Journal of Hazardous Materials 165 (2009) 285–290

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Incorporation of 2,4,6-trinitrotoluene (TNT) transforming bacteria into explosive formulations

G.S. Nyanhongo[a,*], N. Aichernig[a], M. Ortner[a], Walter Steiner[b], G.M. Guebitz[a]

[a] Department of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria
[b] Institute of Biotechnology and Bioprocess Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

ARTICLE INFO

Article history:
Received 19 August 2008
Received in revised form
29 September 2008
Accepted 29 September 2008
Available online 7 October 2008

Keywords:
2,4,6-Trinitrotoluene (TNT)
Explosive
Biotransformation
Immobilization
Humic monomers

ABSTRACT

Pseudomonas putida GG04 and *Bacillus* SF have been successfully incorporated into an explosive formulation to enhance biotransformation of TNT residues and/or explosives which fail to detonate due to technical faults. The incorporation of the microorganisms into the explosive did not affect the quality of the explosive (5 years storage) in terms of detonation velocity while complete biotransformation of TNT moieties upon transfer in liquid media was observed after 5 days. The incorporated microorganisms reduced TNT sequentially leading to the formation of hydroxylaminodinitrotoluenes (HADNT), 4-amino-2,6-dinitrotoluenes; 2-amino-4,6-dinitrotoluenes, different azoxy compounds; 2,6-diaminonitrotoluenes (2,4-DAMNT) and 2,4-diaminonitrotoluenes (2,6-DAMNT). However, the accumulation of AMDNT and DAMNT (major dead-end metabolites) was effectively prevented by incorporating guaiacol and catechol during the biotransformation process.

1. Introduction

TNT is an explosive which has dominated the military production industry since 1902 reaching its peak during the World Wars. It is used in a wide range of application in shells, bombs, grenades, demolition explosives and propellant compositions. In addition to its explosion problems, the electrophillic nature of its nitro groups enables it to readily oxidize biological molecules [1] making it a serious health threat. Human health problems associated with TNT have been documented worldwide during the manufacture, handling, at waste disposal sites, residues from detonation, bombs buried underground and also during dismantlement of munitions [2,3]. For example during the first year of the First World War, 475 munitions workers died in the USA due to poisoning with TNT [4]. Equally important are accidents which occur in the post-war period, where explosives (land mines) buried underground during the war continue to claim innocent victims long after the war is fought.

Although a number of technologies have been developed for TNT waste treatment and the bioremediation of heavily TNT contaminated sites (anaerobic and aerobic bioslurry reactors), destruction of residue explosives arising from civil application, explosives which fail to detonate, or underground buried explosives which persist long after intended period in the soil has not yet been considered. This is so, especially since the explosives residues are spread over wide distances while no one has an idea where they are located. The current work therefore attempts to address the destruction of residue explosives arising from partially detonated explosives during civil and military application, explosives which fail to detonate, or underground buried explosives which persist long after intended period in the soil and leak, resulting in underground water pollution. For the first time we show that this is achieved by incorporating microorganisms into the explosives, which initiate destruction of the explosive without compromising the explosive quality after long term storage.

For a long time biodegradation of TNT has been shown to predominantly result in biotransformation leading to the accumulation of aminodinitrotoluenes (AMDNT), azoxy compounds and diaminonitrotoluenes (DAMNT) which are both toxic and carcinogenic [5–7]. However, several studies including from our lab have shown the possibility of irreversibly binding TNT metabolites onto organic soil constituents leading to detoxification [8,9]. Daun et al. [10] showed that under anaerobic conditions biotransformation products like 2-HADNT, 4-HADNT and 2,4,6-triaminotoluene (TAT) strongly bind to humic acids and clay. Therefore another feature of this work is to investigate the possibility of binding TNT biotransformation products onto humic model substances which are substrates of phenoloxidases produced by the incorporated microorganisms during the reduction process.

* Corresponding author. Tel.: +43 316 873 8312; fax: +43 316 873 8815.
E-mail address: gnyanhongo@yahoo.com (G.S. Nyanhongo).

0304-3894/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2008.09.107

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade; 2,4,6-trinitrotoluene (TNT); 2,6-dinitrotoluene and ρ-nitrotoluene were supplied by a local explosives producer while 2,4-dinitrotoluene; 2,4-diamino-6-nitrotoluene; 2,6-diamino-4-nitrotoluene; 2,2,6,6-tetraazoxynitrotoluene; 4-amino-2,6-dinitrotoluene; 2-amino-4,6-dinitrotoluene; 2,6-dinitrotoluene and 2,4-dinitrotoluene were purchased from Accustandards Inc. (New Haven, USA). Contaminated soils and wastewater samples for isolation of microorganisms were also provided by the local explosives producer and stored at 4 °C. Humic monomers (guaiacol, ferulic acid; syringic acid and catechol were purchased from Sigma. All other chemicals were obtained from Sigma–Aldrich and Merck.

