Int. J. Environ. Sci. Technol. (2016) 13:1357–1366
DOI 10.1007/s13762-016-0976-5

ORIGINAL PAPER

Bioremediation of 2,4,6-trinitrotoluene-contaminated groundwater using unique bacterial strains: microcosm and mechanism studies

Y. T. Sheu[1] · P. J. Lien[1] · C. C. Chen[2] · Y. M. Chang[3] · C. M. Kao[1]

Received: 28 September 2015 / Revised: 12 February 2016 / Accepted: 28 February 2016 / Published online: 15 March 2016
© Islamic Azad University (IAU) 2016

Abstract Groundwater at many military factory, munition storage and maneuver sites is contaminated by explosives chemicals that were released into the subsurface. The 2,4,6-trinitrotoluene (TNT) is among the most common explosive pollutants. In this study, two TNT-degrading strains, isolated from TNT-contaminated soils and wastewater sludge, were applied for TNT biodegradation. Based on the 16S rDNA sequence analyses, these two bacterial strains were identified as *Achromobacter* sp. and *Klebsiella* sp. via biochemical and DNA analyses. Microcosm study was conducted to evaluate the feasibility and efficiency of TNT biodegradation under aerobic conditions. Results indicate that TNT degradation by-products were detected in microcosms (inoculated with *Achromobacter* sp. and *Klebsiella* sp.) with cane molasses addition. *Klebsiella* sp. and *Achromobacter* sp. used TNT as the nitrogen source and caused completely removal of TNT. Two possible TNT biodegradation routes could be derived: (1) part of the TNT was transformed to nitrotoluene then transformed to nitrobenzene followed by the nitro substitute process, and trinitrobenzene, dinitrobenzene, and nitrobenzene were detected; and (2) TNT was transformed via the nitro substitute mechanism, and dinitrotoluene followed by nitrotoluene isomers were detected. The initial TNT degradation involved the reduction or removal of the nitro substitute to an amino derivative or free nitrite. Results show that the second route was the dominant TNT biodegradation pathway. The produced by-products were also degraded without significant accumulation during the degradation process. These findings would be helpful in designing a practical system inoculated with isolated TNT degradation strains for the treatment of TNT-contained groundwater.

Keywords *Achromobacter* sp. · Biodegradation · *Klebsiella* sp. · Groundwater contamination · 2,4,6-Trinitrotoluene (TNT)

✉ C. M. Kao
jkao@mail.nsysu.edu.tw

[1] Institute of Environmental Engineering, National Sun Yat-Sen University, Kaohsiung, Taiwan

[2] Department of Biotechnology, Kaohsiung Normal University, Kaohsiung, Taiwan

[3] Institute of Environmental Engineering and Management, National Taipei University of Technology, Taipei, Taiwan

Introduction

Over the past century, millions of tons of nitroaromatic explosives have been produced for military applications and activities leading to accidental release of energetic materials to the environment (Zou et al. 2014). Soils and groundwater at many of these military factories, munition storages, and maneuver sites are usually contaminated by the explosives chemicals that were released into the subsurface either intentionally or accidentally (Nishino et al. 2010; Douglas et al. 2012). These compounds include 2,4,6-trinitrotoluene (TNT). TNT and its by-products [e.g., 2,6-dinitrotoluene (2,6-DNT), 2,4-dinitrotoluene (2,4-DNT), nitrobenzene (NB)] are reported as highly toxic and carcinogenic (Sheibani et al. 2011), and these chemicals are very difficult to be removed from the environment. Among these explosive compounds, TNT has been extensively used worldwide. Due to its long persistence in the environment and its toxic effects on organisms, efforts have been put to develop effective remediation techniques for TNT-contaminated sites.

Treatment of TNT by physical and chemical treatment processes could increase its water solubility and availability in environments, and more toxic by-products might be produced (Islam et al. 2015). Some of the TNT-contaminated sites applied traditional ex situ remediation methods such as incineration and landfill dumping. However, incineration would destroy soil structure and disrupt ecology and dumping only displaces untreated media to another site (Das et al. 2013). Zhang et al. (2011) investigated the feasibility of using vacuum distillation and activated coke to treat TNT-contained red water. Their results show that most chemical oxygen demand (COD) and DNTs in TNT red water could be removed by this technique (Zhang et al. 2011); however, the fraction and saturated activated coke left were the hazardous wastes and needed to be further treated. Some researchers have investigated the TNT removal by activated carbon and further degradation (Fu et al. 2012). Hwang et al. (2005) conducted laboratory-scale experiments on the alkaline hydrolysis of TNT followed by thermal treatment of the hydrolysates.

Researchers also applied advanced oxidation processes (AOPs) as potential methods for TNT removal from contaminated environment (Matta et al. 2007), Most of these AOP processes are associated with the generation of radical species mainly hydroxyl radicals (OH•) (Rodrigues et al. 2009) for TNT oxidation. Adsorption and wet air oxidation were also applied by researchers for TNT removal (Hao et al. 1993; Lewis et al. 2004; Fu et al. 2012; Islam et al. 2015). However, chemical methods are relatively more expensive and less environmental-acceptable compared to biological technologies and often require additional ex situ treatment systems, which may also produce new chemical by-products or waste gas emissions (Fuller et al. 2005; Ayoub et al. 2010; Douglas et al. 2012; Islam et al. 2015).

Biological treatment has been widely used in wastewater treatment due to its high efficiency and low cost (Qiao et al. 2013; Gao et al. 2014). The most widely used bioremediation procedure is the biostimulation of microorganisms degraders through the addition of nutrients. Primary substrates, electron donors, inorganic nutrients, and trace minerals could stimulate and enhance the degrading activity of the intrinsic microorganisms (Li et al. 2014), and the biodegradation efficiency could be affected according to the amounts of substrate added. In the recent years, the search for a cost-effective, ecologically safe and environmentally sound remediation technique has led to the development of in situ bioremediation processes using TNT-degrading bacteria (Nyanhongo et al. 2005; Lin et al. 2013a).

TNT is one of the toxic explosive chemicals due to its symmetric location of the nitro groups on the aromatic ring, which results in the limited enzyme attack involved in the metabolism of aromatic compounds (Toshinari et al. 2006). Bioremediation of TNT has met with mixed success due to the variability in binding of TNT to various soil types (Larson et al. 2008). Biological processes for degradation of nitroaromatics can be classified as being either aerobic or anaerobic (Lin et al. 2013a). Fungi, anaerobic bacteria, aerobic bacteria and phytoremediation using TNT accumulating plants have been studied to identify efficient techniques for the in situ bioremediation of TNT (Hannink et al. 2002; Gumuscu and Tekinay 2013; Lin et al. 2013a). Muter et al. (2012) added varying concentrations of a nutrient amendment consisting of inorganic salts, plant extracts, and molasses to soil and liquid media and their results show that enhanced TNT bioremediation was achieved and increase in nutrient amendment concentration leads to an increase in TNT degradation. Muter et al. (2012) found both liquid and soil show the stimulating effect of nutrients on overall microbial activity, as well as on TNT biodegradation.

