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MICROBIAL COMMUNITY CHANGES IN TNT SPIKED SOIL BIOREMEDIATION TRIAL USING BIOSTIMULATION, PHYTOREMEDIATION AND BIOAUGMENTATION

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Abstract. Trinitrotoluene (TNT), a commonly used explosive for military and industrial applications, can cause serious environmental pollution. 28-day laboratory pot experiment was carried out applying bioaugmentation using laboratory selected bacterial strains as inoculum, biostimulation with molasses and cabbage leaf extract, and phytoremediation using rye and blue fenugreek to study the effect of these treatments on TNT removal and changes in soil microbial community responsible for contaminant degradation. Chemical analyses revealed significant decreases in TNT concentrations, including reduction of some of the TNT to its amino derivates during the 28-day tests. The combination of bioaugmentation-biostimulation approach coupled with rye cultivation had the most profound effect on TNT degradation. Although plants enhanced the total microbial community abundance, blue fenugreek cultivation did not significantly affect the TNT degradation rate. The results from molecular analyses suggested the survival and elevation of the introduced bacterial strains throughout the experiment.

Keywords: TNT, bioaugmentation, biostimulation, phytoremediation, microbial community.

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Introduction

The nitroaromatic explosive, 2,4,6-trinitrotoluene (TNT), has been extensively used for over 100 years, and this persistent toxic organic compound has resulted in soil contamination and environmental problems at many former explosives and ammunition plants, as well as military areas (Stenuit, Agathos 2010). TNT has been reported to have mutagenic and carcinogenic potential in studies with several organisms, including bacteria (Lachance *et al.* 1999), which has led environmental agencies to declare a high priority for its removal from soils (van Dillewijn *et al.* 2007).

Both bacteria and fungi have been shown to possess the capacity to degrade TNT (Kalderis *et al.* 2011). Bacteria may degrade TNT under aerobic or anaerobic conditions directly (TNT is source of carbon and/or nitrogen) or via co-metabolism where additional substrates are needed (Rylott *et al.* 2011). Fungi degrade TNT via the actions of nonspecific extracellular enzymes and for production of these enzymes growth substrates (cellulose, lignin) are needed. Contrary to bioremediation technologies using bacteria or bioaugmentation, fungal bioremediation requires an *ex situ* approach instead of *in situ* treatment (i.e. soil is excavated, homogenised and supplemented with nutrients) (Baldrian 2008). This limits applicability of bioremediation of TNT by fungi *in situ* at a field scale.

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Bioremediation is increasingly discussed as a useful, eco-friendly and cost-effective strategy for cleaning up explosives-contaminated soils (van Dillewijn *et al.* 2007; Kapley *et al.* 2007; Stenuit, Agathos 2010). Bioremediation techniques including bioaugmentation, biostimulation, and phytoremediation, and combinations of these methods, such as rhizoremediation, as well as landfarming and bioslurry reactors have been tested for TNT removal in the laboratory and in field scale with different levels of success leaving the results still inconclusive (Clark, Boopathy 2007; van Dillewijn *et al.* 2007; Gerth, Hebner 2007). Bioaugmentation involves adding microorganisms with specific degradative properties to the contaminated environment to improve biodegradation and enhance the transformation rate of pollutants (Kuiper *et al.* 2004). Despite problems with the survival of the introduced bacterial inoculum (Ruberto *et al.* 2003), bioaugmentation has been successful in removing TNT (van Dillewijn *et al.* 2007) and so far the majority of studies have focused on discovering microorganisms that could be used to degrade pollutants (Ayoub *et al.* 2010). Biostimulation approaches add nutrients or electron acceptor/donors to the polluted environment to enhance the bioremediation of pollutants like TNT (Gerth, Hebner 2007). Biostimulation can also be used in combination with bioaugmentation to improve the survival and catabolic activity of introduced microorganisms. Phytoremediation utilizes green plants to treat contaminated soil or water and has been successfully applied to remediation of different pollutants, including TNT (Hughes *et al.* 1996; Makris *et al.* 2007). In addition, plants release root exudates and enzymes that stimulate microbial activity and contaminant degradation in the rhizosphere, enabling the application of rhizoremediation, which is considered particularly effective for the treatment of contaminated soil (Schnoor *et al.* 1995; Kuiper *et al.* 2004; Gerhardt *et al.* 2009).

However, despite several studies, bioremediation of TNT is still considered challenging (Stenuit, Agathos 2010). Due to the complexity of the bioremediation processes in contaminated soils, as well as environmental safety considerations, detailed monitoring and final evaluation of remediation efficiency are essential. The abundance of TNT-degrading microorganisms, such as *Pseudomonads,* in explosive containing substrates is traditionally estimated by the CFU method (George *et al.* 2008). Instead we propose the use of *Pseudomonas* and *Stenotrophomonas* group-specific primers in qPCR approach which allows achieving the same goal in less time, avoids the cultivation bias, and broadens the monitoring possibilities for further bioremediation studies.

The objectives of this research were (i) to study the effect of bioremediation methods and combinations (biostimulation, bioaugmentation, phytoremediation) on the degradation of TNT in soil and, (ii) to study changes in microbial community structure and abundance associated with the various bioremediation strategies.

1. Methods

1.1. Bioremediation experiment setup

28-day laboratory bioremediation experiment was performed using individual pots (Table 1), each of which contained a mixture of 70 g industrial quartz (<2 mm) and 8 g peat (dw) forming artificial soil mimicking the soil of explosives contaminated Adazhi military camp, Latvia. The initial TNT concentration in soils of all TNT containing pots was 118 mg TNT/kg (dw). The following amendments with documented enhancing effects on the explosives degradation rate (Clark, Boopathy 2007; Muter *et al.* 2008) were used for biostimulation: 5 ml pot^{-1} molasses (30%, w/v); 5 ml pot^{-1} cabbage leaf extract; and 3 ml pot^{-1} mineral medium stock. The mineral medium stock contained 60 g l^{-1} Na_2HPO_4, 30 g l^{-1} KH_2PO_4, and 5 g l^{-1} NaCl. The cabbage leaf extract contained 4.2 g l^{-1} N_{total}, 10.2 g l^{-1} C; 0.222 g l^{-1} S, 9 g l^{-1} fructose, 11 g l^{-1} glucose, and 1 g l^{-1} sucrose. The molasses (30%, w/v) contained 37.6 g l^{-1} N_{total}, 88.3 g l^{-1} C, 0.841 g l^{-1} S, and 100 g l^{-1} sucrose.

