at the end of the SSR exposure, but this occurred in the controls as well, leading to inconclusive results. These exposures represented only 4% of the UVB of summer sun in subtropical latitudes, and further assessments are recommended if ordnance compounds in concentrations of concern are measured in sediments from shallow, clear water areas or in intertidal sediments.

The phototoxicity of TNT, various diaminotoluenes and DNTs, including 2,6-DNT, and aminodinitrotoluenes to the freshwater cladoceran, *Daphnia magna*, and the marine sea urchin,

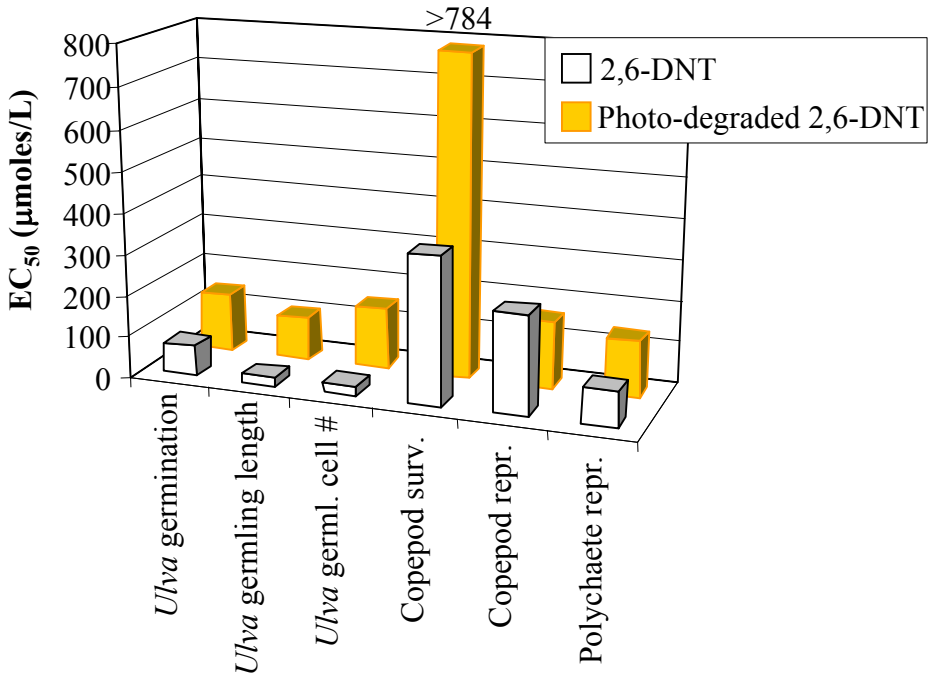

Figure 24. Toxicity of 2,6-DNT and photo-degraded 2,6-DNT. EC50s for photo-degraded 2,6-DNT are based upon concentrations of 2,6-DNT prior to SSR irradiation.

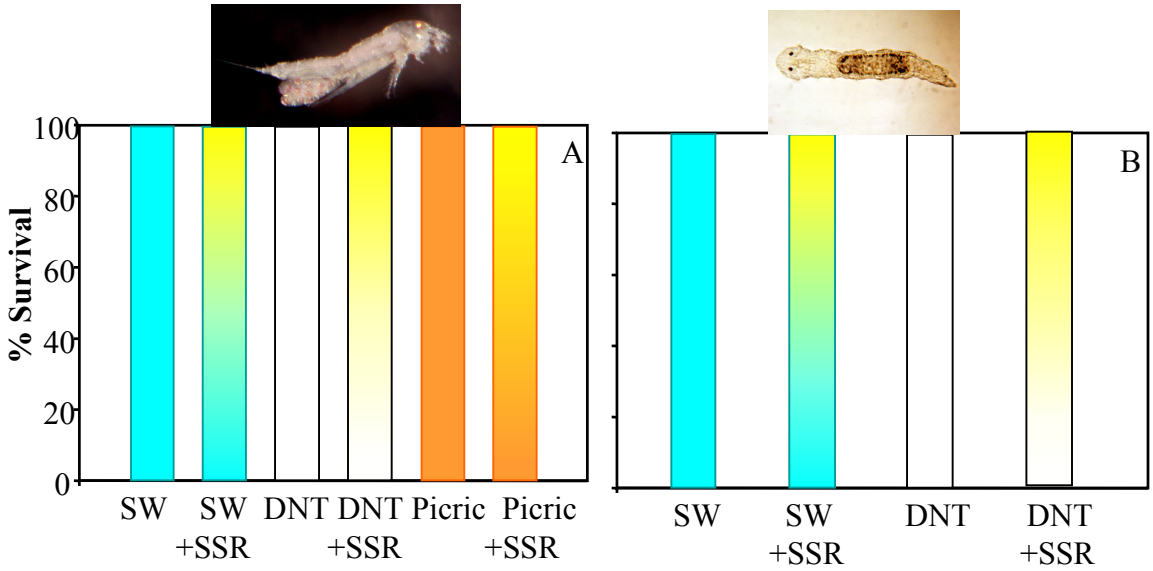

Figure 25. Effect of simulated solar radiation (SSR) on the toxicity of ordnance compounds: A) S. knabeni exposed to ~75% of the 96-h LC_{50} of 2,6-DNT and picric acid prior to SSR exposure. SSR at 280-320 nm = 15.6 $\mu W/cm^2$ = 4% UVB of summer sun; B) D. gyrociliatus exposed to ~50% of the 96-h LC_{50} of 2,6-DNT prior to SSR exposure. SSR at (280-320 nm = 18.7 $\mu W/cm^2$).

Lytechinus variegatus, embryological development, was assessed by Davenport *et al.* (1994). The authors found that all were phototoxic to sea urchins, but their methods did not allow conclusions on whether the effects were due to photo-transformation products or photo-induced toxicity, since near ultraviolet light (emission maximum of 354 nm) and chemical exposure occurred concurrently.

Reference Toxicant Tests

The results of reference toxicant tests with SDS indicated that the sensitivity of the test organisms was within the acceptable range based on our laboratory's warning charts (Environment Canada 1990). EC_{50} values for SDS for *U. fasciata* germination were 5.62, 3.54 and 5.74 mg/L (Appendix D6), within the acceptable range of 1.2 – 5.9mg/L.

The SDS EC_{50} in the *D. gyrociliatus* test run concurrently to the toxicity assessment of photo-transformed 2,6-DNT was 7.07 mg/L, somewhat higher than EC_{50} values observed in previous tests. Despite this fact the test still met the purpose of comparing the toxicity of the photo-transformed and the parent compound.

The copepod test is new to our laboratory and no previous SDS data were available. The results of the numerous tests conducted in this survey were within a relatively narrow margin (Appendix D7), with only one survival LC_{50} value considerably lower than the others, at 15.4 mg/L SDS, whereas the survival LC_{50} values of the remaining tests ranged from 24.6 to 32.3 mg/L. Reproductive EC_{50} values for SDS ranged from 8.2 to 12.9 mg/L, indicating similar sensitivity of the batches of organisms used for all tests.

SUMMARY AND CONCLUSIONS

- Biotransformation of picric acid and 2,6-DNT began during the spiking procedure, and proceeded for several months, generating a variety of different breakdown products. Some of these biotransformation products were identified by GC/MS or LC/MS and found to be more toxic to some species than the original parent compounds.
- The major biotransformation product of 2,6-DNT was 2-amino-6-nitrotoluene (2-A-6-NT). 2-nitrotoluene (2-NT) was also identified as a biotransformation product, and minor components found in 2,6-DNT-spiked samples over time were N,N-dimethyl-3-nitroaniline, benzene nitrile, methylamino-2-nitrosophenol and diaminophenol. 2-A-6-NT was observed to be less toxic than 2,6-DNT in the algal zoospore germination test but was considerably more toxic to the benthic copepod *Schizopera knabeni* nauplii survival.
- Several breakdown products of picric acid were identified by GC/MS, and their nature changed with storage time. Breakdown compounds identified at earlier stages of biotransformation (0 to 56 days) were 2,4-dinitrophenol, amino dinitrophenols (including 2-amino-4,6-dinitrophenol, a.k.a. picramic acid), 3,4-diamino phenol, amino nitrophenol and nitro diaminophenol.
- Picric acid rapidly transforms under aerobic conditions but the toxicity appears to increase significantly in the initial stages of biotransformation, based on both the benthic copepod, *S. knabeni*, nauplii hatching and survival assay and the algal zoospore, *Ulva fasciata*, germination test, which is particularly resistant to high ammonia concentrations. However, after 6 months of storage the toxicity was removed, suggesting that given enough time picric acid is biotransformed into non-toxic products.

- Analyses of total heterotrophic bacteria exhibited a similar behavior in all ordnance-spiked samples: there was a strong initial increase in bacterial abundance relative to the respective blanks, followed by a depression that took bacterial abundance to similar levels of the blanks and lasted a few days, then followed by a remarkable increase in the spiked samples, but not in the blanks. The results suggest that the bacteria were using the nitroaromatic compounds as sources of nutrients and causing their transformation into the measured and other unidentified breakdown products. Microbial community analyses suggested a shift in composition, in addition to the observed changes in total heterotrophs.
- No significant photolysis of picric acid was observed in the current study in up to a 47-day exposure to simulated solar radiation. Photo-transformation of 2,6-DNT in seawater under simulated solar radiation began soon after the initial exposure, with 10% loss in the first 2 hours, 89% photo-transformed in 24 hours, and none remaining after 72 hours.
- LC/MS analyses of the photo-transformation product of 2,6-DNT exhibited high molecular weight chemicals with mass spectra ranging from molecular weight (MW) 200 to 500 compared to the MW 182 for DNT. The photolysis of 2,6-DNT might have promoted polymerization, which formed these high MW compounds.
- 2,6-DNT was more toxic than its photo-transformation product to *U. fasciata* zoospores, copepod female survival, and to the polychaete, *Dinophilus gyrociliatus*, reproduction, but not to copepod nauplii survival, where slightly higher toxicity was observed for the transformation product.
- Photo-induced toxicity was not exhibited by either picric acid or 2,6-DNT.
- It would seem prudent to include some of these degradation products in the list of analytes of concern in field assessments of sediments suspected of contamination by nitroaromatics as these compounds are not identified in the standard HPLC analysis for ordnance compounds.

REFERENCE LIST

Amerkhanova, NN and RP Naumova. 1979. A comparative study of acute toxicity to hydrobionts of a-trinitrotoluene and its degradation products. *Biol. Nauki* 2:26-28.

Atlas, RM and R Barth. 1981. *Microbial Ecology: Fundamentals and Applications*. Reading, MA: Addison-Wesley Publishing Company.

Bausum, HT, WR Mitchell and MA Major. 1992. Biodegradation of 2,4- and 2,6-dinitrotoluene by freshwater microorganisms. *J. Enviro. Sci. Health* A27:663-695.

Bayman, P and GV Radkar. 1997. Transformation and tolerance of TNT (2,4,6-trinitrotoluene) by fungi. *Internat. Biodeterior. Biodegr.* 39:45-53.

Boopathy, R, and CF Kulpa. 1993. Nitroaromatic compounds serve as nitrogen source for *Desulfovibrio* sp. (B strain). *Can. J. Microbiol.* 39:430-433.

Buikema, AL Jr, MJ McGinniss and J Jr Cairns. 1979. Phenolics in aquatic ecosystems: a selected review of recent literature. *Mar. Environ. Res.* 2:87-181.

Carr, RS and M Nipper. 2000. *Toxicity of marine sediments and pore water spiked with ordnance compounds*, Final report prepared for Naval Facilities Engineering Service Center, Port Hueneme, CA, Contract report CR 01-001-ENV. Naval Facilities Engineering Command, Washington, DC, USA.

Chambers, CW. 1963. Degradation of aromatic compounds by phenol-adapted bacteria. *J. Water Poll. Control Fed.* 35:1517-1528.

Clesceri, LS, AE Greenberg and AD Eaton. 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th ed. Baltimore, MD, USA: APHA, AWWA, WEF.

Davenport, R, LR Johnson, DJ Schaeffer and H Balbach. 1994. Phototoxicology. 1. Light-enhanced toxicity of TNT and some related compounds to *Daphnia magna* and *Lytechinus variagatus* embryos. *Ecotoxicol. Environ. Safety* 27:14-22.

Ditsworth, GR, DW Schults and JKP Jones. 1990. Preparation of benthic substrates for sediment toxicity testing. *Environ. Toxicol. Chem.* 9: 1523-1529.

EA Engineering, Science, and Technology, Inc. 1996. Remedial Investigation – Jackson Park Housing Complex/Naval Hospital Bremerton, Engineering Field Activity Northwest, Contract No. N44255-94-D-7309.

Ebert, S., P.G. Rieger and H.-J. Knackmuss. 1999. Function of coenzyme F_{420} in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. *J. Bacteriol.* 181: 2669-2674.

Environment Canada. 1990. *Guidance document on control of toxicity test precision using reference toxicants*. EPS 1/RM/12. Environment Canada, Ottawa, Ontario, Canada.

Fernando, T and SD Aust. 1991. Biodegradation of munition waste, TNT (2,4,6-trinitrotoluene), and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by *Phanerochaete chrysosporium*. *Emerging technologies in hazardous waste management.* DW Tedder, and FG Pohland, 214-232. Vol. II. American Chemical Society.

Fernando, T, JA Bumpus and SD Aust. 1990. Biodegradation TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 56:1666-1671.

Gamo, M and T. Shoji. 1999. A method of profiling microbial communities based on a most-probable-number assay that uses BIOLOG plates and multiple sole carbon sources. *Appl. Environ. Microbiol*. 65:4419-4424.

Garland, JL. 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. *Microbial Ecol*. 24:289-300.

Garland, JL and AL Mills. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon utilization. *Appl. Environ. Microbiol*. 57:2351-2359.

Goodfellow, WL Jr, DT Burton, WC Graves, LW Hall and KR Cooper. 1983. Acute toxicity of picric acid and picramic acid to rainbow trout, *Salmo gairdneri*, and american oyster, *Crassostrea virginica*. *Water Resour. Bull.* 19:641-45.

Gorontzy, T, O Drzyzga, MW Kahl, D Bruns-Nagel, J Breitung, E Von Loew and K-H Blotevogel. 1994. Microbial degradation of explosives and related compounds. *Critical Reviews in Microbiology* 20:265-284.

Gundersen, K and HL Jensen. 1956. A soil bacterium decomposing organic nitro-compounds. *Acta Agricult. Scandinav.* 6:100-114.

Hackett, CA and BS Griffiths. 1997. Statistical analysis of the time-course of Biolog substrate utilization. *J. Microbiol. Methods* 27:183-187.

Haderlein, SB and RP Schwarzenbach. 1995. Environmental processes influencing the rate of abiotic reduction of nitroaromatic compounds in the subsurface. *Biodegradation of nitroaromatic compounds.* JC Spain, 199-225. New York: Plenum Press.

Hallas, LE and M Alexander. 1983. Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* 45:1234-1241.

Hamilton, MA, RC Russo and RV Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.* 11:714-719; Correction 12:417 (1978).

Hooten, RL and RS Carr. 1998. Development and application of a marine sediment pore-water toxicity test using *Ulva fasciata* zoospores. *Environ. Toxicol. Chem.* 17:932-40.

Jensen, HL and K Gundersen. 1955. Biological decomposition of aromatic nitro-compounds. *Nature* 175:341.

Kumar, S and AP Davis. 1997. Heterogeneous photocatalytic oxidation of nitrotoluenes. *Water Environment Research* 69:1238-1245.

Leightley, LE and RA Eaton. 1979. *Nia vibrissa* - a marine white rot fungus. *Trans. Br. Mycol. Soc.* 73:35-40.

Lendenmann, U, JC Spain and BF Smets. 1998. Simultaneous biodegradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in an aerobin fluidized-bed biofilm reactor. *Environ. Sci. Technol.* 32:82-87.

Lenke, H and H-J Knackmuss. 1992. Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. *Appl. Environ. Microbiol.* 58:2933-2937.

Little, EE and DL Fabacher. 1996. Exposure of freshwater fish to simulated solar UVB radiation. In: *Techniques in Aquatic Toxicology*, GK Ostrander (ed.), Lewis Publishers, New York, p. 141-158.

Long, ER, DD MacDonald, SL Smith and FD Calder. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ. Manage.* 19:81-97.

MacDonald, DD, RS Carr, FD Calder and ER Ingersoll CG Long. 1996. Development and evaluation of sediment quality guidelines for Florida coastal waters. *Ecotoxicology* 5:253-78.

McCormick, NG, JH Cornell and AM Kaplan. 1978. Identification of biotranformation products from 2,4-dinitrotoluene. *Appl. Environ. Microbiol.* 35:945-948.

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

Murdoch, MH, PM Chapman, DM Norman and VM Quintino. 1997. Spiking sediment with organochlorines for toxicity testing. *Environ. Toxicol. Chem.* 16:1504-1509.

Nakagawa, M and DG Crosby. 1974. Photodecomposition of nitrophen. *J. Agricult. Food Chem.* 22:849-853.

Nipper, M, RS Carr, JM Biedenbach, RL Hooten and K Miller. 2002. Toxicological and chemical assessment of ordnance compounds in marine sediments and pore waters. *Mar. Pollut. Bull.* 44:789-806.

O I Analytical. 1998. Model 1010 wet oxidation total organic carbon analyzer operator's manual, Revision 8.0. O I Analytical, College Station, TX, 138 pp.

Ostrensky, A and LH Poersch. 1992-1993. Toxidade aguda do nitrito na larvicultura do camarao-rosa *Penaeus paulensis* Perez-Farfante, 1967 (Acute toxicity of nitrite on larval rearing pink shrimp *Penaeus paulensis* Perez-Farfante, 1967). *Neritica* 7:101-107.

Parris, GE. 1980. Environmental and metabolic transformations of primary aromatic amines and related compounds. *Residue Reviews: Residues of pesticides and other contaminants in the total environment.* FA Gunther, and JD Gunther, 1-30. Vol. 76. New York: Springer-Verlag.

Parrish, FW. 1977. Fungal transformation of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. *Appl. Environ. Microbiol.* 34:232-233.

Parsons, TR, Y Maita and CM Lalli. 1984. A manual of chemical and biological methods for seawater analysis. New York, Pergamon Press, 173 p.

Raghukumar, C. 2000. Fungi from marine habitats: an application in bioremediation. *Mycol. Res.* 104:1222-1226.

Raghukumar, C, TM D'Souza, RG Thorn and CA Reddy. 1999. Lignin-modifying enzymes of *Flavodon flavus*, a basidiomycete isloated from a coastal marine environment. *Appl. Environ. Microbiol.* 65:2103-11.

Raghukumar, C, S Raghukumar, A Chinnaraj, D Chandramohan, TM D'Souza and CA Reddy. 1994. Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. *Bot. Mar.* 37:515-523.

Raghukumar, C and G Rivonkar. 2001. Decolorization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat. *Appl. Microbiol. Biotechnol.* 55:510-514.

Rajan, J, K Valli, RE Perkins, FS Sariaslani, SM Barns, A-L Reysenbach, S Rehm, M Ehringer and NR Pace. 1996. Mineralization of 2,4,6-trinitrophenol (picric acid): characterization and phylogenetic identification of microbial strains. *J. Indust. Microbiol.* 16:319-324.

Rand, GM and SR Petrocelli. 1985. *Fundamentals of Aquatic Toxicology*. New York, NY, USA: Hemisphere Publishing Co.

Rieger, P-G, V Sinnwell, A Preuss, W Francke and H-J Knackmuss. 1999. Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. *J. Bacteriol.* 181:1189-1195.

Rosenblatt, DH, EP Burrows, WR Mitchell and DL Parmer. 1991. Organic explosives and related compounds. *The handbook of environmental chemistry.* 3rd ed., O Hutzinger, 195-234. Vol. 3, Part G - Anthropogenic compounds. Berlin: Springer-Verlag.

Russ, R, DM Walters, H-J Knackmuss and PE Rouviere. 2000. Identification of genes involved in picric acid and 2,4-dinitrophenol degradation by mRNA differential display. *Biodegradation of nitroaromatic compounds and explosives.* JC Spain, JB Hughes, and H-J Knackmuss, 127-143. Boca Raton: Lewis Publishers.

SAS Institute Inc. 1989. SAS/STAT® user's guide, Version 6, Fourth Edition, Volume 2. Cary, NC: SAS Institute Inc., 846 p.

SAS Institute Inc. 1992. SAS/LAB® software: User's guide, Version 6, First Edition, Cary, NC: SAS Institute Inc., 291 p.

Scheibner, K and M Hofrichter. 1998. Conversion of aminonitrotoluenes by fungal manganese peroxidase. *J. Basic Microbiol.* 38:51-59.

Shea, PJ, JB Weber and MR Overcash. 1983. Biological activities of 2,4-dinitrophenol in plant-soil systems. *Res. Rev.* 87:1-41.

Simon, EW and GE Blackman. 1953. Studies in the principles of phytotoxicity. *J. Experim. Bot.* 4:235-50.

Solomons, TWG. 1997. *Fundamentals of organic chemistry.* 5th ed. 1068 p. + Glossary and Index. New York, NY, USA: John Wiley & Sons, Inc.

Spanggord, RJ, WR Mabey, TW Chou and JH Smith. 1985. Environmental fate of selected nitroaromatic compounds in the aquatic environment. *Toxicity of nitroaromatic compounds.* DE Rickert, p. 15-33. Washington, DC, USA: Hemisphere Publ. Corp.

Spanggord, RJ, T Mill, T Chuo, W Mabey, J Smith and S Lee. 1980. Environmental fate studies on certain munitions wastewater constituents – Lab studies. ADA 099256. SRI International, Menlo Park, CA.

Spiker, JK, DL Crawford and RL Drawford. 1992. Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58:3199-3202.

Stahl, JD and SD Aust. 1995. Biodegradation of 2,4,6-trinitrotoluene by then white rot fungus *Phanerochaete chrysosporium*. *Biodegradation of nitroaromatic compounds.* JC Spain, 117-133. New York: Plenum Press.

Stillings, A., D. Herzig and B. Roll. 1998. Comparative assessment of the newly developed SimPlate® method with existing EPA-approve pour plate method for the detection heterotrophic plate count bacteria in ozone-treated drinking water.

Proceedings International Ozone Association Conference, Vancouver, Canada.

URS Consultants (1995) Final Remedial Investigation/Feasibility Study Report, U.S. Navy CLEAN Contract, Engineering Field Activity Northwest, Contract No. N62474-89-D-9295.

USEPA. 1994. *SW-846 Method 8330. Nitroaromatics and nitramines by High Performance Liquid Chromatography (HPLC)*. USEPA Office of Solid Waste and Emergency Response, Washington, DC.

Valli, K, BJ Brock, DK Joshi and MH Gold. 1992. Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58:221-228.

Van Aken, B, MD Cameron, JD Stahl, A Plumat, H Naveau, SD Aust and SN Agathos. 2000. Glutathione-mediated mineralization of 14C-labeled 2-amino-4,6-dinitrotoluene by manganese-dependent peroxidase H5 from the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* 54:659-664.

Weast, RC, MJ Astle, and WH Beyer. 1986-1987. *CRC handbook of chemistry and physics*. Boca Raton, FL: CRC Press, Inc.