The recent discovery of nitro explosive-degrading biocatalysts (enzymes or redox-active biomolecules) opened new perspectives for the development and design of bioremediation systems (Stenuit and Agathos 2013). Researchers have reported that beneficial TNT biodegradation pathways with (1) the production of the metabolizable compound 2,4-DNT from monohydride meisenheimer complex of TNT (Ziganshin et al. 2010a) and (2) the evidence using stable isotope probing (SIP) of assimilation of 15 N and 13C from TNT into bacterial DNA (Gallagher et al. 2010).

Taking advantage of the catabolic capabilities of the yeasts Yarrowia lipolytica and Geotrichum candidum and the acidification of the culture medium through the production of organic acids, Ziganshin et al. (2010a, b) reported that the transformation of TNT under acidic conditions ($pH < 4.2$) to 2,4-DNT with concomitant release of nitrite. Gallagher et al. (2010) have reported the utilization of TNT as both carbon and nitrogen sources under anaerobic conditions by a Lysobacter taiwanensis strain initially present in anaerobic organic-rich estuarine sediments. However, the incorporation of TNT into Lysobacter cell biomass as either a primary substrate or a co-substrate (cometabolism) remained to be elucidated. Gumuscu and Tekinay (2013) found rapid microbial-mediated degradation of TNT-contaminated soils by a novel strain, *Achromobacter spanius* STE 11. Complete removal of 100 mg/L of TNT was achieved within 20 h under aerobic conditions by the isolate (Gumuscu and Tekinay 2013).

In recent years, molecular biological techniques [e.g., PCR-DGGE (polymerase chain reaction-denatured gradient gel electrophoresis)] have been used to evaluate the

microbial diversity and dominant bacteria in the environment when it is exposed to the toxic substances. (Kao et al. 2010). In this study, TNT-contaminated soils collected from a munition factory site and aerobic activated sludge collected from the wastewater treatment plant of this studied munition factory were used for TNT-degrading bacteria isolation and microcosm study. Moreover, cane molasses was used as a primary substrate for microorganisms. Cane molasses, a waste product of the sugar industry, is rich in carbon and relatively inexpensive, making it a good substrate for enhancing the biodegradation process (Liang et al. 2013).

The main objectives of this study were to (1) isolate and identify the TNT-degrading bacteria from the TNT-contaminated soils and wastewater sludge, (2) conduct aerobic microcosm experiments to evaluate the feasibility of bioremediation of TNT-contaminated groundwater using the isolated TNT-degrading bacteria, (3) evaluate the TNT biodegradation mechanisms and pathways under aerobic conditions, and (4) determine the dominant microorganisms in microcosms through microbial identification via PCR, DGGE, and nucleotide sequence methods.

Materials and methods

Isolation of TNT-degrading bacteria

Results from our previous studies indicate that soil and groundwater samples collected from a munition factory site located in southern Taiwan were contaminated by TNT (Lin 2014). A wide spread TNT plume was also discovered, and thus, the groundwater contamination needed to be remediated and the plume migration needed to be controlled to protect the downgradient receptors and ecosystem. The detected TNT concentrations ranged from 2 μg/L to 8 mg/L in collected groundwater samples at the contaminated site (Lin 2014). TNT-contaminated soils were collected from the subsurface of the TNT-contaminated site and wastewater sludge was also collected from the final clarifier of the wastewater treatment plant of this munition factory for TNT-degrading bacteria isolation.

TNT-contaminated soils (3 g) or wastewater sludge (3 mL) were added to 100 mL of basal salt medium (BSM) (Rahal and Lobna 2011) containing 10 mg of TNT per liter. The BSM broth contained the following components at the specified concentrations (units are in g/L of water): D-glucose, 0.9; $Mg_2SO_4 \cdot 7H_2O$, 7; KH_2PO_4, 0.2; sodium acetate, 1; sodium citrate, 0.4. After 14 days of incubation at 30 °C (160 rpm) in a Gyrotory Shaker, 1 mL of the enrichment culture was transferred to 100 mL of BSM broth containing 20 mg of TNT per liter. This medium was incubated at 30 °C (160 rpm) for 14 days. The above procedures were repeated until the concentration of TNT in the medium reached 200 mg/L.

After the incubation processes, serial dilutions were plated on the defined medium containing inorganic salts, 200 mg of TNT per liter, and 15 g of BSM agar (Noble class, Difco) per liter. Each separate colony appearing on the defined agar after 2 weeks of incubation at 30 °C was transferred to the liquid nutrient medium (containing 200 mg/L of TNT) separately for further enrichment of the TNT degraders.

PCR analysis and gene identification

One and a half mL of cell suspensions (containing bacterial liquid and liquid medium broth) from each enrichment medium were extracted with DNA Purification kit (GeneMark Co., Taiwan). Bacterial fragments [200 base pairs (bp)] of 16S rDNA V3 region were amplified with the primer sets [341f, forward: 5′-CCTACGGGAGGCAGCAG-3′ containing a guanine-cytosine (GC) clamp of 40-nucleotide GC-rich sequence; 534r, reversed: 5′-ATTACCGCGGCTGCTGG-3′] (Elazhari-Ali et al. 2013).

The mixtures of PCR contained 10 ng of DNA extract, 4 pmol of each primer, and 5 U of Taq DNA polymerase (Takara, Shiga, Japan) in final concentrations of 2.5 mM of MgCl2 and 0.12 mM of deoxyribonucleoside triphosphates in PCR buffer. The PCR amplification was conducted for 35 cycles: denaturation at 94 °C for 1 min, annealing temperature was initially 65.8 °C, and it was decreased by 1 °C per cycle until it was 55.8 °C, after which 25 additional cycles were carried out at 55.8 °C; and extension at 72 °C for 2 min. The PCR-amplified products were electroeluted from gel and then sequenced by MdBio Inc. in Taiwan. Those sequences were compared to the GenBank database using the BLAST algorithm (Hesham et al. 2011).

