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ORIGINAL ARTICLE

Effect of 2,4-dinitrotoluene on the anaerobic bacterial community in marine sediment

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Keywords

2,4-dinitrotoluene, anaerobic, bacterial community, denaturing gradient gel electrophoresis, impact, marine sediment.

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Abstract

Aims: To study the impact of added 2,4-dinitrotoluene (DNT) on the anaerobic bacterial community in marine sediment collected from an unexploded ordnance dumping site in Halifax Harbour.

Methods and Results: Marine sediment was spiked with 2,4-DNT and incubated under anaerobic conditions in the presence and absence of lactate. Indigenous bacteria in the sediment removed 2,4-DNT with subsequent formation of its mono- and diamino-derivatives under both conditions. PCR-DGGE and nucleotide sequencing were used to monitor the change in the bacterial population in sediment caused by the presence of 2,4-DNT. The results showed that denaturing gradient gel electrophoresis banding patterns of sediment microcosms treated with 2,4-DNT were different from controls that did not receive 2,4-DNT. Bacteroidetes, Firmicutes and δ -Proteobacteria were present in sediment incubated in the absence of 2,4-DNT. However, several γ -Proteobacteria became dominant in sediment in the presence of 2,4-DNT, two of which were 99% similar to *Shewanella canadensis* and *Shewanella sediminis*. In the presence of both 2,4-DNT and lactate, two additional δ -Proteobacteria were enriched, one closely related (98% similarity) to *Desulfofrigus fragile* and the other affiliated (96% similarity) to *Desulfovibrio* sp. In contrast, none of the above four Proteobacteria were enriched in sediment incubated with lactate alone.

Conclusions: Presence of 2,4-DNT led to a significant change in bacterial population of marine sediment with the enrichment of several γ - and δ -Proteobacteria.

Significance and Impact of the Study: Our results provided the first evidence on the impact of the pollutant 2,4-DNT on the indigenous bacterial community in marine sediment, and provided an insight into the composition of bacterial community that degrade 2,4-DNT.

Introduction

Dinitrotoluenes (DNTs) are widely used in the manufacturing of explosives, propellants, pesticides and dyes (Hartter 1985). These chemicals have contaminated soil and ground water in the US and worldwide (Feltès and Levsen 1989; Feltès *et al.* 1990; Nishino *et al.* 2000a). Sediments in two marine unexploded ordnance (UXO) sites were investigated, one located near Halifax Harbor of the

Atlantic Ocean that showed the presence of trace amounts of 2,4,6-trinitrotoluene (TNT) (Darrach *et al.* 1998) and another close to the Oahu Island of Hawaii that was found to contain trace amounts of 2,4- and 2,6-dinitrotoluenes (DNTs) (Monteil-Rivera *et al.* 2005). Dinitrotoluenes exhibited acute toxicity to guppy (*Poecilia reticulata*) (Deneer *et al.* 1987), Fisher-344 rats (Rickert *et al.* 1984) and to humans (Rosemond and Wong 1998). Also DNTs transformation products showed toxicity to

bacteria (*Vibrio fischeri*), alga (*Selenastrum capricornutum*, Dodard *et al.* 1999) and copepod (*Copepod nauplii*, Nipper *et al.* 2005). The US Environmental Protection Agency lists DNTs as priority pollutants (Keith and Telliard 1979).

A few aerobic bacteria including strains of *Burkholderia* were previously isolated from an ammunition plant wastewater (Spanggord *et al.* 1991; Suen *et al.* 1996) and activated sludge (Nishino *et al.* 2000b) for their capacity to degrade DNT to CO₂. Some anaerobic bacteria, such as *Clostridium acetobutylicum*, were also reported to reduce 2,4-DNT and 2,6-DNT to their amino derivatives (Hughes *et al.* 1999).

Recently, we found that the mixed indigenous micro-organisms can degrade 2,4-DNT and 2,6-DNT in marine sediment (Yang *et al.* 2008), but little is known about the impact of these chemicals on the microbial community in the sediment. We hypothesized that exposing the sediment to 2,4-DNT followed by incubation under anoxic conditions might induce a change in the composition of the indigenous microbial community. Bacteria that are enriched by the presence of 2,4-DNT would tolerate the presence of the pollutant and degrade the chemical at a contaminated site. Therefore, identifying the types of micro-organism enriched by the presence of 2,4-DNT would help find indicators for the potential occurrence of natural attenuation at the contaminated site.