2.2. Isolation of microorganisms

For isolation of microorganisms TNT contaminated soil and wastewater samples were used as source for the isolation of microorganisms. The enrichment procedure and the detection of TNT removal activity was as described by Gunnison et al. [11]. Pure isolated microorganisms were sent for identification to the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. In addition, TNT biotransformation capability of the thermoalkalophilic *Bacillus* SF strain previously isolated from textile wastewater [12] was investigated.

2.3. Liquid cultures

A medium containing ($g\,l^{-1}$) 10 g glucose, 6 g yeast extract, 5 g $(NH_4)_2SO_4$ was autoclaved at 121 °C for 15 min, cooled and then supplemented with 100 ml sterile basal salt medium. The salt medium contained ($g\,l^{-1}$) K_2HPO_4 0.1, $(NH4)_2SO_4$ 0.4, $MgSO_47H_2O$ 0.05, KCl 0.2, $CaCl_2{\cdot}2H_2O$ 0.005, $FeC1_2{\cdot}H_2O$ 0.005. TNT solution was prepared by suspending 50 g TNT in 500 ml deionized water and heated at 85 °C for 20 min with intermittent stirring. The solution was allowed to cool at room temperature and then filtered to remove undissolved TNT. The culture medium (25 ml) dispensed into a 250 ml buffered flask was then supplemented with 160 μM TNT solution final concentration. Each flask was then inoculated with 10 ml bacterial culture in its exponential phase (approximately 3.2 absorbance units at 600 nm). The inoculated flasks (100 ml working volume) were incubated at 30 °C at 150 rpm. Sampling was done periodically by withdrawing 4 ml which were then centrifuged at 10,000 rpm for 20 min. Apart from using the clear supernatant directly for TLC and HPLC analysis, acetonitrile (99%) in the ratio 1:4 (acetonitrile to sample) was added to the clear supernatant, thoroughly mixed and the mixture frozen at −20 °C over night. The resulting organic top phase was then used for TLC and HPLC analysis.

2.4. Identification of enzymes involved in initial TNT biotransformation

Cells of *Pseudomonas putida* GGO4 and of *Bacillus* SF harvested from a 2-l culture medium in their late exponential phase (approximately 3.2 absorbance units at 600 nm) were washed twice with 50 mM phosphate buffer (pH 7.0) and re-suspended in 100 ml of the same buffer. The suspended cells were cooled on ice and broken with ultrasonic pulses at 2 min intervals (Model Bandelin Sonorex Super RK102H, Berlin, Germany). Cell breakage was monitored microscopically. Cell debris were removed by centrifuging at 20,000 rpm for 20 min at 4 °C. The extracellular fluid, the broken cells and the cytosolic fraction (supernatant) were then separately incubated with TNT in the absence or presence of cofactors (NADPH, FADH, NADH) for 4 h. The reaction mixture contained 150 μl of the individually separated fraction, 800 μl 50 mM phosphate buffer at pH 7.0 containing 200 μM TNT and 1.5 mM cofactor concentration. A control was prepared with denatured fractions (boiled at 100 °C for 10 min) and TNT transformation was also monitored as described below.

2.5. Immobilization of TNT metabolites on humic monomers during TNT transformation

P. putida GG04 and *Bacillus* SF cultures in the mid exponential phase were inoculated with 160 μM TNT solution final concentration and then supplemented with 600 μM of the respective humic monomers (guaiacol, ferulic acid syringic acid and catechol). Three controls were prepared. One control flask was supplemented with TNT only while another was also supplemented with the respective humic monomers in the absence of TNT and the third was boiled at 100 °C for 20 min to inactivate the bacteria and their enzymes before supplementing it with TNT only. The decrease or reduction in the formation of major TNT metabolites in cultures was recognized as the effect caused by the humic monomers. This was justified by comparing cultures supplemented with humic monomers and without humic monomers.

2.6. Monitoring TNT transformation

During incubation periods residual TNT and TNT biotransformation products were monitored using different approaches as described below.

2.6.1. Thin layer chromatography (TLC) analysis of metabolites

Samples were spotted on silica gel 60 plates with fluorescent background (J.T. Backer Inc., Phillipsburg, NJ, USA), and developed in benzene:hexane (3:1) as mobile phase. For identification of metabolites, developed plates were observed under UV at 254 nm. Plates were further sprayed with 10% tetramethylammonium hydroxide [13] and immediately dried. This allowed the differentiation of the metabolites based on color as well as on R_f-values. The metabolites were identified by comparing R_f values and color to authentic standards. Detection of TNT biotransformation products with amino groups was done by immersing developed TLC plates in a solution of $NaNO_2$ (0.1%, w/v) in a 1N HCl for 1–2 min enough to make the plates wet. Afterwards the plates were dried. The plates were then further immersed in N-1-naphthylethylenediamine HCl (0.04%, w/v) solution [14] for 2 min and allowed to stand for another 3 min before drying them. Colors resulting from this reaction were compared with colors from the authentic standards.