DGGE analyses

Total bacterial DNA from groundwater samples was extracted for a DGGE analyses. Before the DGGE analysis, the V3 region of the 16S rDNA was amplified with PCR primer sets (Baldwin et al. 2003). DGGE was performed following the procedures described in Yanru et al. (2005). The equal concentration of each amplified PCR products (2500 ng) was furthermore performed with DGGE using a Bio-Rad D-Code™ universal mutation detection system (Bio-Rad Lab., USA), as described by the manufacturer. The 10 % polyacrylamide gel with a 30–60 % denaturant gradient was used and electrophoresis was performed at 60 °C and 70 V for 14 h. The gels were then stained with SybrGreen I and photographed. This was the effective way to overcome biased amplification by Taq DNA polymerase with multi-template DNA samples for quantitative

structure of the community. The gels were then stained with SybrGreen and photographed (Yanru et al. 2005; Elazhari-Ali et al. 2013). Those sequences were compared to the GenBank database using the BLAST algorithm (Elazhari-Ali et al. 2013).

Biodegradation of TNT: microcosm experiments

Microcosm experiments were conducted to examine the feasibility of TNT biodegradation under aerobic conditions. The inocula used in this microcosm study were isolated TNT-degrading bacteria from the contaminated soils and wastewater sludge. Cane molasses (0.9 or 8 g/L) was added in the microcosms used as the carbon source for the bacterial growth. Each microcosm was constructed with 35 mL of mineral medium, TNT (with final concentration of 100 mg/L), and 20 mL of isolated bacterial solution (with OD = 0.5) as inocula in a 120-mL bottle sealed with Teflon-lined rubber septa. The medium solution was autoclaved before use. The pH of the microcosm solution was 7.5. The mineral medium contained the following components at the specified concentrations (units are in mg per liter of water): KH_2PO_4, 326.4; Na_2HPO_4, 1263.8; $Mg_2SO_4{\cdot}7H_2O$, 98.6; $CaCl_2{\cdot}2H_2O$, 44.1; plus 3.35 mg of trace elements which include $FeSO_4{\cdot}7H_2O$, 1; $MnSO_4{\cdot}4H_2O$, 1; $Na_2B_4O_7{\cdot}10H_2O$, 0.25; $CoCl_2{\cdot}6H_2O$, 0.25; $CuCl_2{\cdot}2H_2O$, 0.25; $ZnCl_2$, 0.25. TNT was the only nitrogen in the microcosm.

No inocula were added in the control microcosms, and nutrient solution was autoclaved before use. Duplicate microcosms were killed at each time point and analyzed for TNT and by-product concentrations. TNT and its degradation by-products were analyzed in accordance with USEPA Method 8330A and Taiwan EPA Method M804.00B using a high-performance liquid chromatography (HPLC) with a separation module (L-2130, HITACHI, Japan) and a UV–VIS detector (L-2200, HITACHI, Japan) with a Thermo Scientific Acclaim™ Explosives E2 column (Dionex, USA). At each time point, results are averages of duplicate microcosm samples.

Results and discussion

PCR-DGGE analysis and bacteria identification

To determine the variations in microbial community patterns in collected soil and sludge samples, the PCR-DGGE techniques were performed to investigate the dominant microorganisms in the samples. Figure 1 presents the DGGE patterns produced by different DNA polymerases for soil and sludge samples. Table 1 shows the comparison of the nucleotide sequences of 16S rDNA of 28 specific

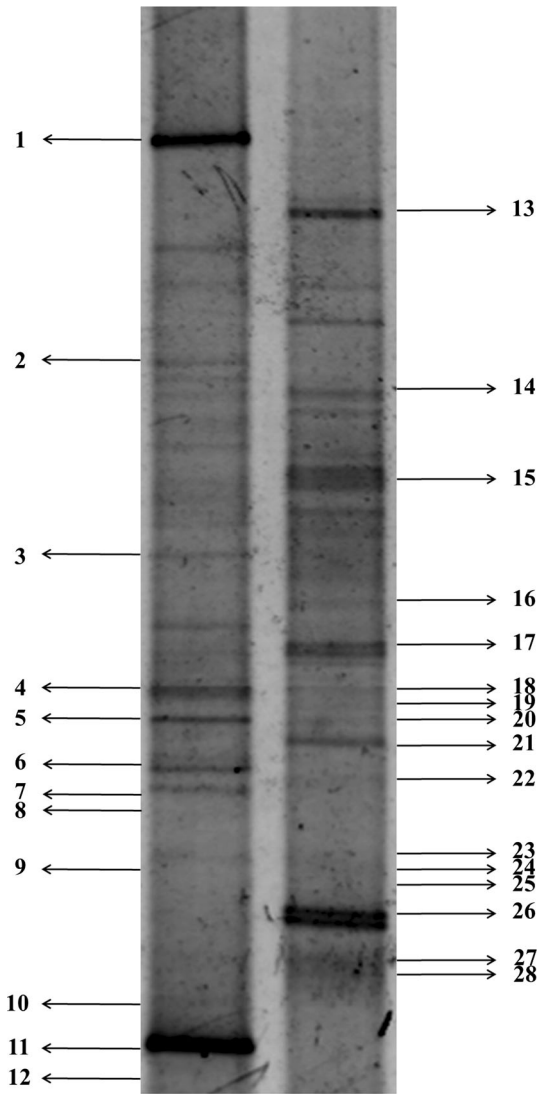

Fig. 1 DGGE patterns of the soil (*right*) and sludge (*left*) samples

microorganisms with the database from GenBank. Table 2 presents the functions of the identified bacteria in microcosms. Results from the DGGE profiles show that both samples had many DGGE bands, and more than 12 and 18 dominant bands were observed in sludge and soil samples, respectively. This indicates that many bacteria existed in the TNT-containing environment.

However, the munition wastewater might contain higher concentrations of toxic chemicals (including TNT) and might inhibit and limit the microbial growth, which resulted in less microbial species in the sludge. To determine the meaning of representatives for bacterial species, the bands of DGGE profiles were eluted and then amplified and sequenced for their nucleotide sequences of 16S rDNA variable V3 regions.