Wyman, JF, HE Guard, WD Won and JH Quay. 1979. Conversion of 2,4,6-trinitrophenol to a mutagen by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 37:222-226.

Appendix A1. Concentration of 2,6-DNT and its major initial transformation product, 2-A-6-NT in sterilized and non-sterilized samples spiked with 2,6-DNT and stored at 10 or 20°C for up to 180 days. Sediments were analyzed wet and dried at room temperature.

			Concentrations (μmoles/L in pore water, μmoles/kg dry weight in sediment)							
		Temp.	Pore Water		Sterile Pore Water		Wet Sediment		Dry Sediment	
Day	Sample	(°C)	2,6-DNT	2-A-6-NT	2,6-DNT	2-A-6-NT	2,6-DNT	2-A-6-NT	2,6-DNT	2-A-6-NT
0	Sandy	-	482.40	254.22	642.70	40.32	555.44	137.40	267.91	95.06
1	Sandy	10	372.11	451.51	509.52	64.94	NM[b]	NM	NM	NM
7			224.49	844.18	432.82	103.76	198.79	526.75	56.07	315.58
14			91.56	1096.44	405.01	106.01	69.72	600.77	24.80	436.62
28			4.62	1092.16	380.41	106.13	4.29	638.50	2.33	412.57
56			1.54	886.70	388.00	124.59	1.07	547.80	BDL	352.91
86			BDL[a]	709.12	376.26	114.21	1.75	419.19	BDL	191.74
180			BDL	54.07	346.85	119.95	BDL	9720.67	NM	NM
1	Sandy	20	370.95	684.73	622.82	139.88	NM	NM	NM	NM
3			144.30	1041.15	601.58	239.84	112.01	604.84	47.50	394.74
7			1.76	1085.44	574.30	309.48	2.08	677.89	0.96	414.48
14			BDL	834.56	497.87	395.88	BDL	528.45	0.47	374.51
28			BDL	294.99	428.45	461.83	BDL	202.31	0.12	120.59
56			BDL	BDL	347.87	416.61	BDL	4.46	BDL	2.25
180			BDL	BDL	NM	NM	BDL	BDL	NM	NM
0	Fine	-	247.71	239.67	356.31	BDL	807.79	327.54	207.77	452.08
1	Fine-grained	10	143.37	491.90	399.58	7.24	73.81	1016.10	6.45	69.40
3			5.88	657.40	345.10	5.29	18.36	1040.32	BDL	760.03
7			BDL	558.49	361.10	8.96	5.16	870.33	BDL	584.03
14			BDL	321.97	355.53	13.01	BDL	576.94	BDL	402.60
28			BDL	94.58	316.51	40.16	3.11	221.07	BDL	128.23
56			BDL	BDL	267.14	97.11	4.50	17.18	BDL	5.15
180			BDL	BDL	NM	NM	BDL	0.53	NM	NM
1	Fine-grained	20	24.59	671.23	401.63	6.98	369.92	748.28	BDL	79.35
3			BDL	499.03	406.18	15.79	2.75	795.95	BDL	584.57
7			BDL	168.16	402.52	28.95	3.79	323.16	BDL	196.38
14			BDL	1.53	350.94	60.76	BDL	34.49	BDL	7.47
28			BDL	BDL	276.65	77.13	BDL	2.56	BDL	2.89

[a]BDL = below detection limit

[b]NM = not measured

Appendix A2. Percent loss of 2,6-DNT over time, in different sediments and at different temperatures.

Day	Sample	Temp. (°C)	%Loss of 2,6-DNT Relative to Initial Concentration			
			Pore Water	Sterile Pore Water	Wet Sediment	Dry Sediment
0	Sandy	-	0.0	0.0*	0.0	0.0
1		10	22.8	20.8	NM[a]	NM
7			53.4	32.7	64.2	79.1
14			81.0	37.0	87.4	90.7
28			99.0	40.8	99.2	99.1
56			99.7	39.7	99.8	100.0
86			100.0	41.5*	99.7	100.0
180			100.0	46.1	100.0	NM
1	Sandy	20	23.0	3.1	NM	NM
3			70.1	6.4	79.8	82.3
7			99.6	10.7	99.6	99.6
14			100.0	22.6	100.0	99.8
28			100.0	33.4	100.0	100.0
56			100.0	45.9	100.0	100.0
180			100.0	NM	100.0	NM
0	Fine-grained	-	0.0	0.0	0.0	0.0
1		10	42.2	-12.2	90.9	96.9
3			97.6	3.1*	97.7	100.0
7			100.0	-1.4	99.4	100.0
14			100.0	0.1	100.0	100.0
28			100.0	11.1	99.6	100.0
56			100.0	25.0	99.4	100.0
180			100.0	NM	100.0	NM
1	Fine-grained	20	90.1	-12.8	54.2	100.0
3			100.0	-14.1	99.7	100.0
7			100.0	-13.1	99.5	100.0
14			100.0	1.4	100.0	100.0
28			100.0	22.3*	100.0	100.0

* Some bacterial contamination observed in sterilized samples

[a]NM = not measured.

Appendix A3. Concentration of picric acid in spiked sediment and corresponding pore waters during different storage times at 10 and 20°C.

Day	Sample	Temp (°C)	Conc. (µmoles/L in pore water, µmoles/kg dry weight in sediment)			
			Pore Water	Sterile Pore Water	Wet Sediment	Dry Sediment
0	Sandy	-	1908.02	1935.36	1456.97	1397.89
1		10	1759.29	1972.15	1360.09	1236.76
7			1230.70	1834.05	959.94	917.46
11			1050.34	NM[a]	NM	NM
14			872.69	1708.26	650.66	481.37
28			90.69	1712.95	49.49	20.23
56			5.77	1734.04	BDL	BDL
86			1.15	1627.19	BDL	BDL
180			BDL[b]	1605.59	BDL	NM
1	Sandy	20	1502.58	1829.82	1087.09	1082.66
7			640.54	1669.93	326.03	320.19
14			8.14	1458.47	BDL	BDL
28			BDL	1399.78	BDL	BDL
0	Fine-grained	-	793.93	1157.04	1256.38	639.61
1		10	489.83	1126.44	546.37	317.85
3			73.09	1069.61	146.21	23.44
7			BDL	1076.89	BDL	BDL
14			BDL	1020.31	BDL	BDL
28			BDL	932.57	BDL	BDL
56			BDL	789.09	BDL	BDL
86			BDL	698.96	BDL	BDL
180			BDL	525.91	BDL	BDL
1	Fine-grained	20	101.00	1114.34	145.00	34.61
3			BDL	1013.18	BDL	BDL
7			BDL	958.09	BDL	BDL

[a]NM = not measured

[b]BDL = below detection limit

Appendix A4. Percent loss of picric acid over time, in different sediments and at different temperatures.

Day	Sample	Temp (^{o}C)	% Loss of Picric Acid Relative to Initial Concentration			
			Pore Water	Sterile Pore Water	Wet Sediment	Dry Sediment
0	Sandy	-	0.0	0.0	0.0	0.0
1		10	7.8	-1.9	6.7	11.5
7			35.5	5.2	34.1	34.4
11[a]			45.0	NM[a]	NM	NM
14			54.3	11.7	55.3	65.6
28			95.2	11.5	96.6	98.6
56			99.7	10.4	100.0	100.0
86			99.9	15.9	100.0	100.0
180			100.0	17.0	100.0	100.0
1	Sandy	20	21.2	5.4	25.4	22.6
7			66.4	13.7	77.6	77.1
14			99.6	24.6	100.0	100.0
28			100.0	27.7	100.0	100.0
0	Fine-grained	-	0.0	0.0	0.0	0.0
1		10	38.3	2.6	56.5	50.3
3			90.8	7.6	88.4	96.3
7			100.0	6.9*	100.0	100.0
14			100.0	11.8	100.0	100.0
28			100.0	19.4*	100.0	100.0
56			100.0	31.8*	100.0	100.0
86			100.0	39.6	100.0	100.0
180			100.0	54.5	100.0	100.0
1	Fine-grained	20	87.3	3.7	88.5	94.6
3			100.0	12.4	100.0	100.0
7			100.0	17.2*	100.0	100.0

* Some bacterial contamination observed in sterilized samples

[a]NM = not measured

Appendix A5. Percent loss of 2,6-DNT and picric acid with time, under simulated solar radiation.

Exposure Time (Hours)	2,6-DNT Measured Concentration (μmoles/L)	% 2,6-DNT loss	Exposure Time (Days)	Picric Measured Concentration (μmoles/L)
0	870.07	-	0	3510.55
1	818.40	5.9	8	3245.25
2	778.85	10.5	14	3337.20
4	707.88	18.6	21	3390.18
6	613.37	29.5	47	3206.05
8	545.75	37.3		
24	94.00	89.2		
48	38.61	95.6		
72	BDL[a]	100.0		

[a]BDL=below detection limit

Appendix A6. Measured concentrations of picric acid and some of its major biotransformation products in pore waters from sediments stored at 10°C and used for toxicity testing.

Sediment	Storage Time (days)	Measured Concentration (μmoles/L)			Sum
		Picric Acid	2,4-DNP	Picramic Acid	
Sandy	0	923.89	23.51	166.32	1113.72
	28	81.90	27.27	3.07	112.24
	56	4.82	16.58	0.36	21.76
Fine-grain	0	800.08	5.71	78.44	884.23
	7	BDL[a]	33.77	0.44	34.21
	56	BDL	1.26	16.87	18.13

[a]BDL=below detection limit

Appendix B1. Water quality data for porewater samples used in toxicity tests with copepods.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Initial Salinity (ppt)	solved Oxygen (mg/L)	% Saturation	pH	Total Ammonia (NH_4+NH_3) (mg/L)	Unionized Ammonia (NH_3) (µg/L)	% of Original Sample
-	MFS[a]-T1	-	-	-	34.0	6.94	94.70	8.21	<0.10	<5.00	88.2
-	TX Ref[b]-T1	-	-	-	30.0	7.32	100.00	8.27	1.34	75.82	100.0
-	MFS[a]-T2	-	-	-	34.0	6.61	89.00	8.27	<0.10	<5.63	88.2
-	TX Ref[b]-T2	-	-	-	30.0	7.50	100.90	8.27	1.12	63.65	100.0
Sandy	MEOH[c]	100.0	0	-	25.5	7.20	98.30	7.92	5.60	146.45	94.8
		100.0	7	20	25.5	7.34	100.50	7.95	7.21	202.56	94.1
Sandy	SW[d]	100.0	0	-	24.0	7.11	96.60	8.09	2.78	105.97	92.8
		100.0	7	20	25.5	7.06	97.20	7.96	3.77	107.35	94.1
Sandy	SW	12.5	28	10	NA[e]	NM[f]	NM	8.39	0.96	70.79	11.9
		25.0			NA	NM	NM	8.46	1.92	164.58	23.8
		50.0			NA	7.97	101.00	8.62	3.89	458.63	47.5
		100.0			25.5	8.10	100.20	8.68	7.82	1042.49	95.0
Sandy	SW	12.5	56	10	NA	NM	NM	8.41	1.05	80.97	11.8
		25.0			NA	NM	NM	8.54	2.12	213.87	23.6
		50.0			NA	7.83	101.70	8.67	4.36	568.59	47.2
		100.0			25.5	8.43	104.90	8.76	8.62	1361.05	94.3
Sandy	SW	50.0	180	20	-	-	-	8.77	5.23	828.99	46.9
		100.0			25.0	7.01	92.90	9.06	8.04	2177.96	93.8
Fine-grain	MEOH	100.0	0	-	30.5	7.88	106.80	8.04	11.10	382.04	100.0
		3.1[g]			30.0	7.09	94.10	8.37	0.46	32.29	3.1
		100.0	7	10	30.5	7.74	104.80	8.49	11.10	1004.87	100.0
Fine-grain	SW	50.0	0	-	30.0	7.09	96.40	8.19	5.42	259.82	50.0
		100.0			30.5	7.40	101.80	8.16	11.40	507.16	100.0
Fine-grain	SW	50.0	7	10	30.0	7.22	98.00	8.12	5.69	234.34	50.0
		100.0			30.5	7.54	101.70	8.10	10.30	402.29	100.0
Fine-grain	SW	25.0	28	10	NA	7.71	100.00	8.23	2.77	144.29	25.0
		50.0			NA	8.10	104.70	8.23	5.34	278.17	50.0
		100.0			31.0	8.51	105.30	8.24	10.60	564.35	100.0

Appendix B1. Continued

Sandy	2,6-DNT	100.0	0	-	25.5	7.27	99.40	8.21	5.46	272.26	94.3
		50.0	7	20	25.5	7.01	96.60	7.76	4.69	85.54	94.4
		100.0	180	20	24.0	7.40	97.60	8.84	12.00	2207.47	93.0
Fine-grain	2,6-DNT	50.0	0	-	30.0	7.26	98.50	8.10	5.80	225.53	50.0
		100.0			30.5	7.73	105.20	8.02	11.40	375.29	100.0
Fine-grain	2,6-DNT	100.0	7	10	30.5	7.29	99.90	7.72	14.50	240.49	100.0
Fine-grain	2,6-DNT	100.0	180	10	31.0	7.59	101.30	7.77	1.12	20.94	100.0
Sandy	Picric	50.0	0	-	26.0	6.99	95.50	8.67	3.21	422.83	95.1
Sandy	Picric	3.1	28	10	NA	NM	NM	8.30	0.41	24.72	5.9
		6.3			NA	NM	NM	8.38	0.79	56.49	11.8
		12.5			NA	NM	NM	8.46	1.59	134.87	23.6
		25.0			NA	7.38	95.80	8.56	3.10	327.95	47.2
		50.0			25.5	7.17	94.00	8.67	6.01	788.49	94.3
Sandy	Picric	12.5	56	10	NA	7.46	96.50	8.53	1.60	157.45	47.6
		25.0			25.5	7.53	95.80	8.67	3.22	422.45	95.1
Fine-grain	Picric	25.0	0	-	30.0	7.22	96.50	8.08	3.01	112.97	25.0
		50.0			30.0	7.39	98.90	8.00	5.97	188.39	50.0
		100.0			31.0	7.47	100.50	7.93	11.50	308.94	100.0
Fine-grain	Picric	50.0	7	10	30.0	7.15	97.00	8.38	7.24	515.98	50.0
		100.0			30.5	7.26	99.10	8.42	15.60	1221.03	100.0
Fine-grain	Picric	12.5	56	10	NA	7.63	98.90	8.46	3.18	271.44	50.0
		25.0			31.0	7.78	100.60	8.54	6.28	633.54	100.0
Fine-grain	Picric	100.0	180	10	30.0	7.16	95.90	8.40	4.95	372.11	100.0

[a]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, concurrent to samples from days 0 and 7; T2 concurrent to samples from days 28 and 56; T3 concurrent to samples from day180.

[b]TX Ref = pore water from standard Texas reference sediment.

[c]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[d]SW = blank spiked with filtered seawater (MFS) in same quantity used in picric acid stock solution

[e]NA = not applicable; [f]NM = not measured

[g]Used as ammonia blank for Day 180 spiked samples

Appendix B2. Water quality data for porewater samples used in toxicity tests with algae.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (^{o}C)	Initial Salinity (ppt)	Dissolved Oxygen (mg/L)	Dissolved Oxygen % Saturation	pH	Total Ammonia (NH_4+NH_3) (mg/L)	Unionized Ammonia (NH_3) (µg/L)	% of Original Sample
-	MFS[a]-T1	-	-	-	26.0	6.43	89.2	8.22	0.20	9.90	100
-	TX Ref[b]-T1	-	-	-	30.0	7.32	100.0	8.27	1.34	75.82	100
-	MFS[a]-T2	-	-	-	34.0	6.61	89.0	8.27	<0.10	<5.63	100
-	TX Ref[b]-T2	-	-	-	30.0	7.50	100.9	8.27	1.12	63.65	100
-	MFS[a]-T3	-	-	-	30.0	6.27	87.5	8.15	0.28	12.02	100
-	TX Ref[b]-T3	-	-	-	30.0	6.66	93.2	8.15	1.44	62.67	100
Sandy	MEOH[c]	100.0	0	-	25.0	7.38	102.8	8.29	1.84	110.16	94
		100.0	7	20	25.0	7.09	99.0	8.20	2.21	106.87	94
Sandy	SW[d]	25.0	0	-	25.0	7.38	102.3	8.31	1.78	109.37	23
		50.0			25.0	7.30	101.5	8.38	3.01	217.75	47
		100.0			25.0	7.66	106.6	8.47	7.34	634.50	93
Sandy	SW	25.0	7	20	25.0	7.01	97.2	7.78	2.54	48.25	24
Sandy	SW	50.0	28	10	25.0	7.26	101.0	8.23	4.61	241.19	47
		100.0			25.0	7.57	105.3	8.23	8.71	457.69	94
Sandy	SW	100.0	28	20	26.0	6.62	91.9	8.72	9.85	1438.40	96
Sandy	SW	50.0	56	10	25.5	7.19	100.1	8.29	5.04	297.19	47
		100.0			25.5	7.47	104.1	8.29	8.50	508.87	94
Fine-grain	MEOH	100.0	0	-	30.0	7.35	102.6	8.22	6.85	348.36	100
		100.0	7	10	30.0	7.36	102.1	7.22	6.90	36.60	100
Fine-grain	MEOH	100.0	180	10	32.0	7.10	98.2	7.67	0.50	7.49	94
Fine-grain	SW	50.0	0	-	30.0	7.30	102.0	8.25	6.65	361.85	50
		100.0			30.0	7.67	107.0	8.34	15.00	995.98	100
Fine-grain	SW	100.0	1	10	30.5	6.52	90.7	8.12	14.00	576.58	100
Fine-grain	SW	50.0	7	10	30.0	7.09	98.9	8.23	6.52	337.42	50
		100.0			30.0	7.64	106.4	8.24	12.70	679.10	100
Fine-grain	SW	50.0	56	10	32.0	7.46	103.7	8.06	1.27	45.19	47
		100.0			32.0	7.60	105.4	7.98	2.00	59.82	94
Sandy	2,6-DNT	25.0	0	-	25.0	7.09	97.0	8.04	1.62	54.90	24
		25.0	7	20	25.0	7.01	96.4	8.19	3.22	154.69	24
Sandy	2,6-DNT	100.0	180	20	26.0	6.56	90.9	8.14	14.10	602.89	95

Appendix B2. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (^{o}C)	Initial Salinity (ppt)	Dissolved Oxygen (mg/L)	Dissolved Oxygen % Saturation	pH	Total Ammonia (NH_4+NH_3) (mg/L)	Unionized Ammonia (NH_3) (µg/L)	% of Original Sample
Fine-grain	2,6-DNT	25.0	0	-	30.0	7.20	98.4	8.07	3.64	134.51	25
		50.0			30.0	7.32	99.6	8.03	7.31	246.61	50
Fine-grain	2,6-DNT	25.0	7	10	30.0	6.82	93.3	7.77	5.27	97.65	25
		50.0			30.0	7.39	101.1	7.67	10.10	151.96	50
Fine-grain	2,6-DNT	100.0	180	10	31.5	6.91	95.9	7.29	2.19	13.76	95
Sandy	Picric	25.0	0	-	25.5	6.72	92.8	8.36	2.03	141.00	24
		50.0			25.5	7.30	100.2	8.44	3.96	323.37	48
		100.0			25.5	7.18	98.8	8.54	8.39	842.91	95
Sandy	Picric	25.0	28	10	25.0	6.94	95.8	8.34	3.71	247.40	23
		50.0			25.0	6.78	93.7	8.38	7.19	517.92	47
		100.0			25.0	6.27	87.5	8.43	14.70	1180.24	93
Sandy	Picric	25.0	56	10	26.0	6.80	93.8	8.54	4.55	456.17	24
		50.0			26.0	7.45	102.2	8.60	8.92	1022.14	48
		100.0			26.0	7.05	97.3	8.70	18.60	2605.91	95
Sandy	Picric	100.0	180	10	26.0	7.21	100.0	7.95	23.20	645.99	95
Fine-grain	Picric	25.0	0	-	30.0	7.14	98.2	8.23	4.11	214.10	25
		50.0			30.0	7.18	99.4	8.21	9.07	449.31	50
		100.0			30.0	6.94	95.9	8.19	19.40	929.97	100
Fine-grain	Picric	25.0	7	10	30.0	6.96	96.1	8.11	5.40	217.54	25
		50.0			30.0	7.36	101.8	8.07	10.90	398.34	50
		100.0			30.0	7.27	100.5	8.01	22.00	705.14	100
Fine-grain	Picric	25.0	56	10	30.0	6.88	95.5	8.38	8.46	609.40	25
		50.0			30.0	7.25	100.3	8.41	16.20	1251.94	50
		100.0			30.0	7.16	99.1	8.45	36.70	3086.81	100
Fine-grain	Picric	100.0	180	10	31.5	6.49	90.1	8.25	9.17	496.80	96

[a]MFS = millipore filtered seawater (0.45µm); T1=Test 1, concurrent to samples from days 0 and 7; T2 concurrent to samples from days 28 and 56; T3 concurrent to samples from day180.

[b]TX Ref = pore water from standard Texas reference sediment.

[c]MEOH = blank spiked methanol in same quantity used in 2,6-DNT stock solution

[d]SW = blank spiked filtered seawater (MFS) in same quantity used in picric acid stock solution

Appendix B3. Water quality data for toxicity tests with single chemicals using *Schizopera knabeni.*

Treatment	Initial Salinity (ppt)	Dissolved Oxygen (mg/L)	Dissolved Oxygen % Saturation	pH	Total Ammonia (NH_4+NH_3) (mg/L)	Unionized Ammonia (NH_3) (µg/L)
MFS[a]	30	6.78	92.1	8.27	<0.10	-
2-A-6NT	30	6.81	94.3	8.24	<0.10	-
PD-MFS[b]	30	7.85	107.3	8.23	<0.10	-
PD-2,6-DNT[b]	25	7.29	110.3	8.06	0.60	21.70
2,6-DNT	30	6.75	93.9	8.24	<0.10	-
Picric Acid	30	6.88	95.5	7.83	<0.10	-
2-NT	30	6.64	90.0	8.36	<0.10	-
2,4-DNP	30	6.75	91.4	8.30	<0.10	-
Picramic Acid	30	6.86	92.7	8.32	<0.10	-
NH_3 (unionized - µg/L)	30	NM	NM	8.24 8.19 8.16 8.11 8.01 7.91	2.00 4.11 8.73 17.30 36.30 77.80	106.5 196.2 390.1 692.3 1163.5 1993.9

[a]MFS = millipore filtered seawater (0.45µm); data are the average of several tests, since the water quality of the control only has minimal variations.

[b]PD-MFS = MFS control exposed to simulated solar radiation (SSR); PD-2,6-DNT = 2,6-DNT exposed to SSR.