Denaturing gradient gel electrophoresis (DGGE) is widely used to characterize the composition of complex microbial communities in sediment and soil (Muyzer *et al.* 1993; Muyzer and Smalla 1998). Earlier studies using DGGE showed that the bacterial composition of sediment (Roeling *et al.* 2002) and soil (Kasai *et al.* 2005; Labbe *et al.* 2007) changed significantly after their exposure to petroleum. In this paper, we used PCR-DGGE and nucleotide sequencing to determine the impact of 2,4-DNT and its metabolism on the bacterial community in marine sediment sampled from an UXO site near Halifax.

Materials and methods

Chemicals and stock solutions

2,4-Dinitrotoluene (2,4-DNT, 97%), 2-amino-4-nitrotoluene (2-A-4-NT, 99%), 4-amino-2-nitrotoluene (4-A-2-NT, 99.8%), 2,4-diaminotoluene (2,4-DAT, 98%) and sodium lactate were obtained from Sigma (Oakville, ON, Canada). A stock solution of 2,4-DNT (100 mg l⁻¹) was prepared in artificial seawater containing 4% w/v sea salt (Sigma), and a sodium lactate stock solution (100 g l⁻¹) was prepared in milliQ water. The two stock solutions were sterilized by filtration through a 0.22-µm pore-sized membrane (Millipore, Billerica, MA). All other chemicals were obtained from Sigma.

Anaerobic treatment of marine sediment with 2,4-DNT

Sediment was sampled from a UXO dumping site, located at Emerald Basin, Halifax Harbor, Canada. The temperature of the sediment site ranged from 4 to 10°C. Characterization of the sediment was previously described (Zhao *et al.* 2004a). Briefly, the sediment had a pH level of 6.5 and was mainly composed of silt (90%, particle size 2.50 µm). The total organic C and NH₃ were 12.0 and 0.0077 g kg (dry sediment)⁻¹ respectively. Neither 2,4-DNT nor its metabolites was detected in the sediment. To understand the effect of 2,4-DNT on the bacterial community structure in sediment, four sets of sediment microcosms, each in duplicate were prepared as shown in Table 1. Two sets were spiked with 2,4-DNT, one of which was supplemented with lactate. The other two sets, one containing sediment and lactate and the second containing sediment alone, served as controls. In a typical experiment, sediment (10 g, wet weight) was suspended in 25 ml sterile artificial seawater (4% sea salts solution) and placed inside a 100-ml serum bottle. The sediment suspension was then treated with 15 ml of the above 2,4-DNT stock solution and 0.4 ml of lactate as shown in

Table 1 Compositions of marine sediment microcosms used in the present study

	Initial amount of components				Amount of second spiking		
	Live sediment (g)	Sea water (ml)	Stock solution		Sea water (ml)	Stock solution	
			2,4-DNT (ml)	Lactate (ml)		2,4-DNT (ml)	Lactate (ml)
Microcosms	Wet weight (62% water)	40 g l ⁻¹	100 mg l ⁻¹	100 g l ⁻¹	40 g l ⁻¹	100 mg l ⁻¹	100 g l ⁻¹
Sediment alone	10	40	0	0	15	0	0
Sediment + 2,4-DNT	10	25	15	0	0	15	0
Sediment + lactate + 2,4-DNT	10	25	15	0.4	0	15	0.5
Sediment + lactate	10	40	0	0.4	15	0	0.5

Table 1. The serum bottles were then capped with rubber stoppers and crimped with aluminium seals under an atmosphere of $N_2 : H_2$ (96 : 4), microcosms were incubated statically at 10°C in the dark. To monitor the long-term effect of 2,4-DNT that is expected to be continuously released from UXO at the sampling site, additional 15 ml of the chemical's stock solution was added to the 2,4-DNT-depleted microcosms after 49 days of incubation. Another 0.5 ml of the lactate stock solution was also added to the lactate-amended microcosms after 56 days. Sampling of microcosms for subsequent analysis of DNT, lactate and their products was conducted as reported previously (Yang *et al.* 2008). Briefly, the liquid phase (0.5 ml) was sampled periodically from the microcosms and centrifuged (16 000 g) for 1 min prior to analysis (see below). For DGGE analysis, aliquots of sediment (0.5 g) including liquid phase were sampled from the bottom of each microcosm with a sterile spoon after 14, 49 and 86 days of incubation. Sampling was conducted in an anaerobic glove box. Sediment samples were frozen at -20°C for subsequent extraction of DNA.