The following bacteria, which could tolerate higher concentrations of TNT, were isolated from the tested soils

Table 1 Comparison of the nucleotide sequences of 16S rDNA of 28 specific microorganisms with the database from GenBank

Clone no.	Closest GenBank match	Identity (%)
1	Uncultured *Geothrix* sp.	99
	Uncultured Acidobacterium group	99
	Geothrix fermentans strain H5	99
	Uncultured delta proteobacterium	99
	Holophaga foetida strain TMBS4	96
2	Uncultured bacterium clone BN104	95
	Uncultured Acidobacteria/Holophaga group bacterium clone Cart-N4	97
3	Uncultured prokaryote clone Td4-4	99
	Uncultured Chlorobi bacterium clone Z53M30B	99
4	Geobacter lovleyi strain Geo7.4B	98
	Uncultured bacterium clone Carex04C	98
	Uncultured *Trichlorobacter* sp.	98
5	Uncultured *Nitrospira* sp. clone L1-83	99
	Candidatus *Nitrospira defluvii*	99
6	Uncultured bacterium clone EA01	99
	Uncultured Nitrospirae bacterium clone Z17M13B	96
7	*Thiobacillus* sp.	99
	Uncultured Hydrogenophilales bacterium clone 3H3	99
	Rhodocyclaceae bacterium FTL11	99
8	*Thiobacillus thioparus* strain THI 115	99
	Uncultured Hydrogenophilaceae bacterium clone D10_45	99
9	*Dyella* sp.	99
	Uncultured Xanthomonadaceae bacterium clone DMS34	99
10	Uncultured Chloroflexi bacterium clone HG-B0248	99
11	*Rhodanobacter* sp.	99
	Dyella sp.	99
	Dyella ginsengisoli	99
	Luteibacter yeojuensis	99
	Fulvimonas sp.	99
12	Uncultured *Rhodanobacter* sp. clone sen297	99
	Uncultured Acidobacteria bacterium clone MBMV1	99
	Frateuria sp.	99
	Uncultured Xanthomonadales bacterium clone MP10A34	99
13	Uncultured Acidobacteria bacterium clone	98
14	Ruminococcaceae bacterium HZ254R	96
15	*Mycobacterium* sp.	99
	Mycobacterium cosmeticum	99
	Mycobacterium frederiksbergense	99
	Mycobacterium neoaurum	99
	Mycobacterium diernhoferi	99
	Mycobacterium aubagnense	99
16	Uncultured alpha proteobacterium clone SI-2M_B02	97
	Uncultured Hyphomicrobiaceae bacterium clon	97
17	Alpha proteobacterium	96
18	Pseudomonas aeruginosa	99
	Pseudomonas mendocina	99
	Pseudomonas fluorescens	99
	Pseudomonas otitidis	99
19	Iron-reducing bacterium	99
	Methylibium petroleiphilum PM1	99
20	Uncultured bacterium clone Filt_MembF08	99
21	Uncultured alpha proteobacterium clone AKYH996	99
22	Alpha proteobacterium	99
	Uncultured *Phenylobacterium* sp. clone XZXXH161	98
	Caulobacter sp.	98
	Brevundimonas sp.	97
23	*Cupriavidus* sp.	99
	Cupriavidus metallidurans	99
24	*Chryseobacterium bovis*	97
25	*Cupriavidus necator*	99
	Wautersia numazuensis	99
	Cupriavidus taiwanensis	99
	Stenotrophomonas maltophilia	99
	Uncultured Burkholderiales bacterium clone IFU_Y1	99
	Ralstonia eutropha	99
26	*Pseudomonas putida*	99
	Pseudomonas corrugata	99
	Pseudomonas thivervalensis	99
	Pseudomonas chlororaphis	99
27	Gamma proteobacterium	99
28	Uncultured beta proteobacterium clone P-R40	97

and sludge. Identification of the bacteria by analyzing its 16S rDNA sequence with the 16S rDNA database in GenBank showed 99 % homologies with *Dyella* sp., *Pseudomonas* sp., Iron-reducing bacterium, *Thiobacillus* sp., Geobacter *lovleyi*, *Rhodanobacter* sp., *Geothrix* sp., *Nitrospira* sp., *Chloroflexi* sp., *Cupriavidus* sp., *Mycobacterium* sp., *Methylibium petroleiphilum*, *alpha proteobacterium*, *beta proteobacterium*, *Klebsiella* sp. and *Achromobacter* sp.

Among them, *Pseudomonas* sp., *Mycobacterium* sp., *Klebsiella* sp. and *Achromobacter* sp. have been reported as the bacteria with capability of TNT biodegradation (Limane et al. 2011; Muter et al. 2012; Gumuscu and Tekinay 2013).

During the process of TNT-degrading bacteria isolation, a total of 30 colonies were found on the agar plate when the TNT concentration was 200 mg/L. All of the 30 high TNT concentration resistant colonies were transferred to the liquid medium separately for further enrichment. Then the DNA of each of the 30 colonies was extracted and the sequences were compared to the GenBank database using the BLAST algorithm for microbial identification. After sequencing process, five different bacterial strains were identified and two of them [*Klebsiella* sp. (isolated from soils) and *Achromobacter* sp. (isolated from sludge)] had the capability for TNT biodegradation. Thus, these two bacteria were applied for the following microcosm study.

Microcosm experiments

Figure 2 presents the variations in TNT concentrations in microcosms with mixed *Klebsiella* sp. and *Achromobacter* sp. inoculation (cane molasses = 0.9 mg/L). Results show that up to 48 % of TNT removal was observed in microcosms with *Klebsiella* sp. and *Achromobacter* sp. inoculation after 21 days of operation when 0.9 g/L of cane molasses was added as the carbon source. This indicates that TNT removal was not effective although different inocula and cane molasses were added. However, significant increase in TNT removal was obtained when 8 g/L of cane molasses was added with *Klebsiella* sp. and *Achromobacter* sp. as the inocula.

Figure 3 presents the variations in TNT concentrations in microcosms with *Klebsiella* sp. or *Achromobacter* sp. inoculation (cane molasses = 8 g/L). Up to 99.9 % of TNT removal was observed within two days of incubation. Results reveal that high concentrations of cane molasses addition could activate the bacterial activities and enhance the biodegradation of TNT. Results also show that no significant TNT removal was observed in control microcosms. Thus, the TNT removal was due to the microbial processes. In the microcosms, TNT was served as the nitrogen source (no other nitrogen was added in the nutrient solution), and thus, microbial processes required higher concentrations of nitrogen when higher concentrations of carbon source was added.

Other researchers indicate that high concentrations (>3 g/L) of glucose molasses addition could enhance TNT degradation processes and shorted the required biodegradation time (Lin et al. 2013b). Sheibani et al. (2011) also reported that 95.2 % of TNT removal was obtained when 6.25 g/L of glucose was supplied. Wang et al. (2010) suggested that the C–N ratio was the key parameter affecting the biodegradation rate of TNT. Wang et al. (2010) proposed that the optimal C/N ratio was 12 to 20 for heterotrophic bacteria. Results from this study imply that sufficient carbon substrate addition is required to enhance TNT biodegradation under aerobic conditions.

