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Fate and effects of picric acid and 2,6-DNT in marine environments: Toxicity of degradation products

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Abstract

The toxicity of transformation products of 2,6-dinitrotoluene (2,6-DNT) and 2,4,6-trinitrophenol (picric acid) were assessed in spiked sandy and fine-grained marine sediments and in seawater. Toxicity of pore water from sediments spiked with 2,6-DNT decreased for the macro-alga, *Ulva fasciata*, zoospores as biotransformation proceeded, but increased for the copepod, *Schizopera knabeni*, nauplii. The primary biotransformation product of 2,6-DNT, 2-amino-6-nitrotoluene, was also more toxic than the parent compound to copepod nauplii, but not to alga zoospores, in spiked seawater tests. Two biotransformation products of picric acid, picramic acid and 2,4-DNP, were more toxic than their parent compound. Porewater toxicity from picric acid-spiked sediments decreased significantly at the end of six-months incubation. Fine-grained sediment spiked with either ordnance compound had lower toxicity than its sandy counterpart after six months, suggesting faster microbial transformation in the former and production of less toxic products. Photo-transformation of 2,6-DNT in seawater resulted in a reduction in toxicity.

Keywords: Ordnance compound; Pore water; Toxicity; Picric acid; 2,6-Dinitrotoluene; Degradation

1. Introduction

Some munitions and explosives of concern (MECs) have been measured in marine sediments near US. Navy facilities in recent years (EA Engineering, Science, and Technology, Inc., 1996). Explosives may reach marine waters and sediments by inappropriate storage or due to the presence of unexploded ordnance. There is a paucity of data in the scientific literature regarding the toxicity of ordnance compounds associated with marine sediments, particularly concerning the fate and the effects of their transformation products. Whereas biotransformation would be the main process of concern in sediments, photo-transformation in the water column could also generate toxic byproducts.

In previous surveys, the toxicity of several MECs in coastal waters and sediments was assessed to a variety of marine organisms (Nipper et al., 2001), and field surveys were conducted in the vicinity of naval facilities in Puget Sound, WA, to assess if sediment contamination by ordnance compounds in those areas posed a hazard to the resident biota (Carr et al., 2001). Such studies

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indicated that the studied ordnance compounds would not be of environmental concern in the concentrations previously measured in marine sediments in Puget Sound (Carr et al., 2001). However, degradation/transformation of some of these compounds enhanced their toxicity to marine organisms (Nipper et al., 2002). 2,6-dinitrotoluene (2,6-DNT) and 2,4,6-trinitrophenol (picric acid) were identified as compounds of concern.

The current study was conducted with the objective of identifying the biological effects of bio- and photo-transformation of 2,6-DNT and picric acid in marine environments. This objective was met by: toxicity assessment of biotransformed 2,6-DNT and picric acid in pore waters of marine sediments; toxicity assessment of photo-transformed 2,6-DNT in filtered seawater; and toxicity assessment of individual biotransformation products in filtered seawater.

The major identified biotransformation product of 2,6-DNT was 2-amino-6-nitrotoluene (2-A-6-NT), followed by 2-nitrotoluene (2-NT) and other minor components, and some of the main breakdown products of picric acid were 2,4-dinitrophenol and amino dinitrophenols, including picramic acid (Nipper et al., 2004).

Bio- and photo-transformed ordnance compounds, as well as isolated biotransformation products, were analyzed for toxicity with the macro-alga, *Ulva fasciata*, and with early-life stages of the benthic copepod, *Schizopera knabeni*. The photo-transformed sample was also assessed with the meiobenthic polychaete, *Dinophilus gyrociliatus*, seven-day life-cycle test, in addition to the other two species.

2. Materials and methods

2.1. Sediment collection and processing

Sediments to be used in this study were collected by grab sampling or coring from two relatively pristine sites, one on the Northwest coast of the USA and one in the Gulf of Mexico. The first 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 second was collected at Redfish Bay, TX, and was selected for its sandy characteristics (79.9% sand, 10.4% silt and 9.7% clay) and low TOC (0.25%). Each sediment was fully characterized for concentrations of metals, polycyclic aromatic hydrocarbons (PAHs) and organochlorine compounds prior to use in the experiments. Very low levels were exhibited by all measured chemicals, although slightly higher in the fine-grained sediment than in the sandy, 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).

Upon arrival at the laboratory each sediment was press-sieved through 1 mm mesh for removal of the indigenous fauna and stored at 4 °C for no more than six weeks prior to spiking with ordnance compounds.