Appendix B4. Water quality data for toxicity tests with single chemicals using *Ulva fasciata.*

Treatment	Initial Salinity (ppt)	Dissolved Oxygen (mg/L)	Dissolved Oxygen % Saturation	pH	Total Ammonia (NH_4+NH_3) (mg/L)	Unionized Ammonia (NH_3) (µg/L)
MFS[a]	28.0	6.47	90.8	8.21	0.194	9.64
2-A-6NT	30.0	6.85	93.5	8.19	<0.1	<4.82
PD-MFS[b]	30.0	7.53	103.4	8.11	0.098	3.90
PD-2,6-DNT[c]	30.0	7.69	108.9	7.92	0.617	16.10
2,6-DNT	30.0	7.02	96.4	8.20	0.175	8.57
Picric Acid	30.0	7.11	98.1	8.28	0.211	12.12
2-NT	30.0	6.56	90.1	8.15	<0.1	<4.38
2,4-DNP	30.0	6.66	91.7	7.41	<0.1	<0.82
Picramic Acid	30.0	6.63	91.4	8.15	<0.1	<4.32

[a]MFS = millipore filtered seawater (0.45µm); data are the average of several tests, since the water quality of the control only has minimal variations.

[b]PD-MFS = MFS control exposed to simulated solar radiation (SSR).

[c]PD-2,6-DNT = 2,6-DNT exposed to SSR.

Appendix B5. DOC concentration in ordnance-spiked pore waters from sandy and fine-grained sediment over time at different temperatures.

Day	Sample	Treatment	Temp. (°C)	Dissolved Organic Carbon (mg/L) Rep 1	Rep 2	Mean	SD[a]	Treatment Blank	Dissolved Organic Carbon (mg/L) Rep 1	Rep 2	Mean	SD
-	-	-	-	-	-	-	-	FSW[b]	1.481	1.552	1.517	0.050
0	Sandy	2,6-DNT	-	97.239	98.262	97.751	0.723	MEOH	38.250	37.520	37.885	0.516
7			20	143.592	154.201	148.897	7.502	MEOH	16.259	16.691	16.475	0.305
180			20	28.088	28.778	28.433	0.488	MEOH	8.151	7.627	7.889	0.371
0	Fine-grained	2,6-DNT	-	60.110	52.058	56.084	5.694	MEOH	12.639	12.544	12.592	0.067
7			10	71.808	76.022	73.915	2.980	MEOH	10.066	8.644	9.355	1.006
180			10	7.878	7.248	7.563	0.445	MEOH	5.714	6.065	5.890	0.248
0	Sandy	Picric Acid	-	181.364	189.616	185.490	5.835	SW[c]	38.257	41.676	39.967	2.418
28			10	71.216	73.599	72.408	1.685	SW	18.435	19.284	18.860	0.600
56			10	51.779	53.883	52.831	1.488	SW	16.862	17.442	17.152	0.410
180			10	23.654	25.385	24.520	1.224	SW	12.787	12.973	12.880	0.132
0	Fine-grained	Picric Acid	-	65.787	71.152	68.470	3.794	SW	11.080	11.213	11.147	0.094
7			10	32.432	34.406	33.419	1.396	SW	8.804	7.865	8.335	0.664
56			10	14.901	14.494	14.698	0.288	SW	8.628	7.100	7.864	1.080
180			10	9.917	10.477	10.197	0.396	SW	NM[d]	NM	NM	NM

[a]SD - Standard deviation

[b]FSW = 0.45µm filtered seawater, in the absence of sediment (DOC control)

[c]SW = Pore water from sediment blank spiked with filtered seawater only.

[d]NM = Not measured

Appendix C1. Total heterotrophic bacteria counts in ordnance-spiked sediments and respective blanks.

Day	Sample	Treat.	Temp. (°C)	# Positive Wells	MPN[a]	95% CI Lower	95% CI Higher	Treat. Blank	# Positive Wells	MPN	95% CI Lower	95% CI Higher
0	Sandy	2,6-DNT	-	83	>738	>476	>1146	MEOH[b]	46	132	98	178
1				84	>738	>476	>1146		58	195	149	257
7				72	324	248	425		68	276	212	361
14				84	>738	>476	>1146		72	324	248	425
28			10	84	>738	>476	>1146		78	440	328	589
56				80	507	371	695		25	59	40	87
86				84	738	476	1146		75	372	282	491
180				82	623	432	899		NM[c]	NM	NM	NM
1	Sandy	2,6-DNT	20	84	>738	>476	>1146	MEOH	56	183	139	241
3				48	141	106	189		38	100	72	138
7				81	555	398	775		66	257	197	335
14				84	>738	>476	>1146		25	202	154	265
28				70	299	229	390		73	231	177	302
56				79	470	348	636		28	68	47	98
180				31	77	54	109		50	151	113	201
0	Fine-grained	2,6-DNT	-	78	440	328	589	MEOH	69	287	220	375
1				64	239	183	312		57	189	144	249
3				60	209	159	273		72	324	249	425
7				76	392	296	519		78	444	328	589
14			10	81	555	398	775		62	223	171	293
28				75	372	282	491		79	470	348	636
56				76	392	296	519		66	257	197	335
180				72	324	249	425		69	287	220	375
1	Fine-grained	2,6-DNT	20	77	414	311	551	MEOH	63	231	177	302
3				71	311	238	407		76	392	296	519
7				73	339	258	444		75	372	282	491
14				84	>738	>476	>1146		61	216	165	283
28				82	623	432	899		64	239	183	312
0	Sandy	Picric acid	-	84	738	476	1146	SW[d]	52	161	121	213
1				51	156	117	207		59	202	154	265
7				81	555	398	775		57	189	144	249
14				84	>738	>476	>1146		75	372	282	491
28			10	46	>738	>476	>1146		77	414	311	551
56				83	>738	>476	>1146		16	35	22	58
86				82	623	432	899		72	324	249	425
180				59	202	154	265		33	83	59	117
1	Sandy	Picric acid	20	47	137	102	183	SW	68	276	212	361
7				81	555	398	775		64	239	183	312
14				84	>738	>476	>1146		66	257	197	335
28				84	>738	>476	>1146		80	507	371	695
56				NM	NM	NM	NM		17	38	23	61

Appendix C1. Continued

Day	Sample	Treat.	Temp. (°C)	# Positive Wells	MPN[a]	95% CI Lower	95% CI Higher	Treat. Blank	# Positive Wells	MPN	95% CI Lower	95% CI Higher
0	Fine-grained	Picric acid	-	81	555	398	775	SW	69	287	220	375
1				71	311	238	407		79	470	348	636
3				77	414	311	551		73	339	258	444
7				83	>738	>476	>1146		74	355	270	466
14			10	76	392	298	519		66	257	197	335
28				73	339	258	444		79	470	348	636
56				79	470	348	636		75	372	282	491
86				78	440	328	589		73	339	258	444
180				74	355	270	466		70	299	229	390
1	Fine-grained	Picric acid	20	75	372	282	491	SW	54	171	130	227
3				59	202	154	265		66	257	197	335
7				82	623	432	899		80	507	371	695
14				NM	NM	NM	NM		67	266	204	347
28				NM	NM	NM	NM		78	440	328	589

[a]MPN=Most Probable Number (bacterial cells/mg sediment wet weight)

[b]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[c]NM = not measured

[d]SW = blank spiked filtered seawater (MFS) in same quantity used in picric acid stock solution

Appendix C2. Ecoplate® data for the degradation experiments conducted with the sandy sediment for control samples (MFS=Millipore filtered seawater, and MeOH = methanol) and picric acid and 2,6-DNT-spiked sediments at two different temperatures.

Appendix C2. Continued

Appendix D1. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to copepod, *Schizopera knabeni,* gravid female survival.

Treatment	Concentration (µmoles/L)		No. Alive Replicate #					Mean % Surv.	St. Dev.	EC_{50} (µmoles/L)	EC_{50} (mg/L)	Signif.
	Initial[a]	Final	1	2	3	4	5					
MFS[b]-T1	-	-	5	5	5	5	5	100.0	0.0	-	-	-
MFS-T2	-	-	5	5	5	5	5	100.0	0.0	-	-	-
MFS-T3	-	-	5	**6**	5	**6**	5	100.0	0.0	-	-	-
MFS-T4	-	-	5	5	5	5	5	100.0	0.0	-	-	-
PD-MFS[c]	-	-	5	5	5	5	5	100.0	0.0	-	-	
Photo-degraded 2,6-DNT (%)[d]	12.5	-	5	5	5	5	5	100.0	0.0	>100%	-	
	25.0		**7**	5	5	5	5	100.0	0.0			
	50.0		5	5	5	5	5	100.0	0.0			
	100.0		5	5	5	4	5	96.0	8.9			
2,6-DNT (µmoles/L)	30.1	27.8	5	5	5	5	5	100.0	0.0	357.00 (350.3-370.0)	65.02 (63.8-67.4)	
	60.1	56.1	5	***4***	5	5	5	100.0	0.0			
	120.2	109.0	4	5	5	5	5	96.0	8.9			
	240.5	214.7	5	5	5	5	5	100.0	0.0			
	480.9	423.1	1	0	0	0	2	12.0	17.9			**
2-A-6NT (µmoles/L)	34.7	35.0	5	5	5	5	5	100.0	0.0	>277.52	>42.22	
	69.4	70.7	5	5	5	5	5	100.0	0.0			
	138.8	134.8	5	5	5	5	5	100.0	0.0			
	277.5	267.0	5	5	5	**6**	4	96.0	8.9			
2-NT (µmoles/L)	72.6	40.7	5	***4***	5	**6**	5	100.0	0.0	798.50 (777.1-820.5)	109.51 (106.6-112.5)	
	145.1	80.9	5	5	5	5	5	100.0	0.0			
	290.3	166.2	5	5	5	5	5	100.0	0.0			
	580.5	334.3	5	**6**	5	4	5	96.0	8.9			
	1161.0	653.5	0	0	0	0	0	0.0	0.0			**
Picric Acid (µmoles/L)	37.1	29.2	5	5	5	5	5	100.0	0.0	>592.72	>135.18	
	74.1	59.3	5	5	5	5	5	100.0	0.0			
	148.2	160.8	5	5	5	5	5	100.0	0.0			
	296.4	356.1	3	2	5	5	5	80.0	28.3			
	592.7	588.5	4	4	2	4	5	76.0	21.9			**
2,4-DNP (µmoles/L)	14.1	15.1	5	5	**6**	5	5	100.0	0.0	77.35 (75.3-79.5)	14.24 (13.9-14.6)	
	28.1	28.8	5	5	5	**6**	5	100.0	0.0			
	56.2	55.0	5	5	5	5	4	96.0	8.9			
	112.5	110.3	0	**0**	0	0	0	0.0	0.0			**
Picramic Acid (µmoles/L)	14.2	13.3	5	5	5	5	5	100.0	0.0	>226.89	>45.17	
	28.4	24.4	5	5	**6**	5	5	100.0	0.0			
	56.7	47.1	5	5	5	***4***	5	100.0	0.0			
	113.4	105.2	***4***	5	***4***	5	5	100.0	0.0			
	226.9	216.5	3	4	2	5	4	72.0	22.8			**

Appendix D1. Continued

Treatment	Concentration (µmoles/L)		No. Alive Replicate #					Mean % Surv.	St. Dev.	EC_{50} (µmoles/L)	EC_{50} (mg/L)	Signif.
	Initial[a]	Final	1	2	3	4	5					
NH_3 (unionized - µg/L)	106.5	160.4	5	5	5	5	5	100.0	0.0	-	1206.9[e] (1145-1272)	
	196.2	280.8	5	5	5	5	5	100.0	0.0			
	390.1	582.3	5	5	5	5	5	100.0	0.0			
	692.3	1119.1	5	5	5	5	5	100.0	0.0		2066.2[f] (1944-2196)	
	1163.5	1918.2	5	2	2	4	1	56.0	32.9			**
	1993.9	3819.7	0	0	0	0	0	0.0	0.0			**

Bold Italics = Only 4 animals in replicate; **Bold = 6 or more animals in replicate**

[a]Highest ordnance concentrations were measured by HPLC at test start; all concentrations were measured at test end.

[b]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, concurrent to 2-A-6-NT and photo-degraded DNT; T2 concurrent to 2,6-DNT and picric acid; T3 concurrent to 2-NT, 2,4-DNP and picramic acid; T4 concurrent to NH3.

[c]PD-MFS = MFS control exposed to simulated solar radiation under identical conditions as the 2,6-DNT.

[d]Presented as percentage of initial 2,6-DNT stock solution ,which had concentration of 783.66 µmoles/L.

[e]Calculated using ammonia concentrations measured at test start

[f]Calculated using ammonia concentrations measured at test ending.

Appendix D2. Toxicity of ordnance compounds, selected biotransformation products, photo-transformed 2,6-DNT and ammonia to copepod, *Schizopera knabeni,* embryo and nauplii survival.

Treatment	Concentration (µmoles/L)		# Alive Nauplii Replicate #					Nauplii/Female Replicate #					Mean Nauplii/ Female	St. Dev.	% of Control[f]	EC_{50} (µmoles/L)	EC_{50} (mg/L)	Signif.
	Initial[a]	Final	1	2	3	4	5	1	2	3	4	5						
MFS[b]-T1	-	-	88	74	74	111	65	17.6	14.8	14.8	22.2	13.0	16.48	3.60	-	-	-	-
MFS-T2	-	-	79	63	66	68	68	15.8	12.6	13.2	13.6	13.6	13.76	1.21	-	-	-	-
MFS-T3			59	77	74	72	70	11.8	12.8	14.8	12.0	14.0	13.09	1.29	-	-	-	-
MFS-T4	-	-	46	46	49	48	52	7.7	9.2	9.8	9.6	10.4	9.33	1.03	-	-	-	-
PD-MFS[c]	-	-	71	73	76	68	92	14.2	14.6	15.2	11.3	23.0	15.67	4.36	-	-	-	-
Photo-degraded 2,6-DNT (%)[d]	12.50	-	42	50	57	84	43	8.4	10.0	11.4	16.8	8.6	11.04	3.44	70.45	-	20.51% (17.4-24.2)	
	25.00		43	46	16	68	2	6.1	9.2	3.2	13.6	0.4	6.51	5.15	41.54			**
	50.00		9	1	34	5	7	1.8	0.2	6.8	1.0	1.4	2.24	2.62	14.29			**
	100.00		0	0	1	2	2	0.0	0.0	0.2	0.4	0.4	0.20	0.20	1.28			**
2,6-DNT (µmoles/L)	30.06	27.81	56	63	63	47	83	11.2	12.6	12.6	9.4	16.6	12.48	2.65	90.70	242.21 (218.1-269.0)	44.12 (39.7-49.0)	
	60.12	56.14	74	52	78	50	51	14.8	13.0	15.6	10.0	10.2	12.72	2.57	92.44			
	120.24	108.96	60	53	71	47	41	12.0	10.6	14.2	9.4	8.2	10.88	2.33	79.07			
	240.47	214.73	46	39	46	52	49	9.2	7.8	9.2	10.4	9.8	9.28	0.97	67.44			**
	480.95	423.12	0	0	1	0	0	0.0	0.0	0.2	0.0	0.0	0.04	0.09	0.29			**
2-A-6NT (µmoles/L)	34.69	34.95	64	31	94	37	51	12.8	6.2	18.8	7.4	10.2	11.08	5.02	67.23	52.18 (46.2-58.9)	7.94 (7.0-9.0)	*
	69.38	70.66	32	8	27	15	36	6.4	1.6	5.4	3.0	7.2	4.72	2.35	28.64			**
	138.76	134.78	22	17	34	0	4	4.4	3.4	6.8	0.0	0.8	3.08	2.76	18.69			**
	277.52	266.98	0	6	17	13	43	0.0	1.2	3.4	2.2	8.6	3.07	3.33	18.65			**
2-NT (µmoles/L)	72.56	40.67	96	58	94	74	47	19.2	14.5	18.8	12.3	9.4	14.85	4.20	113.42	357.34 (332.3-384.3)	49.05 (45.4-52.9)	
	145.12	80.92	61	62	54	67	84	12.2	12.4	10.8	13.4	16.8	13.12	2.26	100.23			
	290.25	166.20	32	34	71	61	36	6.4	6.8	14.2	12.2	7.2	9.36	3.59	71.50			
	580.50	334.34	7	3	6	3	8	1.4	0.6	1.2	0.6	1.6	1.08	0.46	8.25			**
	1161.00	653.46	2	1	2	0	1	0.4	0.2	0.4	0.0	0.2	0.24	0.17	1.83			**
Picric Acid (µmoles/L)	37.06	29.17	60	58	89	83	79	12.0	11.6	17.8	16.6	15.8	14.76	2.80	107.27	191.81 (171.0-215.2)	43.95 (39.2-49.3)	
	74.11	59.32	54	60	66	42	44	10.8	12.0	13.2	8.4	8.8	10.64	2.05	77.33			
	148.18	160.81	54	36	57	31	39	10.8	7.2	11.4	6.2	7.8	8.68	2.29	63.08			**
	296.36	356.13	27	28	44	25	35	5.4	5.6	8.8	5.0	7.0	6.36	1.56	46.22			**
	592.72	588.46	0	1	2	0	2	0.0	0.2	0.4	0.0	0.4	0.20	0.20	1.45			**

Appendix D2. Continued

Treatment	Concentration (µmoles/L)		# Alive Nauplii					Nauplii/Female					Mean Nauplii/ Female	St. Dev.	% of Control[f]	EC_{50} (µmoles/L)	EC_{50} (mg/L)	Signif.
			Replicate #					Replicate #										
	Initial[a]	Final	1	2	3	4	5	1	2	3	4	5						
2,4-DNP (µmoles/L)	14.06	15.06	65	81	91	81	112	13.0	16.2	15.2	16.2	22.4	16.59	3.50	126.76			
	28.12	28.83	69	73	75	55	66	13.8	14.6	15.0	9.2	13.2	13.15	2.34	100.48	53.85	9.91	
	56.23	54.97	22	24	20	36	28	4.4	4.8	4.0	7.2	5.6	5.20	1.26	39.72	(43.8-66.2)	(8.1-12.2)	**
	112.47	110.27	23	22	8	31	18	4.6	3.7	1.6	6.2	3.6	3.93	1.67	30.05			**
Picramic Acid (µmoles/L)	14.18	13.29	83	76	69	61	75	16.6	15.2	13.8	12.2	15.0	14.56	1.65	111.23			
	28.36	24.39	62	21	44	43	45	12.4	4.2	7.3	8.6	9.0	8.31	2.96	63.46	47.69	9.50	
	56.72	47.08	30	14	20	32	19	6.0	2.8	4.0	8.0	3.8	4.92	2.08	37.59	(42.5-53.5)	(8.47-10.6)	**
	113.44	105.22	12	16	11	14	19	3.0	3.2	2.8	2.8	3.8	3.11	0.42	23.76			**
	226.89	216.54	0	0	0	0	2	0.0	0.0	0.0	0.0	0.4	0.08	0.18	0.61			**
NH_3 (unionized - µg/L)	106.50	160.40	48	69	75	43	64	9.6	13.8	15.0	8.6	12.8	11.96	2.75	124.07	-	386.6[e]	
	196.20	280.80	52	51	34	46	50	10.4	10.2	6.8	9.2	10.0	9.32	1.48	96.68	-	(356.3-419.5)	
	390.10	582.30	9	30	13	14	-	1.8	6.0	2.6	2.8	-	3.30	1.85	34.23	-		**
	692.30	1119.10	4	3	9	8	8	0.8	0.6	1.8	1.6	1.6	1.28	0.54	13.28	-	587.2[f]	**
	1163.50	1918.20	2	1	6	2	2	0.4	0.2	1.2	0.4	0.4	0.52	0.39	5.39	-	(536.7-642.4)	**
	1993.90	3819.70	0	6	3	3	4	0.0	1.2	0.6	0.6	0.8	0.64	0.43	6.64	-		**

[a]Highest ordnance concentrations were measured by HPLC at test start; all concentrations were measured at test end.

[b]MFS = Millipore filtered seawater (0.45 µm); T1=Test 1, concurrent to 2-A-6-NT and photo-degraded DNT; T2 concurrent to 2,6-DNT and picric acid; T3 concurrent to 2-NT, 2,4-DNP and picramic acid; T4 concurrent to NH_3.

[c]PD-MFS = MFS control exposed to simulated solar radiation under identical conditions as the 2,6-DNT.

[d]Presented as percentage of initial 2,6-DNT stock solution, which had concentration of 783.66 µmoles/L.

[e]Calculated using ammonia concentrations measured at test start.

[f]Calculated using ammonia concentrations measured at test ending.

Appendix D3. Toxicity of ordnance compounds, selected biotransformation products, and photo-transformed 2,6-DNT to algae, *Ulva fasciata,* zoospore germination.

Treatment	Meas. Conc. (µmoles/L)		% Germination Replicate #					Mean		% of		EC_{50}	EC_{50}
	Initial[a]	Final	1	2	3	4	5	% Germ.	St. Dev.	Control	Signif.	(µmoles/L)	(mg/L)
MFS[b]-T1	NA[c]	NA	88	95	92	96	95	93.2	3.3	-	-	-	-
MFS-T2	NA	NA	92	90	93	91	96	92.4	2.3	-	-	-	-
MFS-T3	NA	NA	81	78	87	85	88	83.8	4.21	-	-	-	-
PD-MFS[d]	NA	NA	90	91	87	92	95	91.0	2.9	97.6	-	-	-
PD-DNT[e] (% of stock)	3.10	-	93	93	94	92	91	92.6	1.1	100.2			
	6.30	-	93	94	92	94	91	92.8	1.3	100.4			
	12.50	-	80	87	89	87	84	85.4	3.5	92.4	**	20.17%	-
	25.00	-	27	29	22	21	20	23.8	4.0	25.8	**	(18.8-21.6)	
	50.00	-	0	0	0	0	0	0.0	0.0	0.0	**		
2,6-DNT (µmoles/L)	14.50	12.49	95	96	99	98	94	96.4	2.1	103.4			
	29.00	25.13	92	94	97	95	96	94.8	1.9	101.7		73.41	13.37
	58.00	51.36	84	80	69	82	76	78.2	5.9	83.9	**	(69.8-77.2)	(12.7-14.1)
	115.99	99.50	0	0	0			0.0	0.0	0.0	**		
2-A-6NT (µmoles/L)	9.99	10.40	98	97	95	97	95	96.4	1.3	103.4			
	19.98	21.12	96	94	94	91	97	94.4	2.3	101.3			
	39.96	41.08	92	94	88	96	94	92.8	3.0	99.6		>159.82	>24.32
	79.91	81.83	94	96	96	95	94	95.0	1.0	101.9			
	159.82	159.17	74	72	84	82	83	79.0	5.6	84.8	**		
2-NT (µmoles/L)	99.92	39.18	90	92	84	86	84	87.2	3.63	104			
	199.84	79.17	82	78	84	83	81	81.6	2.30	97		367.77	50.44
	399.67	152.03	37	46	24	48	18	34.6	13.26	41	**	(342.2-395.3)	(46.9-54.2)
	799.35	306.18	0	0	0	0	0	0	0.00	0	**		
	1598.69	610.34	0	0	0	0	0	0	0.00	0	**		
Picric Acid (µmoles/L)	192.87	317.64	93	91	88	87	95	90.8	3.3	97.4			
	385.75	545.15	94	92	86	93	88	90.6	3.4	97.2			
	771.50	892.43	91	83	79	82	83	83.6	4.4	89.7	**	2029.10	464.90
	1542.99	1587.47	73	63	56	61	60	62.6	6.3	67.2	**	(1728.9-2381.4)	(396.1-545.6)
	3085.99	2940.03	28	18	15	35	16	22.4	8.7	24.0	**		

Appendix D3. Continued

Treatment	Meas. Conc. (µmoles/L) Initial[a]	Final	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)	EC_{50} (mg/L)
	17.67	21.71	88	82	90	90	86	87.2	3.35	104			
	35.34	34.40	85	82	74	72	81	78.8	5.54	94			
2,4-DNP	70.67	71.08	61	66	63	58	78	65.2	7.73	78	**	165.00	30.52
(µmoles/L)	141.34	138.81	57	67	49	55	48	55.2	7.63	66	**	(147.9-185.8)	(27.2-34.2)
	282.68	271.07	22	32	23	41	30	29.6	7.70	35	**		
	565.36	543.96	0	0	0	0	0	0	0.00	0	**		
Picramic	142.80	132.57	76	80	91	90	86	84.6	6.47	101		>571.22	>113.74
Acid	285.61	272.85	81	88	92	93	92	89.2	4.97	106			
(µmoles/L)	571.22	590.92	40	62	83	57	68	67.5	11.27	81	**		

[a]Highest ordnance concentrations were HPLC measured at test start; all concentrations were measured at test end.