Figure 4 presents the variations in TNT and its degradation by-products using mixed *Klebsiella* sp. and *Achromobacter* sp. as the inocula in microcosms during the operational period (cane molasses = 0.9 mg/L). Figure 5 presents the variations in TNT and its degradation by-products using *Klebsiella* sp. as the inocula in microcosms during the operational period (cane molasses = 8 mg/L). Figure 6 shows the variations in TNT and its by-products concentrations in microcosms with *Achromobacter* sp. as inocula (cane molasses = 8 mg/L). Results indicate that several TNT degradation by-products were detected in microcosms with high and low concentrations of cane molasses addition. The production of by-products also confirmed the occurrence of TNT biodegradation.

Results show that the following by-products were detected during the TNT degradation process: 2-amino-2,6-dinitrotoluene (2-A-2,6-DNT), 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT), 1,3,5-trinitrobenzene (1,3,5-TNB), 1,3-dinitrobenzene (1,3-DNB), NB, 2,6-DNT, 2,4-DNT, 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT) and 4-nitrotoluene (4-NT). Results also indicate that the produced by-products were also degraded without significant accumulation during the degradation process.

Because cane molasses is a waste of sugar industry, the application of cane molasses as the carbon source could effectively reduce the operational and maintenance cost for the future practical application.

TNT biodegradation routes and by-product analyses

The trends of by-products production indicate that two possible TNT biodegradation routes could be derived: (1) During the TNT biodegradation, part of the TNT was transformed to nitrotoluene then transformed to nitrobenzene followed by the nitro substitute process, and small amount of TNB, DNB, and NB were detected (Fig. 6a); and (2) during the TNT biodegradation, TNT was transformed via the nitro substitute mechanism, and DNT followed by NT isomers were detected (Fig. 6b).

Results also show that the second route could be the dominant TNT biodegradation pathway. The initial TNT degradation involved the reduction or removal of the nitro substitute to an amino derivative or free nitrite. The identified amino derivatives included 2-A-2,6-DNT and 4-A-2,6-DNT.

Researches had been done to elucidate the pathways of TNT transformation under aerobic conditions (Cohen et al. 2007). The most effective route of TNT reduction involves the conversion of nitro groups into nitroso, hydroxylamino and amino groups. Several by-products

Table 2 Functions of the identified bacteria in aerobic and anaerobic microcosms

Microorganism	Metabolism	References
Mycobacterium vaccae	50 % radioactivity from [^{14}C]TNT incorporated into lipids	Vanderberg et al. (1995)
Mycobacterium sp. Strain HL4NT-1	Cells grown in 4-nitrotoluene transformed TNT to hydride and dihydride Meisenheimer complex	Vorbeck et al. (1994)
Pseudomonas aeruginosa	Nitrite releasing from TNT and transformed to 2A-4NT	Kalafut et al. (1998)
Pseudomonas fluorescens	NADPH flavoprotein oxidoreductase catalyzing multiple transformations, generating ArNHOH, H-TNT and H_2-TNT	Pak et al. (2000)
Pseudomonas pseudoalcaligenes	Reduction of nitro groups and nitrite releasing from 2,4-dihydroxylamino-6-nitrotoluene	Fiorella and Spain (1997)
Pseudomonas savastanoi	Denitration of TNT to release nitrite and reduction of nitro group to amino	Martin et al. (1997)
Pseudomonas sp.	TNT as a sole nitrogen source; transformation of TNT to 2ADNT and 4ADNT	Jones et al. (1995)
Rhodococcus rythropolis	Cells growing on picric acid catalyze ring hydrogenation, forming hydride and dihydride TNT-Meisenheimer complexes	Vorbeck et al. (1994)
Serratia marcensens	TNT as a sole carbon and energy source in the presence of Tween 80	Montpas et al. (1997)
Staphylococcus sp.	Nitrite releasing from TNT and transformation of TNT to 2A4NT	Kalafut et al. (1998)
Pseudomonas sp. strain JLR11	TNT as a nitrogen source; TNT as a final electron acceptor	Esteve-Núñez et al. (2001)
Clostridium sp.	Bamberger rearrangement of dihydroxylaminodinitrotoluene	Hughes et al. (1997)
Desulfovibrio sp.	Transformation of TNT into TAT and DANT	Drzyzga et al. (1998)
Lactobacillus sp.	Reduction of TNT to TAT	Ederer et al. (1997)
Methanococcus sp. strain B	Reduction of TNT to DANT	Boopathy et al. (1998)
Clostridium sordelii	Reduction of TNT to TAT	Ederer et al. (1997)
Clostridium bifermentans LJP-1	Transformation of TNT into TAT and phenolic compounds	Lewis et al. (2004)
Clostridium acetobutylicum	Reduction of TNT to TAT	Ederer et al. (1997), Khan et al. (1997)

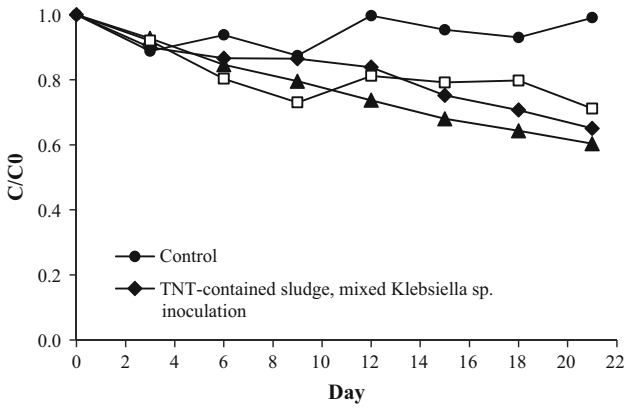

Fig. 2 Variations in TNT concentrations in microcosms with mixed *Klebsiella* sp. and *Achromobacter* sp. inoculation (cane molasses = 0.9 mg/L) (C/Co is the ratio of remaining TNT concentration to initial TNT concentration (100 mg/L)) (at each time point, results are averages of duplicate microcosm samples)

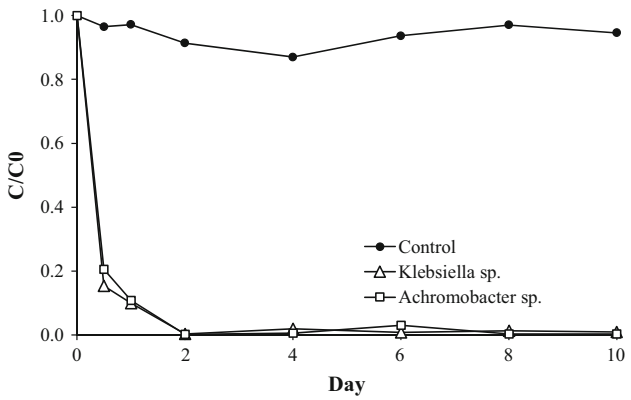

Fig. 3 Variations in TNT concentrations in microcosms with *Klebsiella* sp. or *Achromobacter* sp. inoculation (cane molasses = 8 g/L) (C/Co is the ratio of remaining TNT concentration to initial TNT concentration (100 mg/L)) (at each time point, results are averages of duplicate microcosm samples)

and metabolites such as 2,4-DNT, 2,6-DNT, 2-A-2,6-DNT, and 4-A-2,4-DNT were formed during this process. Other studies demonstrated that the complete TNT degradation by aerobic bacteria resulted in accumulation of ADNTs as key metabolites (Solyanikova et al. 2012). Recent studies found that *Achromobacter spanius* STE 11