PCR-DGGE analysis of bacterial 16S rDNA in the marine sediment

DNA was extracted from 0.5 g frozen sediment slurry samples with PowerSoil™ DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). PCR amplification of partial 16S rDNA (approx. 420 bp) was performed with bacterial primers 341F containing a GC clamp (5'-CGCCCCCGCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') (Roelleke *et al.* 1996) and 758R (5'-CTACCAGGGTA TCTAATCC-3') (Lee *et al.* 1993). PCR was performed using a MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA) in 50 µl mixture containing 0.2 µmol l⁻¹ primer, 0.2 mmol l⁻¹ of each dNTP, 1 µl DNA, 1 mmol l⁻¹ MgCl₂ and 1.0 U of Taq DNA polymerase (GE Healthcare, Piscataway, NJ) using a touchdown PCR protocol as described previously (Labbe *et al.* 2007).

DGGE was performed using the Bio-Rad DCode system (Bio-Rad). PCR product (500–600 ng) was loaded onto an 8% (wt/vol) polyacrylamide gel containing a 40–60% denaturing gradient. The 100% denaturant was 7 mol l⁻¹ urea and 40% deionized formamide. Gels were run at 85 V, 60°C in 1× TAE buffer (pH 8.0) for 16 h. The gels were then stained with Vistra Green (Amersham Biosciences, Piscataway, NJ) and visualized with a FluorImager System (Model 595; Molecular Dynamics, Sunnyvale, CA).

Numerical analysis of DGGE banding patterns was performed using GELCOMP II software package (Ver. 2.0; Applied Maths, Austin, TX). Similarity of banding patterns was calculated based on the Pearson correlation coefficient (Pearson 1926). Cluster analysis was performed

using the unweighted pair group method with arithmetic means based on Dice correlation coefficient.

Sequencing and phylogenetic analyses of 16S rDNA

DGGE bands were excised from the gels and eluted in 60 µl of sterile distilled water at 37°C overnight. Eluted DNA was purified with DNA and Gel Band Purification kit from GE Healthcare. Purified DNA solution (1 µl) was re-amplified using the same primers as mentioned earlier (341F and 758R) but without GC clamp. PCR was performed using the following protocol: an initial denaturation at 95°C for 5 min, followed by 30 cycles of amplification reactions under the following conditions: 94°C, 1 min; 64°C, 1 min; 72°C, 1 min. The PCR product was purified with the same Purification kit from GE Healthcare and sequenced at University of Laval (Quebec, Canada) using the ABI Prism 3100XL DNA Analyzer system (Applied Biosystems). In cases where no sequences can be obtained from the isolated bands using the method mentioned, PCR fragments from the re-amplification with primers 341F and 758R were introduced into the pGEM-T easy vector system (Promega, Madison, WI) according to the manufacturer's instructions. Transformation was performed in *E. coli* DH5α cells. Plasmids were extracted by QIAprep Spin Miniprep kit (Qiagen, Mississauga, ON). DNA inserts were sequenced on both strands with M13 forward and reverse primers as described earlier.

All sequences were checked for chimeras using the Chimera Check Program from the Ribosomal database Project II (<http://rdp8.cme.msu.edu>). Sequences (360–464 bp) obtained were compared with the published sequences in Genbank using BLAST from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). The 16S rDNA sequences were aligned with CLUSTAL X (1.83). A phylogenetic tree was constructed with neighbour-joining method in MEGA3 package (Kumar *et al.* 2004) based on the pairwise nucleotide distance of Kimura 2-parameter. Bootstrap analysis with 500 replicates was performed to determine the statistical significance of the branching order.

Analytical methods

2,4-DNT and its amino derivatives 2-A-4-NT, 4-A-2-NT and 2,4-DAT were analysed by HPLC-UV (Waters, Milford, MA) using a C18 Discovery column (25 cm × 4.6 mm, 5 µm; Supelco, Oakville, ON, Canada) and acetonitrile/water gradient as described previously (Yang *et al.* 2008). Lactate and its acetate metabolite were analysed by HPLC (Waters) using an ICsep ICE-ION-300 column (Transgenomic, San Jose, CA) (Yang *et al.* 2008). All experiments were run in duplicates and the concentration of

chemicals presented in the article is the average values of the duplicates.