[b]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, concurrent to 2-A-6-NT, 2,6-DNT, and picric acid; T2 concurrent to photo-degraded DNT; T3 concurrent to 2-NT, 2,4-DNP and picramic acid.

[c]NA=not applicable.

[d]PD-MFS = MFS control exposed to simulated solar radiation under identical conditions as the 2,6-DNT.

[e]Photo-degraded 2,6-DNT, with concentrations in percentage of stock solution. 2,6-DNT concentration of stock prior to degradation = 705.29 mmoles/L.

Appendix D4. Toxicity of ordnance compounds, selected biotransformation products, and photo-transformed 2,6-DNT to algae, *Ulva fasciata,* germling length.

Treatment	Meas. Conc. (µmoles/L)		Germling Length[b] Replicate #					Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)	EC_{50} (mg/L)
	Initial[a]	Final	1	2	3	4	5						
MFS[c]-T1	NA[d]	NA	67.4	62.4	65.9	59.8	67.4	64.6	3.4	-	-	-	-
MFS-T2	NA	NA	64.9	71.0	67.4	40.6	65.4	61.9	12.1	-	-	-	-
MFS-T3	NA	NA	41.6	40.1	43.6	36.0	42.6	40.8	3.0	-	-	-	-
PD-MFS[e]	NA	NA	57.8	58.8	61.9	60.8	66.9	61.2	3.6	-	-	-	
PD-DNT[f] (% of stock)	3.10	-	63.4	58.3	71.0	70.0	70.0	66.5	5.5	108.7			
	6.30	-	38.0	55.8	54.2	48.2	53.7	50.0	7.3	81.7	**	14.88%	-
	12.50	-	39.0	40.1	39.5	35.5	31.0	37.0	3.8	60.0	**	(13.4-16.6)	
	25.00	-	22.8	13.7	20.8	21.3	15.7	18.9	3.9	30.8	**		
	50.00	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
2,6-DNT (µmoles/L)	14.50	12.49	33.0	42.1	44.6	36.5	41.6	39.5	4.7	61.2	**		
	29.00	25.13	23.3	21.3	25.4	24.3	26.9	24.2	2.1	37.5	**	20.2	3.68
	58.00	51.36	11.7	10.6	11.7	10.6	9.6	10.8	0.8	16.8	**	(16.4-24.9)	(3.0-4.5)
	115.99	99.50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
2-A-6NT (µmoles/L)	9.99	10.40	49.7	44.6	42.1	49.2	38.5	44.8	4.7	69.4	**		
	19.98	21.12	41.1	38.0	35.5	35.5	42.6	38.5	3.2	59.6	**	123.04	18.72
	39.96	41.08	54.2	58.3	39.5	65.4	61.3	55.8	9.9	86.3		(113.2-133.7)	(17.2-20.3)
	79.91	81.83	52.2	46.1	55.3	54.2	59.8	53.5	5.0	82.9			
	159.82	159.17	15.7	22.3	15.2	22.3	22.3	19.6	3.8	30.3	**		
2-NT (µmoles/L)	99.92	39.18	28.9	34.0	35.0	31.9	37.5	33.5	3.2	82.1			
	199.84	79.17	22.3	17.2	22.8	21.8	20.8	21.0	2.2	51.5	**	206.66	28.34
	399.67	152.03	8.1	9.1	8.6	8.1	6.6	8.1	1.0	19.9	**	(182.0-234.6)	(25.0-32.2)
	799.35	306.18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
	1598.69	610.34	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
Picric Acid (µmoles/L)	192.87	317.64	48.7	51.7	51.7	54.8	61.9	53.7	5.0	83.2			
	385.75	545.15	45.6	47.7	52.7	50.7	54.2	50.2	3.5	77.7	**	1875.12	429.62
	771.50	892.43	43.6	42.6	41.1	48.2	53.2	45.7	5.0	70.8	**	(1504.0-2337.8)	(344.6-535.6)
	1542.99	1587.47	34.5	38.5	38.5	37.5	41.1	38.0	2.4	58.9	**		
	3085.99	2940.03	16.2	19.8	25.1	22.8	20.8	20.9	3.3	32.4	**		

Appendix D4. Continued

Treatment	Meas. Conc. (µmoles/L)		Germling Length[b] Replicate #					Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)	EC_{50} (mg/L)
	Initial[a]	Final	1	2	3	4	5						
2,4-DNP (µmoles/L)	17.67	21.71	33.0	30.4	34.5	48.7	41.1	37.5	7.4	92.0		117.94 (102.0-136.3)	21.71 (18.8-25.1)
	35.34	34.40	41.6	28.9	34.0	36.5	45.1	37.2	6.4	91.3			
	70.67	71.08	22.3	26.9	23.3	23.8	29.9	25.2	3.1	61.9	**		
	141.34	138.81	12.7	20.8	15.7	15.2	16.2	16.1	2.9	39.6	**		
	282.68	271.07	15.2	9.6	14.2	11.2	11.7	12.4	2.3	30.3	**		
	565.36	543.96	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
Picramic Acid (µmoles/L)	142.80	132.57	37.5	37.5	40.6	43.6	36.0	39.0	3.0	95.8	**	414.42 (383.8-447.5)	86.52 (76.4-89.1)
	285.61	272.85	35.0	30.9	31.9	31.9	30.9	32.1	1.7	78.9	*		
	571.22	590.92	10.7	9.6	10.7	11.2	9.6	10.3	0.7	25.4			

[a]Highest ordnance concentrations were measured by HPLC at test start; all concentrations were measured at test end.

[b]Germling length = mean of 10 measurements in each replicate.

[c]MFS = Millipore filtered seawater (0.45mm); T1=Test 1, concurrent to 2-A-6-NT, 2,6-DNT, and picric acid; T2 concurrent to photo-degraded DNT; T3 concurrent to 2-NT, 2,4-DNP and picramic acid.

[d]NA=not applicable.

[e]PD-MFS = MFS control exposed to simulated solar radiation under identical conditions as the 2,6-DNT.

[f]Photo-degraded 2,6-DNT: concentrations in percentage of stock solution. 2,6-DNT conc. of stock (100%) prior to degradation = 705.29 mmoles/L.

Appendix D5. Toxicity of ordnance compounds, selected biotransformation products, and photo-transformed 2,6-DNT to algae, *Ulva fasciata,* germling cell number.

Treatment	Meas. Conc. (µmoles/L)		Germling Cell No.[b] Replicate #					Mean Germling Cell No.	St. Dev	% of Control	Signif.	EC_{50} (µmoles/L)	EC_{50} (mg/L)
	Initial[a]	Final	1	2	3	4	5						
MFS[c]-T1	NA[d]	NA	6.6	6.2	5.9	5.5	6.4	6.1	0.4	-	-	-	-
MFS-T2	NA	NA	6.3	6.3	6.2	3.3	6.0	5.6	1.3	-	-	-	-
MFS-T3	NA	NA	5.9	5.4	5.9	5.5	5.7	5.7	0.2	-	-	-	-
PD-MFS[e]	NA	NA	5.8	5.9	5.9	6.3	6.6	6.1	0.3	-	-	-	-
PD-DNT[f] (% of stock)	3.10	-	6.1	6.5	7.4	7.3	7.3	6.9	0.6	123.0		21.33% (19.7-23.1)	-
	6.30	-	3.1	6.9	6.5	6.3	6.5	5.9	1.6	104.6			
	12.50	-	5.6	5.4	5.3	4.4	4.2	5.0	0.6	88.9	*		
	25.00	-	2.8	1.4	2.4	2.3	1.8	2.1	0.5	38.2	**		
	50.00	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
2,6-DNT (µmoles/L)	14.50	12.49	3.1	3.8	4.0	3.2	3.8	3.6	0.4	58.7	**	24.3 (17.3-34.0)	4.43 (3.2-6.2)
	29.00	25.13	2.8	2.8	3.2	2.7	3.0	2.9	0.2	47.5	**		
	58.00	51.36	1.5	1.2	1.3	1.4	1.1	1.3	0.2	21.3	**		
	115.99	99.50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
2-A-6NT (µmoles/L)	9.99	10.40	3.3	3.5	3.4	3.7	3.1	3.4	0.2	55.7	**	137.39 (124.7-151.3)	20.90 (19.0-23.0)
	19.98	21.12	3.5	3.2	3.1	3.1	3.7	3.3	0.3	54.4	**		
	39.96	41.08	5.4	5.1	3.5	5.0	4.9	4.8	0.7	78.4	**		
	79.91	81.83	5.8	4.8	5.6	5.6	6.4	5.6	0.6	92.5			
	159.82	159.17	1.9	2.7	1.8	2.7	2.4	2.3	0.4	37.7	**		
2-NT (µmoles/L)	99.92	39.18	3.5	4.4	5.1	4.8	5.8	4.7	0.9	83.1		206.46 (182.6-233.4)	28.31 (25.0-32.0)
	199.84	79.17	3.3	2.3	3.2	3.0	3.0	3.0	0.4	52.1	**		
	399.67	152.03	1.0	1.0	1.2	1.1	1.0	1.1	0.1	18.7	**		
	799.35	306.18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
	1598.69	610.34	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
Picric Acid (µmoles/L)	192.87	317.64	4.6	4.9	5.2	5.1	5.5	5.1	0.3	83.0	**	2759.57 (2284.1-3334.1)	632.26 (523.3-763.9)
	385.75	545.15	4.4	4.6	5.8	4.6	5.6	5.0	0.6	82.0	**		
	771.50	892.43	4.5	5.0	5.3	5.0	5.7	5.1	0.4	83.6	**		
	1542.99	1587.47	4.5	4.8	4.8	4.6	4.6	4.7	0.1	76.4	**		
	3085.99	2940.03	2.3	3.0	3.3	2.8	2.3	2.7	0.4	44.9	**		

Appendix D5. Continued

Treatment	Meas. Conc. (µmoles/L)		Germling Cell No.[b] Replicate #					Mean Germling Cell No.	St. Dev	% of Control	Signif.	EC_{50} (µmoles/L)	EC_{50} (mg/L)
	Initial[a]	Final	1.0	2.0	3.0	4.0	5.0						
2,4-DNP (µmoles/L)	17.67	21.71	4.8	4.1	5.3	6.1	5.4	5.1	0.7	90.5		129.97 (113.2-149.2)	23.93 (20.8-27.5)
	35.34	34.40	5.8	4.2	5.0	5.0	6.6	5.3	0.9	93.7			
	70.67	71.08	3.3	4.9	3.3	3.8	4.8	4.0	0.8	70.8	**		
	141.34	138.81	1.9	3.0	2.1	2.1	2.5	2.3	0.4	40.8	**		
	282.68	271.07	2.2	1.4	2.0	1.4	1.5	1.7	0.4	29.9	**		
	565.36	543.96	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**		
Picramic Acid (µmoles/L)	142.80	132.57	5.6	4.7	6.0	6.0	5.4	5.5	0.5	97.5		412.49 (390.0-436.3)	82.13 (77.6-86.9)
	285.61	272.85	5.2	4.8	4.7	4.1	5.2	4.8	0.5	84.5			
	571.22	590.92	1.0	1.0	1.1	1.2	1.0	1.1	0.1	18.7	**		

[a]Highest ordnance concentrations were HPLC measured at test start; all concentrations were measured at test end.

[b]Cell number = mean of 10 measurements in each replicate.

[c]MFS = Millipore filtered seawater (0.45 µm); T1=Test 1, concurrent to 2-A-6-NT, 2,6-DNT, and picric acid; T2 concurrent to photo-degraded DNT; T3 concurrent to 2-NT, 2,4-DNP and picramic acid.

[d]NA=Not applicable.

[e]PD-MFS = MFS control exposed to simulated solar radiation under identical conditions as the 2,6-DNT.

[f]Photo-degraded 2,6-DNT, with concentrations in percentage of stock solution. 2,6-DNT concentration of stock (100%) prior to degradation = 705.29 mmoles/L.

Appendix D6. Results of macroalgae toxicity tests with sodium dodecyl sulfate.

Test No.	Initial Conc. (mg/L)	% Germination Replicate No. 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control[d]	EC_{50}
1[a]	1.3	92	91	92	90	94	91.8	1.5	98.5	5.62 (5.2-6.0)
	2.5	88	90	87	83	88	87.2	2.6	93.6	
	5.0	73	65	69	65	63	67.0	4.0	71.9	
	10.0	0	0	0	0	0	0.0	0.0	0.0	
2[b]	1.3	93	94	93	91	93	92.8	1.1	100.4	3.54 (3.4-3.7)
	2.5	95	90	86	82	93	89.2	5.3	96.5	
	5.0	3	4	1	1	4	2.6	1.5	2.8	
	10.0	0	0	0	0	0	0.0	0.0	0.0	
3[c]	1.3	91	86	93	95	97	92.4	4.2	110.0	5.74 (5.4-6.1)
	2.5	87	87	92	95	91	90.4	3.4	108.0	
	5.0	52	61	69	49	64	59.0	8.3	70.0	
	10.0	0	0	0	0	0	0.0	0.0	0.0	
		Germling Length[e]								
1[a]	1.3	34.0	28.4	27.4	27.4	31.4	29.7	2.9	46.0	<1.3
	2.5	33.0	28.4	22.8	26.9	32.4	28.7	4.2	44.4	
	5.0	16.5	18.8	18.8	18.8	18.3	18.2	1.0	28.2	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
2[b]	1.3	31.4	39.5	39.0	37.0	37.5	36.9	3.2	59.7	2.15 (1.5-3.0)
	2.5	36.5	38.5	17.7	20.8	33.5	29.4	9.5	47.5	
	5.0	10.1	23.3	12.7	14.7	25.9	17.3	6.9	28.0	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
3[c]	1.3	40.1	34.0	33.5	39.0	33.5	36.0	3.3	88.0	2.98 (2.6-3.4)
	2.5	19.8	21.8	24.8	24.3	21.3	22.4	2.1	55.0	
	5.0	12.7	11.7	13.7	11.2	12.2	12.3	1.0	30.0	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		Germling Cell No.[e]								
1[a]	1.3	2.9	2.6	2.9	2.6	2.7	2.7	0.2	44.8	<1.3
	2.5	2.8	2.6	2.3	2.7	3.0	2.7	0.3	43.8	
	5.0	1.9	2.2	2.2	2.1	2.3	2.1	0.2	35.0	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
2[b]	1.3	2.9	3.7	3.2	3.3	3.0	3.2	0.3	57.3	2.34 (1.4-3.8)
	2.5	3.5	3.8	1.8	2.1	3.2	2.9	0.9	51.4	
	5.0	1.1	2.4	1.4	1.5	2.5	1.8	0.6	31.8	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
3[c]	1.3	5.1	4.5	3.9	4.4	4.1	4.4	0.5	77.0	2.42 (2.1-2.8)
	2.5	2.3	2.6	3.0	2.6	2.9	2.7	0.3	47.0	
	5.0	1.4	1.3	1.5	1.3	1.4	1.4	0.1	24.0	
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

[a]Test 1: concurrent to porewater tests with samples from days 0, 7, 28 and 56 and single chemical tests with 2,6-DNT, 2-A-6-NT and picric acid; [b]Test 2: concurrent test with photo-degraded 2,6-DNT. [c]Test 3: concurrent to porewater tests with samples from day 180 and single chemical tests with 2-NT, 2,4-DNP and picramic acid; [d]Percent of control using MFS results from each respective test: see corresponding appendices; [e]Germling length and cell no.= mean of 10 measurements in each replicate.

Appendix D7. Results of copepod toxicity tests with the reference toxicant, sodium dodecyl sulfate.

Test No.	Initial Conc. (mg/L)	No. Alive Replicate # 1	2	3	% Survival Replicate # 1	2	3	Mean % Surv.	St. Dev.	LC_{50}	# Alive Nauplii Replicate # 1	2	3	Nauplii/Female Replicate # 1	2	3	Mean Nauplii/ Female	St. Dev.	% of Control[i]	EC_{50}
	2.5	5	5	5	100	100	100	100	0		41	40	50	8.2	8.0	10.0	8.73	1.10	71.9	
	5	5	5	5	100	100	100	100	0	28.3	35	43	48	7.0	8.6	9.6	8.40	1.31	61.4	10.3
1[a]	10	5	5	5	100	100	100	100	0	(NC)[h]	34	43	29	6.8	8.6	5.8	7.07	1.42	59.6	(8.6-12.2)
	20	5	5	5	100	100	100	100	0		2	0	0	0.4	0.0	0.0	0.13	0.23	3.5	
	40	0	0	0	0	0	0	0	0		0	1	0	0.0	0.2	0.0	0.07	0.12	0.0	
	2.5	5	5	5	100	100	100	100	0		56	54	68	11.2	10.8	13.6	11.87	1.51	89.9	
	5	5	5	5	100	100	100	100	0	24.6	44	48	47	8.8	9.6	9.4	9.27	0.42	70.2	8.2
2[b]	10	5	5	5	100	100	100	100	0	(23.3-26.0)	35	42	24	7.0	8.4	4.8	6.73	1.81	51.0	(7.3-9.3)
	20	4	5	3	80	100	60	80	20		0	1	3	0.0	0.2	0.6	0.27	0.31	2.0	
	40	0	0	0	0	0	0	0	0		2	3	0	0.4	0.6	0.0	0.33	0.31	2.5	
3[c]	2.5	5	5	**6**	100	100	100	100	0		68	42	56	13.6	8.4	9.3	10.444	2.8	79.7	
	5	5	5	5	100	100	100	100	0	28.3	76	60	57	15.2	12.0	11.4	12.867	2.0	98.2	7.81
	10	**6**	5	5	100	100	100	100	0	(NC)	13	14	21	2.2	2.8	4.2	3.056	1.0	23.3	(7.31-8.33)
	20	5	**7**	5	100	100	100	100	0		0	4	0	0.0	0.6	0.0	0.190	0.3	1.5	
	40	0	0	0	0	0	0	0	0		0	0	0	0.0	0.0	0.0	0	0.0	0.0	
4[d]	2.5	5	5	5	100	100	100	100	0		77	88	82	15.4	17.6	16.4	16.47	1.10	125.8	
	5	5	5	5	100	100	100	100	0	28.3	55	44	68	11.0	8.8	13.6	11.13	2.40	85.1	8.4
	10	5	5	5	100	100	100	100	0	(NC)	28	24	27	5.6	4.0	5.4	5.00	0.87	38.2	(7.7-9.2)
	20	5	5	5	100	100	100	100	0		2	2	1	0.4	0.4	0.2	0.33	0.12	2.5	
	40	0	0	0	0	0	0	0	0		4	1	1	0.8	0.2	0.2	0.40	0.35	3.1	
5[e]	5	5	5	5	100	100	100	100	0		73	64	35	14.6	12.8	7.0	11.47	3.97	69.6	
	10	4	5	5	80	100	100	93.3	11.5	32.5	73	41	64	14.6	8.2	12.8	11.87	3.30	72.0	12.9
	20	5	5	5	100	100	100	100	0	(30.7-34.3)	0	0	0	0.0	0.0	0.0	0.00	0.00	0.0	(NC)
	40	0	1	2	0	20	40	20.0	20.0		0	0	0	0.0	0.0	0.0	0.00	0.00	0.0	
	80	0	0	0	0	0	0	0	0		1	1	0	0.2	0.2	0.0	0.13	0.12	0.8	
	2.5	5	5	5	100	100	100	100.0	0.0		94	68	77	18.8	13.6	15.4	15.93	2.64	115.8	
	5	4	5	5	80	100	100	93.3	11.5	15.4	73	54	76	14.6	10.8	15.2	13.53	2.39	98.4	8.3
6[f]	10	5	5	5	100	100	100	100.0	0.0	(14.7-16.1)	27	11	10	5.4	2.2	2.0	3.20	1.91	23.3	(7.8-8.8)
	20	**1**	***0***	1	17	0	20	12.2	10.7		0	1	0	0.0	0.3	0.0	0.08	0.14	0.6	
	40	0	0	0	0	0	0	0.0	0.0		1	2	0	0.2	0.4	0.0	0.20	0.20	1.5	

Appendix D7. Continued

Test No.	Initial Conc. (mg/L)	No. Alive Replicate # 1	2	3	% Survival Replicate # 1	2	3	Mean % Surv.	St. Dev.	LC_{50}	# Alive Nauplii Replicate # 1	2	3	Nauplii/Female Replicate # 1	2	3	Mean Nauplii/ Female	St. Dev.	% of Control[f]	EC_{50}
	2.5	5	5	5	100	100	100	100.0	0.0		30	57	55	6.0	11.4	11.0	9.47	3.01	101.5	
	5	5	5	5	100	100	100	100.0	0.0	30.8	47	38	48	9.4	7.6	9.6	8.87	1.10	95.0	11.3
7[g]	10	5	5	**6**	100	100	100	100.0	0.0	(29.5-32.2)	22	37	52	4.4	7.4	8.7	6.82	2.19	73.1	(10.4-12.2)
	20	5	5	5	100	100	100	100.0	0.0		0	0	0	0.0	0.0	0.0	0.00	0.00	0.0	
	40	2	0	1	40	0	20	20.0	20.0		2	0	0	0.4	0.0	0.0	0.13	0.23	1.4	

Bold Italics = Fewer than 5 animals in replicate; **Bold = More than 5 animals in replicate**

[a]Test 1: concurrent to porewater tests with samples from days 0 and 7; [b]Test 2: concurrent to porewater tests with samples from days 28 and 56; [c]Test 3: concurrent to porewater tests with samples from day 180; [d]Test 4 concurrent to single chemical tests with 2-NT, 2,4-DNP and sodium picramate; [e]Test 5: concurrent to single chemical tests with 2-A-6-NT and photo-degraded 2,6-DNT; [f]Test 6: concurrent to single chemical tests with 2,6-DNT and picric acid; [g]Test 7: concurrent to test with ammonia.

[h]NC = not calculable

[i]Percent of control using MFS results from each respective test (see corresponding appendices).

Appendix E1. Toxicity of pore waters from sediments spiked with 2,6-DNT to copepod, *Schizopera knabeni,* female survival.