2.6.2. HPLC analysis of TNT and its biotransformation products

For detection and confirmation of products of TNT and its metabolites already identified by TLC, above described methods were used for HPLC analysis. The analysis was performed by HPLC system from Dionex with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector (UV 220 nm). Identification and quantitative determination of TNT and its transformation products was done by reversed phase HPLC, on a Discovery HS C18 column (5 μm; 25 cm × 4.6 mm, Supelco, Bellefonte, USA) using 50/50 methanol: deionized water as solvent at a flow rate of 1 ml min^{-1} and at an oven temperature of 40 °C.

2.7. Preparation of microorganisms for incorporation into explosives

2.7.1. Preparation of Bacillus SF for incorporation into explosives

A sporulation medium for *Bacillus* SF containing per liter 8 g bacterial nutrient broth, 1 g KCl, 0.25 g $MgSO_4$ and 0.5 ml NaOH (1 M) was prepared and autoclaved at 121 °C for 15 min. The medium was left to cool down to 50 °C before supplementing it with 1 ml of the medium containing $Ca(NO_3)_2{\cdot}4H_2O$ (1 M); MnCl (0.01 M) and 1 ml $FeSO_4$ (1 mM) [15]. The medium was inoculated by aseptically transferring the microorganisms from an agar plate into a 1 l Erlenmeyer flask containing 500 ml medium and incubated at 40 °C at 150 rpm for 2 days to allow the formation of high biomass. The cultures were then supplemented with 50 g wood flour (component of explosive formulation) to enable the organism to adsorb on to the flour and further incubated while monitoring spore formation. Spore formation was monitored microscopically in the following way. A drop from the culture was mixed with malachite green on a glass slide and fixed by gently heating until for 2–3 min. The so fixed slide was then washed with distilled water and further counter stained with safranin for 1 min. The spores stained green while the vegetative cells stained red. Finally the cultures were harvested by carefully decanting excess medium. The remaining filter cake was transferred into 1-l plastic bottles and frozen at −20 °C.

2.7.2. Preparation of P. putida GG04 for incorporation into explosives

For incorporation of *P. putida* GG04 into the explosive, different cryo-protective media were compared. The screened cryoprotectants were milk, honey, raffinose, Na-glutamate, trehalose [16]. Preliminary screening in cryovials showed 5% raffinose as the best protective media evidenced by the viability test of 49% and was therefore chosen for incorporation. *P. putida* GG04 culture (500 ml) was grown in the medium described before up to mid-exponential phase (approximately 1.2 absorbance units monitored at 600 nm). Two times of 500 ml culture were then transferred into a 2-l Erlernmeyer flask containing 50 g sterile wood flour to enable the organism to adsorb on the wood flour. This was further incubated and later harvested in the late exponential phase by filtering excess water through a 0.45 μm filter membrane under sterile conditions. The filter cake (wood flour and microorganisms) was then packed into 1-l plastic bottles which were supplemented with 200 ml 5% raffinose solution as a protective medium and frozen at −20 °C.

For the lyophilization and incorporation of the filter cake of both cultures into the explosive, frozen samples were then freeze dried using the Labconco Freeze Dry System/FreeZone© 4.5 Liter Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. The freeze dried filter cakes of *P. putida* GG04 and of *Bacillus* SF were mixed and then incorporated into a TNT based explosive by the local explosives producer. The quality of the explosive was assessed by measuring the detonation velocity of the explosive.

2.8. Recovery of microorganisms from detonation residues and explosive formulation

To investigate whether *P. putida* GG04 and *Bacillus* SF were affected due to incorporation into the explosive formulation, 3 g of the TNT based explosive was inoculated in 200 ml culture medium and incubated under similar conditions described before. The TNT biotransformation process was monitored by TLC and HPLC. Agar plates overlaid with TNT crystal as in the isolation procedure were also inoculated with the explosive formulation containing microorganism. The agar plates were scored for growth and no growth. Long term survival of the microorganism was monitored over a period of 3 years by inoculating 1 g of the ammonium nitrate explosive containing microorganism onto agar plates and then scoring the colony forming units.