Nucleotide sequence accession number

The nucleotide sequences of 16S rRNA genes obtained from isolated DGGE bands have been deposited at GenBank under accession numbers EU617339–EU617357.

Results

Treatment of marine sediment with 2,4-DNT

The potential effect of 2,4-DNT on sediment bacterial community was tested anaerobically at 10°C under two conditions: the first experiment was conducted by incubating the sediment with the nitroaromatic compound in the absence of any cosubstrate, and the second was conducted in the presence of lactate (Table 1).

As shown in Fig. 1, 2,4-DNT ($139 \mu\text{mol l}^{-1}$ initial concentration in the aqueous phase) was completely removed from the aqueous phase of the live sediment whether lactate was present or not. In the abiotic control prepared using autoclaved sediment, approx. 30% of 2,4-DNT was lost during the first day of incubation (Fig. 1). Previously, we attributed such initial loss to reversible sorption onto sediment, which should be bioavailable to the degrading bacteria (Yang *et al.* 2008). Figure 1 also shows that the disappearance of 2,4-DNT in live sediment was accompanied with concurrent formation of 2-amino-4-nitrotoluene (2-A-4-NT), 4-amino-2-nitrotoluene (4-A-2-NT) and 2,4-diaminotoluene (2,4-DAT). None of these products were detected in the sterile sediment controls. In a previous study, using uniformly ring labelled [^{14}C]-2,4-DNT, <10% of total radioactivity was found in the sediment after 86 days of incubation (Yang *et al.* 2008), further supporting the exclusion of sorption as a major route for the removal of DNT. Experimental evidences gathered thus far indicate that most of 2,4-DNT lost from the aqueous phase of the live sediment are because of biotransformation of the chemical by the mixed indigenous micro-organisms.

After 49 days of incubation, when most of DNT was depleted, additional amount of the chemical was thus added to the sediment to further induce changes in the indigenous bacterial community. Interestingly, we found that the disappearance rate of DNT in the presence of lactate (1.2 micromoles per day, Fig. 1b) was faster than the rate in the absence of lactate (0.4 micromoles per day) (Fig. 1a). The loss of DNT in the lactate-supplemented microcosm was accompanied by the loss of lactate and the production of acetate (Fig. 2). In contrast, only slow removal (28%) of lactate was observed in the sterile sediment control (Fig. 2), further indicating the

Figure 1 Anaerobic metabolism of 2,4-DNT by indigenous micro-organisms in the absence (a) or presence (b) of lactate in the cold marine sediment. (a,b) (□) 2,4-DNT; (△) 2A4NT; (○) 4A2NT; (◇) 2,4-DAT and (■) 2,4-DNT/abiotic control.

involvement of bacteria in the loss of lactate and the production of acetate in 2,4-DNT-amended live sediment microcosms. Figure 1 also shows that the presence of lactate led to faster biotransformation of the initially formed monoaminotoluene to 2,4-diaminotoluene.

DGGE analysis of bacterial 16S rDNA in 2,4-DNT-treated marine sediment

PCR and DGGE were employed to determine the composition of bacteria in sediment sampled during the above anaerobic incubation. The PCR–DGGE banding patterns of the PCR-amplified 16S rDNA fragments in duplicate microcosms were similar for each treatment; therefore, only one of the duplicates was chosen for further analysis. In a sediment microcosm control that did not contain

Figure 2 Conversion of lactate to acetate during 2,4-DNT metabolism in anaerobic marine sediment. (—■—) lactate/abiotic control; (---□---) lactate; (—△—) acetate.

either 2,4-DNT or lactate, the DGGE banding patterns did not differ from the ones observed in the original sediment at 0 h as shown in Fig. 3a. However, dendrogram analysis (Fig. 3b) showed that the banding patterns of 2,4-DNT-amended microcosms were different from the banding pattern of control sediment microcosms that were not treated with 2,4-DNT. Similarly, the banding patterns of lactate-amended sediment microcosms incubated with 2,4-DNT were different from the ones incubated without 2,4-DNT (Fig. 3b). Both results indicate that 2,4-DNT and its metabolism induce a change in bacterial community in sediment.