To each bioaugmented pot, 10 ml of inoculum that contained a bacterial consortium ($3*10^8$ colony forming units (CFU) ml^{-1}) was introduced. The bacterial consortium used as the inoculum was previously isolated from soil contaminated with explosives at the Adazhi military camp, Latvia (Limane *et al.* 2011), thus fulfilling the suggestion that in order to increase the survival of introduced consortium, the strains used should originate from a similar ecological niche as the study material (El Fantroussi, Agathos 2005). Using 16S rRNA gene full-length sequencing (BCCM/LMG, Belgium), the predominant bacterial strains in the inoculum were determined to be members of *Pseudomonas* and *Stenotrophomonas* genus (Table 2) which are well-established TNT (and its metabolites) degraders and have been shown to be

Table 1. Variants used in vegetation pot experiments

Variants			Nitro-aromatics	Inoculum	Amendments
N	R	B			
1	1R	1A	+	–	–
2	2R	2A	+	+	+
3	3R	3A	+	–	+
4	4R	4A	–	–	–
5	5R	5A	–	+	+
6	6R	6A	–	–	+

N – non-planted; R – rye; B – blue fenugreek
+ included with variant
– not included with variant

Table 2. Sequencing results of bacterial strains used for inoculum preparation

Bacterial strain	Size (bp)[a]	Closest relative	% identity
PS 11	1259	*Pseudomonas palleroniana*	99.9
ST 10	1263	*Stenotrophomonas maltophilia*	99.0
ST 13	1252	*Stenotrophomonas maltophilia*	99.4

[a]Size in bp of the sequenced PCR products

among dominant groups in TNT-contaminated soil microbial communities (Esteve-Nuñez, Ramos 1998; Oh, Kim 1998; Snellinx *et al.* 2003; Cho *et al.* 2008; Travis *et al.* 2008b).

For phytoremediation two different plants, rye (*Secale cereale)* and nitrogen fixing blue fenugreek (*Trigonella caerulea)* were chosen and cultivated using 10 seeds in each pot. A temperature of 22 °C and a light period of 12 h were maintained throughout the experiment. Plant biomass was harvested 14 days after the beginning of the experiment, the plant roots were homogenized with soil, and the experiment was continued for an additional 14 days. All treatments were performed in two replicates.

1.2. Analytical methods

TNT and its metabolites were detected and quantified using high pressure liquid chromatography (HPLC) according to EPA method (US EPA method 8330, 1994). The standard mixtures of explosives MixA (EPA 8330, SUPELCO Bellefonte, PA), Nitroaromatics/ExplosiveMix1, and Nitroaromate-Nitroamine-Mix4 (Dr. Ehrenstorfer Reference Materials) were used for calibration.

1.3. Microbial community metabolic profile by Biolog EcoPlates

The functional diversity of the artificial soil microbial communities was determined using Biolog EcoPlates (Biolog Inc., USA). Tetrazolium dye used in the Biolog EcoPlates wells is not metabolized by fungi, so fungi do not contribute to color formation on these plates (Preston-Mafham *et al.* 2002). From each soil sample a 10^{-5} dilution in sterile 0.85% NaCl (w/v) was prepared, and 150 µl of the dilution was inoculated into each of the 96 wells (31 carbon sources and control in three replicates) of the micro-plate and incubated at 25 °C. The color development was measured as absorbance at 590 nm using a microplate reader (Multiscan 340C).

1.4. PCR-DGGE analyses of bacterial communities

Microbial DNA was extracted from 0.3 g (ww) soil samples using PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Inc.) and stored at −20 °C. Bacterial community structure was assessed with 16S rDNA sequence specific primer pair 338F-GC/518R (Table 3). The PCR mixture included 1 × PCR buffer (75 mM Tris-HCl, pH 8.8; 20 mM $(NH_4)_2SO_4$; and 0.01% Tween 20), 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 2.5 mM $MgCl_2$, 0.006 mg ml^{-1} bovine serum albumin (BSA), 0.0008 mM of each primer, and 0.5 U of *Taq* DNA polymerase (MBI Fermentas, Lithuania). After 5 min of denaturation at 95 °C, 30 thermal cycles of 2 min at 95 °C, 1 min at 53 °C, and 1 min at 72 °C, the PCR was completed with an extension step at 72 °C for 10 min.

A denaturing gradient gel electrophoresis (DGGE) system Dcode (BioRad, Inc. Hercules, CA, USA) was used to separate the amplified gene fragments. Prior DGGE analysis PCR products from two replicates were pooled. PCR products were applied for the DGGE analysis and electrophoresis was performed as described by Muyzer *et al.* (1993) with 10% (vol/vol) polyacrylamide gel (acrylamide : bisacrylamide = 37.5 : 1 in 1 × TAE buffer). A linear DNA denaturing gradient of 35–65% was produced with deionized formamide and urea (100% denaturant agent is 7 M urea and 40% [vol/vol] deionized formamide). Electrophoresis was performed using 1 × TAE buffer for 13 h at a constant temperature of 60 °C and constant voltage of 100 V. The gel was stained in MilliQ water (Millipore, Billerica, MA, USA) containing 0.5 µg l^{-1} ethidium bromide and de-stained twice in MilliQ water. The DGGE gel was digitized with Gel Doc System (Bio-Rad, Inc. Hercules, CA, USA).