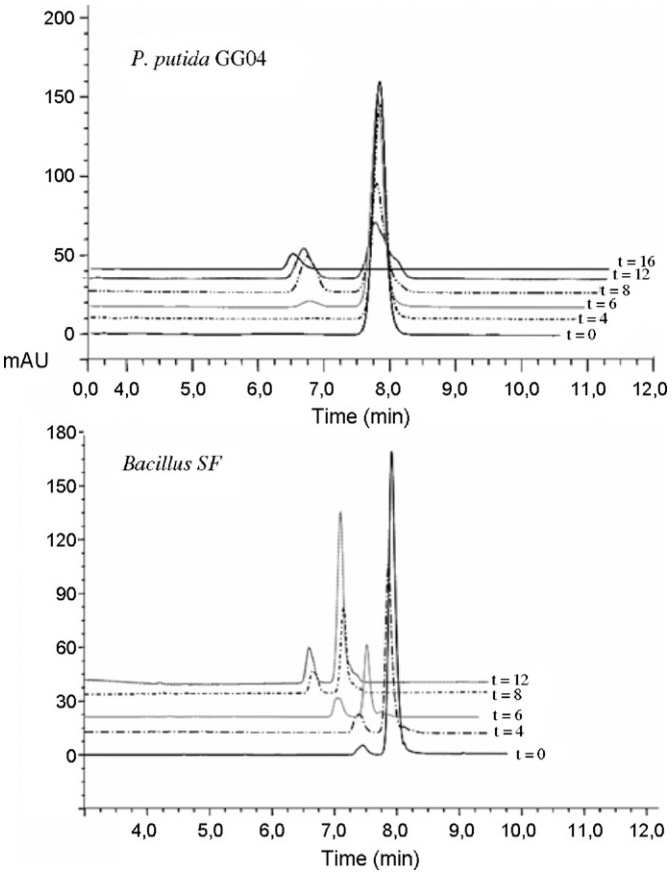

Fig. 1. HPLC chromatograms of TNT (160 μM) biodegradation profiles by *Bacillus* SF and *P putida* GG04 in liquid cultures at different times (*t*) in hours.

3. Results and discussion

A bacterial strain (GG04) growing on TNT overlaid crystal was isolated and identified as *P. putida*. *P. putida* GG04 attacked the TNT crystals forming dark red colonies on the agar plates. TNT transformation by *P. putida* GG04 and *Bacillus* SF (thermoalkalophilic *Bacillus* SF strain isolated previously from textile wastewater [12] showed different profiles in liquid cultures as shown by HPLC chromatograms in Fig. 1 and color of cultures. TNT biotransformation by *P. putida* GG04 turned the color of the culture medium to a deep red color while *Bacillus* SF cultures turned yellow. Complete TNT biotransformation of 160 μM TNT was achieved in less than 12 h in *Bacillus* SF although a much longer time (16 h) was required by *P. putida* GG04 (Fig. 1).

Therefore from the color of the culture medium, HPLC chromatograms profiles (Fig. 1) and TLC profiles it is clear that *P. putida* GG04 and *Bacillus* SF use different pathways during TNT biotransformation. Similarly, Kalafut et al. [17] observed different ability of three aerobic bacterial strains (*Pseudomonas aeruginosa*, *Bacillus* sp. and *Staphylococcus* sp.) to grow in the presence of TNT and transform it. In another similar screening study involving Gram negative and Gram positive bacteria, the former were found to be more efficient [18,19]. However, in agreement with Kalafut et al. [17], *Bacillus* SF (a Gram positive bacterium) was shown to be more efficient in degrading TNT than *Pseudomonas* (Fig. 1) in this study.

Table 1
Identification of TNT degradation metabolites during TNT degradation by *P. putida* GG04 and *Bacillus* SF.

TNT metabolites	*Bacillus* SF	*P. putida* GG04
4-HADNT	–	+
2-HADNT	–	+
2-AMDNT	+	+
4-AMDNT	+	+
3 Azoxy-compound	+	+
2,2,6,6-Tetraazoxytetranitrotoluene	+	+
2,6-DAMNT	+	+
2,4-DAMNT	+	+
Deep red metabolite	–	+

(–) Metabolite not detected; (+) metabolite detected.