Meanwhile, the DGGE banding patterns of sediment microcosms containing both DNT and lactate were different from the ones incubated with DNT alone (Fig. 3b). Lactate was indeed found to metabolize to acetate by sediment micro-organisms in the presence of 2,4-DNT as shown in Fig. 3. The presence of lactate also led to the rapid disappearance of the initially produced monoaminonitrotoluene to the corresponding diamino-toluene (DAT). This observation suggests the supporting role of lactate as a cosubstrate in enriching certain types of sediment bacteria.

Approximately 15 bacterial bands (bands 1, 2 and 4–10) were observed (lane A, Fig. 3a) in the sediment controls. Treatment with 2,4-DNT resulted in the appearance of new bands. During incubation with 2,4-DNT alone, four new bands (bands 11–14, lanes B and E) appeared, while some bacterial bands (bands 4–6, lanes B, C) that were dominant in sediment controls (lane A) became faint or disappeared (lanes B, C, F and G) (Fig. 3). In sediment microcosms incubated with both 2,4-DNT and lactate, two new bands 15 and 16 (lanes C

Figure 3 (a) DGGE analyses of PCR-amplified bacterial 16S rDNA fragments derived from original or 2,4-DNT-treated sediment. Lane A indicates untreated sediment; lanes B, E and H indicate the respective sediments sampled after 14, 49 and 86 days of incubation with 2,4-DNT only; lanes C, F and I indicate the respective sediment sampled after 14, 49 and 86 days of incubation with both 2,4-DNT and lactate; lanes D, G and J indicate the respective sediments sampled after 14, 49 and 86 days of incubation in the control with lactate. Numbers in the gel indicate the position of sequenced bands. Stars or circles indicate the bands excised and sequenced from each lane. The three circles indicate band 19. (b) Dendrogram of the DGGE banding patterns obtained using Pearson correlation and the algorithm of UPGMA. The scale indicates the similarity coefficient of band clusters.

and F) in addition to bands 13 and 14 (lanes B and E), were observed. None of the above-mentioned new bands were observed in sediment controls containing lactate alone (lanes D, G, J).

Phylogeny of bacteria in 2,4-DNT-treated marine sediment

Well-separated DGGE bands as shown in Fig. 3a were selected to determine the phylogenetic affiliation of the indigenous bacteria in the tested marine sediment.

Following amplification of the DGGE fragments and subsequent sequencing, the sequences (360–464 bp) were successfully obtained from most of the selected DGGE bands. For bands 7 and 9, no readable sequences were produced likely because of a background interference caused by the presence of minor fragments with similar electrophoretic mobility (Gafan and Spratt 2005). In these cases, we used the cloning technique to obtain their sequences (464 bp). Furthermore, although bands 15 and 19 had similar migration positions, the two bands were found to be different in their sequences. In total, 19 distinct bacterial 16S rDNA sequences were obtained.

Phylogenetic analyses of these 19 bacterial 16S rDNA sequences showed that the dominant sediment bacteria were affiliated with Bacteroidetes, Firmicutes and Proteobacteria (Fig. 4). Overall, most of the 19 bacteria were

related to uncultured bacteria or isolates from cold marine sediment, consistent with the low temperature (4–10°C) nature of the present Halifax sediment (Table 2). Those bacteria in original sediment and not affected by the DNT-treatment were Bacteroidetes (bands 1, 2) and δ -Proteobacteria (bands 7–10). The new bacteria appeared after incubation with 2,4-DNT were found to be members of either γ - or δ -Proteobacteria.

The 16S rDNA sequences of the two bacteria (bands 13, 14, in lanes B, C, E, F and H, Fig. 3a) enriched in all DNT-treated sediment, with and without lactate, were 99% similar to those of *Shewanella sediminis* HAW-EB3 and *Shewanella canadensis* HAW-EB2, previously isolated from the same sediment as degraders of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Zhao *et al.* 2005, 2007). The two transient bands 11 and 12 (Fig. 3a, lane

Figure 4 Phylogenetic tree of 16S rDNA sequences of DGGE bands obtained in this study and those related ones. Bootstrap values (500 replicates) higher than 50% were indicated. The scale bar indicated 5% sequence divergence.