2.2. Preparation of stock solutions and sediment spiking

Picric acid and 2,6-DNT (⩾98% purity) were purchased from ChemService (West Chester, PA, USA). Stock solutions were prepared under sterile conditions, in a laminar flow hood using autoclaved glassware and seawater sterilized by filtration through a 0.45 μm pore-sized membrane in a sterile Millipore® apparatus. Stock solutions of picric acid and 2,6-DNT used for sediment spiking were prepared in sterilized seawater at 30 ppt salinity and HPLC grade methanol, respectively. Sediments were spiked following the procedures described in Nipper et al. (2004), using the rolling jar method (Ditsworth et al., 1990). In summary, the stock solution of picric acid in seawater was added directly to the sediment in glass jars, vigorously shaken, and then rolled at 1.1 rpm for 6 h. The stock solution of 2,6-DNT dissolved in methanol was added to each jar, which was then rolled at 1.1 rpm with an open lid, until the methanol evaporated and 2,6-DNT crystals could be seen coating the walls of the jar (approximately 1.5 h). Sediment was then added to each jar and the jars were rolled at 1.1 rpm for 6 h. Sediment blanks used as controls were treated in the same manner, but in the absence of ordnance compounds.

2.3. Porewater sampling and chemical analyses

Sediment subsamples were taken for porewater sampling prior to incubation, for HPLC and microbiological analyses, and additional subsamples were subsequently collected at pre-determined time intervals (based on the results of chemistry experiments—Nipper et al., 2004), for pore water collection for toxicity and dissolved organic carbon (DOC) analyses. Porewater sampling was performed by vacuum, using disposable syringes attached to filtering media (Nipper et al., 2004), and then subdivided into samples for toxicity testing, chemical and DOC analyses. Samples for toxicity testing and chemical analyses were frozen and porewater samples for DOC analyses were filtered through a 0.45 μm nylon syringe filter prior to freezing.

Prior to toxicity testing porewater samples were analyzed by HPLC following USEPA Method 8330 (USEPA, 1994) with some modifications (Nipper et al., 2004).

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 an analyzed as described in Nipper et al. (2002).

Porewater samples collected from picric acid-spiked sediments after a 56-day incubation 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.

2.4. Photo-transformation of 2,6-DNT and picric acid in seawater

Filtered seawater at 30 ppt salinity was spiked separately with 2,6-DNT and picric acid and exposed to simulated solar radiation (SSR) for the assessment of photo-transformation. Exposures were conducted 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. For comparison purposes, the solar UVB irradiance measured in mid-July in Corpus Christi, Texas was 221 $\mu W/cm^2$. The UVA and UVB irradiance measured in the 2,6-DNT photo-transformation experiment at the surface of the exposure vessel were 1398 and 292 $\mu W/cm^2$, respectively. Therefore, the measured UVB irradiance was approximately 132% of the mid-July solar maximum in Corpus Christi. Samples were analyzed by HPLC after SSR exposure and, when photo-transformation occurred, tested for toxicity. No ordnance compound was left in the solutions when these were used in toxicity tests.

2.5. Water quality measurements for toxicity testing

Porewater samples for use in toxicity tests were moved from the freezer to a refrigerator at 4 °C two days prior to the tests. 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 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.

2.6. Toxicity tests

Toxicity tests were performed using the macro-alga (*U. fasciata*) 96-h zoospore test, the harpacticoid copepod (*S. knabeni*) 96-h test, and the polychaete (*D. gyrociliatus*) seven-day test.

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 (US Army Corps of Engineers, Engineer Research and Development Center, 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. *D. gyrociliatus* has been in culture in our laboratory for over six years, and original organisms were isolated from material obtained from Long Beach Harbor, CA.

The copepod, *S. knabeni*, 96-h test had adult female survival and embryo hatching and survival as endpoints. It was conducted in 20 mL glass scintillation vials with Teflon®-lined caps containing five ovigerous females in 5 mL sample per replicate, with five replicates per treatment. At the end of the exposure period the number of surviving females was enumerated under a dissecting microscope and removed from the sample, which was then preserved with 4% formaldehyde containing rose Bengal. No less than two days after preservation and staining, the number of stained (alive at test end) nauplii in each vial was counted. The number of nauplii/stocked female was calculated for each replicate.

The alga 96-h zoospore test, with germination and germling growth as endpoints, was conducted following standardized methodologies described by Hooten and Carr (1998). Released zoospores were exposed to the samples in 20 mL glass chambers with plastic snap lids and a round cover-slide inserted onto the bottom. Each replicate contained 5 mL pore water, with five replicates per treatment. At test termination the glass cover slides were analyzed for percent germination of the zoospores, and germling length and cell number.

The polychaete seven-day survival and reproduction test was performed according to the basic methodology described in Carr et al. (1989). It was conducted in 20 mL stender dishes containing four 1- to 2-day post-emergence females in 5 mL sample per replicate, with five replicates per treatment. Each chamber was examined after four and seven days. Mortality and number of eggs produced per surviving female were used as endpoints. The copepod and polychaete test were conducted in complete darkness to minimize photo-degradation of the ordnance compounds during the exposure period. A 12 h light:12 h darkness photoperiod was used for the macro-alga tests due to the need of light for zoospore settlement and germination.

Seawater containing photo-transformed 2,6-DNT was assessed for toxicity using the *U. fasciata*, *S. knabeni* and *D. gyrociliatus* tests. Pore waters and purified ordnance compounds and their major identified biotransformation products spiked into dilution water were assessed for toxicity with the *U. fasciata* and *S. knabeni* tests only.