1.5. Evaluation of bacterial gene copy numbers by quantitative PCR

Primer sets 785FL/919R, SteF/SteR, and PseF2/PseR (Table 3) were used for total and *Stenotrophomonas* and *Pseudomonas* genus-specific 16S rRNA gene detection and enumeration on SYBR green qPCR. A new *Stenotrophomonas* genus-specific primer set was designed using the Primer3Plus program (Untergasser *et al.* 2007) based on conserved motifs M1 and M4 proposed by Verma *et al.* (2010). Primer properties were calculated with OligoAnalyzer 3.0 software (Integrated DNA Technologies, IA, USA) and the specificity of the primer pair was checked by sequence alignment using BLAST and NCBI entries. For standard curve creation the DNA of reference strains *Pseudomonas mendocina* PC1, *Pseodomonas fluorescens* T11, and *Stenotrophomonas maltophilia* T9 (Collection of Environmental and Laboratory Microbial Strains, University of Tartu) were used for total, *Pseudomonas*-specific, and *Stenotrophomonas*-specific 16S rRNA PCR-amplifications, respectively.

The PCR reaction mixture was prepared as described in Section 1.4, with the exclusion of BSA from the mixture. The PCR reactions were performed with the following reaction conditions: preheating at

Table 3. Characteristics of PCR primers used in DGGE and quantitative PCR methods

Primer	Primer sequence (5′→3′)	Amplicon size (bp)	Annealing temperature (°C)	References
338F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGACTCCTACGG GAGGCAGCAG	236	53	Muyzer *et al.* 1993
518R	ATTACCGCGGCTGCTGG			Øvreås *et al.* 1997
785FL	ggactacGGATTAGATACCCTGGTAGTCC[a]	156	63	Nõlvak *et al.* 2012
919R	CTTGTGCGGGTCCCCGTCAAT			
Ste-F	TTGTCCTTAGTTGCCAGCAC	192	58	This study
Ste-R	CCGGACTGAGATAGGGTTTC			
Pse-F2	GGTCTTCGGATTGTAAAGCAC	184	58	Juhanson *et al.* 2009
Pse-R	CCGGGGMTTTCACATCCAAC			

[a]can be used as LUX™ primer when appropriate fluorophore is attached to the primer

95 °C for 5 min; 30 thermal cycles of 30 s at 94 °C, 30 s at an annealing temperature of the primer pair used (Table 3), and 45 s at 72 °C. The resultant PCR products were cloned using InsT/Aclone PCR cloning kit (Fermentas); plasmid-DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, CA, USA); and nucleotide sequenced using BigDye™ chemistry with M13-primers (Fermentas). The numbers of copies of standards plasmids were calculated according to plasmid (2886 bp) plus insert lengths (Table 3), assuming a molecular mass of 660 Da for a base pair. Standard DNA stock solutions of 10^9 plasmid copies/µl were prepared and serial dilutions ranging from 25 to 10^8 target gene copies were used for standard curve creation on qPCR. The detection limit for all assays was 25 target gene copies μl^{-1} of template.

The qPCR assays were performed on the real-time PCR system, Rotor-Gene® Q (Qiagen), and the data was analyzed using Rotor-Gene Series software, version 2.0.2. The optimized reaction mixture contained 5 µl Maxima SYBR Green Master Mix (Fermentas); 0.0002 mM of forward and reverse primer, 1 µl template DNA, and 3.6 µl sterile distilled water for a total volume of 10 µl. The optimized reaction conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at the annealing temperature of a primer pair used (Table 3), and 30 s at 72 °C. Immediately after the real-time PCR assay, melting curve analyses were performed ramping temperatures from 65 °C to 90 °C using a 3 s and 0.35 °C interval with continuous fluorescence recording. The initial target gene copy numbers in pot experiment samples were deduced from the standard curves.

1.6. Data analysis

On the basis of an examination of the kinetic curves of average well color development (AWCD) in each Biolog plate, 48 h measurements were chosen for further data analysis. Optical density values from 48 h measurements divided by AWCD were processed by Principal Component Analysis (PCA). Centroids of two replicate samples per treatment are shown on PCA ordination plot. Digitized DGGE gel image banding pattern was analysed using GelCompar II ver.4.0 program (Applied Maths NV, Belgium). Relationship between samples was visualized on ordination plot using PCA based on the Pearson correlation coefficient of densitometric curves obtained from the DGGE fingerprints. One-way permutational multivariate analysis (PERMANOVA) with 9999 permutations was used to test for differences in microbial community due to plant presence, treatment type and TNT addition (Anderson 2001). Before conducting PERMANOVA, the distance-based test for the homogeneity of multivariate dispersions was performed.

The Kruskal-Wallis one-way analysis of variance by ranks was applied to compare gene copy numbers and relative abundances between plant treatments (nonplanted, rye, blue fenugreek; $n = 12$ in each group) and bioaugmentation/biostimulation treatments (no treatment, biostimulation, biostimulation combined with bioaugmentation, $n = 12$ in each group). The Mann-Whitney test was applied to compare gene copy numbers and relative abundances between TNT-spiked and non-spiked samples.

2. Results

2.1. Fate of TNT and metabolites

The concentration of residual contaminants in the soil during and after bioremediation is an important and readily measurable criterion for remediation technology evaluation. Thus, the presence and concentration of TNT and its biodegradation metabolites were determined in the artificial soil samples after the 28-day experiment. TNT and two of its metabolites (2-Am-4, 6-DNT and 4-Am-2, 6-DNT) were present, even after 28 days of incubation, in all variants that contained TNT; however, there was a notable decrease from the initial TNT-contamination, even without any bioremediation (Fig. 1). Rye cultivation had the most

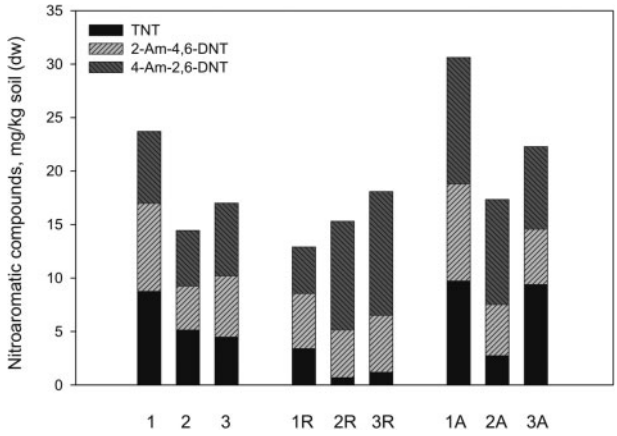

Fig. 1. Fate of TNT after 28 days of treatment (initial TNT concentration in soil 118 mg/kg). Sample codes are given in Table 1

positive effect on TNT removal in all tested variants, especially in those samples that also had inoculum and amendments. In comparison, blue fenugreek cultivation had positive effect on TNT-degradation only in the variant with inoculum and amendments. Higher degradation of TNT was observed in those samples subjected to both biostimulation and bioaugmentation.