Table 2 The sequences of DGGE-separated 16S rDNA bands detected in the present marine sediment and those closely related ones

DGGE band	Accession no.	Phylogenetic affiliation	Matched sequences in Genbank		Similarity (%)
			Clone or isolate/accession no.	Site	
1	EU617339	Bacteroidetes	Unc. <i>Cytophaga</i> clone (AB188788)	Sediment, Sagami Bay, Japan, >1000 m	98
2	EU617340		Unc. <i>Cytophaga</i> clone FE2TopBac05 (AY768965)	Sediment, Gulf of Mexico, >3000 m	97
3	EU617341		Unc. <i>Cytophaga</i> clone FE2TopBac41 (AY768987)		96
4	EU617342	Firmicutes	Unc. <i>Fusibacter</i> sp. (AB189368)	Sediment, Japan Trench	98
5	EU617343		Unc. Firmicutes clone (AB015270)		99
6	EU617344		Unc. clone GoM GC019S6 (AY211742)	Sediment, Gulf of Mexico, 550–575 m	96
11	EU617349	Proteobacteria, γ -division	Unc. <i>Marinomonas</i> clone SIMO-4371 (DQ421736)	Salt marsh tidal creek, North Atlantic Ocean	95
12	EU617350		DMSP-degrading bacterium JA9 (AF296153)	Sediment, Atlantic Ocean	95
13	EU617351		<i>Shewanella sediminis</i> HAW-EB3 (AY579750)	Sediment, Halifax Harbor, North Atlantic	99
14	EU617352		<i>Shewanella canadensis</i> HAW-EB2 (AY579749)	Ocean, >200 m	99
7	EU617345	Proteobacteria, δ -division	δ -Proteobacteria isolates EtOHpelo (AY771935)	Sediment, Wadden Sea	90
18	EU617356		<i>Desulfovibrio</i> sp. G5VII (AJ786057)		96
9	EU617347		Unc. myxobacterium clone Sva0537 (AJ297473)	Sediment, Arctic Ocean	96
15	EU617353		<i>Desulfofrigus fragile</i> (AF099065)		98
17	EU617355		<i>Desulfobacterium</i> sp. LSV25 (AF099056)		89
16	EU617354		<i>Desulfovibrio</i> sp. HS2 (AY274444)	Sediment, German North Sea	96
8	EU617346		Unc. δ -Proteobacteria clone Hyd89-23 (AJ535245)	Sediment, Oregon margin, Pacific Ocean, 780 m	98
10	EU617348		Unc. <i>Desulfobacteraceae</i> clone (AB294969)	Coral reef, a Japan island, Pacific Ocean	96
19	EU617357		<i>Desulfobaba fastidiosa</i> (AY268891)	Sediment, North Atlantic Ocean	93

B) detected in sediment incubated with 2,4-DNT (no lactate) were most closely related to either uncultured *Marinomonas* clone SIMO-4371 (band 11, 95%) or to the dimethylsulfoniopropionate (DMSP)-degrading γ -Proteobacterial isolate JA9 (band 12, 95% similar) (Table 2). In contrast, the two bacteria (bands 15, 16), which emerged only from the sediment microcosms incubated with both 2,4-DNT and lactate, but not from the control (sediment + lactate), were found affiliated with δ -Proteobacteria. Band 15 was 98% similar to *Desulfofrigus fragile* isolated earlier from the Arctic Ocean (Sahm *et al.* 1999), and band 16 was 96% similar to *Desulfovibrio* sp. HS₂ found in marine sediment from the North Sea (Dinh *et al.* 2004). Two other δ -Proteobacteria (bands 17–18) were enriched by lactate in 86-day-old lactate-amended sediment in the absence or presence of 2,4-DNT. Interestingly, a new bacterium (Fig. 3a, band 19, lane J) was noted in the lactate sediment microcosm that did not contain 2,4-DNT.

Discussion

The present DGGE and phylogeny results provide an insight into the types of bacteria indigenous to the deep (200 m), cold (10°C) marine sediment sampled from UXO dumping site, near Halifax Harbor. In general, the detected indigenous bacteria including Bacteroidetes, Firmicutes and Proteobacteria at this site are similar to those in other deep-sea sediments (Ravenschlag *et al.* 2000; van

der Wielen and Heijs 2007). However, three of the detected δ -Proteobacteria (bands 7, 17, 19) only displayed a low similarity by 16S rDNA sequence to δ -Proteobacteria EtOHpelo (90%), *Desulfobacterium* sp. LSV25 (89%) and *Desulfobaba fastidiosa* (94%) respectively, indicating the presence of novel clades of δ -Proteobacteria in the Halifax UXO site.