3.1. TNT biotransformation metabolites

Several TNT biotransformation metabolites were observed on TLC and HPLC although only a total of 8 different TNT metabolites were positively identified (Table 1). Hydroxylaminodinitrotoluenes (2-HADNT and 4-HADNT) were the first detectable metabolites observed in *P. putida* GG04 cultures. In *Bacillus* SF the products detected during the first early incubation periods were 2-AMDNT and 4-AMDNT and azoxytetranitrotoluene compounds. The amino group containing metabolites (aminodinitrotoluenes and diaminonitrotoluenes) could only be differentiated after treating the TLC plate with $NaNO_2$ followed by *N*-1-naphthylethylenediamine solution. Aminodinitrotoluenes gave a characteristic intense red color while 2,6-diamino-4-nitrotoluene gave a violet color. Although monoaminodinitrotoluenes isomers could not be separated by TLC, they were separated by HPLC. 2-Amino-4,6-dinitrotoluene had a retention time of 12 min while 4-amino-2,6-dinitrotoluene had a retention time of 11.4 min. Diaminonitrotoluenes (2,6-diamino-4-nitrotoluene and 2,4-diamino-6-nitrotoluene) with elution time of 3.3 and 3.0, respectively were also resolved using HPLC. Furthermore it was possible to identify and separate 2,4-dinitrotoluene by HPLC which had similar R_f value as TNT on TLC plates. Other products identified using the HPLC included 2,6-dinitrotoluene and 2,2,6,6-tetranitro-4,4-azoxytoluene.

The highly hydrophilic, deep red metabolite in *P. putida* GG04 which could not be extracted using most of the organic solvents like methylchloride, chloroform, ethylacetate and hexane was only recovered by lowering pH below 2.5. The properties of the red metabolite in *P. putida* GG04 cultures are consistent with data reported by previous researchers for TNT–Meiseinheimer complexes [20–23]. Although AMDNTs and DAMNTs started accumulating at the same time in *P. putida* GG04 cultures, DAMNTs (2,4-DAMNT and 2,6-DAMNT) were the predominant metabolites, which steadily increased achieving 58 and 43 μM in *P. putida* GG04 cultures respectively (Fig. 2). Similarly 2,4-DAMNT and 2,6-DAMNT were the predominant metabolites in *Bacillus* SF cultures reaching 71 and 48 μM, respectively (Fig. 2). Although the TNT reduction was slow during the first 4 h in *Bacillus* SF cultures, thereafter the conversion proceeded faster achieving complete degradation of TNT during 12 h of incubation.

3.2. Identification of TNT degrading enzymes

To identify the enzymes involved in TNT reduction, the extracellular fractions, the cell debris and the cytosolic fractions were incubated with TNT in the presence and absence of cofactors. TNT transformation activity was detected in intracellular fractions only for both *P. putida* GG04 and *Bacillus* SF in the presence of NADPH and NADH (Table 2). In particular, *P. putida* GG04 transformed 78 μM TNT in the presence of NADPH and 33 μM in the presence of NADH. However, the amount of TNT transformed by *Bacillus* SF intracellular fractions in the presence of NADPH and NADH was almost the same (Table 2). Nitroreductases have been identified as the enzymes involved in initial TNT degradation in all organisms where enzymatic studies have been carried out [8,9]. The preferred reduction of TNT is attributed to the strong electron withdrawing properties of the nitro group which confers a high electron deficiency on the TNT molecule [24]. The fact that TNT transformation starts immediately after adding TNT to cultures clearly shows that the enzymes responsible are produced constitutively.

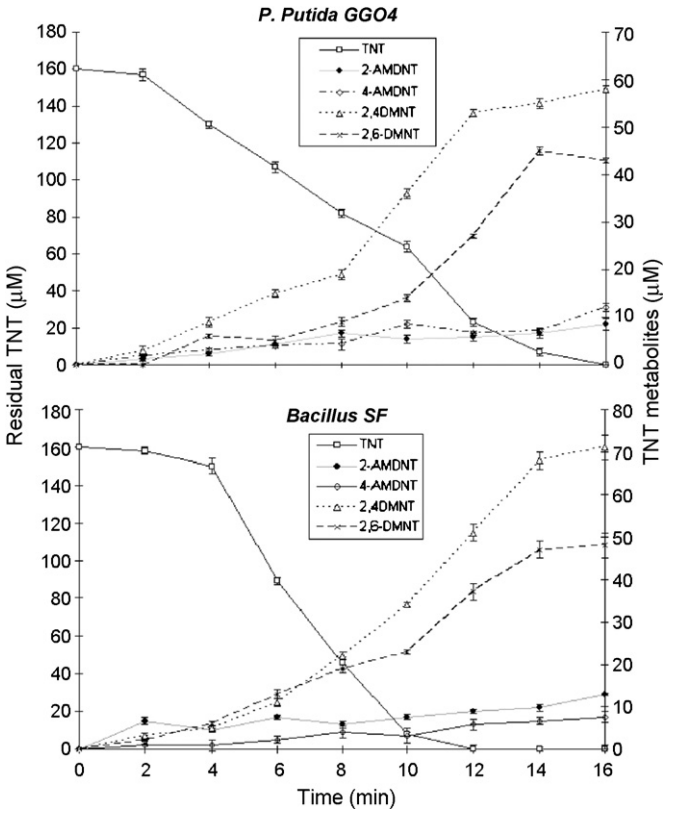

Fig. 2. Formation of ADMNT and DAMNT during TNT degradation by *P. putida* GG04 and *Bacillus* SF.