2.2. Changes in microbial community functional diversity

Biolog EcoPlates were used to estimate how TNT and the different biotechnological applications affected the functional diversity of the culturable microbial community. PCA results of the substrate utilization patterns of all studied microbial communities indicated the formation of several distinct clusters (Fig. 2), especially in samples treated with both microbial inoculum and amendments. This indicates that the functional abilities (intensity and diversity of substrate assimilation) of the soil microbial communities were affected by the addition of the active consortium of microorganisms. Impact of bioaugmentation on microbial community functional pattern was confirmed by PERMANOVA analysis ($P < 0.01$). Bioaugmented samples aside, all tested variants without plant cultivation clustered together irrespective of the treatment applied. The type of plant cultivated (rye or blue fenugreek) resulted in no significant variance in microbial communities. The effect of TNT on the functional diversity of soil microbial communities was not statistically significant ($P > 0.05$).

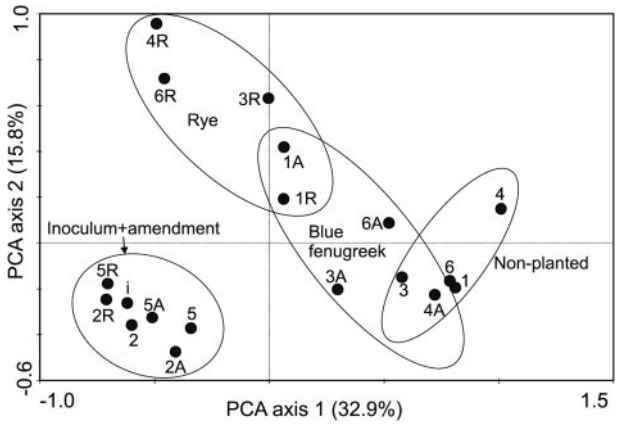

Fig. 2. Grouping of soil samples based on principal component analysis of the 48 h substrate utilization patterns obtained with Biolog EcoPlates. Sample codes are given in Table 1. Abbreviations: i – inoculum

2.3. Changes in microbial community phylogenetic structure

Multivariate analysis of DGGE data showed that the samples fell into two clusters along the first PCA axis on the basis of bioaugmentation treatment, and that a distinct group of samples was formed when the consortium of microorganisms (inoculum) was added, as shown in samples 2, 2R, 2A, 5, 5A, and 5R (Fig. 3). This group of samples was characterized by a bacterial community structure which had more similarity to the introduced bacterial consortium pattern than the rest of the samples. Strong impact of bioaugmentation on microbial community phylogenetic structure was confirmed by PERMANOVA analysis ($P < 0.001$).

The second PCA axis separated samples according to the plant treatment; samples with blue fenugreek plants were discernible from both non-planted samples and from samples with rye cultivation. Impact of plant treatment on microbial community structure was revealed by PERMANOVA analysis ($P < 0.01$). Most of the soil samples with no plant cultivation had very little variation in bacterial community phylogenetic structure and these were clustered with the rye cultivated samples, indicating that rye had a rather weak effect on the overall bacterial community structure in the soil. The PERMANOVA analysis did not

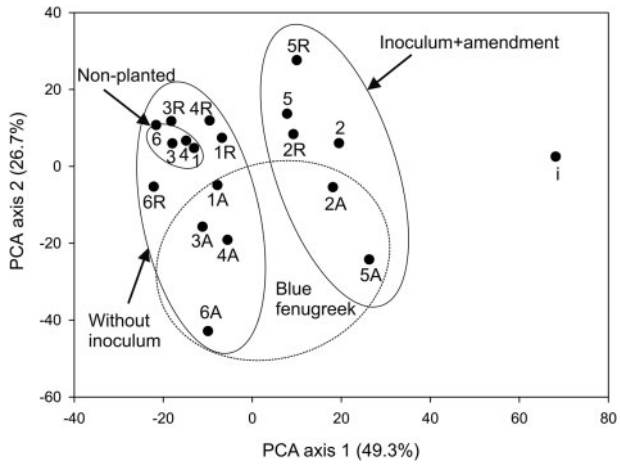

Fig. 3. Grouping of soil samples based on principal component analysis of DGGE fingerprints. Sample codes are given in Table 1. Circle with dashed line indicates soil samples with blue fenugreek cultivation. Abbreviations: i – inoculum

reveal any TNT-induced effects on the bacterial community phylogenetic structure ($P > 0.05$).

2.4. Bacterial abundances influenced by TNT and different treatments

Quantitative PCR targeting 16S rRNA genes was used to evaluate the abundance of microbial populations in the bioremediation experiments and to determine the effect of different treatments.

Table 4 shows that TNT addition to soil had no effect on the total bacterial community abundance; however, selective pressure likely enhanced the abundance of *Pseudomonas* and *Stenotrophomonas* groups (on average 2.5 and 1.4 times, respectively) compared to unspiked parallels (Fig. 4). Biostimulated samples generally showed slightly higher estimated gene copy numbers with all three primer pairs used in TNT spiked variants, as compared to unspiked parallels.