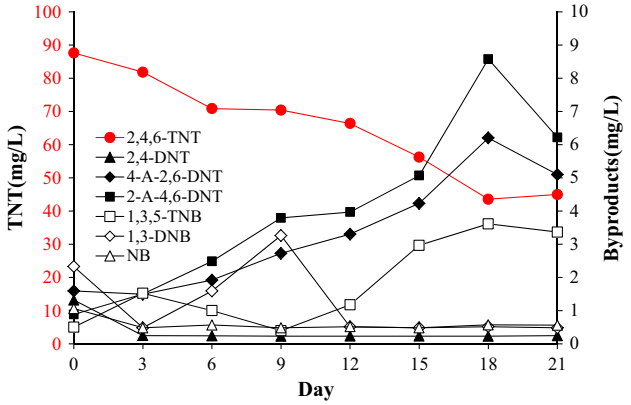

Fig. 4 Variations in TNT and its by-products concentrations in microcosms with mixed *Klebsiella* sp. and *Achromobacter* sp. inoculation (cane molasses = 0.9 mg/L) (at each time point, results are averages of duplicate microcosm samples)

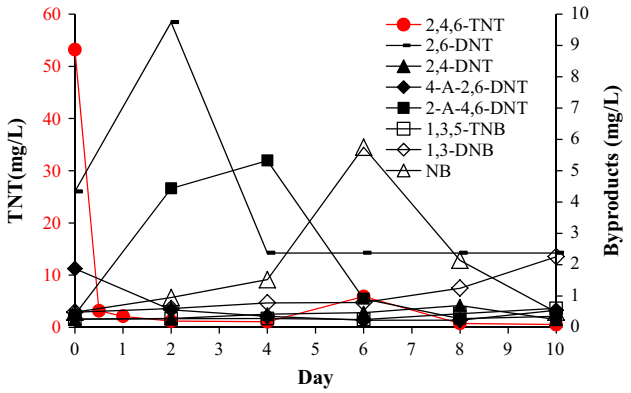

Fig. 5 Variations in TNT and its by-products concentrations in microcosms with *Klebsiella* sp. inoculation (cane molasses = 8 g/L) (at each time point, results are averages of duplicate microcosm samples)

strain could transform TNT completely through denitrification and nitro group reduction. The results showed that TNT could be incorporated into the cell biomass and reduced to DNT and ADNT isomers together with an unknown metabolite through enzymatic reactions (Gumuscu and Tekinay 2013).

Results from this study indicate that the nitro groups in TNT molecule were reduced on the ring to their amino analogs via a hydroxylamine intermediate, and 4-A-2,6-DNT and 2-A-4,6-DNT were the main intermediates. The results matched with the findings from Maeda et al. (2006), who indicated that 4-A-2,6-DNT and 2-A-4,6-DNT were detected during the TNT biodegradation by the isolated six strains (five *pseudomonas* sp. and one *Sphingomonas* sp.) from soils polluted by TNT. Esteve-Nunez et al. (2001) also reported that most but not all aerobic microorganisms reduced TNT to the corresponding amino derivatives via the formation of hydroxylamine intermediates. This result

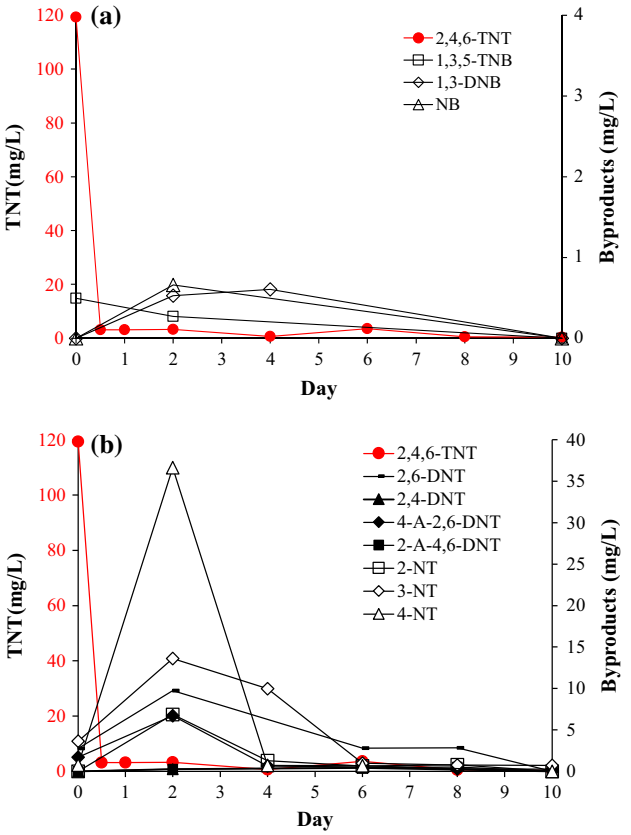

Fig. 6 Variations in TNT and its by-products concentrations in microcosms with *Achromobacter* sp. inoculation (cane molasses = 8 mg/L). **a** During the TNT biotransformation process, TNB, DNB, and NB were detected; **b** During the TNT biotransformation process, NT isomers were detected (at each time point, results are averages of duplicate microcosm samples)

confirmed that the nitrate reductase was induced by the presence of TNT. Results from this study show that both *Klebsiella* sp. and *Achromobacter* sp. consumed TNT as sole source of nitrogen and possibly produced nitrate reductase enzyme, which converted the nitro group to amino derivatives.

Conclusion

In this study, two TNT-degrading bacteria were isolated from the TNT-contaminated soils and wastewater sludge and identified as *Klebsiella* sp. and *Achromobacter* sp. via biochemical and DNA analyses. Conclusions of this study include the following:

(1) Results from the batch microcosm experiments indicate that *Klebsiella* sp. and *Achromobacter* sp. used TNT as the nitrogen source and caused completely removal of TNT with the supplement of cane molasses as the carbon and energy source.