3.3. Immobilization of TNT metabolites onto humic monomers

Since AMDNT (2-AMDNT and 4-AMDNT) and DAMNT (2,4-DAMNT and 2,6-DAMNT) constituted the major TNT metabolites which persisted during the incubation period, incorporation of humic monomers (catechol, ferulic, syringic acid and guaiacol) was investigated to prevent their formation (Table 3). Preliminary experiments showed the ability of *P. putida* GG04 and *Bacillus* SF to transform all the humic monomers. In both cases, cultures of *P. putida* GG04 and *Bacillus* SF supplemented with guaiacol were more efficient in preventing the formation of AMDNT and DAMNT

Table 2
Percentage of 100 μM TNT degradation by intracellular fractions from *P. putida* GG04 und *Bacillus* SF after 4 h of incubation.

Enzyme preparation	No cofactor	NADH	FADH	NADPH
P. putida GG04	4.2 ± 0.8	33 ± 4	3.5 ± 2	78 ± 2
Bacillus SF	3.8 ± 1	66 ± 3	4.9 ± 0.5	64 ± 5

Table 3
Effect of incorporating humic monomers (guaiacol, ferulic acid, syringic acid and catechol) during TNT degradation process on the formation of AMDNT and DAMNT in *Bacillus* SF cultures and *P. putida* GG04 cultures.

Products	Concentrations of AMDNTs and DAMNTs in control (μM)	Percentage decrease in the formation of AMDNTs and DAMNTs in the presence of different humic monomers			
		Ferulic acid	Guaiacol	Catechol	Syringic acid
Bacillus SF					
2-AMDNT	24 ± 3	23 ± 1	90 ± 4	63 ± 2	35 ± 4
4-AMDNT	31 ± 1	33 ± 2	82 ± 1	68 ± 2	24 ± 4
2,4-DAMNT	58 ± 2	17 ± 1	95 ± 4	71 ± 3	39 ± 4
2,6-DAMNT	43 ± 2	21 ± 2	96 ± 3	60 ± 1	37 ± 3
P. putida GG04					
2-AMDNT	29 ± 4	22 ± 3	88 ± 3	61 ± 4	21 ± 3
4-AMDNT	17 ± 1	19 ± 4	84 ± 2	71 ± 3	27 ± 5
2,4-DAMNT	71 ± 2	45 ± 1	92 ± 4	74 ± 3	48 ± 1
2,6-DAMNT	48 ± 3	25 ± 5	90 ± 2	65 ± 4	38 ± 2

(Table 3). The ability to oxidize humic monomers is not surprising since several bacterial species including those from the same *Bacillus* sp. and *P. putida* have been shown to produce different phenol oxidizing enzymes [25]. It is possible that the radicals generated by the bacterial phenoloxidases from humic monomers may have reacted with HADNT thereby reducing the formation of AMDNT and DAMNT. Using different TNT metabolites, Nyanhongo et al. [26] demonstrated complete immobilization of HADNT in the presence different humic monomers tested in the presence of fungal laccases. However, ferulic acid and syringic acid supplemented cultures did not effectively prevent the formation AMDNT and DAMNT in both cultures (Table 3). This is in contrast with earlier observation by Nyanhongo et al. [27] where incorporation of ferulic acid in *Trametes modesta* cultures was shown to prevent the accumulation of all AMDNT by at least 92%. This maybe attributed to the culture conditions since fungal cultures are in the acidic range while bacterial cultures are usually near neutral pH range. Independently of the culture conditions different humic monomers have also been shown to have different binding capability. For example, a total of 71% TNT was immobilized with catechol as co-substrate while only 25 was immobilized with humic acid as co-substrate [28]. Further, Achtnich et al. [29] using ^{14}C-labelled TNT observed that between 11 and 16% of the radioactivity was in the fulvic and humic acid fraction while 71% was in the humin fraction. Thorn et al. [30] also provided evidence for different binding capacity of TNT metabolites by different humic fractions. Summing up the immobilization studies in this work and that of previous researchers, it is obvious that humic monomers have different efficiencies in immobilizing TNT biotransformation metabolites.