As expected, cultivation of plants, especially blue fenugreek, generally had positive effect on the total bacterial 16S rRNA copy number per gram of soil irrespective of other treatment types used (Kruskal-Wallis test, $P < 0.01$). Cultivation of blue fenugreek also enhanced the total abundance of *Pseudomonas* and *Stenotrophomonas* groups (up to 16.7 and 6.4 times, respectively; Kruskal-Wallis test, $P < 0.05$), whereas cultivation of rye did not have such a profound effect. However, when these results were assessed as functional group proportions in the total community, no clear trend resulting from the presences of plants was revealed. TNT had a statistically significant positive effect on *Stenotrophomonas* species abundance

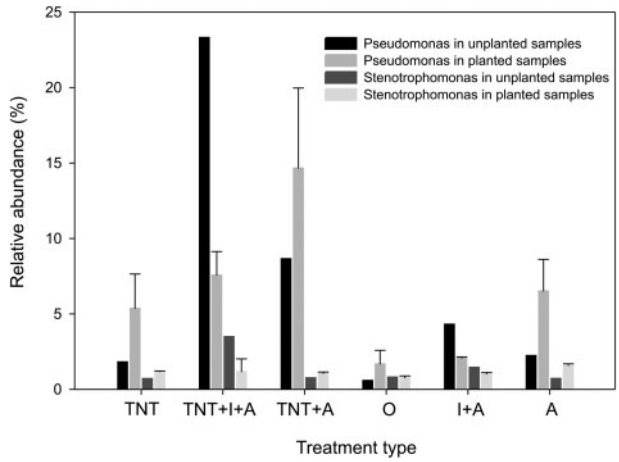

Fig. 4. Changes in relative abundance of *Pseudomonas* and *Stenotrophomonas* groups recorded by 16S rRNA gene enumerations using qPCR in samples with different treatments.
Shown are group mean and standard deviation. Treatment type abbreviations: TNT – TNT spiked soil, I – bioaugmentation, A – biostimulation, O – only planted or nonplanted samples, plus indicates combination of different treatments.

(Mann-Whitney test, $P < 0.05$). Both *Pseudomonas* and *Stenotrophomonas* groups were enhanced in TNT spiked soils treated using the combined (simultaneous bioaugmentation and biostimulation) method (Fig. 4).

3. Discussion

In an attempt to contribute to the filling of the void in TNT biodegradation research, for this study biostimulation, bioaugmentation, phytoremediation, and com-

Table 4. Quantification of 16S rRNA genes of total and selected functional groups of microbial community in soil samples. Sample labels are given in Table 1. Standard deviation is shown in brackets

Sample	Total 16S rRNA gene copies per g of soil	*Pseudomonas* 16S rRNA gene copies per g of soil	*Stenotrophomonas* 16S rRNA gene copies per g of soil
1	$7.65*10^8$ ($0.02*10^7$)	$1.37*10^7$ ($0.02*10^6$)	$5.29*10^6$ ($0.02*10^5$)
2	$3.11*10^8$ ($0.04*10^7$)	$9.42*10^7$ ($0.02*10^6$)	$1.08*10^7$ ($0.03*10^5$)
3	$6.54*10^8$ ($0.06*10^7$)	$5.64*10^7$ ($0.06*10^6$)	$4.84*10^6$ ($0.04*10^5$)
4	$5.83*10^8$ ($0.05*10^7$)	$3.28*10^6$ ($0.00*10^6$)	$4.62*10^6$ ($0.01*10^5$)
5	$3.82*10^8$ ($0.04*10^7$)	$1.64*10^7$ ($0.01*10^6$)	$5.45*10^6$ ($0.02*10^5$)
6	$5.24*10^8$ ($0.02*10^7$)	$1.16*10^7$ ($0.01*10^6$)	$3.69*10^6$ ($0.05*10^5$)
1R	$5.56*10^8$ ($0.05*10^7$)	$1.69*10^7$ ($0.01*10^6$)	$6.05*10^6$ ($0.03*10^5$)
2R	$4.24*10^8$ ($0.01*10^7$)	$3.87*10^7$ ($0.01*10^6$)	$9.75*10^6$ ($0.02*10^5$)
3R	$7.95*10^8$ ($0.08*10^7$)	$7.39*10^7$ ($0.03*10^6$)	$9.46*10^6$ ($0.02*10^5$)
4R	$7.11*10^8$ ($0.03*10^7$)	$5.16*10^6$ ($0.01*10^6$)	$4.12*10^6$ ($0.02*10^5$)
5R	$6.37*10^8$ ($0.06*10^7$)	$1.23*10^7$ ($0.01*10^6$)	$2.64*10^6$ ($0.02*10^5$)
6R	$4.52*10^8$ ($0.02*10^7$)	$3.89*10^7$ ($0.01*10^6$)	$7.63*10^6$ ($0.02*10^5$)
1A	$1.91*10^9$ ($0.01*10^7$)	$1.46*10^8$ ($0.07*10^6$)	$2.33*10^7$ ($0.15*10^5$)
2A	$3.44*10^9$ ($0.19*10^7$)	$2.04*10^8$ ($0.34*10^6$)	$1.89*10^7$ ($0.04*10^5$)
3A	$8.58*10^8$ ($0.02*10^7$)	$1.71*10^8$ ($0.03*10^6$)	$1.27*10^7$ ($0.12*10^5$)
4A	$2.12*10^9$ ($0.51*10^7$)	$5.48*10^7$ ($0.05*10^6$)	$1.89*10^7$ ($0.04*10^5$)
5A	$3.24*10^9$ ($0.07*10^7$)	$6.97*10^7$ ($0.01*10^6$)	$2.15*10^7$ ($0.01*10^5$)
6A	$1.66*10^9$ ($0.09*10^7$)	$7.23*10^7$ ($0.12*10^6$)	$2.35*10^7$ ($0.20*10^5$)

binations of these approaches for TNT-removal were selected for investigation at a laboratory scale.

The research indicates that all treatments used resulted in decreased concentrations of TNT in soil. Similar to previous reports (van Dillewijn *et al*. 2007; Travis *et al*. 2008a, b), TNT and its aminoderivates 2ADNT and 4ADNT were the only compounds detectable by HPLC; all of these were reduced to a considerable degree of the initial concentrations. The isomeric aminoderivates (2ADNT and 4ADNT) are formed during microbial transformation of TNT (Williams *et al*. 2004); these compounds exhibit less toxicity than TNT and bind more tightly to soil clay particles and organic matter, thereby reducing bioavailability as well as toxicity in soil (Pennington *et al*. 1995; Price *et al*. 1997). Of the treatments used, the combined bioaugmentation-biostimulation approach coupled with plant (especially rye) cultivation was the most effective treatment resulting in the lowest final TNT concentrations.