Porewater treatments for use in the toxicity tests were prepared by 50% serial dilutions of each sample, and some of the blank treatments with higher ammonia concentrations were also diluted by a factor of two (50%) to provide some dilutions where ammonia would be below toxic levels for the different test organisms. A copepod test was also performed with ammonium chloride in seawater to establish the toxic levels of unionized ammonia to this test's endpoints. The sensitivity of the macro-alga zoospore test had been established previously (Hooten and Carr, 1998). The solution containing photo-transformed 2,6-DNT was also tested in a 50% dilution series. Millipore (0.45 μm) filtered seawater (MFS) was used as the diluent for all tests. A control series with MFS and a positive control series with sodium dodecyl sulfate were prepared concurrently to each toxicity test.

Single chemical tests were conducted with 2,6-DNT, picric acid, and some of their main biotransformation products, 2-amino-6-nitrotoluene (2-A-6-NT), 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) kindly donated some sodium picramate (97% purity), formerly available at Sigma (St. Louis, MO). Although the dissociation constant (pK_a) 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 pK_a values of its parent compound, picric acid and of 2,4-DNP, respectively (Weast et al., 1986–1987). The pK_a 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 the range of that of 2,4-DNP. Therefore, the dissociated form that would be found in seawater would be the same for sodium picramate and picramic acid, thus resulting in the same toxicity values. Due to this 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 dilution water and stirred on magnetic stirrer for 24 h prior to use in experiments. Chemical analyses of the stock solutions were performed at the beginning of each toxicity test, and for all treatments at test end, to assess the 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, but in the absence of organisms.

2.7. Statistical analyses

The results of the toxicity tests were used for calculation of the inhibitory or lethal concentration to 50% of the test organisms (IC_{50} or LC_{50}), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values. These were calculated using initial measured chemical concentrations for single chemical tests and percent porewater for the porewater tests. Concentrations of single chemicals were also measured at test ending, but changes from initial to final concentrations were considered negligible for the purpose of these test endpoint calculations, with the exception of 2-NT, where 40–60% loss occurred in the copepod and macro-alga zoospore tests, respectively. In a previous study using purified 2,6-DNT and picric acid in seawater toxicity tests, both 2,6-DNT and picric acid loss during exposure periods ranging from 48 h to seven days were never higher than 14% (Nipper et al., 2001), which is within the error range of the analysis method.

In the porewater experiments, blank treatments from the sediment type with equivalent water quality values (particularly unionized ammonia) were used as controls for statistical analyses. This minimized the possibility of false positives, i.e., samples that could have been toxic because of their ammonia concentrations rather than to the ordnance compounds or their biotransformation products. However, ammonia may have acted as an additional stressor, particularly for the copepod embryo hatching endpoint, which was the most sensitive to this confounding factor.

The data for each sample and corresponding blanks were isolated as an independent dataset, i.e., data from each sediment spiked with each ordnance compound, and so forth, were analyzed separately. 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 endpoint. 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(dfError, .05/(2 × n)). Only one outlier occurred in copepod tests and five in macro-alga tests, including one for zoospore germination in single chemical tests and two in porewater tests, and one each for germling length and cell number in porewater tests.

After omitting outliers but prior to further analysis, the data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). When necessary, the data were arcsine square root, square root, or $\log_{10}$ transformed, in order to meet these assumptions. 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 transformed data with the aid of SAS (1989).

The Trimmed Spearman–Karber method (Hamilton et al., 1977) with Abbott's correction (Morgan, 1992) was used to calculate LC_{50} in the copepod test and the IC_p method (Norberg-King, 1993) was used for calculation of IC_{50} for endpoints with continuous values, such as copepod hatching success and polychaete reproduction (number of hatched nauplii or of laid eggs/adult female), and alga germination and germling length and cell number.

3. Results

3.1. Porewater toxicity

Some of the porewater samples exhibited elevated levels of ammonia. Therefore, the toxic concentrations of unionized ammonia to *S. knabeni* survival and hatching success endpoints were calculated. LC/IC_{50} values to female survival and nauplii hatching and survival were 1207 and 346 μg/L, respectively. Some of the tested pore waters contained ammonia in toxic levels. The identification of false positives due to ammonia was minimized by 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, although in some cases it could have contributed to observed adverse effects as an additional stressor. Some of the most critical examples of the effects of porewater blanks containing ammonia and corresponding ordnance spiked samples are given in Table 1. This table shows that although ammonia could have been a stressor in a number of samples, inhibition of embryo hatching and survival was much stronger in pore waters containing ordnance compounds and their biotransformation products. The possibility of additive effects, however, should not be ruled out.

U. fasciata zoospores have extremely low sensitivity to ammonia, with germination EC_{50} of 1650 μg/L unionized ammonia (Hooten and Carr, 1998). No false positives due to ammonia toxicity should have occurred in the macro-alga tests. Table 1 shows that porewater blanks containing up to 996 μg/L of ammonia had 82.4% zoospore germination, suggesting that effects observed in pore waters from sediments spiked with 2,6-DNT or picric acid would be due to these chemicals and/or their biotransformation products, with no or very little influence of ammonia.