3.4. Incorporation of microorganisms into explosive formulation

The ability of *Bacillus* SF to produce spores was taken as advantage for its incorporation into the explosive resulting in 81×10^{10} colony forming units (CFU) per gram wood flour. Since *P. putida* GG04 does not produce spores, lyophilization was chosen as the best option. Several potential cryoprotectants (milk, honey, Na-glutamate, trehalose, saccharose, lactose meso-inositol and sucrose) were tested and raffinose was observed to offer better protection achieving a survival rate of 49% after lyophilization. Among the cryoprotectants used, 5% raffinose gave the highest yield of 39.3×10^7 CFU g^{-1} wood flour while the worst was inositol which gave a yield of 0.5×10^2 CFU g^{-1} wood flour. A large number of different cryoprotectants and bulking agents have been reported for stabilizing bacterial suspensions including saccharose, lactose, trehalose, glycerol, sodium glutamate, meso-inositol, honey, raffinose, sucrose and skimmed milk [17,31]. These substances generally improve resistance of organism to freezing but their performances depend on the organism. There is therefore an additional need for further studies to find the best protective medium. However, for this study 5% raffinose was chosen as protective media for the incorporation of *P. putida* GG04 into the explosive. The wood flour samples coated with microorganisms (*Bacillus* SF and *P putida* GG04) were incorporated into TNT/ammonium nitrate based explosive.

After incubation of bioexplosives on agar-plates overlaid with TNT crystals, the crystals disappeared forming a mixture of dark red colonies and watery transparent indicating reduction of TNT. TNT (160 μM) was completely degraded within 4 days when 3 g of explosive formulation containing *Bacillus* SF or *P. putida* GG04 were added into liquid cultures. Long-term storage at room temperature of wood flour coated with the *Bacillus* SF incorporated in explosive formulations did not affect the organism over the 5 years of storage. Colony forming units recorded 1 day after preparation of the explosive were 29×10^5 CFU g^{-1} wood flour as compared to 28.5×10^5 CFU g^{-1} wood flour after 5 years of storage at room temperature. However after 2 years of storage all *P. putida* GG04 cells were inactive. There is still a need to optimize the protective media and lyophilization conditions for *P. putida* GG04. To investigate whether the incorporation of the microorganisms into the explosive formulation did affect the quality of the explosive, the detonation velocity which is one of the most important parameter of an explosive was tested. This was compared to the reference, which in this case was a normal explosive without microorganisms. Compared to the reference with a detonation velocity of 1701 m s^{-1} the explosive containing *Bacillus* SF (stored for 5 years) showed even a better performance with a detonation velocity increase of 17%. This increase in detonation velocity could be due to the partial replacement of wood flower in the explosive formulation with biomass.

Summarizing our results we were able to show for the first time that *Bacillus* spores can survive in an explosive formulation allowing immediate TNT transformation upon addition of water. While the incorporated bacteria can initiate biotransformation, the presence of humic monomers (guaiacol and catechol) can prevent the accumulation of toxic metabolites (AMDNT and DAMNT). Incorporation of *Bacillus* SF spores into explosives is a very attractive option especially for formulations that are used in the mining and construction industry since incompletely detonated parts constitute a great occupational health hazard to workers and are polluting the environment.

Acknowledgement

This work was made possible by the support offered by the Austrian Academic Exchange Service (ÖAD) and Austin Powder GmbH.

References

[1] R.P. Mason, P.D. Josephy, Free radical mechanism of nitroreductase, in: D.E. Rickert (Ed.), Toxicity of Nitroaromatic Compounds, Hemisphere, New York, 1985, pp. 121–140.

[2] G.R. Chaundry, Biological Degradation and Bioremediation of Toxic Chemicals, Dioscorides Press, Portland, Oregon, 1994, pp. 87–98.

[3] S.B. Funk, R.L. Crawford, R.L. Crawford, Bioremediation of nitroaromatic compounds, in: Don L. Crawford, Ronal L. Crawford (Eds.), Bioremediation Principles and Applications, Cambridge University Press, Cambridge, 1996, pp. 195–205.

[4] J. Yinon, Toxicity and Metabolism of Explosives, CRC Press, Boca Raton, FL, 1990, pp. 128–145.

[5] J. Hawari, S. Beauet, S. Halasz, S. Thiobout, G. Ampleman, Microbial degradation of explosives: biotransformation versus mineralization, Appl. Microbiol. Biotechnol. 54 (2000) 605–618.

[6] A. Esteve-Núñez, A. Caballero, J.L. Ramos, Biodegradation of 2,4,6-trinitrotoluene, Microbiol. Mol. Biol. Rev. 65 (3) (2001) 335–352.

[7] G. Heiss, H.J. Knackmuss, Bioelimination of trinitroaromatic compounds: immobilization versus mineralization, Curr. Opin. Microbiol. 5 (2002) 282–287.

[8] G.S. Nyanhongo, M. Schroeder, W. Steiner, G.M. Gübitz, Biodegradation of 2,4,6-trinitrotoluene (TNT): an enzymatic perspective, Biocatal. Biotransform. 23 (2) (2005) 53–69.

[9] F.B. Smets, H. Yin, A. Esteve-Nuñez, TNT biotransformation: when chemistry confronts mineralization, Appl. Microbiol. Biotechnol. 76 (2007) 267–277.