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	No. Alive Replicate # 1	2	3	4	5	% Survival Replicate # 1	2	3	4	5	Mean % Surv.	St. Dev	Signif.	EC_{50} (µmoles/L)[e]
-	MFS[b]		-	-	NA[h]	NA	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
-	TX Ref[c]		-	-	NA	NA	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
Sandy	MEOH[d]	100	0	-	NA	NA	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		100	7	20	NA	NA	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
Sandy	Seawater	6.25	28[f]	10	NA	NA	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		12.5							5	5	5	**7**	***4***	100	100	100	100	100	100.0	0.0		
		25							**6**	**6**	**6**	5	***4***	100	100	100	100	100	100.0	0.0		
		50							5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	MEOH	100	0	-	NA	NA	NA	NA	5	5	5	**6**	5	100	100	100	100	100	100.0	0.0	-	-
		100	7	10	NA	NA	NA	NA	***4***	***4***	2	4	4	100	100	40	80	80	80.0	24.5	-	-
		3.1	0[g]	-	NA	NA	NA	NA	**6**	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
Sandy	2,6-DNT	6.25	0	-	28.51	15.28	25.27	17.25	5	5	5	5	5	100	100	100	100	100	100.0	0.0		269.26 (251.8-287.9)
		12.5			57.03	30.57	52.26	29.80	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25			114.06	61.14	97.19	57.01	5	5	5	4	4	100	100	100	80	80	92.0	11.0	*	
		50			228.12	122.28	207.78	121.13	2	1	0	0	2	40	20	0	0	40	20.0	20.0	**	
		100			456.24	244.55	429.67	243.37	0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	
Sandy	2,6-DNT	3.12	7	20	BDL[i]	33.21	BDL	33.48	5	5	5	5	5	100	100	100	100	100	100.0	0.0		>531.14
		6.25			BDL	66.43	BDL	65.98	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		12.5			BDL	132.85	BDL	128.12	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25			BDL	265.70	BDL	254.88	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			BDL	531.41	BDL	528.01	**5**	5	5	5	5	83	100	100	100	100	96.7	7.5		
Sandy	2,6-DNT	6.25	180	20	BDL	BDL	BDL	BDL	5	5	**6**	5	5	100	100	100	100	100	100.0	0.0		-
		12.5			BDL	BDL	BDL	BDL	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25			BDL	BDL	BDL	BDL	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			BDL	BDL	BDL	BDL	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	2,6-DNT	6.25	0	-	14.80	14.63	13.85	14.58	5	5	5	5	5	100	100	100	100	100	100.0	0.0		342.78 (335.3-350.5)
		12.5			29.60	29.25	27.70	28.81	**6**	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25			59.20	58.50	55.41	57.10	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			118.40	117.01	110.82	117.20	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		100			236.81	234.01	221.64	231.42	2	0	***0***	0	0	40	0	0	0	0	8.0	17.9	**	

Appendix E1. Continued

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	No. Alive Replicate # 1	2	3	4	5	% Survival Replicate # 1	2	3	4	5	Mean % Surv.	St. Dev	Signif.	EC_{50} (µmoles/L)[e]
Fine-grain	2,6-DNT	6.25	7	10	BDL	35.09	BDL	35.31	5	5	5	5	5	100	100	100	100	100	100.0	0.0		396.97 (NC)[j]
		12.5			BDL	70.17	BDL	66.53	5	**6**	5	5	5	100	100	100	100	100	100.0	0.0		
		25			BDL	140.35	BDL	132.98	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			BDL	280.70	BDL	271.73	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		100			BDL	561.39	BDL	547.94	0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	
Fine-grain	2,6-DNT	6.25	180	10	BDL	BDL	BDL	BDL	5	5	***4***	**6**	5	100	100	100	100	100	100.0	0.0		-
		12.5			BDL	BDL	BDL	BDL	**6**	5	***4***	5	5	100	100	100	100	100	100.0	0.0		
		25			BDL	BDL	BDL	BDL	**6**	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			BDL	BDL	BDL	BDL	5	5	5	**6**	5	100	100	100	100	100	100.0	0.0		
		100			BDL	BDL	BDL	BDL	5	5	5	**6**	5	100	100	100	100	100	100.0	0.0		

Bold Italics = Only 4 animals in replicate; **Bold = 6 animals in replicate**

[a]PW = Porewater

[b]MFS = Millipore filtered seawater (0.45µm)

[c]TX Ref = pore water from standard Texas reference sediment.

[d]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[e]EC_{50} expressed as sum 2,6-DNT and 2-A-6-NT concentrations a test start

[f]Used as blank for sandy sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[g]Used as blank for fine-grained sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[h]NA = not applicable

[i]BDL=below detection limit

[j]NC = not calculable

Appendix E2. Toxicity of pore waters from sediments spiked with 2,6-DNT to copepod, *Schizopera knabeni,* embryo and nauplii survival.

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Total Number of Nauplii Replicate # 1	2	3	4	5	Nauplii/Female Replicate # 1	2	3	4	5	Mean No. Nauplii	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[i]
-	MFS[b]		-	-	NA[d]	NA	NA	NA	52	58	54	72	48	10.4	11.6	10.8	14.4	9.6	11.4	1.85	-	-	-
-	TX Ref[c]		-	-	NA	NA	NA	NA	77	63	59	52	67	15.4	12.6	11.8	10.4	13.4	12.7	1.86	-	-	-
Sandy	MEOH[e]	100	0	-	NA	NA	NA	NA	63	35	52	28	39	12.6	7.0	10.4	5.6	7.8	8.7	2.80	-	-	-
		100	7	20	NA	NA	NA	NA	24	33	23	29	29	4.8	6.6	4.6	5.8	5.8	5.5	0.82	-	-	-
Sandy	SW[f]	6.25	28[g]	10	NA	NA	NA	NA	50	51	86	55	62	10.0	10.2	17.2	11.0	12.4	12.16	2.97	-	-	-
		12.5							48	46	52	61	68	9.6	9.2	10.4	8.7	17.0	10.98	3.42			
		25							51	66	69	74	55	8.5	11.0	11.5	14.8	13.8	11.91	2.47			
		50							25	41	35	18	29	5.0	8.2	7.0	3.6	5.8	5.92	1.78			
Fine-grain	MEOH	100	0	-	NA	NA	NA	NA	17	8	24	20	10	3.4	1.6	4.8	3.3	2.0	3.0	1.27	-	-	-
		100	7	10	NA	NA	NA	NA	0	3	0	0	0	0.0	0.8	0.0	0.0	0.0	0.2	0.34	-	-	-
Fine-grain	MEOH	3.1	0[h]	-	NA	NA	NA	NA	71	61	64	68	57	11.8	12.2	12.8	13.6	11.4	12.37	0.86	-	-	-
Sandy	2,6-DNT	6.25	0	-	28.51	15.28	25.27	17.25	67	52	63	49	53	13.4	10.4	12.6	9.8	10.6	11.4	1.55	130.88		377.38 (352.0-404.6)
		12.5			57.03	30.57	52.26	29.80	77	65	62	51	49	15.4	13.0	12.4	10.2	9.8	12.2	2.27	140.09		
		25			114.06	61.14	97.19	57.01	49	57	38	42	72	9.8	11.4	7.6	8.4	14.4	10.3	2.70	118.89		
		50			228.12	122.28	207.78	121.13	10	10	39	37	35	2.0	2.0	7.8	7.4	7.0	5.2	2.97	60.37		
		100			456.24	244.55	429.67	243.37	0	0	1	0	0	0.0	0.0	0.2	0.0	0.0	0.0	0.09	0.00	**	
Sandy	2,6-DNT	3.12	7	20	BDL	33.21	BDL	33.48	67	54	59	71	80	13.4	10.8	11.8	14.2	16.0	13.2	2.04	107.47		178.40 (148.3-214.7)
		6.25			BDL	66.43	BDL	65.98	72	38	61	49	62	14.4	7.6	12.2	9.8	12.4	11.3	2.63	91.56		
		12.5			BDL	132.85	BDL	128.12	55	32	40	39	28	11.0	6.4	8.0	7.8	5.6	7.8	2.07	62.99	*	
		25			BDL	265.70	BDL	254.88	12	48	5	17	11	2.4	9.6	1.0	3.4	2.2	3.7	3.40	30.19	**	
		50			BDL	531.41	BDL	528.01	27	2	7	11	16	4.5	0.4	1.4	2.2	3.2	2.3	1.59	26.35	**	
Sandy	2,6-DNT	6.25	180	20	BDL	BDL	BDL	BDL	48	53	46	71	68	9.6	10.6	7.7	14.2	13.6	11.13	2.75	91.56		21.6% PW[j] (20.1-23.3)
		12.5			BDL	BDL	BDL	BDL	24	59	49	61	67	4.8	11.8	9.8	12.2	13.4	10.40	3.39	94.72		
		25			BDL	BDL	BDL	BDL	22	4	24	10	39	4.4	0.8	4.8	2	7.8	3.96	2.71	33.25	**	
		50			BDL	BDL	BDL	BDL	2	0	0	0	0	0.4	0	0	0	0	0.08	0.18	1.35	**	
Fine-grain	2,6-DNT	6.25	0	-	14.80	14.63	13.85	14.58	49	71	81	51	62	9.8	14.2	16.2	10.2	12.4	12.6	2.70	144.70		326.58 (255.4-417.5)
		12.5			29.60	29.25	27.70	28.81	38	76	61	40	78	6.3	15.2	12.2	8.0	15.6	11.5	4.18	132.10		
		25			59.20	58.50	55.41	57.10	34	56	54	60	73	6.8	11.2	10.8	12.0	14.6	11.1	2.81	127.65		
		50			118.40	117.01	110.82	117.20	12	40	33	35	28	2.4	8.0	6.6	7.0	5.6	5.9	2.15	68.20		
		100			236.81	234.01	221.64	231.42	2	4	12	1	1	0.4	0.8	3.0	0.2	0.2	0.9	1.19	30.36	*	
Fine-grain	2,6-DNT	6.25	7	10	BDL	35.09	BDL	35.31	47	78	82	60	63	9.4	15.6	16.4	12.0	12.6	13.2	2.84	152.07		204.38 (189.0-221.0)
		12.5			BDL	70.17	BDL	66.53	35	51	47	57	41	7.0	8.5	9.4	11.4	8.2	8.9	1.64	102.53		
		25			BDL	140.35	BDL	132.98	55	32	43	17	39	11.0	6.4	8.6	3.4	7.8	7.4	2.81	85.71		
		50			BDL	280.70	BDL	271.73	9	2	4	17	3	1.8	0.4	0.8	3.4	0.6	1.4	1.24	16.13	**	
		100			BDL	561.39	BDL	547.94	0	12	1	0	0	0.0	2.4	0.2	0.0	0.0	0.5	1.05	5.99	**	

Appendix E2. Continued

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Total Number of Nauplii Replicate # 1	2	3	4	5	Nauplii/Female Replicate # 1	2	3	4	5	Mean No. Nauplii	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[h]
Fine-grain	2,6-DNT	6.25	180	10	BDL	BDL	BDL	BDL	77	55	85	72	94	15.4	11.0	21.3	12.0	18.8	15.69	4.37	126.84		93.6% PW[i] (88.6-108.7)
		12.5			BDL	BDL	BDL	BDL	87	75	71	71	69	14.5	15.0	17.8	14.2	13.8	15.05	1.57	121.67		
		25			BDL	BDL	BDL	BDL	74	60	62	62	84	12.3	12.0	12.4	12.4	16.8	13.19	2.03	106.60		
		50			BDL	BDL	BDL	BDL	49	50	53	77	57	9.8	10.0	10.6	12.8	11.4	10.93	1.23	88.33		
		100			BDL	BDL	BDL	BDL	26	25	25	59	17	5.2	5.0	5.0	9.8	3.4	5.69	2.43	45.97	**	

[a]PW = Porewater

[b]MFS = millipore filtered seawater (0.45µm).

[c]TX Ref = pore water from standard Texas reference sediment.

[d]NA = Not applicable

[e]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution.

[f]SW = blank spiked filtered seawater (MFS) in same quantity used in 2,6-DNT spiked sediment.

[g]Used as blank for sandy sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[h]Used as blank for fine-grained sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[i]EC_{50} expressed as sum 2,6-DNT and 2-A-6-NT concentrations at test start.

[j]EC_{50} expressed as percent porewater, since no chemicals were measured in samples.

Appendix E3. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata,* zoospore germination.

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev	% of Control	Signif.	EC_{50} (µmoles/L)[b]
-	MFS-T1[c]		-	-	NA[g]	NA	NA	NA	88	95	92	96	95	93.2	3.3	-	-	-
-	TX Ref-T1[d]		-	-	NA	NA	NA	NA	91	96	-	90	92	92.3	2.6	-	-	-
-	MFS-T2[c]	-	-	-	NA	NA	NA	NA	81	78	87	85	88	83.8	4.21	-	-	-
-	TX Ref-T2[d]	-	-	-	NA	NA	NA	NA	84	80	83	90	86	84.6	3.71	-	-	-
Sandy	MEOH[e]	100	0	-	NA	NA	NA	NA	96	97	95	91	95	94.8	2.3	-	-	-
		100	7	20	NA	NA	NA	NA	95	93	90	96	87	92.2	3.7	-	-	-
Sandy	SW[f]	50	28	20	NA	NA	NA	NA	90	89	94	84	85	88.4	4.04	-	-	-
Fine-grain	MEOH	50.0	0	-	NA	NA	NA	NA	95	91	92	96	94	93.6	2.1	-	-	-
		50.0	7	10	NA	NA	NA	NA	96	97	98	95	96	96.4	1.1	-	-	-
Fine-grain	MEOH	100.0	180	10	NA	NA	NA	NA	82	66	64	72	61	69.0	8.31	-	-	-
Sandy	2,6-DNT	0.78	0	-	3.18	1.84	2.06	1.99	96	97	93	97	94	95.4	1.8	100.6		80.32 (74.9-86.1)
		1.56			6.36	3.68	5.03	3.49	94	96	92	93	97	94.4	2.1	99.6		
		3.13			12.73	7.35	10.42	7.10	96	96	98	96	93	95.8	1.8	101.1		
		6.25			25.45	14.71	20.76	14.19	94	97	98	92	97	95.6	2.5	48.9		
		12.5			50.91	29.41	42.75	29.15	35	42	32	62	61	46.4	14.3	49.0	**	
		25.0			101.81	58.82	83.68	57.67	0	0	-	-	-	0.0	0.0	0.0	**	
Sandy	2,6-DNT	0.78	7	20	BDL	7.27	BDL	7.64	95	98	97	91	96	95.4	2.70	103.5		182.27 (173.4-191.6)
		1.56			BDL	14.54	BDL	14.82	93	96	92	97	95	94.6	2.07	102.6		
		3.13			0.06	29.09	BDL	29.18	94	93	97	97	98	95.8	2.17	103.9		
		6.25			0.12	58.17	BDL	60.67	91	96	96	95	90	93.6	2.88	101.5		
		12.5			0.24	116.34	0.07	117.47	94	93	93	87	94	92.2	2.95	100.0		
		25.0			0.48	232.68	0.19	232.05	6	18	27	18	37	21.2	11.56	22.9	**	
Sandy	2,6-DNT	6.25	180	10	BDL	BDL	BDL	BDL	73	88	79	77	68	77.0	7.45	87.1	**	68.63%[g] (53.0-8.8)
		12.5			BDL	BDL	BDL	BDL	82	86	84	83	92	85.4	3.97	96.6		
		25			BDL	BDL	BDL	BDL	82	92	85	87	86	86.4	3.65	97.7		
		50			BDL	BDL	BDL	BDL	64	48	53	70	56	58.2	8.79	65.8	**	
		100			BDL	BDL	BDL	BDL	22	27	12	71	36	24.3	10.01	27.4	**	
Fine-grain	2,6-DNT	1.56	0	-	3.61	3.60	3.04	3.75	96	98	96	97	99	97.2	1.30	103.8		94.34 (89.2-99.8)
		3.13			7.22	7.20	5.98	7.49	97	98	96	92	93	95.2	2.59	101.7		
		6.25			14.44	14.39	12.88	15.00	98	95	96	95	95	95.8	1.30	102.4		
		12.5			28.88	28.79	25.62	29.57	89	98	94	94	89	92.8	3.83	99.1		
		25.0			57.76	57.58	49.18	58.26	19	15	28	20	18	20	4.85	21.4	**	
		50.0			115.53	115.16	96.30	115.38	0	0	-	-	-	0.0	0.0	0.0	**	

Appendix E3. Continued

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev	% of Control	Signif.	EC_{50} (µmoles/L)[b]
Fine-grain	2,6-DNT	3.13	7	10	BDL	16.83	BDL	17.92	99	97	98	97	96	97.4	1.14	101.0		117.36 (109.8-125.5)
		6.25			BDL	33.66	BDL	35.66	91	95	94	96	98	94.8	2.59	98.3		
		12.5			BDL	67.32	BDL	68.91	92	94	93	98	98	95.0	2.83	98.5		
		25.0			BDL	134.65	BDL	133.60	37	33	23	29	20	28.4	6.99	29.5	**	
		50.0			BDL	269.29	BDL	259.98	2	2	1	0	3	1.6	1.14	1.7	**	
Fine-grain	2,6-DNT	3.1	180	10	BDL	BDL	BDL	BDL	85	92	90	93	92	90.4	3.21	131.0		74.81%[h] (72.4-77.3)
		6.25			BDL	BDL	BDL	BDL	91	88	92	98	90	91.8	3.77	133.0		
		12.5			BDL	BDL	BDL	BDL	89	91	89	94	93	91.2	2.28	132.2		
		25			BDL	BDL	BDL	BDL	84	88	87	88	91	87.6	2.51	127.0		
		50			BDL	BDL	BDL	BDL	59	65	74	85	80	72.6	10.64	105.2		
		100.0			BDL	BDL	BDL	BDL	6	7	19	11	5	9.6	5.73	13.9	**	

[a]PW = Porewater

[b]EC_{50} expressed as sum 2,6-DNT and 2-A-6-NT concentrations at test start

[c]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, conducted concurrently to samples from days 0 and 7; T2=Test 2, conducted concurrently to samples from day 180.

[d]TX Ref = pore water from standard Texas reference sediment.

[e]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[f]Sample spiked with seawater used as blank for sandy sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[g]NA=Not applicable.

[h]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E4. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata,* germling length.

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Germling Length[b] Replicate # 1	2	3	4	5	Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[c]
-	MFS[d]		-	-	NA[h]	NA	NA	NA	67.4	62.4	65.9	59.8	67.4	64.6	3.4	-	-	-
-	TX Ref[e]		-	-	NA	NA	NA	NA	46.1	57.3	-	54.8	69.5	56.9	9.6	-	-	-
-	MFS-T2[d]	-	-	-	NA	NA	NA	NA	41.6	40.1	43.6	36	42.6	40.76	2.97	-	-	-
-	TX Ref-T2[e]	-	-	-	NA	NA	NA	NA	37.5	45.6	45.1	46.1	48.7	44.62	4.20	-	-	-
Sandy	MEOH[f]	25	0	-	NA	NA	NA	NA	84.5	85.2	81.6	94.3	88.2	86.8	4.8	-	-	-
		25	7	20	NA	NA	NA	NA	69.0	68.4	73.0	78.6	87.2	75.2	7.8	-	-	-
Sandy	SW[g]	50	28	20	NA	NA	NA	NA	69.3	66.4	68.5	76.1	65.9	69.22	4.06	-	-	-
Fine-grain	MEOH	50.0	0	-	NA	NA	NA	NA	71.0	73.0	66.9	69.0	80.6	72.1	5.3	-	-	-
		50.0	7	10	NA	NA	NA	NA	79.1	77.6	80.1	82.6	77.1	79.3	2.2	-	-	-
Fine-grain	MEOH	100.0	180	10	NA	na	na	na	10.7	9.63	9.13	10.1	10.7	10.0	0.66	-	-	-
Sandy	2,6-DNT	0.78	0	-	3.18	1.84	2.06	1.99	66.4	63.9	61.9	51.7	55.8	59.9	6.0	69.0	**	22.39 (16.9-29.7)
		1.56			6.36	3.68	5.03	3.49	60.8	62.4	51.2	50.2	55.3	56.0	5.5	64.5	**	
		3.13			12.73	7.35	10.42	7.10	44.6	47.2	63.4	40.6	43.6	47.9	9.0	55.1	**	
		6.25			25.45	14.71	20.76	14.19	31.9	35.5	30.4	31.4	32.4	32.3	1.9	37.3	**	
		12.5			50.91	29.41	42.75	29.15	10.1	10.1	9.1	14.4	13.2	11.4	2.3	13.1	**	
		25.0			101.81	58.82	83.68	57.67	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	2,6-DNT	0.78	7	20	BDL[i]	7.27	BDL	7.64	53.2	60.3	56.8	44.6	60.8	55.2	6.6	73.4	**	56.82 (47.6-67.8)
		1.56			BDL	14.54	BDL	14.82	44.1	65.9	71	58.3	73	62.5	11.7	83.1	*	
		3.13			0.06	29.09	BDL	29.18	53.2	55.8	57.8	62.9	53.2	56.6	4.0	75.2	**	
		6.25			0.12	58.17	BDL	60.67	32.4	31.9	30.4	34.5	39	33.7	3.3	44.8	**	
		12.5			0.24	116.34	0.07	117.47	23.3	26.4	19.3	18.8	23.8	22.3	3.2	29.7	**	
		25.0			0.48	232.68	0.19	232.05	9.63	8.62	7.1	8.62	8.62	8.5	0.9	11.3	**	
Sandy	2,6-DNT	6.25	180	10	BDL	BDL	BDL	BDL	26.9	40.1	28.9	41.1	34	34.2	6.39	49	**	>100%[j]
		12.5			BDL	BDL	BDL	BDL	36.5	38	37	40.1	39	38.1	1.45	55	**	
		25			BDL	BDL	BDL	BDL	26.4	38	31.9	43.1	32.5	34.4	6.39	50	**	
		50			BDL	BDL	BDL	BDL	23.8	25.9	29.4	29.4	21.8	26.1	3.38	38	**	
		100			BDL	BDL	BDL	BDL	20.3	17.2	21.3	19.3	17.2	19.1	1.81	67	**	

Appendix E4. Continued

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Germling Length[b] Replicate # 1	2	3	4	5	Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[c]
Fine-grain	2,6-DNT	1.56	0	-	3.61	3.60	3.04	3.75	67.4	80.6	82.1	78.3	78.6	77.4	5.8	107.4		45.25 (40.3-50.8)
		3.13			7.22	7.20	5.98	7.49	57.8	61.9	61.3	65.4	66.9	62.7	3.6	86.9	*	
		6.25			14.44	14.39	12.88	15.00	49.7	43.6	50.7	50.2	55.8	50.0	4.3	69.3	**	
		12.5			28.88	28.79	25.62	29.57	30.4	33.5	34	35.5	30.9	32.9	2.1	45.6	**	
		25.0			57.76	57.58	49.18	58.26	8.62	8.62	11.2	10.1	9.13	9.5	1.1	13.2	**	
		50.0			115.53	115.16	96.30	115.38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	2,6-DNT	3.13	7	10	BDL	16.83	BDL	17.92	80.6	87.2	76.1	90.2	85.2	83.9	5.6	105.7		65.80 (58.6-73.9)
		6.25			BDL	33.66	BDL	35.66	57.3	75	61.3	64.9	67.9	65.3	6.7	82.3	**	
		12.5			BDL	67.32	BDL	68.91	38.5	40.1	36	42.6	44.6	40.4	3.4	50.9	**	
		25.0			BDL	134.65	BDL	133.60	9.38	8.62	9.63	9.63	10.9	9.6	0.8	12.1	**	
		50.0			BDL	269.29	BDL	259.98	7.1	8.11	7.1	0	8.37	6.1	3.5	7.7	**	
Fine-grain	2,6-DNT	3.1	180	10	BDL	BDL	BDL	BDL	43.6	54.3	50.7	42.1	43.6	46.8	5.33	467		>100%[j]
		6.25			BDL	BDL	BDL	BDL	45.1	48.7	55.8	37	50.7	47.5	6.99	473		
		12.5			BDL	BDL	BDL	BDL	41.1	44.6	41.6	53.2	47.2	45.5	4.96	453		
		25			BDL	BDL	BDL	BDL	31.9	42.6	29.4	29.4	27.4	32.1	6.06	320		
		50			BDL	BDL	BDL	BDL	11.2	10.7	17.8	20.3	19.8	15.9	4.68	159		
		100.0			BDL	BDL	BDL	BDL	7.1	5.07	6.08	5.58	5.07	5.8	0.85	58	**	

[a]PW = Porewater

[b]Germling length = mean of 10 measurements in each replicate.