Water quality in the porewater tests was otherwise within acceptable levels, with sulfide below detection (<0.01 mg/L) in all samples, DO >88% saturation, and pH ranging from 7.2 to 9.1. The highest pH values, of 8.8 and 9.1 in the copepod tests, and 8.7 in the alga tests, occurred in the sandy sample spiked with 2,6-DNT and the respective blank spiked with filtered seawater after a 180-day incubation.

DOC concentration in the pore waters of picric acid-spiked sediments tended to decrease with incubation time, whereas in the 2,6-DNT-spiked samples an initial increase in DOC occurred, followed by a sharp decrease after a six-month incubation (Table 2). DOC concentrations were always higher in the pore waters from the ordnance-spiked sediments than in their respective blanks.

The LC/IC_{50} values calculated in porewater tests were based on porewater dilutions (% porewater) rather than on chemical concentrations. This allows comparisons among samples after transformation of 2,6-DNT to 2-A-6-NT, and of picric acid to 2,4-DNP, picramic acid, and possibly other unidentified products. Measured concentrations of the different chemicals are given in micromoles per liter rather than milligram per liter, also to allow direct comparisons. Molecular weights of 2,6-DNT, 2-A-6-NT, 2-NT, 2,4-DNP, picric and picramic acids are 182, 152, 137, 184, 229 and 199, respectively.

3.1.1. 2,6-DNT

The sensitivity of the two different test organisms and endpoints used in tests with pore waters from sediments spiked with 2,6-DNT was very distinct. Copepod hatching success, expressed as number of surviving nauplii hatched per female, tended to be a more sensitive endpoint than adult female survival, with few exceptions (Table 3). Samples in which 2,6-DNT was totally metabolized and only 2-A-6-NT could be measured (seven-day incubation) were the most toxic to copepod nauplii (Table 3). The opposite occurred with female survival, where samples with only 2-A-6-NT and no 2,6-DNT had lower or similar toxicity (sandy and fine-grained sediment, respectively) relative to the initial samples (Day 0). After 180 days of incubation, no 2-A-6-NT was detectable in either sediment type and no significant toxicity to copepod female survival was exhibited in the highest test concentration (Table 3).

Table 1
Concentration of unionized ammonia (NH_3) and toxicity of some porewater blanks and ordnance spiked samples

Sample	Treatment	Incubation time (days)	% Pore water	NH_3 (μg/L)	Mean no. alive nauplii
Copepod, *S. knabeni*					
Filt. seawater	Control			67.8	12.7
Fine-grained	Blank–Picric Test	0	100	507.2	5.6
	Picric acid	0	100	308.9	0.5
		7	50	516.0	0.1
		56	12.5	271.4	0.0
		180	100	372.1	0.0
Fine-grained	Blank–DNT Test	0	100	382.0	3.0
	2,6-DNT	0	100	375.3	0.9
		7	100	240.5	0.5
Sandy	Blank–Picric Test	28	100	1042.5	1.8
		28	50	458.6	9.6
	Picric acid	0	50	422.8	0.0
		28	25	328.0	0.7
		56	25	422.4	0.2
Sandy	Blank–DNT Test	0	50	458.6	5.9
	2,6-DNT	0	100	272.3	0.0
Macro-alga, *U. fasciata*					
					Mean % germination
Filt. seawater	Control			9.9	92.3
Fine-grained	Blank–Picric Test	0	100	996.0	82.4
	Picric	0	100	930.0	0.0
Fine-grained	Blank–Picric & DNT Test	7	100	679.1	86.8
	Picric acid	7	100	705.1	0.0
		56	25	609.4	0.0
	2,6-DNT	0	50	246.6	0.0
Sandy	Blank–Picric & DNT Test	0	100	634.5	73.6
	Picric acid	0	50	323.4	0.0
		28	50	517.9	37.2
		56	50	1022.1	64.0
		180	100	646.0	20.6
	2,6-DNT	180	100	602.9	24.3

Toxicity to copepod embryo hatching success decreased in the pore water from the fine-grained sediment, but did not change significantly in the sample from the sandy substrate.

Porewater tests with macro-alga zoospores exhibited a slight decrease in toxicity with 2,6-DNT transformation to 2-A-6-NT (Table 3, Days 0 and 7). More prolonged sediment incubation (180 days) with further transformation of the 2,6-DNT resulted in strongly reduced toxicity to macro-alga zoospores. Although the sensitivity of the three endpoints in the macro-alga tests differed, they exhibited the same trend in their response to the different samples (decreased toxicity with sediment incubation time). Germling length and cell number were more sensitive to the initial samples, which contained measurable 2,6-DNT and 2-A-6-NT, whereas germination was the more sensitive endpoint after 180 days of sediment incubation, when no transformation products were identified (Table 3).