(2) Results indicate that TNT degradation by-products including 2,4-DNT, 2-A-2,6-DNT, 4-A-2,6-DNT, 1,3,5-TNB, 1,3-DNB, NB, 2,6-DNT, 2,4-DNT, 2-NT, 3-NT, and 4-NT were detected in microcosms. The production of these by-products confirmed the occurrence of TNT biodegradation. Results also indicate that the produced by-products were degraded without significant accumulation during the degradation process.

(3) Two possible TNT biodegradation routes could be derived: (1) During the TNT biodegradation, part of the TNT was transformed to nitrotoluene then transformed to nitrobenzene followed by the nitro substitute process, and small amount of TNB, DNB, and NB were detected; and (2) during the TNT biodegradation, TNT was transformed via the nitro substitute mechanism, and DNT followed by NT isomers were detected. Results show that the second route was the dominant TNT biodegradation pathway. The initial TNT degradation involved the reduction or removal of the nitro substitute to an amino derivative or free nitrite, and the identified amino derivatives included 2-A-2,6-DNT and 4-A-2,6-DNT.

(4) Results from the microbial analyses indicate that more than 28 different TNT-degrading bacteria were observed in soils and sludge indicating that bioremediation could be a feasible technology for site cleanup.

(5) Results imply that the in situ or on-site aerobic bioremediation using the isolated TNT-degrading bacteria would be a feasible technology to clean up TNT-contaminated sites if sufficient carbon sources (e.g., cane molasses) could be supplied. Results from this study will be useful in designing a scale-up in situ or on-site TNT bioremediation system (e.g., enhanced in situ bioremediation, on-site bioreactor) for field application.

Acknowledgments This project was funded in part by Ministry of Science and Technology, Taiwan. The authors would like to thank the personnel at Ministry of Science and Technology and researchers at the Department of Biological Science, National Sun Yat-Sen University, Taiwan for their assistance and support throughout this project.

References

Ayoub K, van Hullebusch ED, Cassir M, Bermond A (2010) Application of advanced oxidation processes for TNT removal: a review. J Hazard Mater 178(1–3):10–28

Baldwin BR, Nakatsu CH, Nies L (2003) Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ Microbiol 69:3350–3358

Boopathy R, Manning J, Kulpa CF (1998) A laboratory study of the bioremediation of 2,4,6-trinitrotoluene-contaminated soil using aerobic/anoxic soil slurry reactor. Water Environ Res 70:80–86

Cohen R, Zeiri Y, Wurzberg E, Kosloff R (2007) Mechanism of thermal unimolecular decomposition of TNT (2,4,6-trinitrotoluene): a DFT study. J Phys Chem A 111:11074–11083

Das P, Sarkar D, Makris KC, Punamiya P, Datta R (2013) Effectiveness of urea in enhancing the extractability of 2,4,6-trinitrotoluene from chemically variant soils. Chemosphere 93:1811–1817

Douglas TA, Walsh ME, Weiss CA, McGrath CJ, Trainor TP (2012) Desorption and transformation of nitroaromatic (TNT) and nitramine (RDX and HMX) explosive residues on detonated pure mineral phases. Water Air Soil Pollut 223:2189–2200

Drzyzga O, Bruns-Nagel D, Gorontzy T, Blotevogel KH, Gemsa D (1998) Mass balance studies with ^{14}C-labeled 2,4,6-trinitrotoluene (TNT) mediated by an anaerobic *Desulfovibrio* species and an aerobic *Serratia* species. Curr Microbiol 37:380–386

Ederer MM, Lewis TA, Crawford RL (1997) 2,4,6-Trinitrotoluene (TNT) transformation by Clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J Ind Microbiol Biotechnol 18:82–88

Elazhari-Ali A, Singh AK, Davenport RJ, Head IM, Werner D (2013) Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. Environ Pollut 173:125–132

Esteve-Nunez A, Caballero A, Ramos JL (2001) Biological degradation of 2,4,6-trinitrotoluene. Microbiol Mol Biol 65(3):335–352

Fiorella PD, Spain JC (1997) Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl Environ Microbiol 63(5):2007–2015

Fu D, Zhang Y, Lv FZ, Chu PK, Shang J (2012) Removal of organic materials from TNT red water by Bamboo Charcoal adsorption. Chem Eng J 193–194:39–49

Fuller ME, Lowey JM, Schaefer CE, Steffan RJ (2005) A peat moss-base technology for migrating residues of explosives TNT, RDX, and HMX in soil. Soil Sediment Contam 14:373–385

Gallagher EM, Young LY, McGuinness LM, Kerkhof LJ (2010) Detection of 2,4,6-trinitrotoluene-utilizing anaerobic bacteria by 15N and 13C incorporation. Appl Environ Microbiol 76:1695–1698

Gao DW, Hu Q, Yao C, Ren NQ, Wu WM (2014) Integrated anaerobic fluidized bed membrane bioreactor for domestic wastewater treatment. Chem Eng J 240:362–368

Gumuscu B, Tekinay T (2013) Effective biodegradation of 2,4,6-trinitrotoluene using a novel bacterial strain isolated from TNT-contaminated soil. Int Biodeterior Biodegrad 85:35–41

Hannink N, Rosser SJ, Nicklin S, Bruce NC (2002) Phytoremediation of explosives. Crit Rev Plant Sci 21(5):511–538

Hao OJ, Phull KK, Chen JM, Davis AP, Maloney SW (1993) Factors affecting wet air oxidation of TNT red water-rate studies. J Hazard Mater 34:51–68

Hesham A, Qi R, Yang M (2011) Comparison of bacterial community structures in two systems of a sewage treatment plant using PCR-DGGE analysis. J Environ Sci 23(12):2049–2054

Hughes JB, Shanks M, Vanderford J, Lauritzen BR (1997) Transformation of TNT by aquatic plants and plant tissue cultures. Environ Sic Technol 31:266–271

Hwang S, Ruff TJ, Bouwer EJ, Larson SL, Davis JL (2005) Applicability of alkaline hydrolysis for remediation of TNT-contaminated water. Water Res 39(18):4503–4511

Islam MN, Shin MS, Jo YT, Park JH (2015) TNT and RDX degradation and extraction from contaminated soil using subcritical water. Chemosphere 119:1148–1152

Jones AM, Grier CW, Ampleman G, Thiboutot S, Lavigne J, Halawari J (1995) Biodegradability of selected highly energetic pollutants under aerobic conditions. Battelle, Columbus, pp 251–258

Kalafut T, Wales ME, Rastogi VK, Naumova RP, Zaripova SK, Wild JR (1998) Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr Microbiol 36(1):45–54