[10] G. Daun, H. Lenke, M. Reuss, H.J. Knackmuss, Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components, Environ. Sci. Technol. 32 (1998) 1956–1963.

[11] D. Gunnison, J.C. Pennington, C.B. Price, G.B. Myrick, "Screening test and isolation procedure for TNT-degrading microorganisms" Technical Report IRRP-93-2, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS, NTIS No. AD A269 124, 1993, pp. 1–32.

[12] J. Maier, A. Kandelbauer, A. Erlacher, A. Cavaco-Paulo, G.M. Gübitz, A new alkali-thermostable azoreductase from *Bacillus* sp. strain SF, Environ. Microbiol. 70 (2) (2004) 837–844.

[13] N.G. McCormick, F.E. Feeherry, H.S. Levinson, Microbial transformation of 2,4,6-TNT and other nitroaromatic compounds, Appl. Environ. Microbiol. 31 (1976) 949–958.

[14] A.M. Tope, K. Jamil, T.R. Baggi, Transformation of 2,4,6-trinitrotoluene (TNT) by immobilized and resting cells of *Arthrobacter* sp, J. Hazard. Substances Res. 2 (1999) 3–9.

[15] C. Held, A. Kandelbauer, M. Schroeder, A. Cavaco-Paulo, G.M. Guebitz, Biotransformation of phenolics with laccase containing bacterial spores, Environ. Chem. Lett. 3 (2005) 74–77.

[16] A.M. Malik, Long-term preservation of some Rhodospirillaceae by freeze-drying, J. Microbiol. Methods 8 (1998) 259–271.

[17] T. Kalafut, M.E. Wales, V.K. Rastogi, R.P. Naumova, S.K. Zaripova, J.R. Wild, Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria, Curr. Microbiol. 36 (1998) 45–54.

[18] S.A. Zaripov, A.V. Naumov, E.S. Suvorova, A.V. Garusov, R.P. Naumov, Initial stages of 2,4,6-Trinitrotoluene transformation by microorganisms, Microbiology 73 (4) (2004) 398–403.

[19] M.E. Fuller, J.F. Manning Jr., Microbiological changes during bioremediation of explosives-contaminated soils in laboratory and pilot-scale bioslurry reactors, Bioresour. Technol. 91 (2004) 123–133.

[20] J.W. Pak, K.L. Knoke, D.R. Noguera, B.G. Fox, G.H. Chambliss, Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C, Appl. Environ. Microbiol. 66 (2000) 4742–4750.

[21] A. Haidour, J.L. Ramos, Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene; 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Pseudomonas* sp, Environ. Sci. Technol. 30 (1996) 2365–2370.

[22] C. Vorbeck, H. Lenke, P. Fischer, H.J. Knackmuss, Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a Mycobacterium strain, J. Bacteriol. 176 (1994) 932–934.

[23] C.E. French, S. Nicklin, N.C. Bruce, Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase, Appl. Environ. Microbiol. 64 (1998) 2864–2868.

[24] G. Heiss, H.J. Knackmuss, Bioelimination of trinitroaromatic compounds: immobilization versus mineralization, Cur. Opin. Microbiol. 5 (2002) 282–287.

[25] P. Sharma, R. Goel, N. Capalash, Bacterial laccases, World J. Microbiol. Biotechnol. 23 (2007) 823–832.

[26] G.S. Nyanhongo, S. Rodrıguez Couto, G.M. Guebitz, Coupling of 2,4,6-trinitrotoluene (TNT) metabolites onto humic monomers by a new laccase from *Trametes modesta*, Chemosphere 64 (2006) 359–370.

[27] G.S. Nyanhongo, A. Erlacher, M. Schroeder, G.M. Gubitz, Enzymatic immobilization of 2,4,6-trinitrotoluene (TNT) biodegradation products onto model humic substances, Enzyme Microbiol. Technol. 39 (2006) 1197–1204.

[28] S. Thiele, E. Fernandes, J.M. Bollag, Enzymatic transformation and binding of labelled 2,4,6-trinitrotoluene to humic substances during an anaerobic/aerobic incubation, J. Environ. Qual. 31 (2002) 437–444.

[29] C. Achtnich, U. Sieglen, H.J. Knackmuss, H. Lenke, Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil, Environ. Toxicol. Chem. 18 (11) (1999) 2416–2423.

[30] K.A. Thorn, J.C. Pennington, C.A. Hayes, ^{15}N NMR Investigation of the reduction and binding of TNT in an aerobic bench scale reactor simulating windrow composting, Environ. Sci. Technol. 36 (2002) 3797–3805.

[31] A.P. Mackenzie, Comparative studies on the freeze-drying survival of various bacteria: gram type, suspending medium and freezing rate, Dev. Biol. Stand. 36 (1977) 263–277.