3.1.2. Picric acid

The toxicity of pore waters from picric acid-spiked sediments varied with sediment incubation time and toxicity test endpoint (Table 4). Copepod embryo hatching and survival was more sensitive than adult female survival in all samples. Toxicity to adult survival decreased significantly in the samples from the sandy sediment incubated for $\geqslant$28 days, as picric acid transformation progressed. No adverse effect occurred after 180 days of sediment incubation, when no biotransformation products were identified. Embryo hatching and survival was strongly reduced in samples from sandy sediment incubated for up to 56 days, with a nearly fourfold decrease in toxicity after 180 days of incubation (Table 4).

Pore waters from the fine-grained sediment exhibited the strongest effect to both copepod female survival and embryo hatching success after 56 days of sediment incubation, when only a small amount of picramic acid was measured (Table 4). Toxicity to both endpoints

Table 2
Dissolved organic carbon (DOC) concentration in pore waters from ordnance-spiked sandy and fine-grained sediments at different incubation times and temperatures

Storage time (Days)	Temperature (°C)	Sample	Treatment	Treatment blank	DOC (mg/L)	
					Treatment	Blank
–	–	–	–	FSW[a]	–	1.517
0	–	Sandy	2,6-DNT	MEOH[b]	97.751	37.885
7	20				148.897	16.475
180	20				28.433	7.889
0	–	Fine-grained	2,6-DNT	MEOH	56.084	12.592
7	10				73.915	9.355
180	10				7.563	5.890
0	–	Sandy	Picric acid	SW[c]	185.490	39.967
28	10				72.408	18.860
56	10				52.831	17.152
180	10				24.520	12.880
0	–	Fine-grained	Picric acid	SW	68.470	11.147
7	10				33.419	8.335
56	10				14.698	7.864
180	10				10.197	NM[d]

[a] FSW = 0.45 μm filtered seawater (DOC control).
[b] MEOH = pore water from sediment blank spiked with methanol only.
[c] SW = pore water from sediment blank spiked with filtered seawater only.
[d] NM = not measured.

decreased dramatically after 180 days. Major aromatic compounds in the 180-day sample were benzoic and benzene acetic acids, the source of which is not clear (Carr and Nipper, 2003; Nipper et al., 2004).

Toxicity of pore waters from picric acid-spiked sandy sediment to *U. fasciata* zoospore germination did not change significantly with picric acid biotransformation during the initial 28 days of sediment incubation. This was followed by a 60% decrease in toxicity after 56 days of incubation, and a minor increase after 180 days at 20 °C (Table 4). Germling growth (length and cell number), however, was greatly reduced during the initial stages of picric acid transformation in the sandy sediment (Days 0–56, Table 4), with a marked decrease in toxicity in the pore waters from the most aged sediment (180 days).

Samples from the fine-grained sediment reached their highest toxicity to all *U. fasciata* test endpoints after 56 days of incubation, when no germination occurred (Table 4). Further sediment incubation up to 180 days resulted in further transformation of the spiked chemical and transformation products, leading to total loss of toxicity.

3.2. Toxicity 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. NOEC values of 2,6-DNT and 2-A-6-NT were 277 and 240 μmol/L, respectively, with LC_{50} values of 357 and >277 μmol/L (Fig. 1). The copepod nauplii hatching and survival endpoint, however, indicated significantly higher toxicity of the biotransformation product, with IC_{50} values of 50 and 303 μmol/L for 2-A-6-NT and 2,6-DNT, respectively (Fig. 1). The opposite happened in *U. fasciata* zoospore germination tests, where the toxicity of the parent compound, 2,6-DNT, was markedly higher than that of 2-A-6-NT (Fig. 2). Another biotransformation product of 2,6-DNT, 2-NT, was somewhat less toxic than the parent compound to both endpoints in the copepod test (Fig. 1), and an order of magnitude less toxic in all endpoints of the macro-alga test (Fig. 2). However, 40–60% of the 2-NT was lost during the 96-h tests, possibly due to volatilization. Results of toxicity tests with 2-NT are reported as measured initial concentration.

The main identified breakdown products of picric acid, 2,4-DNP and picramic acid, were more toxic to copepods and macro-alga zoospores than their parent compound (Figs. 1 and 2). The toxicity of 2,4-DNP was higher than that of picramic acid to all test endpoints except copepod nauplii hatching and survival, where both chemicals had similar toxicity.

3.3. 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

Table 3
Toxicity of pore waters from sediments spiked with 2,6-DNT in copepod and macro-algae tests