Kao CM, Chen CS, Tsa FU, Yang KH, Chien CC, Liang SH, Yang CA, Chen SC (2010) Application of real-time PCR, DGGE fingerprinting, and culture-based method to evaluate the effectiveness of intrinsic bioremediation on the control of petroleum-hydrocarbon plume. J Hazard Mater 178:409–416

Khan TA, Bhadra R, Hughes J (1997) Anaerobic transformation of 2,4,6-TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*. J Ind Microbiol Biotechnol 18:198–203

Larson SL, Martin WA, Scalon BL, Thompson M (2008) Dissolution, sorption, and kinetics involved in systems containing explosives, water, and soil. Environ Sci Technol 42:786–792

Lewis TA, Newcombe DA, Crawford RL (2004) Bioremediation of soils contaminated with explosives. J Environ Manage 70:291–307

Li T, Wang HJ, Dong WY, Liu TZ, Ouyang F, Zhang Q, Dong XQ (2014) Performance of an anoxic reactor proposed before BAF: effect of ferrous sulfate on enhancing denitrification during simultaneous phosphorous removal. Chem Eng J 248:41–48

Liang SH, Kuo YC, Chen SH, Surampalli RY, Kao CM (2013) Development of a slow polycolloid-releasing substrate (SPRS) biobarrier to remediate TCE-contaminated aquifers. J Hazard Mater 254–255:107–115

Limane B, Muter O, Juhanson J, Truu M, Truu J, Nolvak H (2011) Characterization of Microbial community structure after application of different bioremediation approaches in TNT contaminated Soil. Environ Eng Vilnius Lith 19–20:188–194

Lin YJ (2014) Biodegradation of TNT-contaminated groundwater. Master Thesis, National Sun Yat-Sen University, Kaohsiung, Taiwan

Lin H, Chen Z, Megharaj M, Naidu R (2013a) Biodegradation of TNT using *Bacillus mycoides* immobilized in PVA–sodium alginate–kaolin. Appl Clay Sci 83–84:336–342

Lin HY, Yu CP, Chen ZL (2013b) Aerobic and anaerobic biodegradation of TNT by newly isolated Bacillus mycoides. Ecol Eng 52:270–277

Maeda T, Nagafuchi N, Kubota A, Kadokami K, Ogawa HI (2006) One-step isolation and identification of hydroxylamino-dinitrotoluenes, unstable products from 2,4,6-trinitrotoluene metabolites, with thin-layer chromatography and laser time-of-flight mass spectrometry. J Chromatogr Sci 44:96–100

Martin JL, Comfort SD, Shea PJ, Kokjohn TA, Drijber RA (1997) Denitrification of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. Can J Microbiol 43:447–455

Matta R, Hanna K, Chiron S (2007) Fenton-like oxidation of trinitrotoluene using different irons. Sci Total Environ 385:242–251

Montpas S, Samson J, Langlois E, Lei J, Piche Y, Chêvenert R (1997) Degradation of 2,4,6-trinitrotoluene by *Serratia marcescens*. Biotechnol Lett 19:291–294

Muter O, Potapova K, Limane B, Sproge K, Jakobsone I, Cepurnieks G, Bartkevics V (2012) The role of nutrients in the biodegradation of 2,4,6-trinitrotoluene in liquid and soil. J Environ Manage 98:51–55

Nishino SF, Shin KA, Payne RB, Spain JC (2010) Growth of bacteria on 3-nitropropionic acid as a sole source of carbon, nitrogen, and energy. Appl Environ Microbiol 76:3590–3598

Nyanhongo GS, Schroeder M, Stenier W, Gubitz GM (2005) Biocatal Biotransform 23(2):53–69

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

Qiao S, Tian T, Duan X, Zhou JT, Chen YJ (2013) Novel single-stage autotrophic nitrogen removal via co-immobilizing partial nitrifying and anammox biomass. Chem Eng J 230:19–26

Rahal AG, Lobna AM (2011) Degradation of 2,4,6-trinitrotoluene (TNT) by soil bacteria isolated from TNT contaminated soil. Aust J Basic Appl Sci 55(2):8–17

Rodrigues M, Silva F, Paiva T (2009) Combined zero-valent iron and Fenton processes for the treatment of Brazilian TNT industry wastewater. J Hazard Mater 165:1224–1228

Sheibani G, Naeimpoor F, Hejazi P (2011) Statistical factor-screening and optimization in slurry phase bioremediation of 2,4,6-trinitrotoluene contaminated soil. J Hazard Mater 188:1–9

Solyanikova IP, Baskunov BP, Baboshin MA, Saralov AI, Golovleva LA (2012) Detoxification of high concentrations of trinitrotoluene by bacteria. Appl Biochem Microbiol 48:21–27

Stenuit BA, Agathos SN (2013) Biodegradation and bioremediation of TNT and other nitro explosives. Ref Modul Earth Syst Environ Sci 6:167–181

Toshinari M, Kiwao K, Hiroaki IO (2006) Characterization of 2,4,6-trinitrotoluene (TNT)-metabolizing bacteria isolated from TNT-polluted soils in the Yamada Green Zone, Kitakyushu, Japan. J Environ Biotechnol 6:33–39

Vanderberg LA, Perry JJ, Unkefer PJ (1995) Catabolism of 2,4,6-trinitrotoluene by *Mycobacterium vaccae*. Appl Microbiol Biotechnol 43(5):937–945

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

Wang ZY, Ye ZF, Zhang MH, Bai X (2010) Degradation of 2,4,6-trinitrotoluene (TNT) by immobilized microorganism-biological filter. Process Biochem 45:993–1001

Yanru Y, Manuel P, William S, Josef Z (2005) Identification of microorganisms involved in reductive dehalogenation of chlorinated ethenes in an anaerobic microbial community. Water Res 39:3954–3966

Zhang MH, Zhao QL, Ye ZF (2011) Organic pollutants removal from 2,4,6-trinitrotoluene (TNT) red water using low cost activated coke. J Environ Sci China 23:1962–1969

Ziganshin AM, Gerlach R, Naumenko E, Naumova RP (2010a) Aerobic degradation of 2,4,6-trinitrotoluene by the yeast strain Geotrichum candidum AN-Z4. Microbiology 79:178–183

Ziganshin AM, Naumova RP, Pannier AJ, Gerlach R (2010b) Influence of pH on 2,4,6-trinitrotoluene degradation by Yarrowia lipolytica. Chemosphere 79:426–433

Zou WS, Zou FH, Shao Q, Zhang J, Wang YQ, Xie FZ, Ding Y (2014) A selective fluorescent resonance energy transfer quenching and resonance light scattering enhancement dual-recognition probe for 2,4,6-trinitrotoluene. J Photochem Photobiol A 278:82–88