The PCA analyses based on the substrate utilization patterns and DGGE analysis results of studied soil microbial communities showed very distinct clustering of bioaugmented samples. This suggests that the introduced TNT-degrading microbial community was able to survive and dominate the community, which fulfills one very important prerequisite for bioaugmentation application (Thompson *et al*. 2005). This was confirmed by a community structure analysis, which showed a shift in community structures and clustering of bioaugmented samples in both planted and non-planted versions. Previous studies have established that TNT-pollution causes shifts in soil microbial community structure, usually towards the *Pseudomonadaceae* and *Xanthomonadaceae* families (George *et al*. 2008; Travis *et al*. 2008b). However, this shift was not as strong (compared to uncontaminated parallels) in this study as was the shift caused by different bioremediation treatment approaches. Furthermore, the quantification of 16S rRNA gene copy numbers of the total microbial community and two of its phylogenetic groups (*Pseudomonas* and *Stenotrophomonas*) showed that despite the stability in total community numbers, these two groups grew quite significantly in TNT-contaminated and, subsequently, bioaugmented soils. This indicates the survival of introduced microbial consortium in contaminated samples, as well as the selective pressure of TNT.

Contrary to previous findings where rhizoremediation by maize overshadowed the effect of bioaugmentation in TNT removal from soil (van Dillewijn *et al*. 2007), we found that the simultaneous application of biostimulation and bioaugmentation treatments had more profound effects on both TNT-removal and microbial community composition than the cultivation of plants. Also, great differences in the impact of selected plant species on TNT-removal, as well as on soil microbial communities were observed. Even though the cultivation of nitrogen-fixing blue fenugreek greatly enhanced the total bacterial abundance in soil, it had no significant enhancing effect on the overall proportions of the studied phylogenetic groups, resulting in less efficient removal of TNT. This could be attributed to microbes in the rhizosphere that prefer to use the compounds provided by the plant instead of attacking the complex molecule of the pollutant. On the other hand, rye cultivation, especially combined with bioaugmentation, had the most positive effect on TNT removal. The analyses of the microbial community revealed that the cultivation of rye did not affect the structure of microbial communities, but slightly enhanced the overall abundance of microbes. Rye has been used successfully in oil-sludge contaminated soil bioremediation (Muratova *et al*. 2010) and it appears that it can also be efficient in enhancing the removal of TNT by microorganisms.

Gong *et al*. (1999) have proposed that TNT can inhibit microbial growth and activity, even at very low concentrations. However, no TNT-inhibitory effect on the soil bacterial community was observed; the 16S rRNA gene abundance was comparable in TNT spiked samples and uncontaminated parallels. This corresponds well to other more recent results with similar TNT contamination rates (Travis *et al*. 2008b) and suggests that it may be possible to use even greater TNT-loads for remediation without harming the microbial community responsible for pollutant degradation.

Several recent TNT bioremediation experiments have used a methodology consisting of CFU enumeration, community level physiological profiling (CLPP), and microbial community structure analyses using DGGE to assess the microbial community parameters in tested samples (George *et al*. 2008; Travis *et al*. 2008a, b). The application of these methods has provided many interesting findings, but has also revealed several shortcomings. The only method free of cultivation bias in this selection is DGGE, which does not allow direct quantifications of parts of the community of interest. In this study, we applied quantitative PCR (qPCR) to evaluate the abundance of the total bacterial community, as well as two functionally important groups in TNT removal from soil. *Pseudomonads* are usually estimated by the CFU method, which is time-consuming and has possible cultivation bias. As an alternative, we suggest the use of *Pseudomonas* group-specific primers on a qPCR approach to achieve the same goal more accurately and in less time. Secondly *Xanthomonadaceae* is known as a family that becomes dominant in TNT-contaminated soils (George *et al*. 2008). We designed and successfully used a new *Stenotrophomonas*-specific primer set for evaluation of the abundance of the members of one genus of this family. This new primer set can be used for monitoring this bacterial group in further bioremediation studies.

Likewise to cultivation based enumeration methods, results of qPCR can be affected by workflow bias (i.e. the specificy/universality of the primers used, insufficient amplification program optimization and data handling procedure). It also has to be kept in mind, that the absolute gene copy numbers from different studies gained by using different workflow steps (i.e. different DNA extraction methods or amplification kits) are not readily comparable (Smith, Osborn 2009). Nevertheless, careful selection or design of primers, appropriate optimization of amplification programs used and meticulous data quality evaluation can minimize possible bias arising from the method used. As always, it is recommended to use qPCR in combination with other methods to achieve comprehensive overview of the ongoing processes.

Combined bioaugmentation-biostimulation treatment, coupled with rye cultivation, showed the most promise for TNT bioremediation at a laboratory scale. This assumption should further be tested at a field scale. Also, more precise notions regarding the actions of the microbial community responsible for TNT degradation could be gained with further development of the qPCR approach by designing appropriate primers to determine which nitroreductases are present and active in microbial communities of TNT-contaminated environment undergoing bioremediation treatment. In our study we used microbiological methods which address only bacteria as we were interested in impact of added bacterial strains on soil bacterial community. It is possible that applied treatments affected also fungal community in soil, and further research in this field is advisable.

Conclusions

1. Chemical analyses revealed significant decreases in TNT concentrations, including the reduction of TNT to its less toxic amino derivates. The combination of bioaugmentation-biostimulation coupled with rye cultivation had the most profound effect on TNT degradation.

2. Results from the physiological, structural, and quantitative microbial community analyses suggest that TNT had no inhibitory effect on microbes. In fact, the survival and elevation of *Pseudomonas* and *Stenotrophomonas* strains, especially *Pseudomonas,* was noted in TNT-contaminated samples.