Although dinitrotoluenes are known to be widely dispersed in various environments such as waters and sediments (Lendenmann *et al.* 1998), our DGGE and phylogeny data provided the first evidence on the impact of the toxic 2,4-DNT pollutant on the bacterial community of marine environment. Only very recently another nitroaromatic compound, TNT, was reported for its impact on soil bacterial composition with the enrichment of a couple of soil bacterial species such as *Pseudomonas* (George *et al.* 2008; Travis *et al.* 2008a,b). In our study, we found that the presence of 2,4-DNT led to enrichment of marine γ -Proteobacteria such as *Shewanella*.

Our DGGE data also showed that the presence of cosubstrates influenced the effect of 2,4-DNT on the microbial community in sediment. In this study, we chose lactate as a cosubstrate, because it is a common bacterial fermentation product in anoxic environment (Widdel 1988; Parkes *et al.* 1989). Also, lactate is widely recognized as a carbon and an energy source (Llobet-Brossa *et al.* 2002) for many respiratory anaerobic bacteria including sulfate-reducing bacteria and *Shewanella* that are known to be present in marine sediment (Zhao *et al.*

2004b). In the present sediment, lactate was indeed found to be metabolized by sediment bacteria with the production of acetate as a metabolite (Fig. 2). As a result, the number of some δ -Proteobacteria such as bands 15, 16 in sediment was increased as revealed by DGGE and phylogenetic analyses (Fig. 3).

Two other δ -Proteobacteria bands 17 and 18 (lanes I and J) were also detected during incubation with lactate and 2,4-DNT and with lactate alone, and they appear to tolerate 2,4-DNT. However, the DGGE band (band 19, lane J) detected only in the lactate-alone sediment microcosms seemingly did not tolerate the toxic effect of DNT.

These bacteria that became dominant during incubation with 2,4-DNT are likely to be involved in metabolism of the toxic chemical and tolerate its metabolites (Sayama *et al.* 1998; Dodard *et al.* 1999). This is supported by the detection of 2,4-DNT metabolites including mono and diamino derivatives in the live sediment. Some δ -Proteobacteria, including *Desulfovibrio*, have also been reported for their capacity to reduce 2,4-DNT and TNT (Boopathy and Kulpa 1993; Esteve-Nunez *et al.* 2001) and for reduction of the cyclic nitramine RDX (Zhao *et al.* 2004b). Previously, strains of *Shewanella* were reported for their ability to reduce the nitro groups of cyclic nitramine explosive RDX (Zhao *et al.* 2004b). In a separate experiment, we also found that strains of *Shewanella* previously isolated from the same sediment reduced the nitro groups of 2,4-DNT to its mono- and diamino derivatives (H. Yang, J.-S. Zhao and J. Hawari; unpublished data) as found in the present study (Fig. 1). *Shewanella* are also known to produce biogenic Fe(II) that has been reported to play a role in the removal of pollutants including nitro-containing explosives such as RDX in sediment (Bhushan *et al.* 2006; Roh *et al.* 2006; Zhang and Webber 2009).

Shewanella and sulfate-reducing δ -Proteobacteria are known to play important roles in mineralizing marine organic matter (Dhillon *et al.* 2003). Detection of both *Shewanella* and δ -Proteobacteria using the present DGGE approach further suggests that these bacteria may participate in the *in situ* metabolism of organic matter including RDX, DNT and TNT if they leak from UXO at this site.

Conclusions

PCR-DGGE and subsequent sequencing showed that the marine sediment from a UXO site near Halifax Harbor contained Bacteroidetes, Firmicutes and Proteobacteria as found in other marine sediments. If the toxic 2,4-DNT is leaked from the UXO found at this site, a shift of bacterial population would occur, leading to enrichment of several γ - and δ -Proteobacteria. The present data should provide an insight into the types of indigenous marine bacteria adapted to 2,4-DNT presence and that would

likely participate in the degradation of 2,4-DNT *in situ* (natural attenuation) in marine sediment.

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