[c]EC_{50} expressed as sum 2,6-DNT and 2-A-6-NT concentrations at test start

[d]MFS = millipore filtered seawater (0.45µm)

[e]TX Ref = pore water from standard Texas reference sediment.

[f]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[g]Sample spiked with sewawater used as blank for sandy sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[h]NA=Not applicable

[i]BDL=below detection limit

[j]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E5. Toxicity of pore waters from sediments spiked with 2,6-DNT to macroalga, *Ulva fasciata,* germling cell number.

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Germling Cell No.[b] Replicate # 1	2	3	4	5	Mean Germling Cell No.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[c]
-	MFS-T1[d]		-	-	NA[h]	NA	NA	NA	6.6	6.2	5.9	5.5	6.4	6.1	0.4	-	-	-
-	TX Ref-T2[e]		-	-	NA	NA	NA	NA	4.3	5.7	-	5.1	6.3	5.4	0.9	-	-	-
-	MFS-T2[d]	-	-	-	NA	NA	NA	NA	5.9	5.4	5.9	5.5	5.7	5.68	0.23	-	-	-
-	TX Ref-T2[e]	-	-	-	NA	NA	NA	NA	4.9	5.1	4.8	5.3	6.0	5.22	0.48	-	-	-
Sandy	MEOH[f]	100	0	-	NA	NA	NA	NA	9.6	10.9	10.0	12.5	12.3	11.1	1.3	-	-	-
		100	7	20	NA	NA	NA	NA	7.3	7.1	7.8	9.6	11.7	8.7	1.9	-	-	-
Sandy	SW[g]	50	28	20	NA	NA	NA	NA	9.44	9.1	9.9	9.78	9.7	9.58	0.32	-	-	-
Fine-grain	MEOH	50.0	0	-	NA	NA	NA	NA	7.6	7.6	8.0	8.2	10.9	8.5	1.4	-	-	-
		50.0	7	10	NA	NA	NA	NA	9.0	9.2	9.2	9.3	8.7	9.1	0.2	-	-	-
Fine-grain	MEOH	100.0	180	10	NA	NA	NA	NA	1.1	1.1	1.1	1.2	1.1	1.12	0.04	-	-	-
Sandy	2,6-DNT	0.78	0	-	3.18	1.84	2.06	1.99	5.6	5.7	5.1	4.4	5.0	5.2	0.5	46.7		<5.020
		1.56			6.36	3.68	5.03	3.49	5.8	5.5	4.9	4.0	5.2	5.1	0.7	45.9	**	
		3.13			12.73	7.35	10.42	7.10	5.1	5.6	5.7	4.8	4.7	5.2	0.5	46.8	**	
		6.25			25.45	14.71	20.76	14.19	3.5	4.3	3.8	3.9	3.6	3.8	0.3	34.5	**	
		12.5			50.91	29.41	42.75	29.15	1.1	1.3	1.2	1.9	1.8	1.5	0.4	13.2	**	
		25.0			101.81	58.82	83.68	57.67	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	2,6-DNT	0.78	7	20	BDL[i]	7.27	BDL	7.64	4.2	4.9	4.9	4.6	6	4.9	0.7	56.6	**	38.23 (30.9-47.3)
		1.56			BDL	14.54	BDL	14.82	4	6.1	6	5.1	6.3	5.5	1.0	63.2	**	
		3.13			0.06	29.09	BDL	29.18	4.9	5.2	5	5.6	5.1	5.2	0.3	59.3	**	
		6.25			0.12	58.17	BDL	60.67	3.1	3	2.7	3.5	3.4	3.1	0.3	36.1	**	
		12.5			0.24	116.34	0.07	117.47	2.6	3.1	2.5	2.4	2.8	2.7	0.3	30.8	**	
		25.0			0.48	232.68	0.19	232.05	1.0	1.0	1.0	1.0	1.1	1.0	0.0	11.7	**	
Sandy	2,6-DNT	6.25	180	10	BDL	BDL	BDL	BDL	3.6	5.5	3.9	5.3	4.3	4.5	0.84	47	**	>100%[j]
		12.5			BDL	BDL	BDL	BDL	4.8	5.7	5.2	5.9	5.4	5.4	0.43	56	**	
		25			BDL	BDL	BDL	BDL	3.4	5.4	4.7	5.4	4.3	4.6	0.84	48	**	
		50			BDL	BDL	BDL	BDL	3.2	3.3	3.8	4.4	2.9	3.5	0.59	37	**	
		100			BDL	BDL	BDL	BDL	2.3	1.8	2.7	2.7	2.3	2.4	0.37	63	**	

Appendix E5. Continued

Sediment	Treat.	PW[a] Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial 2,6-DNT	Initial 2-A-6-NT	Final 2,6-DNT	Final 2-A-6-NT	Germling Cell No.[b] Replicate # 1	2	3	4	5	Mean Germling Cell No.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)[c]
Fine-grain	2,6-DNT	1.56	0	-	3.61	3.60	3.04	3.75	7.6	9.2	9.2	6.9	9.9	8.6	1.3	101.2		50.55 (45.2-56.6)
		3.13			7.22	7.20	5.98	7.49	6.4	6.8	6.5	7.5	8.4	7.1	0.8	84.2	*	
		6.25			14.44	14.39	12.88	15.00	6.6	5.8	6.5	6.5	6.7	6.4	0.4	75.9	**	
		12.5			28.88	28.79	25.62	29.57	4.8	4.9	5	5.5	4.7	5.0	0.3	58.9	**	
		25.0			57.76	57.58	49.18	58.26	1.0	1.0	1.2	1.0	1.0	1.0	0.1	12.3	**	
		50.0			115.53	115.16	96.30	115.38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	2,6-DNT	3.13	7	10	BDL	16.83	BDL	17.92	9.1	11.1	7.8	11.4	9.3	9.7	1.5	107.3		76.33 (68.6-85.0)
		6.25			BDL	33.66	BDL	35.66	6.8	11.2	8.2	8.4	9.6	8.8	1.7	97.4		
		12.5			BDL	67.32	BDL	68.91	5.2	5.5	4.9	6.2	5.7	5.5	0.5	60.6	**	
		25.0			BDL	134.65	BDL	133.60	1.0	1.0	1.2	1.0	1.1	1.1	0.1	11.7	**	
		50.0			BDL	269.29	BDL	259.98	1.0	1.0	1.0	-	1.0	1.0	0.0	11.0	**	
Fine-grain	2,6-DNT	3.1	180	10	BDL	BDL	BDL	BDL	6.0	6.5	6.7	5.6	6.0	6.2	0.44	550		>100%[j]
		6.25			BDL	BDL	BDL	BDL	6.0	7.4	6.5	3.8	5.9	5.9	1.33	529		
		12.5			BDL	BDL	BDL	BDL	5.4	6.8	6.0	6.8	7.4	6.5	0.78	579		
		25			BDL	BDL	BDL	BDL	5.4	6.0	4.6	4.8	4.2	5.0	0.71	446		
		50			BDL	BDL	BDL	BDL	1.2	1.2	2.1	2.8	2.6	2.0	0.76	177		
		100.0			BDL	BDL	BDL	BDL	1.0	1.0	1.0	1.0	1.0	1.0	0.00	89		

[a]PW = Porewater

[b]Germling cell number = mean of 10 measurements in each replicate.

[c]EC_{50} expressed as sum 2,6-DNT and 2-A-6-NT concentrations at test start

[d]MFS = Millipore filtered seawater (0.45µm)

[e]TX Ref = pore water from standard Texas reference sediment.

[f]MEOH = blank spiked with methanol in same quantity used in 2,6-DNT stock solution

[g]Sample spiked with seawater used as blank for sandy sediment spiked with 2,6-DNT and stored for 180 days, due to similar ammonia content.

[h]NA=Not applicable

[i]BDL=below detection limit

[j]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E6. Toxicity of pore waters from sediments spiked with picric acid to copepod, *Schizopera knabeni,* female survival.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	No. Alive Replicate # 1	2	3	4	5	% Survival Replicate # 1	2	3	4	5	Mean	St. Dev.	Signif.	EC_{50} (µmoles/L)
-	MFS[a]-T1	-	-	-	NA[d]	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
-	TX Ref[b]-T1	-	-	-	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
-	MFS[a]-T2	-	-	-	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
-	TX Ref[b]-T2	-	-	-	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
Sandy	SW[c]	25	0	-	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		50					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Sandy	SW	12.5	28	10	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		25					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		100					5	5	**6**	5	5	100	100	100	100	100	100.0	0.0		
Sandy	SW	12.5	56	10	NA	NA	4	**6**	5	5	5	80	100	100	100	100	96.0	8.9	-	-
		50					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		100					3	5	5	4	5	60	100	100	80	100	88.0	17.9		
Sandy	SW	6.25	28[e]	10	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		12.5					5	5	5	**7**	***4***	100	100	100	100	100	100.0	0.0		
		25					**6**	**6**	**6**	5	***4***	100	100	100	100	100	100.0	0.0		
		50					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	SW	50	0	-	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		100					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	SW	50	7	10	NA	NA	**6**	5	5	***4***	5	100	100	100	100	100	100.0	0.0	-	-
		100					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	SW	12.5	28	10	NA	NA	5	5	5	5	5	100	100	100	100	100	100.0	0.0	-	-
		25					5	5	**6**	5	5	100	100	100	100	100	100.0	0.0		
		50					**6**	5	5	**6**	5	100	100	100	100	100	100.0	0.0		
		100					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Fine-grain	SW	25	0[f]	-	NA	NA	5	5	5	6	5	100	100	100	100	100	100.0	0.0	-	-
		50					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		100					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
Sandy	Picric acid	6.25	0	-	123.23	151.19	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		12.5			246.45	337.99	2	4	4	3	1	40	80	80	60	20	56.0	0.0	**	256.91
		25			492.89	580.31	0	0	**0**	0	0	0	0	0	0	0	0.0	0.0	**	(239.8-275.2)
		50			985.78	980.48	0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	

Appendix E6. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L)		No. Alive Replicate #					% Survival Replicate #					Mean	St. Dev.	Signif.	EC_{50} (µmoles/L)
					Initial	Final	1	2	3	4	5	1	2	3	4	5				
Sandy	Picric acid	3.13	28	10	2.00	BDL	5	5	5	5	5	100	100	100	100	100	100.0	0.0		>32.01 (NC)[g]
		6.25			4.00	6.03	**6**	5	**6**	5	5	100	100	100	100	100	100.0	0.0		
		12.5			8.00	5.52	**6**	5	5	***4***	5	100	100	100	100	100	100.0	0.0		
		25			16.01	13.19	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			32.01	30.00	3	4	5	5	1	60	80	100	100	20	72.0	0.0		
Sandy	Picric acid	1.56	56	10	BDL	BDL	5	**6**	5	5	5	100	100	100	100	100	100.0	0.0		>25% PW[h]
		3.13					5	5	5	***4***	5	100	100	100	100	100	100.0	0.0		
		6.25					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		12.5					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25					5	5	5	5	***4***	100	100	100	100	100	100.0	0.0		
Sandy	Picric acid	1.56	180	20	BDL	BDL	5	5	5	5	5	100	100	100	100	100	100	0.0		-
		3.13					5	5	5	5	5	100	100	100	100	100	100	0.0		
		6.25					5	5	5	5	5	100	100	100	100	100	100	0.0		
		12.5					**7**	**6**	5	5	***5***	100	100	100	100	100	100	0.0		
		25					**6**	**6**	**6**	5	***5***	100	100	100	100	100	100	0.0		
		50					5	**6**	5	**6**	***5***	100	100	100	100	100	100	0.0		
		100					***4***	5	5	**6**	5	100	100	100	100	100	100	0.0		
Fine-grain	Picric acid	6.25	0	-	45.68	38.37	5	5	5	5	5	100	100	100	100	100	100.0	0.0		258.39 (NC)
		12.5			91.35	93.75	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		25			182.71	234.63	5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50			365.42	486.61	0	0	**0**	0	**0**	0	0	0	0	0	0.0	0.0	**	
		100			730.83	776.28	0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	7	10	0.00	0.00	5	5	5	5	5	100	100	100	100	100	100.0	0.0		68.78% PW (66.9-70.7)
		12.5					5	5	5	5	***4***	100	100	100	100	100	100.0	0.0		
		25					5	5	5	5	5	100	100	100	100	100	100.0	0.0		
		50					4	5	5	5	5	80	100	100	100	100	96.0	8.9		
		100					0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	
Fine-grain	Picric acid	1.56	56	10	0.00	0.00	5	5	5	5	5	100	100	100	100	100	100.0	0.0		6.88% PW (6.4-7.4)
		3.13					5	5	5	***4***	5	100	100	100	100	100	100.0	0.0		
		6.25					3	3	3	4	3	60	60	60	80	60	64.0	8.9	**	
		12.5					0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	
		25					0	0	0	0	0	0	0	0	0	0	0.0	0.0	**	

Appendix E6. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L)		No. Alive Replicate #					% Survival Replicate #					Mean	St. Dev.	Signif.	EC_{50} (µmoles/L)
					Initial	Final	1	2	3	4	5	1	2	3	4	5				
Fine-grain	Picric acid	1.56	180	10	BDL	BDL	5	5	5	5	5	100	100	100	100	100	100	0.0		-
		3.13					5	5	5	5	5	100	100	100	100	100	100	0.0		
		6.25					**7**	5	5	5	5	100	100	100	100	100	100	0.0		
		12.5					5	5	5	5	5	100	100	100	100	100	100	0.0		
		25					**6**	5	5	5	**6**	100	100	100	100	100	100	0.0		
		50					5	5	5	5	5	100	100	100	100	100	100	0.0		
		100					5	**6**	5	5	5	100	100	100	100	100	100	0.0		

Bold Italics = Fewer than 5 animals in replicate; **Bold = More than 5 animals in replicate**

[a]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, concurrent to samples from days 0 and 7, T2=Test 2 concurrent to samples from days 28 and 56.

[b]TX Ref = pore water from standard Texas reference sediment.

[c]SW = blank spiked filtered seawater (MFS) in same quantity used in picric acid stock solution

[d]NA=Not applicable.

[e]Used as blank for sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[f]Used as blank for fine-grained sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[g]NC = not calculable

[h]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E7. Toxicity of pore waters from sediments spiked with picric acid to copepod, *Schizopera knabeni,* embryo and nauplii survival.

Sediment	Treatment	PW Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L)		Total Number of Nauplii					Nauplii/Female					Mean	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
							Replicate #					Replicate #									
					Initial	Final	1	2	3	4	5	1	2	3	4	5					
-	MFS[a]-T1		-	-	NA[c]	NA	52	58	54	72	48	10.4	11.6	10.8	14.4	9.6	11.36	1.85	-	-	-
-	TX Ref[b]-T1		-	-	NA	NA	77	63	59	52	67	15.4	12.6	11.8	10.4	13.4	12.72	1.86	-	-	-
-	MFS[a]-T2	-	-	-	NA	NA	59	66	61	68	76	11.8	13.2	12.2	13.6	15.2	13.20	1.33	-	-	-
-	TX Ref[b]-T2	-	-	-	NA	NA	77	55	86	60	85	15.4	11.0	17.2	12.0	17.0	14.52	2.87	-	-	-
Sandy	SW[d]	25	0	-	NA	NA	75	53	66	47	67	15.0	10.6	13.2	9.4	13.4	12.32	2.27	-	-	-
		50					56	36	37	51	42	11.2	7.2	7.4	10.2	8.4	8.88	1.76			
Sandy	SW	12.5	28	10	NA	NA	63	77	71	61	62	12.6	15.4	11.8	12.2	12.4	12.89	1.43	-	-	-
		25					76	55	91	78	68	15.2	11.0	18.2	15.6	13.6	14.72	2.66			
		50					53	40	50	46	51	10.6	8.0	10.0	9.2	10.2	9.60	1.03			
		100					12	5	1	26	1	2.4	1.0	0.2	5.2	0.2	1.80	2.10			
Sandy	SW	12.5	56	10	NA	NA	54	67	60	52	62	10.8	11.2	12.0	10.4	12.4	11.35	0.83	-	-	-
		25					53	58	44	39	47	10.6	11.6	8.8	7.8	9.4	9.64	1.49			
		50					28	16	44	11	45	5.6	3.2	8.8	2.2	9.0	5.76	3.12			
		100					0	0	2	1	4	0.0	0.0	0.4	0.2	0.8	0.28	0.33			
Sandy	SW	6.25	28[e]	10	NA	NA	50	51	86	55	62	10.0	10.2	17.2	11.0	12.4	12.16	2.97	-	-	-
		12.5					48	46	52	61	68	9.6	9.2	10.4	8.7	17.0	10.98	3.42			
		25					51	66	69	74	55	8.5	11.0	11.5	14.8	13.8	11.91	2.47			
		50					25	41	35	18	29	5.0	8.2	7.0	3.6	5.8	5.92	1.78			
Fine-grain	SW	50	0	-	NA	NA	41	46	58	35	37	8.2	9.2	11.6	7.0	7.4	8.68	1.84	-	-	-
		100					36	38	50	9	8	7.2	7.6	10.0	1.8	1.6	5.64	3.75			
Fine-grain	SW	50	7	10	NA	NA	76	37	52	28	26	12.7	7.4	10.4	7.0	5.2	8.53	2.97	-	-	-
		100					1	9	4	5	10	0.2	1.8	0.8	1.0	2.0	1.16	0.74			
Fine-grain	SW	12.5	28	10	NA	NA	74	62	91	73	88	14.8	12.4	18.2	14.6	17.6	15.52	2.38	-	-	-
		25					76	43	71	68	71	15.2	8.6	11.8	13.6	14.2	12.69	2.59			
		50					47	32	29	24	39	7.8	6.4	5.8	4.0	7.8	6.37	1.59			
		100					12	25	19	9	11	2.4	5.0	3.8	1.8	2.2	3.04	1.33			
Fine-grain	SW	25	0[f]	-	NA	NA	44	56	67	69	68	8.8	11.2	13.4	11.5	13.6	11.70	1.95	-	-	-
		50					83	54	47	59	11	16.6	10.8	9.4	11.8	2.2	10.16	5.21			
		100					6	20	5	15	12	1.2	4	1	3	2.4	2.32	1.25			
Sandy	Picric acid	6.25	0	-	123.23	151.19	40	49	32	31	47	8.0	9.8	6.4	6.2	9.4	7.96	1.66	89.64		
		12.5			246.45	337.99	6	4	23	3	3	1.2	0.8	4.6	0.6	0.6	1.56	1.72	17.57	**	184.02
		25			492.89	580.31	3	2	1	0	0	0.6	0.4	0.2	0.0	0.0	0.23	0.26	4.23	**	(172.3-196.6)
		50			985.78	980.48	0	1	0	0	0	0.0	0.2	0.0	0.0	0.0	0.04	0.09	0.72	**	

Appendix E7. Continued.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (^{o}C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	Total Number of Nauplii Replicate # 1	2	3	4	5	Nauplii/Female Replicate # 1	2	3	4	5	Mean	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
		3.13			2.00	BDL	66	62	55	23	68	13.2	12.4	11.0	4.6	13.6	10.96	3.69	82.04		
		6.25			4.00	6.03	21	1	27	17	0	3.5	0.2	4.5	3.4	0.0	2.32	2.07	17.37	**	2.81
Sandy	Picric acid	12.5	28	10	8.00	5.52	23	16	17	9	7	3.8	3.2	3.4	1.8	1.4	2.73	1.06	18.52	**	(2.7-3.0)
		25			16.01	13.19	3	6	13	9	7	0.6	1.2	2.6	1.8	1.4	1.52	0.74	15.83	**	
		50			32.01	30.00	8	4	1	1	9	1.6	0.8	0.2	0.2	1.8	0.92	0.76	51.40[f]	**	
		1.56					36	48	63	19	83	7.2	8.0	12.6	3.8	16.6	9.64	5.00	81.08		
		3.13					60	53	51	69	71	12.0	10.6	10.2	17.3	14.2	12.85	2.92	108.07		9.04% PW[g]
Sandy	Picric acid	6.25	56	10	0.00	0.00	57	58	26	40	48	11.4	11.6	5.2	8.0	9.6	9.16	2.65	77.04		(8.3-9.8)
		12.5					0	15	16	7	15	0.0	3.0	3.2	1.4	3.0	2.12	1.39	21.99	**	
		25					1	0	0	0	2	0.2	0.0	0.0	0.0	0.5	0.14	0.22	2.43	**	
		1.56					75	65	66	52	59	15	13	13.2	10.4	11.8	12.68	1.71	104.28		
		3.13					58	59	68	44	70	11.6	11.8	13.6	8.8	14	11.96	2.06	98.36		
		6.25					38	62	53	59	62	7.6	12.4	10.6	11.8	12.4	10.96	2.02	90.13		28.4% PW
Sandy	Picric acid	12.5	180	20	BDL	BDL	92	34	36	54	63	13.1	5.7	7.2	10.8	12.6	9.88	3.31	81.27		(25.1-32.2)
		25					31	47	34	44	51	5.2	7.8	5.7	8.8	10.2	7.53	2.11	68.61	**	
		50					3	13	2	17	23	0.6	2.2	0.4	2.8	4.6	2.12	1.73	17.80	**	
		100					5	3	1	0	0	1.3	0.6	0.2	0.0	0	0.41	0.53	6.91	**	
		6.25			45.68	38.37	42	9	50	59	10	8.4	1.8	10.0	11.8	2.0	6.80	4.63	78.34		
		12.5			91.35	93.75	29	7	17	17	2	5.8	1.4	3.4	3.4	0.4	2.88	2.09	33.18	**	71.93
Fine-grain	Picric acid	25	0	-	182.71	234.63	5	11	0	4	10	1.0	2.2	0.0	0.8	2.0	1.20	0.91	13.82	**	(63.9-81.0)
		50			365.42	486.61	0	0	1	1	0	0.0	0.0	0.2	0.2	0.0	0.07	0.10	0.84	**	
		100			730.83	776.28	2	3	0	4	3	0.4	0.6	0.0	0.8	0.6	0.48	0.30	8.51	**	
		6.25					45	60	40	63	44	9.0	12.0	8.0	12.6	8.8	10.08	2.07	118.17		
		12.5					65	14	25	30	11	13.0	2.8	5.0	6.0	2.8	5.91	4.21	69.28		17.41% PW
Fine-grain	Picric acid	25	7	10	0.00	0.00	22	6	10	12	7	4.4	1.2	2.0	2.4	1.4	2.28	1.28	26.73	**	(15.9-19.1)
		50					0	0	0	2	1	0.0	0.0	0.0	0.4	0.2	0.12	0.18	2.13	**	
		100					0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	**	
		1.56					24	50	62	32	67	4.8	10.0	12.4	6.4	13.4	9.40	3.73	60.57	**	
		3.13					8	9	0	0	18	1.6	1.8	0.0	0.0	3.6	1.40	1.50	9.02	**	1.84% PW
Fine-grain	Picric acid	6.25	56	10	0.00	0.00	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	**	(1.7-2.0)
		12.5					0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	**	
		25					0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	**	

Appendix E7. Continued.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (^{o}C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	Total Number of Nauplii Replicate # 1	2	3	4	5	Nauplii/Female Replicate # 1	2	3	4	5	Mean	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
Fine-grain	Picric acid	1.56	180	20	BDL	BDL	66	71	61	45	68	13.2	14.2	12.2	9	13.6	12.44	2.06	106.32		41.6% PW (38.2-45.4)
		3.13					73	54	18	53	74	14.6	10.8	3.6	10.6	14.8	10.88	4.54	92.99		
		6.25					67	71	49	62	58	9.6	14.2	9.8	12.4	11.6	11.51	1.92	98.41		
		12.5					51	43	72	41	76	10.2	8.6	14.4	8.2	15.2	11.32	3.28	96.75		
		25					58	62	36	56	69	9.67	12.4	7.2	11.2	11.5	10.39	2.04	88.83		
		50					11	9	17	22	24	2.2	1.8	3.4	4.4	4.8	3.32	1.32	32.68	**	
		100					0	0	0	0	0	0	0	0	0	0	0.00	0.00	0.00	**	

[a]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, concurrent to samples from days 0 and 7, T2=Test 2 concurrent to samples from days 28 and 56.