3. Plants enhanced the total microbial community abundance, but in the case of blue fenugreek, cultivation did not significantly affect the TNT degradation rate.

4. Abundance of *Pseudomonads* in explosive containing substrates are traditionally estimated by the CFU method; instead the use of *Pseudomonas* group-specific primers in a qPCR approach allows to achieve the same goal in less time and avoids the cultivation bias.

5. A new *Stenotrophomonas*-specific primer set was designed and successfully used for evaluation of this genus abundance broadening the monitoring possibilities for further bioremediation studies.

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References

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance, *Austral Ecology* 26: 32–46.

Ayoub, K.; van Hullebusch, E. D.; Cassir, M.; Bermond, A. 2010. Application of advanced oxidation processes for TNT removal: a review, *Journal of Hazardous Materials* 178: 10–28. http://dx.doi.org/10.1016/j.jhazmat.2010.02.042

Baldrian, P. 2008. Wood-inhabiting ligninolytic basidiomycetes in soils: ecology and constraints for applicability in bioremediation, *Fungal Ecology* 1(1): 4–12. http://dx.doi.org/10.1016/j.funeco.2008.02.001

Cho, Y.-S.; Lee, B.-U.; Oh, K.-H. 2008. Simultaneous degradation of nitroaromatic compounds TNT, RDX, atrazine, and simazine by Pseudomonas putida HK-6 in bench-scale bioreactors, *Journal of Chemical Technology and Biotechnology* 83: 1211–1217. http://dx.doi.org/10.1002/jctb.1924

Clark, B.; Boopathy, R. 2007. Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana, *Journal of Hazardous Materials* 143: 643–648. http://dx.doi.org/10.1016/j.jhazmat.2007.01.034

van Dillewijn, P.; Caballero, A.; Paz, J. A.; González-Pérez, M. M.; Oliva, J. M.; Ramos, J. L. 2007. Bioremediation of 2,4,6-trinitrotoluene under field conditions, *Environmental Science & Technology* 41: 1378–1383. http://dx.doi.org/10.1021/es062165z

El Fantroussi, S.; Agathos, S. N. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation?, *Current Opinion in Microbiology* 8: 268–275. http://dx.doi.org/10.1016/j.mib.2005.04.011

Esteve-Nuñez, A.; Ramos, J. L. 1998. Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas sp.* JLR11, *Environmental Science & Technology* 32: 3802–3808. http://dx.doi.org/10.1021/es9803308

George, I.; Eyers, L.; Stenuit, B.; Agathos, S. N. 2008. Effect of 2,4,6-trinitrotoluene on soil bacterial communities, *Journal of Industrial Microbiology and Biotechnology* 35: 225–236. http://dx.doi.org/10.1007/s10295-007-0289-2

Gerhardt, K. E.; Huang, X.-D.; Glick, B. R.; Greenberg, B. M. 2009. Phytoremediation and rhizoremediation of organic

soil contaminants: potential and challenges, *Plant Science* 176: 20–30. http://dx.doi.org/10.1016/j.plantsci.2008.09.014

Gerth, A.; Hebner, A. 2007. Risk assessment and remediation of military and ammunition sites, in Marmiroli, N.; Samotokin. B.; Marmiroli, M. (Eds.). *Advanced science and technology for biological decontamination of sites affected by chemical and radiological nuclear agents.* NATO science series, vol 75, Springer, Heidelberg, 45–57.

Gong, P.; Siciliano, S. D.; Greer, C. W.; Paquet, L.; Hawari, J.; Sunahara, G. I. 1999. Effects and bioavailability of 2,4,6-trinitrotoluene in spiked and field-contaminated soils to indigenous microorganisms, *Environmental Toxicology and Chemistry* 18: 2681–2688. http://dx.doi.org/10.1002/etc.5620181205

Hughes, J. B.; Shanks, J.; Vanderford, M.; Lauritzen, J.; Bhadra, R. 1996. Transformation of TNT by aquatic plants and plant tissue cultures, *Environmental Science & Technology* 31: 266–271. http://dx.doi.org/10.1021/es960409h

Juhanson, J.; Truu, J.; Heinaru, E.; Heinaru, A. 2009. Survival and catabolic performance of introduced *Pseudomonas* strains during phytoremediation and bioaugmentation field experiment, *FEMS Microbiology Ecology* 70: 446–455. http://dx.doi.org/10.1111/j.1574-6941.2009.00754.x

Kalderis, D.; Juhasz, A. L.; Boopathy, R.; Comfort, S. 2011. Soils contaminated with explosives: environmental fate and evaluation of state-of-the-art remediation processes (IUPAC technical report), *Pure and Applied Chemistry* 83(7): 1407–1484. http://dx.doi.org/10.1351/PAC-REP-10-01-05

Kapley, A.; Prasad, S.; Purohit, H. J. 2007. Changes in microbial diversity in fed-batch reactor operation with wastewater containing nitroaromatic residues, *Bioresource Technology* 98: 2479–2484. http://dx.doi.org/10.1016/j.biortech.2006.09.012

Kuiper, I.; Lagendijk, E. L.; Bloemberg, G. V.; Lugtenberg, B. J. J. 2004. Rhizoremediation: a beneficial plant-microbe interaction, *Molecular Plant-Microbe Interactions* 17(1): 6–15. http://dx.doi.org/10.1094/MPMI.2004.17.1.6

Lachance, B.; Robidoux, P. I.; Hawari, J.; Ampleman, G.; Thiboutot, S.; Sunahara, G. I. 1999. Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro, *Mutation Research: Genetic Toxicology and Environmental Mutagenesis* 444(1): 25–39. http://dx.doi.org/10.1016/S1383-5718(99)00073-X

Limane, B.; Muter, O.; Juhanson, J.; Truu, M.; Truu, J.; Nõlvak, H. 2011. Characterization of microbial community structure after application of different bioremediation approaches in TNT contaminated soil, in *Proceedings of the 8th International Conference Environmental Engineering*, 19–20 May, 2011, Vilnius, Lithuania, 188–194.

