Improved RDX detoxification with starch addition using a novel nitrogen-fixing aerobic microbial consortium from soil contaminated with explosives

ARTICLE *in* JOURNAL OF HAZARDOUS MATERIALS · JANUARY 2015

Impact Factor: 4.33 · DOI: 10.1016/j.jhazmat.2015.01.058

4 AUTHORS, INCLUDING:

Muhammad Imran Khan
Yonsei University
29 PUBLICATIONS **284** CITATIONS
SEE PROFILE

Joonhong Park
Yonsei University
85 PUBLICATIONS **983** CITATIONS
SEE PROFILE

Journal of Hazardous Materials 287 (2015) 243–251

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Improved RDX detoxification with starch addition using a novel nitrogen-fixing aerobic microbial consortium from soil contaminated with explosives

Muhammad Imran Khan[a,b], Jihoon Yang[a], Byungun Yoo[a], Joonhong Park[a,*]

[a] School of Civil and Environmental Engineering, College of Engineering, Yonsei University, Seoul 120–749, Republic of Korea
[b] Department of Agronomy, University of Agriculture, Faisalabad 38040, Pakistan

HIGHLIGHTS

- A novel nitrogen-fixing RDX-degrading aerobic microbial consortium was developed.
- Complete detoxification of RDX was achieved by co-addition of starch and RDX.
- A positive correlation between RDX degradation and nitrogen fixation was found.

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 13 January 2015
Accepted 26 January 2015
Available online 28 January 2015

Keywords:
Rhizoremediation
Explosives
Microbial degradation
Pyrosequencing
Nitrogen fixation
Rhizobium

ABSTRACT

In this work, we developed and characterized a novel nitrogen-fixing aerobic microbial consortium for the complete detoxification of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Aerobic RDX biodegradation coupled with microbial growth and nitrogen fixation activity were effectively stimulated by the co-addition of starch and RDX under nitrogen limiting conditions. In the starch-stimulated nitrogen-fixing RDX degradative consortium, the RDX degradation activity was correlated with the *xplA* and *nifH* gene copy numbers, suggesting the involvement of nitrogen fixing populations in RDX biodegradation. Formate, nitrite, nitrate, and ammonia were detected as aerobic RDX degradation intermediates without the accumulation of any nitroso-derivatives or NDAB (4-nitro-2,4-diazabutanal), indicating nearly complete mineralization. Pyrosequencing targeting the bacterial 16S rRNA genes revealed that the *Rhizobium*, *Rhizobacter* and *Terrimonas* population increased as the RDX degradation activity increased, suggesting their involvement in the degradation process. These findings imply that the nitrogen-fixing aerobic RDX degrading consortium is a valuable microbial resource for improving the detoxification of RDX-contaminated soil or groundwater, especially when combined with rhizoremediation.

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is one of the most widespread and powerful cyclic nitramine explosives in the world [1,2]. In addition, RDX is known as a possible human carcinogen and a toxicant to many other biological systems [3]. Because RDX is recalcitrant to microbial biodegradation and has a relatively high solubility, the toxic RDX from military bomb training and manufacturing activities leaches rapidly into groundwater and surface water environments [1,4]. To protect water from contamination by explosive, contaminated sites and groundwater must be cleaned up. One option for the RDX remediation is the use of RDX-degrading microorganisms.

Although anaerobic RDX biodegradation has been reported [5–7], a number of aerobic RDX degrading bacteria have been isolated that use RDX as a sole nitrogen source [8–11]. Aerobic RDX degradation may have several advantages relative to anaerobic RDX biodegradation due to its faster degradation rate [10,12,13], and lower toxicity of degradation intermediates [14,15]. Currently, the known bacterial systems for aerobic RDX degradation include (i) cytochrome P450-like monooxygenase systems encoded by *xplAB* (XplAB) [16] from Actinomycetales (Actinobacteria class [17]), (ii) xenobiotic reductase systems encoded by *xenAB* (XenAB) from Pseudomonadales (Gammaproteobacteria class [18]), and (iii) type-I-nitroreductase system from Enterobacteriales (Gammaproteobacteria class [19]). The XplAB system, which is probably the

* Corresponding author. Tel.: +82 2 312 5798; fax: +82 2 312 5798.
E-mail address: parkj@yonsei.ac.kr (J. Park).

http://dx.doi.org/10.1016/j.jhazmat.2015.01.058
0304-3894/

best-characterized pathway among the known aerobic RDX degradation pathways [16], was reported to only be shared among the Actinomycetales members [17]. Meanwhile, ^{15}N-RDX stable isotope probing (SIP) of mixed cultures from soils or groundwater revealed that not only Actinobacteria and Gammaproteobacteria but also Alphaproteobacteria members assimilated nitrogen from RDX [4,20]. However, it is unclear if the Alphaproteobacteria assimilating nitrogen from the RDX was capable of aerobically degrading RDX. These results led to a speculation that novel aerobic RDX degrading bacteria exist that belong to taxonomic groups other than the Actinobacteria and Gammaproteobacteria classes.