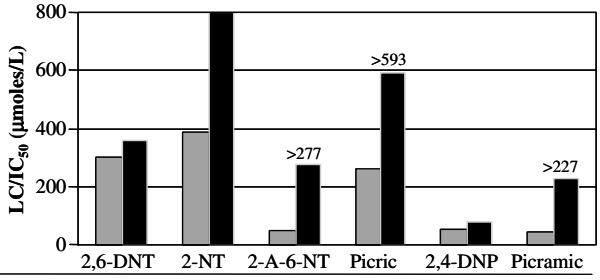
Sediment	Storage		Measured conc. in 100% PW (μmol/L)		% of each chemical in 100% pore water		LC/IC_{50} (% pore water)[a]				
	Time (days)	Temperature (°C)					Copepod survival		Macro-alga germination		
			2,6-DNT	2-A-6-NT	2,6-DNT	2-A-6- NT	Females	Hatched nauplii	% Germination	Germling	
										Length	Cell no.
Sandy	0	–	456.24	244.55	65.1	34.9	38.4 (35.9–41.1)	63.9 (49.8–75.0)	12.3 (11.2–14.4)	4.1 (3.0–4.9)	2.94 (2.7–3.6)
	7	20	BDL[b]	531.41	0.0	100.0	>50 (3.3)	16.7 (12.3–21.6)	20.6 (19.8–21.4)	5.7 (5.1–6.2)	4.38 (3.0–5.4)
	180	20	BDL	BDL	0.0	0.0	>50 (0.0)	21.8 (19.1–24.7)	73.0 (64.4–83.4)	>100 (33.0)	>100 (36.6)
Fine-grained	0	–	236.81	234.01	50.3	49.7	73.5 (NC)[c]	74 (NC)	20.4 (20.0–20.8)	11.3 (10.2–12.2)	14.9 (12.6–16.4)
	7	10	BDL	561.39	0.0	100.0	70.7 (NC)	37.6 (31.8–40.6)	21.3 (20.7–22.2)	12.8 (11.9–13.6)	15.2 (14.0–16.0)
	180	10	BDL	BDL	0.0	0.0	>100 (0.0)	95.2 (NC)	78.9 (76.4–81.4)	95.1 (NC)	>100 (11.0)

[a] 95% confidence interval in parenthesis. Where LC/IC_{50} is expressed as > or <, the number represents the highest or lowest tested porewater concentration, and the value in parenthesis is the percent observed effect at that concentration relative to the control.
[b] BDL = below detection limit.
[c] NC = not calculable.

Table 4
Toxicity of pore waters from sediments spiked with picric acid in copepod and macro-algae tests

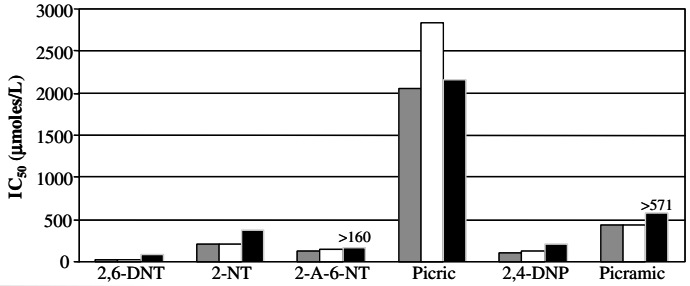
Sediment	Storage		Measured concentration 100% PW (μmol/L)			% of each chemical in 100% pore water			LC/IC_{50} (% pore water)[a]				
	Time (days)	Temperature (°C)							Copepod survival		Macro-alga germination		
			Picric	Picramic	2,4-DNP	Picric	Picramic	2,4-DNP	Gravid females	Hatched nauplii	% Germin.	Germling	
												Length	Cell no.
Sandy	0	–	170.99	16.05	2.43	90.2	8.5	1.3	13.0 (12.2–14.0)	9.7 (8.7–10.3)	34.4 (31.1–35.7)	4.47 (4.1–4.9)	8.83 (8.0–9.7)
	28	10	6.37	0.55	3.39	61.8	5.3	32.9	>50 (0.3)	5.1 (4.9–5.6)	39.6 (23.8–56.7)	4.57 (4.4–4.8)	4.1 (4.0–4.4)
	56	10	BDL[b]	1.03	1.80	0.0	36.4	63.6	>25 (28.0)	9.5 (7.8–10.5)	67.3 (60.8–71.0)	4.65 (4.3–5.1)	4.2 (4.1–4.4)
	180	20	BDL	BDL	BDL	0.0	0.0	0.0	>100 (0.0)	35.0 (28.8–39.2)	54.0 (48.3–60.0)	>100 (33.1)	>100 (37.6)
Fine-grain	0	–	61.27	8.06	0.90	87.2	11.5	1.3	35.4 (NC)[c]	10.2 (4.1–12.4)	42.4 (35.6–59.1)	4.38 (4.1–4.8)	4.0 (3.8–4.1)
	7	10	BDL	BDL	2.78	0.0	0.0	100.0	68.8 (66.9–70.7)	18.2 (10.0–21.6)	58.9 (48.8–66.4)	4.33 (4.0–4.8)	4.0 (3.8–4.4)
	56	10	BDL	1.69	BDL	0.0	100.0	0.0	6.9 (6.4–7.4)	1.9 (1.3–2.2)	<6.25 (100.0)	<6.25 (100.0)	<6.25 (100.0)
	180	10	BDL	BDL	BDL	0.0	0.0	0.0	>100 (0.0)	42.7 (38.8–45.8)	>100 (0.8)	>100 (0.0)	>100 (0.0)

[a] 95% confidence interval in parenthesis. Where LC/IC_{50} is expressed as > or <, the number represents the highest or lowest tested porewater concentration, and the value in parenthesis is the percent observed effect at that concentration relative to the control.
[b] BDL = below detection limit.
[c] NC = not calculable.