[b]TX Ref = pore water from standard Texas reference sediment.

[c]NA=Not applicable.

[d]SW = blank spiked filtered seawater (MFS) in same quantity used in picric acid stock solution

[e]Used as blank for fine-grained sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[f]Compared to second 28-day control at 50%, with 5.92 nauplii/female due to confounding factors (ammonia).

[g]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E8. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata,* zoospore germination.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
-	MFS-T1[a]	-	-	-	NA[c]	NA	88	95	92	96	95	93.2	3.3	100.0	-	-
-	TX Ref-T1[b]	-	-	-	NA	NA	91	96	-	90	92	92.3	2.6	99.0	-	-
-	MFS-T2[a]	-	-	-	NA	NA	5.9	5.4	5.9	5.5	5.7	5.68	0.23	100.0	-	-
-	TX Ref-T2[b]	-	-	-	NA	NA	4.9	5.1	4.8	5.3	6.0	5.22	0.48	100.0	-	-
Sandy	SW[d]	25.0	0	-	NA	NA	90	87	86	89	83	87.0	2.7	-	-	-
		50.0					88	87	91	86	-	88.0	2.2			
		100.0					66	77	80	71	74	73.6	5.4			
Sandy	SW	50.0	28	10	NA	NA	59	81	63	77	na	70.0	10.6	-	-	-
		100.0					36	58	61	47	39	48.2	11.1			
Sandy	SW	50.0	56	10	NA	NA	79	82	85	89	na	83.8	4.3	-	-	-
		100.0					51	43	45	50	78	53.4	14.2			
Sandy	SW	50.0	28[e]	20	NA	NA	90	89	94	84	85	88.4	4.04	-	-	-
Fine-grain	SW	50.0	0	-	NA	NA	91	90	91	93	85	90.0	3.0	-	-	-
		100.0					77	80	83	80	92	82.4	5.8			
Fine-grain	SW	50.0	7	10	NA	NA	91	92	94	-	-	92.3	1.5	-	-	-
		100.0					94	80	83	88	89	86.8	5.4			
Fine-grain	SW	50.0	56	10	NA	NA	81	83	87	84	86	84.2	2.4	-	-	-
		100.0					70	88	87	85	74	80.8	8.2			
Fine-grain	SW	100.0	1[f]	10	NA	NA	93	97	98	93	94	95.0	2.35	-	-	-
Sandy	Picric acid	6.25	0	-	115.49	158.71	87	77	66	69	83	76.4	8.9	87.8	*	569.24 (514.8-629.4)
		12.5			230.97	363.78	72	77	71	80	75	75.0	3.7	86.2	*	
		25			461.94	606.28	77	70	67	50	85	69.8	13.1	80.2	**	
		50			923.89	990.57	0	0	0	0	0	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	28	10	5.12	4.50	82	77	87	71	79	79.2	5.9	113.1		35.16 (32.1-38.5)
		12.5			10.24	4.60	77	65	79	80	81	76.4	6.5	109.1		
		25.0			20.47	18.91	37	30	55	71	69	52.4	18.5	74.9		
		50.0			40.95	39.53	18	25	61	32	50	37.2	17.9	53.1		
		100.0			81.90	71.52	0	0	-	-	-	0.0	0.0	0.0	**	

Appendix E8. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
Sandy	Picric acid	6.25	56	10	0.30	BDL	87	89	88	85	93	88.4	3.0	105.5		2.89 (2.72-3.06)
		12.5			0.60	BDL	85	80	94	87	92	87.6	5.6	104.5		
		25.0			1.21	0.93	86	87	81	82	89	85.0	3.4	101.4		
		50.0			2.41	2.84	57	44	78	70	71	64.0	13.5	76.4	**	
		100.0			4.82	3.53	0	0	-	-	-	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	180	20	BDL	BDL	74	67	72	76	58	69.4	7.20	79	**	53.12% PW[g] (44.7-63.1)
		12.5			BDL	BDL	46	69	48	66	57	57.2	10.33	65	**	
		25.0			BDL	BDL	64	67	71	72	70	68.8	3.27	78	**	
		50.0			BDL	BDL	40	46	52	44	48	46.0	4.47	52	**	
		100.0			BDL	BDL	16	12	21	28	26	20.6	6.69	27	**	
Fine-grain	Picric acid	6.25	0	-	50.01	34.74	93	92	94	89	91	91.8	1.9	102.0		307.41 (280.5-336.9)
		12.5			100.01	94.29	87	81	91	90	84	86.6	4.2	96.2		
		25.0			200.02	252.71	61	60	72	78	86	71.4	11.1	79.3	*	
		50.0			400.04	479.18	12	13	65	11	66	33.4	29.3	37.1	**	
		100.0			800.08	767.19	0	0	-	-	-	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	7	10	BDL	BDL	87	91	88	93	96	91.0	3.7	98.6		50.89 % PW[g] (46.9-55.2)
		12.5			BDL	BDL	83	71	89	90	91	84.8	8.3	91.9		
		25.0			BDL	BDL	87	92	93	93	89	90.8	2.7	98.4		
		50.0			BDL	BDL	36	41	54	73	77	56.2	18.4	60.9	**	
		100.0			BDL	BDL	0	0	-	-	-	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	56	10	BDL	BDL	0	0	0	-	-	0.0	0.0	0.0	**	<6.25 % PW[g]
		12.5			BDL	BDL	0	0	-	-	-	0.0	0.0	0.0	**	
		25.0			BDL	BDL	0	0	-	-	-	0.0	0.0	0.0	**	
		50.0			BDL	BDL	0	0	-	-	-	0.0	0.0	0.0	**	
		100.0			BDL	BDL	0	0	-	-	-	0.0	0.0	0.0	**	

Appendix E8. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	% Germination Replicate # 1	2	3	4	5	Mean % Germ.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
Fine-grain	Picric acid	6.25	180	10	BDL	BDL	96	94	93	90	94	93.4	2.19	98		>100% PW[g]
		12.5			BDL	BDL	96	95	97	97	92	95.4	2.07	100		
		25.0			BDL	BDL	95	98	99	97	97	97.2	1.48	102		
		50.0			BDL	BDL	91	98	95	97	99	96.0	3.16	101		
		100.0			BDL	BDL	97	94	95	90	95	94.2	2.59	99		

[a]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, conducted concurrently to samples from days 0, 7, 28 and 56; T2=Test 2, conducted concurrently to samples from day 180.

[b]TX Ref = pore water from standard Texas reference sediment.

[c]NA=Not applicable.

[d]SW = blank spiked with MFS in same quantity used in picric acid stock solution

[e]Used as blank for sandy sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[f]Used as blank for fine-grained sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[g]EC_{50} given in % porewater when chemical concentrations were below detection.

Appendix E9. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata,* germling length.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L)		Germling Length[a] Replicate #					Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
					Initial	Final	1	2	3	4	5					
-	MFS-T1[b]	-	-	-	NA[d]	NA	67.4	62.4	65.9	59.8	67.4	64.6	3.4	-	-	-
-	TX Ref-T1[c]	-	-	-	NA	NA	46.1	57.3	-	54.8	69.5	56.9	9.6	-	-	-
-	MFS-T2[b]	-	-	-	NA	NA	41.6	40.1	43.6	36	42.6	40.76	2.97	-	-	-
-	TX Ref-T2[c]	-	-	-	NA	NA	37.5	45.6	45.1	46.1	48.7	44.62	4.20	-	-	-
Sandy	SW[e]	25.0	0	-	NA	NA	58.8	69.0	80.1	69.0	71.0	69.6	7.6	-	-	-
		50.0					68.4	59.8	63.4	65.4	-	64.3	3.6			
		100.0					60.3	55.8	52.2	49.2	55.8	54.7	4.2			
Sandy	SW	50.0	28	10	NA	NA	77.6	87.2	84.7	83.1	-	83.1	4.1	-	-	-
		100.0					50.7	60.3	74.0	63.9	63.9	62.6	8.4			
Sandy	SW	50.0	56	10	NA	NA	73.0	78.6	81.1	76.1	-	77.2	3.5	-	-	-
		100.0					55.3	38.5	70.0	54.8	63.4	56.4	11.8			
Sandy	SW	50.0	28[f]	20	NA	NA	69.3	66.4	68.5	76.1	65.9	69.22	4.06	100.0	-	-
Fine-grain	SW	50.0	0	-	NA	NA	85.7	94.3	83.1	83.7	88.7	87.1	4.6	-	-	-
		100.0					16.2	25.4	51.7	22.8	37.5	30.7	14.0			
Fine-grain	SW	50.0	7	10	NA	NA	68.4	65.4	82.6	-	-	72.2	9.2	-	-	-
		100.0					47.7	43.6	54.8	53.2	58.8	51.6	6.0			
Fine-grain	SW	50.0	56	10	NA	NA	14.7	13.2	14.2	46.1	16.7	21.0	14.1	-	-	-
		100.0					10.4	9.9	11.2	9.6	10.1	10.2	0.6			
Fine-grain	SW	100.0	1[g]	10	NA	NA	59.8	54.3	61.4	63.9	65.9	61.04	4.46	-	-	-
Sandy	Picric acid	6.25	0	-	115.49	158.71	12.7	19.8	20.8	18.8	28.9	20.2	5.8	29.0	**	<115.486
		12.5			230.97	363.78	9.6	16.7	23.3	20.8	21.3	18.4	5.4	26.4	**	
		25.0			461.94	606.28	10.1	10.4	14.2	8.1	18.3	12.2	4.0	17.6	**	
		50.0			923.89	990.57	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	28	10	5.12	4.50	21.3	26.1	27.4	28.4	28.4	26.3	3.0	31.7	**	<5.119
		12.5			10.24	4.60	10.1	12.2	17.2	18.3	19.3	15.4	4.0	18.5	**	
		25.0			20.47	18.91	7.6	9.6	10.6	12.2	11.7	10.3	1.8	12.4	**	
		50.0			40.95	39.53	8.6	6.6	8.6	8.6	8.1	8.1	0.9	9.8	**	
		100.0			81.90	71.52	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	56	10	0.30	BDL	17.2	29.4	22.3	26.9	30.7	25.3	5.5	32.8	**	<0.301
		12.5			0.60	BDL	11.2	19.8	28.9	23.8	20.8	20.9	6.5	27.1	**	
		25.0			1.21	0.93	11.2	12.2	14.7	12.4	11.7	12.4	1.4	16.1	**	
		50.0			2.41	2.84	6.3	7.6	9.1	9.6	8.4	8.2	1.3	10.6	**	
		100.0			4.82	3.53	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	

Appendix E9. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	Germling Length[a] Replicate # 1	2	3	4	5	Mean Germling Length	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
Sandy	Picric acid	6.25	180	20	BDL	BDL	46.1	47.7	58.3	47.2	31.9	46.2	9.39	67	**	>100%[h]
		12.5			BDL	BDL	48.7	48.7	35.5	43.6	53.7	46.0	6.90	67	**	
		25.0			BDL	BDL	48.2	58.3	64.4	50.2	52.7	54.8	6.59	79	**	
		50.0			BDL	BDL	36	30.9	37	38	61.9	40.8	12.10	59	**	
		100.0			BDL	BDL	14.2	15.2	19.8	21.8	20.8	18.4	3.43	64	**	
Fine-grain	Picric acid	6.25	0	-	50.01	34.74	21.3	18.8	22.8	27.9	34.5	25.0	6.2	28.8	**	<50.005
		12.5			100.01	94.29	9.6	11.2	13.2	23.8	24.8	16.5	7.2	19.0	**	
		25.0			200.02	252.71	8.1	9.6	9.1	13.2	18.3	11.7	4.1	13.4	**	
		50.0			400.04	479.18	6.6	7.6	7.1	9.1	11.7	8.4	2.0	9.7	**	
		100.0			800.08	767.19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	7	10	BDL	BDL	13.7	13.7	16.7	26.9	29.4	20.1	7.5	27.8	**	<6.25%[h]
		12.5			BDL	BDL	9.4	11.7	13.7	23.8	24.3	16.6	7.0	23.0	**	
		25.0			BDL	BDL	9.6	11.2	10.6	21.8	22.8	15.2	6.5	21.1	**	
		50.0			BDL	BDL	8.6	8.4	6.6	11.2	12.2	9.4	2.3	13.0	**	
		100.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	56	10	BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	<6.25%[h]
		12.5			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		25.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		50.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		100.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	180	10	BDL	BDL	66.9	70	83.2	72	80.6	74.5	7.01	122		>100%[h]
		12.5			BDL	BDL	72	69	86.2	78.6	79.1	77.0	6.73	126		
		25.0			BDL	BDL	81.6	77.1	80.1	94.8	77.6	82.2	7.27	135		
		50.0			BDL	BDL	66.4	82.6	73	75.5	73	74.1	5.84	121		
		100.0			BDL	BDL	63.9	59.3	58.3	63.9	62.4	61.6	2.60	101		

[a]Germling length = mean of 10 measurements in each replicate.
[b]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, conducted concurrently to samples from days 0, 7, 28 and 56; T2=Test 2, conducted concurrently to samples from day 180.
[c]TX Ref = pore water from standard Texas reference sediment.
[d]NA=Not applicable.
[e]SW = blank spiked with MFS in same quantity used in picric acid stock solution
[f]Used as blank for sandy sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.
[g]Used as blank for fine-grained sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.
[h]Result in % pore water, since measured chemical was below detection.

Appendix E10. Toxicity of pore waters from sediments spiked with picric acid to macroalga, *Ulva fasciata,* germling cell number.

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	Germling Cell No.[a] Replicate # 1	2	3	4	5	Mean Germling Cell No.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
-	MFS-T1[b]	-	-	-	NA[d]	NA	6.6	6.2	5.9	5.5	6.4	6.1	0.4	-	-	-
-	TX Ref-T1[c]	-	-	-	NA	NA	4.3	5.7		5.1	6.3	5.4	0.9	-	-	-
-	MFS-T2[b]	-	-	-	NA	NA	5.9	5.4	5.9	5.5	5.7	5.68	0.23	100.0	-	-
-	TX Ref-T2[c]	-	-	-	NA	NA	4.9	5.1	4.8	5.3	6.0	5.22	0.48	100.0	-	-
Sandy	SW[e]	25.0	0	-	NA	NA	6.2	7.3	8.8	7.4	6.9	7.3	1.0	-	-	-
		50.0					8.1	7.4	7.8	7.1		7.6	0.4			
		100.0					7.4	6.6	6.5	5.2	7.1	6.6	0.8			
Sandy	SW	50.0	28	10	NA	NA	9.5	12.3	11.3	11.8		11.2	1.2	-	-	-
		100.0					6.1	7.7	8.1	6.9	7.5	7.3	0.8			
Sandy	SW	50.0	56	10	NA	NA	9.5	10.7	10.9	10.2		10.3	0.6	-	-	-
		100.0					6.4	4.2	9.3	6.4	8.5	7.0	2.0			
Sandy	SW	50.0	28[f]	20	NA	NA	9.44	9.1	9.9	9.78	9.7	9.58	0.32	100.0	-	-
Fine-grain	SW	50.0	0	-	NA	NA	11.1	12.7	11.0	10.2	11.9	11.4	1.0	-	-	-
		100.0					1.7	2.7	4.9	2.2	4.6	3.2	1.4			
Fine-grain	SW	50.0	7	10	NA	NA	8.1	8.5	10.4			9.0	1.2	-	-	-
		100.0					5.0	4.9	6.2	6.1	6.7	5.8	0.8			
Fine-grain	SW	50.0	56	10	NA	NA	1.8	1.3	1.5	3.9	2.0	2.1	1.0	-	-	-
		100.0					1.0	1.0	1.0	1.0	1.1	1.0	0.0			
Fine-grain	SW	100.0	1[g]	10	NA	NA	7.1	7.6	8.7	8.5	8.4	8.06	0.68	100.0	-	-
Sandy	Picric acid	6.25	0	-	115.49	158.71	1.5	2.1	2.3	2.0	2.7	2.1	0.4	29.0	**	<115.486
		12.5			230.97	363.78	1.0	2.1	2.6	2.7	2.7	2.2	0.7	30.4	**	
		25			461.94	606.28	1.0	1.2	1.7	1.0	2.1	1.4	0.5	19.2	**	
		50			923.89	990.57	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	28	10	5.12	4.50	2.2	2.7	2.8	3.0	3.0	2.7	0.3	3.8	**	<5.119
		12.5			10.24	4.60	1.0	1.5	2.3	2.4	2.3	1.9	0.6	2.7	**	
		25.0			20.47	18.91	1.0	1.0	1.3	1.6	1.5	1.3	0.3	1.8	**	
		50.0			40.95	39.53	1.1	1.0	1.0	1.0	1.0	1.0	0.0	1.4	**	
		100.0			81.90	71.52	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Sandy	Picric acid	6.25	56	10	0.30	BDL	2.0	3.2	2.4	2.7	3.1	2.7	0.5	26.0	**	<0.301
		12.5			0.60	BDL	1.2	2.4	2.8	2.6	2.5	2.3	0.6	22.3	**	
		25.0			1.21	0.93	1.1	1.3	1.6	1.3	1.1	1.3	0.2	12.4	**	
		50.0			2.41	2.84	1.0	1.0	1.0	1.0	1.0	1.0	0.0	9.7	**	
		100.0			4.82	3.53	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	

Appendix E10. Continued

Sediment	Treatment	Pore water Conc. (%)	Storage Time (days)	Storage Temp. (°C)	Measured Conc. (µmoles/L) Initial	Measured Conc. (µmoles/L) Final	Germling Cell No.[a] Replicate # 1	2	3	4	5	Mean Germling Cell No.	St. Dev.	% of Control	Signif.	EC_{50} (µmoles/L)
Sandy	Picric acid	6.25	180	20	BDL	BDL	6.1	6.5	8.0	6.1	3.5	6.0	1.62	63	**	>100% PW[h]
		12.5			BDL	BDL	7.4	7.0	5.1	5.8	7.6	6.6	1.08	69	**	
		25.0			BDL	BDL	6.8	8.8	9.5	7.3	8.1	8.1	1.09	85		
		50.0			BDL	BDL	4.7	4.5	5.0	5.1	8.7	5.6	1.75	58	**	
		100.0			BDL	BDL	1.7	1.9	2.4	2.8	2.8	2.3	0.51	62	**	
Fine-grain	Picric acid	6.25	0	-	50.01	34.74	2.2	1.9	2.2	2.7	3.0	2.4	0.4	21.1	**	<50.005
		12.5			100.01	94.29	1.0	1.0	1.4	2.4	2.7	1.7	0.8	14.9	**	
		25.0			200.02	252.71	1.0	1.0	1.0	1.5	1.9	1.3	0.4	11.2	**	
		50.0			400.04	479.18	1.0	1.0	1.0	1.0	1.2	1.0	0.1	9.1	**	
		100.0			800.08	767.19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	7	10	BDL	BDL	1.3	1.3	1.8	2.8	2.9	2.0	0.8	22.4	**	<6.25% PW[h]
		12.5			BDL	BDL	1.0	1.2	1.4	2.7	2.8	1.8	0.9	20.2	**	
		25.0			BDL	BDL	1.0	1.2	1.1	2.7	2.7	1.7	0.9	19.3	**	
		50.0			BDL	BDL	1.0	1.0	1.0	1.3	1.6	1.2	0.3	13.1	**	
		100.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	56	10	BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	<6.25% PW[h]
		12.5			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		25.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		50.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
		100.0			BDL	BDL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**	
Fine-grain	Picric acid	6.25	180	10	BDL	BDL	9.0	10.4	11.1	9.4	11.0	10.2	0.94	126		>100% PW[h]
		12.5			BDL	BDL	10.4	9.4	11.8	10.8	11.5	10.8	0.95	134		
		25.0			BDL	BDL	10.7	10.9	10.1	14.3	11.2	11.4	1.65	142		
		50.0			BDL	BDL	9.7	12.4	10.6	10.4	10.2	10.7	1.03	132		
		100.0			BDL	BDL	9.2	7.8	8.0	9.2	8.8	8.6	0.66	107		

[a]Germling cell number = mean of 10 measurements in each replicate.

[b]MFS = Millipore filtered seawater (0.45µm); T1=Test 1, conducted concurrently to samples from days 0, 7, 28 and 56; T2=Test 2, conducted concurrently to samples from day 180.

[c]TX Ref = pore water from standard Texas reference sediment.

[d]NA=Not applicable.

[e]SW = blank spiked with MFS in same quantity used in picric acid stock solution

[f]Used as blank for sandy sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[g]Used as blank for fine-grained sediment spiked with picric acid and stored for 180 days, due to similar ammonia content.

[h]Result in % pore water, since measured chemical was below detection.

ATTACHMENT 1

SOP 10.24

Extraction and storage of porewater samples using *in situ* devices

Date Prepared: October 1, 2001

Date Revised: October 11, 2001

EXTRACTION AND STORAGE OF POREWATER SAMPLES USING *IN SITU* DEVICES

1.0 OBJECTIVE

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

2.0 PREPARATION

2.1 Description of the Porewater Extraction System

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

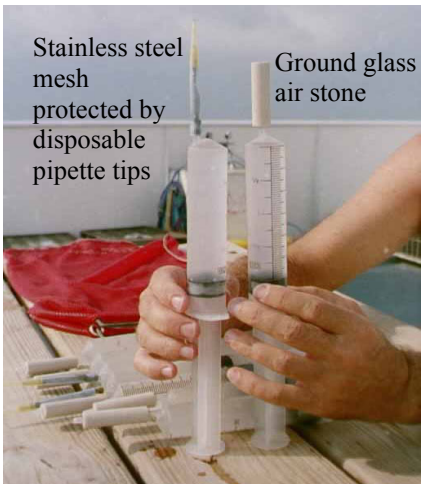

Figure 1. Porewater extraction devices with two types of filtration media.