Makris, K. C.; Shakya, K. M.; Datta, R.; Sarkar, D.; Pachanoor, D. 2007. Chemically catalyzed uptake of 2,4,6-trinitrotoluene by *Vetiveria zizanioides*, *Environmental Pollution* 148: 101–106. http://dx.doi.org/10.1016/j.envpol.2006.10.047

Muratova, A. Y.; Bondarenkova, A. D.; Panchenko, L. V.; Turkovskaya, O. V. 2010. Use of integrated phytoremediation for cleaning-up of oil-sludge-contaminated soil, *Applied Biochemistry and Microbiology* 46(8): 789–794. http://dx.doi.org/10.1134/S0003683810080090

Muter, O.; Versilovskis, A.; Scherbaka, R.; Grube, M.; Zarina, D. 2008. Effect of plant extract on the degradation of nitroaromatic compounds by soil microorganisms, *Journal of Industrial Microbiology and Biotechnology* 35: 1539–1543. http://dx.doi.org/10.1007/s10295-008-0455-1

Muyzer, G.; Waal, E. C. D.; Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Applied and Environmental Microbiology* 59: 695–700.

Nõlvak, H.; Sildvee, T.; Kriipsalu, M.; Truu, J. 2012. Application of microbial community profiling and functional gene detection for assessment of natural attenuation of petroleum hydrocarbons in boreal subsurface, *Boreal Environmental Research* 17: 113–127.

Oh, K.-H.; Kim, Y.-J. 1998. Degradation of explosive 2,4,6-trinitrotoluene by s-triazine degrading bacterium isolated from contaminated soil, *Bulletin of Environmental Contamination and Toxicology* 61: 702–708. http://dx.doi.org/10.1007/s001289900818

Øvreås, L.; Forney, L.; Daae, F. L.; Torsvik, V. 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA, *Applied and Environmental Microbiology* 63(9): 3367–3373.

Pennington, J. C.; Hayes, C. A.; Myers, K. F.; Ochmann, M.; Gunnison, D.; Felt, D. R.; McCormick, E. F. 1995. Fate of 2,4,6-trinitrotoluene in a simulated compost system, *Chemosphere* 30: 429–438. http://dx.doi.org/10.1016/0045-6535(94)00422-Q

Preston-Mafham, J.; Boddy, L.; Randerson, P. F. 2002. Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles – a critique, *FEMS Microbiology Ecology* 42(1): 1–14.

Price, C. B.; Brannon, J. M.; Hayes, C. A. 1997. Effect of redox potential and pH on TNT transformation in soil-water slurries, *Journal of Environmental Engineering* 123(10): 988–992.

Ruberto, L.; Vazquez, S. C.; Mac Cormack, W. P. 2003. Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil, *International Biodeterioration & Biodegradation* 52: 115–125. http://dx.doi.org/10.1016/S0964-8305(03)00048-9

Rylott, E. L.; Lorenz, A.; Bruce, N. C. 2011. Biodegradation and biotransformation of explosives, *Current Opinion in Biotechnology* 22(3): 434–440. http://dx.doi.org/10.1016/j.copbio.2010.10.014

Schnoor, J. L.; Light, L. A.; McCutcheon, S. C.; Wolfe, N. L.; Carreira, L. H. 1995. Phytoremediation of organic and nutrient contaminants, *Environmental Science & Technology* 29: 318–323.

Smith, C. J.; Osborn, A. M. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in

microbial ecology, *FEMS Microbiology Ecology* 67: 6–20. http://dx.doi.org/10.1111/j.1574-6941.2008.00629.x

Snellinx, Z.; Taghavi, S.; Vangronsveld, J.; van der Lelie, D. 2003. Microbial consortia that degrade 2,4-DNT by interspecies metabolism: isolation and characterization, *Biodegradation* 14: 19–29. http://dx.doi.org/10.1023/A:1023539104747

Stenuit, B. A.; Agathos, S. N. 2010. Microbial 2,4,6-trinitrotoluene degradation: could we learn from (bio) chemistry for bioremediation and vice versa?, *Applied Microbiology and Biotechnology* 88: 1043–1064. http://dx.doi.org/10.1007/s00253-010-2830-x

Thompson, I. P.; van der Gast, C. J.; Ciric, L.; Singer, A. C. 2005. Bioaugmentation for bioremediation: the challenge of strain selection, *Environmental Microbiology* 7: 909–915. http://dx.doi.org/10.1111/j.1462-2920.2005.00804.x

Travis, E. R.; Bruce, N. C.; Rosser, S. J. 2008a. Microbial and plant ecology of a long-term TNT-contaminated site, *Environmental Pollution* 153: 119–126. http://dx.doi.org/10.1016/j.envpol.2007.07.015

Travis, E. R.; Bruce, N. C.; Rosser, S. J. 2008b. Short term exposure to elevated trinitrotoluene concentrations induced structural and functional changes in the soil bacterial community, *Environmental Pollution* 153: 432–439. http://dx.doi.org/10.1016/j.envpol.2007.08.006

Untergasser, A.; Nijveen, H.; Rao, X.; Bisseling, T.; Geurts, R.; Leunissen, J. A. M. 2007. Primer3Plus, an enhanced web interface to Primer3, *Nucleic Acids Research* 35(supp. 2): W71–W74. http://dx.doi.org/10.1093/nar/gkm306

Verma, V.; Raju, S. C.; Kapley, A.; Kalia, V. C.; Daginawala, H. F.; Purohit, H. J. 2010. Evaluation of genetic and functional diversity of *Stenotrophomonas* isolates from diverse effluent treatment plants, *Bioresource Technology* 101(20): 7744–7753. http://dx.doi.org/10.1016/j.biortech.2010.05.014

Williams, R. E.; Rathbone, D. A.; Scrutton, N. S.; Bruce, N. C. 2004. Biotransformation of explosives by the old yellow enzyme family of flavoproteins, *Applied and Environmental Microbiology* 70: 3566–3574. http://dx.doi.org/10.1128/AEM.70.6.3566-3574.2004

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