	2,6-DNT	2-NT	2-A-6-NT	Picric	2,4-DNP	Picramic
Nauplii survival	303 (286-321)	389 (286-438)	50.2 (32-60)	263 (158-322)	51.4 (48-54)	43.1 (25-59)
Female survival	357 (350-370)	798 (777-820)	>277	>593	77.4 (75-80)	>227

Fig. 1. Survival (LC_{50}) and reproduction inhibitory concentrations (IC_{50}) in copepod toxicity tests with ordnance compounds and transformation products; 95% confidence interval in parenthesis.



	2,6-DNT	2-NT	2-A-6-NT	Picric	2,4-DNP	Picramic
Length	21.4 (20-23)	209 (190-232)	130 (125-137)	2061 (1927-2245)	108 (93-128)	440 (424-454)
Cell No.	25.6 (21-30)	212 (187-237)	142 (133-149)	2829 (NC)	120 (108-132)	435 (419-446)
% Germ.	81.4 (79-84)	369 (335-407)	>160	2157 (2019-2351)	215 (193-244)	>571

Fig. 2. Inhibitory concentrations (IC_{50}) in *Ulva fasciata* zoospore toxicity tests with ordnance compounds and transformation products; 95% confidence interval in parenthesis.

the photo-transformed product are expressed as percent of stock solution. 2,6-DNT concentrations in the stock solutions, prior to photo-transformation, were 783.7 µmol/L in the copepod and polychaete tests and 705.3 µmol/L in the macro-alga test. Toxicity tests were performed after 2,6-DNT was completely photo-transformed, with none remaining in the stock solution.

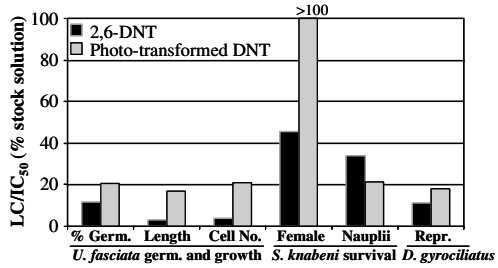


Fig. 3. Toxicity of 2,6-DNT and photo-transformed 2,6-DNT to copepod survival and reproduction, macro-alga zoospore germination and germling growth and polychaete reproduction. Results are expressed as percent stock solution, in LC_{50} for adult copepod survival and IC_{50} for all other endpoints.

LC/IC_{50} values were calculated as percent of these stock solutions for both photo- and non-photo-transformed 2,6-DNT. Toxicity of 2,6-DNT was higher than that of the photo-transformed solution in all tests and endpoints but copepod nauplii hatching and survival, in which case the parent compound had slightly lower toxicity (Fig. 3). 2,6-DNT breakdown products could not be identified, although the presence of high molecular weight chemicals with mass spectra ranging from molecular weight 200–500 (compared to 182 for DNT) was detected (Nipper et al., 2004).

4. Discussion

In a general sense, given sufficient time (180 days), 2,6-DNT and picric acid in marine sediments incubated under controlled conditions were biotransformed to less toxic forms. Toxicity decrease was stronger in samples from fine-grained sediment than in those from the sandy substrate, probably due to a faster rate of

transformation leading to less toxic compounds in the first. Such data suggest that six months would be sufficient time for both 2,6-DNT and picric acid transformation into less toxic forms in fine-grained sediments with TOC >1% and a healthy microbial community (Carr and Nipper, 2003), even at temperatures as low as 10 °C. However, in sandier sediments with lower TOC and reduced microbial activity, the transformation of both chemicals occurred at a slower rate and longer periods of time would be needed for toxicity reduction. It is expected that further microbial transformation would occur over time in the sandy sediment, in which such processes typically exhibited slower rates (Carr and Nipper, 2003; Nipper et al., 2004).

Toxicity test results with pore waters from 2,6-DNT-spiked sediments were corroborated by single chemical tests with 2,6-DNT and 2-A-6-NT. The latter was identified as the main biotransformation product of 2,6-DNT (Nipper et al., 2004). Copepod nauplii hatching and survival in single chemical tests was more strongly impaired by 2-A-6-NT than 2,6-DNT, similarly to what occurred in pore waters. Another arthropod, the midge *Chironomus tentans*, was more sensitive to the aminated derivative of trinitrotoluene (TNT), 2,4-diamino nitrotoluene, than to its parent compound in sublethal sediment exposures (Steevens et al., 2002). The opposite occurred in *U. fasciata* zoospore germination tests, where toxicity decreased with the transformation of 2,6-DNT in 2-A-6-NT in porewater samples, with the same occurring in experiments with single chemicals. 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; *in* Stahl and Aust, 1995), thus demonstrating the importance of using several species and endpoints for toxicity assessments.