2.2 Equipment List

Supplies and equipment needed are listed in Attachment 1.

3.0 PROCEDURE

3.1 *In Situ* Procedures

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

3.1.1 *In situ* porewater extraction

This procedure was developed for use at sites with coarse sands, typically in the vicinity of coral reefs. Eight to ten 60 mL syringes are usually sufficient to obtain the necessary amount of pore water for toxicity testing and water quality assessment. The syringes with attached connectors and air stones attached to a short length (~ 0.3 inch) of aquarium air tubing are placed in a mesh bag or other suitable container for transport to the sampling location by divers. Once at the correct location, one air stone (or other selected filtration medium) is attached to each syringe. The device is gently inserted into the sediment until at least the whole filtration medium and connecting parts are buried in the sediment, ideally until the bottom 10 or 20 mL of the syringe are also buried. The total buried depth should approximate 10 cm. Once this is achieved, vacuum should be applied through the syringe, promoting the suction of pore water into the syringe. When the syringe is full, the filtration medium should be carefully removed from the sediment, the whole device placed in the bag, and taken to the surface. Should the air stone be broken or lost in the sediment, the extracted pore water will still remain in the syringe and can be taken to the surface and transferred to appropriate jars as described below. If necessary, the same air stone can be removed from a full syringe and attached to an empty one for further porewater extraction.
Once on board of the research vessel, the filtration medium is removed from the tip of the syringe and the pore water is gently squirted into clean, pre-labeled polycarbonate centrifuge bottles. This procedure has to be done very slowly to avoid excessive aeration of the sample, which may promote alterations of the equilibrium between water, particles, and contaminants. Samples must then be stored on ice until arrival at the laboratory. Pore water should be centrifuged as soon as possible after extraction (section 3.2).

3.1.2 Sediment coring

This procedure should be used when fine grain sediments are encountered and *in situ* porewater extraction is not practicable. The upper 10 cm of sediment are collected by multiple cores at each sampling station. Prior to leaving the research vessel, the coring tubes may be placed inside a bucket with screw cap and holes in the lid to allow water intake and easy

submersion. Once the divers reach the sampling location, the lexane™ coring tubes are inserted in the sediment to a depth of approximately 10 cm. Once the insertion is complete, the stoppers are placed into the upper part of the tube and the core is extracted from the surrounding sediment. The cap is placed in the lower part of the tube as soon as it emerges from the sediment. The filled cores are placed in the bucket and taken back to the research vessel.

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

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

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

3.2 Centrifugation of Porewater Samples

Porewater samples used for toxicity testing at the Marine Ecotoxicology Research Station are usually stored frozen until tested. The porewater samples should be centrifuged shortly after they are collected and before they are frozen.

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

2. Place the polycarbonate bottle in the centrifuge and spin at ∃1200 *g* for 20 minutes. Decant the supernatant from the centrifuged sample to a pre-cleaned and labeled jar, taking care not to disturb any material that may have settled on the bottom/sides of the centrifuge bottle.

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

3.3 Storage of Porewater Samples

If the porewater samples are not to be used on the day of collection, they should be frozen for storage. Sufficient room for freeze expansion should be left in the jars (for example, 200 mL maximum sample in a 250 mL jar). If the volume needed for testing is known in advance, it is prudent to allocate only that specific volume plus a little excess (−10 mL) to each jar in order to conserve pore water (once thawed, the pore water cannot be refrozen and reused), and to simplify the volume measurements required for Water Quality Adjustment of Samples (SOP F10.12) performed the day prior to testing. Frozen porewater samples may be shipped with dry ice.

4.0 QUALITY CONTROL

A sample tracking system is maintained for each sediment sample collected and porewater sample extracted. All actions taken with that respective sample are recorded on the Sample History Data Form (Attachment 2). This information includes, but not exclusively: a) the date of collection or receipt, b) the date of porewater extraction, c) the date of porewater centrifugation and relevant information about this procedure, d) the volume or number of jars of pore water collected, e) date frozen and location (freezer number or other relevant information), and f) date and jar number thawed and used in which test. The Sample History Forms are kept in a three-ring binder at the same location where the samples are stored.

5.0 TRAINING

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

6.0 SAFETY

The sediment and porewater samples handled may contain contaminants. Care should be taken to avoid contact with the samples. Protective gloves, glasses and clothing may be worn. Waste sediment should be properly disposed.

7.0 ATTACHMENTS

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

8.0 REFERENCES

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

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

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

Prepared by:

_____
Marion Nipper
Senior Research Scientist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Laverne Cleveland
Chief, Field Research Division

Attachment 1

REQUIRED EQUIPMENT AND MATERIALS

For porewater extraction:

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

For sediment coring:

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

Other required supplies/equipment:

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

Attachment 2

SAMPLE HISTORY DATA FORM

Sample Designation: _____ Study Protocol: _____ Initials: _____

Date of acquisition: _____ Sample type: _____

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

Initials	Date	Action Taken

ATTACHMENT 2

SOP 10. 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 200C 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 20^0C 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 20oC.

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 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.)

$$\text{Ocular Units} * 2.57 = \text{germling length } (\mu m)$$

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.

Kapraun, D.F. 1970. Field and cultural studies of *Ulva* and *Enteromorpha* in the vicinity of Port Aransas, Texas. Contr Mar Sci 15:205-285

Reed DC, Neushul M, Ebeling AW (1991) Role of settlement density on gametophyte growth and reproduction in the kelps *Pterygophera californica* and *Macrocystis pyrifera* (Phaeophyceae) J Phycol 27:361-366

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

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

Prepared by:

_____
Russell Hooten
Research Assistant

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Laverne Cleveland
Branch Chief

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	

ATTACHMENT 3

SOP 10.25

Culture and toxicity testing methods for *Schizopera knabeni* (Copepoda, Harpacticoida, Diosaccidae)

Date Prepared: February 7, 2002

Date Revised: April 23, 2003

CULTURE AND TOXICITY TESTING METHODS FOR *Schizopera knabeni* (COPEPODA, HARPACTICOIDA, DIOSACCIDAE)

1.0 OBJECTIVE

The purpose of the *Schizopera knabeni* toxicity test is to determine if a sediment, pore water, sea water, sea surface microlayer, or other sample reduces survival and/or reproduction in exposed *S. knabeni* copepods relative to those exposed to a reference sample. The test may also be used to determine the concentration of a test substance that reduces survival or reproduction. Reproduction is expressed as nauplii hatching and survival after exposure of gravid females to the test treatments. Test results are reported as treatment (or concentration) that produces statistically significant reduced survival or reproduction.

2.0 LABORATORY CULTURE

The maintenance of test organisms in permanent laboratory cultures facilitates their use in toxicity tests. The culture kept at MERS, initiated in February 2002, was donated by Dr. Guilherme Lotufo (ACE, Vicksburg, Mississipi). Organisms were originally obtained from the surface sediment of intertidal mudflats in a *Spartina alterniflora* salt marsh at Port Fourchon, Louisiana, and have been in laboratory culture since 1993. Organisms arrived at MERS in seawater at 15 ppt salinity, and were kept at this salinity for a week, after which salinity was gradually increased to 30 ppt, with no more than a 5 ppt increase per week.

Cultures are maintained in 750 ml of 30 ppt seawater (Millipore® filtered through 0.45 μm mesh) in 1 L Erlenmeyer flasks sealed with cotton or gauze stoppers, with mild aeration, at room temperature (22-28°C). If a different salinity is needed for a toxicity test, cultures should be slowly acclimated to the desired salinity, with changes of no more that 5 ppt per week. The seawater in the cultures should be fully exchanged weekly. For water exchange, pour all the content from each flask through a 48 μm mesh into a container with some filtered seawater at 30 ppt to minimize stress to the animals. All developmental stages of *S. knabeni* are retained in this pore size. Rinse the material retained on the sieve into the container, gently applying seawater with a squirt bottle if necessary. Add approximately 700 ml filtered seawater at 30 ppt to a 1L Erlenmeyer flask and add the animals, bringing the volume up to 750 ml.

Each culture flask should be fed twice a week with approximately 36mg dry weight of a mixture of at least 3 microalgae species. Microalgae species to be used for feeding can be species of the genus *Chaetoceros, Phaeodactylum, Isochrysis, Tetraselmis, Dunaliella, Nannochloropsis* or *Thallasiosira*. In July 2002 the cultures were being fed *Nannochloropsis oculata*, *Thalassiosira pseudonana* and *Isochrysis sp*., or spinach only. This procedure was modified in September 2002, when cultures started to be fed a pre-prepared mixture called pre-set 1800, from Reed Mariculture (San Jose, CA), composed of 30% *Isochrysis* sp., 40% *Pavlova* sp. 15% *Thalassiosira weissflogii*, and 15% *Nannochloropsis oculata*, with a dry weight of 9%.

Cultures not used to obtain copepods for toxicity tests should be renewed every 10 weeks. The contents of a flask should be sieved through 125 µm mesh and mature, preferably egg-bearing, females sorted under a dissecting microscope. Groups of 100 females are transferred to new culture flasks.

2.1 Preparation of algae to feed cultures

The algae mix needs to be diluted in seawater prior to addition to the cultures, for osmotic equilibration. Firstly mix equal volumes of the algae mix and Millipore filtered seawater (MFS). Swirl and let sit for 30 seconds. Add twice as much MFS to the mixture and swirl. Add 1.6 ml of this mixture to each culture flask.

3.0 TOXICITY TEST

Copepods are initially obtained by sieving the contents of one or more culture flasks through a 125 µm mesh. The retained material is analyzed under a dissecting microscope to identify egg-bearing females. Although most adult copepods are retained on the sieve, nauplii and copepodites (juvenile stages) are not. The latter stages can be obtained separated from most adults, if desired, by sieving the material sequentially through a 125 µm and a 48 µm mesh.

Copepods retrieved from the culture system are placed on cell culturing dishes and observed under a dissecting microscope. A light source from underneath is preferable. Picking a specific number of copepods is difficult and requires patience and practice. Having few copepods in the dish at a time facilitates the work. Swimming copepods can be picked with a Pasteur pipette and transferred to another dish half-filled with seawater.

3.1 Liquid Phase Toxicity Tests

Disposable 20-ml scintillation vials should be used as test vials, and a minimum of 5 replicates per treatment should be prepared. Each vial receives 5 ml of the test sample and after all vials are prepared they are transferred to an incubator at 20ºC.

Pore water can be obtained as described in SOP F10.9 or F10.24, and water quality measured and following procedures in SOP F10.12. Test salinity should be the same as that of the cultures, i.e., 30 ppt.

Egg-bearing females should be sorted from the cultures no more that 5 hours before test initiation, and transferred with a wide-mouthed pipette to a Petri dish. Five egg-bearing females are added to each scintillation vial, with the minimum possible amount of water. Test vials are kept at 20ºC, in the dark, for 96 hours after the addition of the females. Animals should be fed 0.24 mg dry weight of live micro-algae per vial at the beginning of the test, i.e., 10 µl of the diluted algae mix (see section 2.1). If a different food source is used, algae must be concentrated in a volume ≤ 100 µl.

A reference chemical test should be performed concurrently with every series of tests conducted with environmental samples. The LC_{50}-96h in the test with the reference chemical should be within the limits established by the laboratory's warning chart.

Endpoints of the aqueous phase toxicity test are female survival and egg hatching success (embryo survival). The contents of the test vials should be transferred to small dishes and observed under dissecting microscope at test termination. Alive adult females (presenting spontaneous movement or reacting to a gentle touch or blow of air from the tip of a pipette) are counted and registered as alive. For counts at the end of the test, alive females can be registered into 3 catgories: egg-bearing, non-egg-bearing or moribund. The latter are females that are mobile but not freely swimming, just presenting slight movement after a gentle touch or blow of air. The contents of the vial should then be preserved with 1 ml of formaldehyde at 10% mixed with rose Bengal. After 2-3 days the copepods should have an intense red color and the contents of the test vial should then be sieved through a 48 µm mesh and washed with filtered seawater to remove the formaldehyde. Handling of samples containing formaldehyde must the done in a fume hood and wearing protective gear. Retained material is analyzed under a dissecting microscope and the nauplii are counted on plankton counting slides or dishes. The plankton counting slide ordish must be totally scanned and the number of nauplii registered with counters. Due to their small size, nauplii observation requires a minimum magnification of 30x and careful observation of the whole bottom of the dish.

A test should be discarded if: control mortality is >70%; no nauplii hatch in the control; the LC_{50}-96h in the test with the reference chemical is not within acceptable limits.

3.2 Tests with Reference Chemicals

Several chemicals, such as sodium dodecyl sulfate (SDS), potassium dichromate, zinc sulfate and cadmium chloride have been used as reference chemicals with other species. For the establishment of result repeatability, a minimum of 5 tests with a same chemical is recommended (Weber *et al.*, 1989). Tests with reference chemicals should have a

minimum of 3 replicates per treatment. The procedure must be identical to that of other aqueous phase tests. The EC_{50}-96h is then calculated and plotted on a warning chart.

3.3 Toxicity Tests with Sediments

Approximately 10 g of sediment per treatment are sufficient for toxicity tests. Sediments should be press-sieved through a 0.5 mm mesh prior to storage and testing. A non-contaminated sediment with which the copepods are compatible should be used as control, and reference sediments with similar features to those of the test sediments should also be used.

Toxicity tests can also be done with dilution of the contaminated sediment to be tested with the control sediment. The concentrations for serial dilutions are prepared by mixing the control and the contaminated sediment on a volume/volume or weight/weight basis, prior to test initiation. Sediments must be mixed until homogeneous color and texture are observed.

Twenty-ml glass scintillation vials can be used as test vials. At least 5 replicates per treatment are recommended. Approximately 2 g of sediment are added to each vial, creating an approximately 0.5 cm sediment layer. A 15 ml volume of the same water used for the culture is then gently added to each vial, making sure that the sediment is not disturbed. Boxes containing the test vials are kept at 20ºC for 24 hours prior to test initiation.

Egg-bearing females, or those with mature gonads, visible as two brownish longitudinal stripes, should be used as test organisms. Females should be sorted from the cultures less than 5 hours prior to test initiation, and transferred with a pipette to Petri dishes. If enough egg-bearing females are available, only those should be utilized. Ten females are added to each test vial. If it is necessary to use non-egg-bearing females, it is recommended that the proportion to of egg-bearing and mature females is kept constant among replicates.

After copepod addition, the test vials should be kept at 20ºC for 10 days. Food should be provided immediately after copepod addition to the vials. A volume of 0.1 ml of a mixture of yeast and water (3 mg/ml) is used as food. No aeration is necessary.

A reference chemical test in aqueous phase should be run concurrently to each sediment test, following the procedure described previously. The LC_{50}-96h in this test must be within the established acceptable limits.

Test endpoints are female survival and reproductive success. During the 10-day exposure period females in the control sediment typically produce 3 generations of offspring.

Effects of test treatments on the formation, development and survival of embryos, larvae and juveniles will define the number of offspring produced during the 10-day exposure.

At test termination 1 ml of formaldehyde at 10% mixed with rose bengal or floxin is added to each vial each vial for copepod preservation and dyeing. After 2-3 days the copepods should have an intense red color and the contents of the test vial should then be sieved through a 48 µm mesh to remove fine silt and clay. Retained material is analyzed under a dissecting microscope and the adult copepods, nauplii and copepodites are counted on plankton counting slides. Alive adult females are removed, registered as egg-bearing or not, and counted. The plankton counting slide must be totally scanned and the number of nauplii and copepodites registered with counters. Handling of samples containing formaldehyde must the done in a fume hood and wearing protective gear. As an additional endpoint, eggs contained in the egg-bearing females brood sacs can also be counted and added to the fecundity results of each replicate (nauplii + copepodites). However, some of the eggs may not be viable and, therefore, the use of this endpoint might lead to incorrect interpretation of the results. If eggs are counted, the egg sac must be removed from the females and the number of eggs counted under the maximum magnification of the dissecting microscope. A test should be discarded if control survival is <70% and/or if the LC_{50}-96h in the liquid phase test with the reference chemical is not within the established acceptable limits.

4.0 INTERPRETATION AND EXPRESSION OF RESULTS

Two endpoints are assessed in *S. knabeni* toxicity tests: adult mortality (or survival) and nauplii hatching and survival.

Results can be assessed in two manners:

a) With a dose-response curve, and calculation of LC_{50}-96h and/or LC_{25}-96h. The use of regression models allows the calculation of the concentration that causes a 25% or 50% reduction in nauplii hatching and survival relative to the control group (U.S.EPA, 1994; Bailer *et al.*, 2000). There is not a minimum number of offspring for a test to be valid; it is only necessary that hatching of nauplii occurs in the control.
b) By analysis of variance (ANOVA) or equivalent, and comparison of the test results with the control for calculation of NOEC (no observable effect concentration) and LOEC (lowest observable effect concentration).

Results can be expressed through plots representing adult survival (or mortality) and/or nauplii survival in each treatment shown as NOEC, LOEC and LC_{50}.

Results of sediment toxicity tests with no dilutions should be analyzed by ANOVA followed by multiple comparisons tests, comparing survival and fecundity in each treatment to the control. Usually the data are expressed on tables or plots presenting female or nauplii

survival in each treatment, and including standard deviation and indicating the treatments that were significantly different from the control.

Results of copepod toxicity tests can be classified as toxic if they meet the following requisites:

a) Survival or mortality and/or nauplii sruvival are significantly different from the control, and
b) If there is a minimum 20% difference between the control mean result and a test treatment (based on the MSD – minimum significant difference – principle).

If the results do not meet these two requisites the sample should be considered non-toxic.

5.0 REFERENCES

Bailer, AJ, Hughes,MR, Denton,DL and Oris JT. 2000. An empirical comparison of effective concentration estimators for evaluating aquatic toxicity test responses. *Environ. Toxicol. Chem.* 19:141-150.

U.S. Environmental Protection Agency. 1994. Methods for measuring the toxicity of sediment-associated contaminants with estuarine and marine amphipods. EPA-600/R-94/025, Narragansett, RI.

Weber, CI, Peltier, WH, Norberg-King, TJ, Horning II, WB, Kessler, FA, Menkedick, J, Neiheisel, TW, Lewis, PA, Klemm, DJ, Pickering, QH, Robinson, EL, Lazorchak, JM, Wymer, LJ and Freyberg, RW. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 2nd Edition, U.S. Environmental Protection Agency, EPA/600/4-89/001.

Prepared by:

_____
Marion Nipper
Senior Research Scientist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
Laverne Cleveland
Chief, Field Research Division

Attachment 1

REQUIRED EQUIPMENT AND MATERIALS

1 L Erlen Meyer flasks
Cotton and gauze for stoppers
Plastic film
Scintillation vials
Dissecting microscope with illuminator
Pasteur pipettes (with latex bulbs)
5-mL Oxford-type pipette (with tips)
10-100 µL pipette
48-µm mesh sieve with 8 cm diameter (for cultures)
48-µm mesh sieve with 2.5 cm diameter (for use with formaldehyde)
125-µm mesh sieve with 8 cm diameter (for cultures)
2 to 3 small Carolina type dishes or Petri dishes
Filtering apparatus (with 0.45-µm filters) to prepare MFS
Vacuum pump
Colored labeling tape
Pens and markers
Data sheets
Manual counter
Kimwipes

Attachment 2

CULTURE MAINTENANCE RECORD

Organism: *Schizopera knabeni* Month and Year: _____
Flask number and date started: _____
Flask number and date terminated: _____

Date	Time	Salinity (ppt)	Temp. (°C)		DO (% sat.)	Food (mg dry wt)	Water change	Comments	Initials
			Air Min-Max	Water					

Attachment 3

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL_____ INITIALS_____
SAMPLE DESIGNATION_____ DATE_____

A. Salinity Adjustment:

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

B. Character of Sample (after salinity adjustment):

Volume (ml) _____

Salinity (‰) _____

pH _____

Dissolved oxygen (mg/L) _____

DO saturation (%) _____

Total ammonia (mg/L) _____

Sulfide (mg/L) _____

COMMENTS_____

_____

_____

_____

_____

_____

_____

Attachment 4

Schizopera knabeni TOXICITY TEST ENVIRONMENTAL CONDITIONS

Test Material_____ Test Description_____

Date/Time Test Started_____ Date/Time Test Completed_____

Observation Period_____ Date_____ Time_____

Parameter

Treatment	Temp (°C)	Salinity(ppt)	DO (mg/L)	pH	mV (mg/L)	Ammonia (mg/L)

Method: _____ Entered by:_____ Date: _____

Observation Period: _____ Date: _____ Time: _____

Attachment 5

Schizopera sp. BIOLOGICAL MONITORING DATA

Test Material_____

Test Description _____

Date and Time Test Started_____ Date and Time Test Ended_____

	# females										# alive nauplii				
	egg-bearing/without eggs					moribund/dead									
Treatment	Rep1	Rep2	Rep3	Rep4	Rep5	Rep1	Rep2	Rep3	Rep4	Rep5	Rep1	Rep2	Rep3	Rep4	Rep5

Organism Source: _____ Data Entered By: _____

Approval: _____ Comments: _____

ATTACHMENT 4

SOP 10.10

***Dinophilus gyrociliatus* toxicity test**

For users other than USGS staff, this
Document is for reference only. THIS IS
NOT A CITABLE DOCUMENT.

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 caneasily 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	$\geq$ 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 l. 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

Daniel, W.W. 1978. Applied non-parametric statistics. Houghton Mifflin Company, Boston, MA. 503 pp.

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

Snedecor G.W., and Cochran, G.C. 1980. Statistical methods. 7th edition. Iowa State University Press. Ames, IA. 507 pp.

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

Prepared by:

_____
Duane Chapman
Fishery Biologist

Approved by:

_____
R. Scott Carr
Field Station Leader

_____
A. Dennis Lemly
Chief, Field Research Program

_____
James W. Hogan
Quality Assurance Officer

Attachment 1

CULTURE MAINTENANCE RECORD

Organism: *Dinophilus gyrociliatus* Culture Designation _____

Date Culture Started:_____ Project Number _____

Date Culture Terminated:_____

Date/Time	Salinity (‰)	Temp (^{o}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 (°C)	Salinity (‰)	DO (mg/L)	pH	Ammonia (mg/L) mv	Ammonia (mg/L) mg/L
Method						

Entered by:_____ Date: _____

Observation Period: _____ Date: _____ Time: _____

Parameter

Treatment	Temp (°C)	Salinity (‰)	DO (mg/L)	pH	Ammonia (mg/L) 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						
							Day 7		
Treatment/ Replicate	No. Observed Day 1	No. Observed Day 4	No. Observed Day 7	Day 4 Total No. Eggs	Total No. Eggs	No. Eggs Cases	No. Eggs In Coelom	No. Eggs/ Adult	Comments

Organism Source: _____

Data Entered By: _____Approval: _____

Comments:_____