The measured toxicity of pore waters from picric acid-spiked sediments incubated for six months at 10 °C to copepod nauplii indicates that toxic chemicals resulting from the picric acid biotransformation were still present. GC/MS analyses were qualitative in nature but only indicated the presence of a very small amount of nitro diaminophenol in the pore water from the fine-grained sediment (Carr and Nipper, 2003), suggesting that unidentified compounds would have been responsible for the observed results. The high toxicity, to *U. fasciata* zoospores, of the sample from the fine-grained sediment after 56 days of incubation could neither be explained by measured ordnance compounds or their metabolites (Table 4), nor by elevated ammonia, as blanks with similar levels of ammonia were used to help with results interpretation. Nitrite concentrations in those samples (2.6 and 87.6 μmol/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-h LC_{50} of 67.4 μmol/L (Ostrensky and Poersch, 1992–1993). The same authors reported irregular sensitivity to nitrite in toxicity tests with the micro-alga *Tetraselmis chuii*, where the highest toxic concentration was 3,456 μmol/L. Thus it is concluded that transient highly toxic unidentified products were generated by the picric acid biotransformation and were present at that stage of the process.

Two of the major biotransformation products of picric acid, 2,4-DNP and picramic acid, had higher toxicity than its parent compound in copepod and macro-alga tests (Figs. 1 and 2). These compounds could have contributed to the increased toxicity in the aged pore waters from picric acid-spiked sediments, but do not fully explain the highly elevated toxic effects of several samples. For instance, the high porewater toxicity to copepod hatching success of the sample from the sandy sediment after 28 days of incubation cannot be explained by the measured 6.4 μmol picric acid/L, well below the NOEC which was 74 μmol/L (Carr and Nipper, 2003) or the IC_{50} of 263 μmol/L. This sample also contained 3.4 μmol 2,4-DNP/L and 0.55 μmol picramic acid/L (Table 4), which may have contributed to, but do not explain, the observed toxicity. Unidentified biotransformation products are expected to have caused the strong effects at the initial sediment incubation period (up to 56 days), as well as the milder effects observed after 180 days. Possible chemicals related to picric acid found at Day 180 were products containing amino, nitro and hydroxyl groups, in addition to nitrophenol isocyanate (Carr and Nipper, 2003; Nipper et al., 2004), whose toxicity to aquatic organisms has not been established.

The toxicity ranking of picric acid and its transformation products 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 μmol/L in 48 and 24-h tests, respectively. These values are comparable to the 96-h adult LC_{50} and embryo hatching and survival IC_{50} values for the copepod used in the current study. 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 μmol/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 twofold higher toxicity than picric acid in oyster tests, with 144-h LC_{50} values of 251 and 478 μmol/L, respectively, and about threefold higher in trout tests, with 96-h LC_{50} values of 379 and 1112 μmol/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 and the copepod embryo hatching and survival somewhat more sensitive to picramic acid (Figs. 1 and 2). Sublethal effects to oyster shell deposition were at a toxicity level closer to that observed for copepod nauplii in the present study, with EC_{50} values of 30 and 122 μmol/L for picramic and picric acid, respectively (Goodfellow et al., 1983).

The higher toxicity of 2,6-DNT, relative to its photo-irradiated solution, to *U. fasciata* zoospores, copepod adult female survival and polychaete reproduction, resembles what was observed with TNT photolysis in freshwater, which caused toxicity decrease to several species of freshwater fish, the cladoceran, *Daphnia magna*, and the mudworm, *Lumbriculus variegatus*, although it did not modify the toxicity to a freshwater amphipod and midge (Rosenblatt et al., 1991). 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, *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.

The high variability of water quality in porewater tests was likely due to the varied matrix (sediment) and conditions to which they were exposed prior to porewater sampling. The pH elevation may have been caused by sedimentary processes taking place during the incubation at 20 °C. The higher DOC concentrations in pore waters from the ordnance-spiked sediments than in their respective blanks suggests that the degradation of the ordnance compounds acted as a source of organic carbon.

5. Conclusions

Biological effects of microbial- and photo-transformation products of 2,6-DNT and picric acid varied with toxicity test species and endpoint. For example, the toxicity of pore water from sediments spiked with 2,6-DNT decreased for *U. fasciata* zoospores as biotransformation proceeded, but tended to increase for copepod nauplii. The same occurred with the primary 2,6-DNT biotransformation product, 2-A-6-NT, in single chemical solutions, but the opposite occurred with 2-nitrotoluene. Two biotransformation products of picric acid, picramic acid and 2,4-DNP, were more toxic than their parent compound to both toxicity test species and all endpoints, but this was not sufficient to explain the extremely elevated toxicity of several porewater samples. Therefore, it would seem prudent to include some of the identified transformation products in the list of analytes of concern in field assessments of sediments suspected of contamination by nitroaromatics, while also applying multiple-species toxicity analyses.

The influence of solar radiation on environmental effects of 2,6-DNT does not seem to be reason for major concern. Toxicity to three species and five endpoints decreased after 2,6-DNT was exposed to SSR, and the sixth endpoint was only slightly changed between pre- and post-SSR exposure.

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