The aerobic degradation of RDX by known bacteria is generally induced under nitrogen limiting growth conditions (i.e., conditions with few fixed nitrogen sources other than RDX) [11]. According to previous studies using *Gordonia* sp. strain KTR9, the expression of *xplA* and RDX degradation were induced by limited nitrogen rather than by RDX relative to the nitrogen-assimilation regulatory protein GlnR, which occurs in diverse Actinobacteria [21,22]. Bacterial nitrogen fixation is a possible nitrogen assimilation mechanism when no fixed nitrogen is available. If nitrogen-fixing RDX degrading bacteria are present, they would be a useful microbial resource for RDX bioremediation by plants because they can grow at high biomass concentrations within the rhizosphere [23]. However, little is known regarding the involvement of nitrogen fixation in aerobic RDX degradation.

Microbial consortia may be more stable and efficient for bioremediation than pure cultures (microbial isolates) because of the microbial diversity and synergetic activities that occur in microbial communities [24–29]. In addition, more opportunities for the complete mineralization of pollutants may occur when more diverse biodegrading populations are present [17,30]. So far, incomplete RDX mineralization (or accumulation of 4-nitro-2,4-diazabutanal [NDAB], a dead-end product) has been reported in previous studies involving aerobic RDX degradation in a microbial consortium [31] and in microbial communities [13,32,33]. To improve detoxification by aerobic RDX degrading microbial consortia, it is important to develop novel microbial consortia that can achieve complete RDX mineralization.

To develop microbial consortium, it is important to ensure the presence of sufficient toxicant degrading bacteria. For pure cultures, simple carbon sources, such as glucose and succinate, are typically used as external carbon sources. However, for mixed culture conditions, simple carbon sources may not be effective for stimulating aerobic RDX degradative bacteria because they are mainly used by copiotrophic non-degradative bacteria rather than by slow-growing degraders [34,35]. To maintain microbial diversity and degradation activities in microbial consortia or communities, it is practical and cost-effective to use slow-release complex carbon sources, such as starch and molasses [36–38]. Particularly, when selectively enriching rhizospheric nitrogen fixing bacteria, starch (a plant-produced carbohydrate polymer) may be an effective stimulant [39]. However, to our knowledge, no studies have used starch to stimulate nitrogen-fixing aerobic RDX degrading bacteria.

In this work, we attempted to develop a nitrogen-fixing microbial consortium with the ability to aerobically degrade RDX using starch. Next, we examined the completeness of RDX detoxification and the microbial community characteristics of the developed consortium.

2. Materials and methods

2.1. Chemicals

The RDX (purity > 99%) was purchased from AccuStandard, Inc. (New Haven, CT). In addition, the high performance liquid chromatography (HPLC) grade acetone (for preparation of RDX stock solution), methanol, isopropyl alcohol, and acetonitrile (for HPLC mobile phase) were purchased from J.T. Baker (Phillipsburg, NJ). The organic carbon sources for microbial growth, including sodium acetate (>98%), glucose (extra-pure) and soluble starch (extra pure), and inorganic nitrogen sources, including sodium nitrite (98.5%), sodium nitrate (99%), and ammonium chloride (>99%) were obtained from Junsei Chemicals Co., Ltd. (Japan). To prepare the culture medium and the HPLC mobile phase, water was purified using an EASYpure Reverse Osmosis System and a Compact Ultrapure Water System from Barnstead/Thermolyne (Dubuque, IA).

2.2. Microcosm experiments

The 1st enrichment microcosm experiment was conducted using soil samples that were collected from a military shooting site at Darokdae (Kangwon province, South Korea). Aerobic incubation experiments were conducted in 1-L Erlenmeyer flasks containing 190 mL of an aqueous growth medium comprised of (in gram per liter unless specified otherwise): $NaHCO_3$ (2.5), $NaH_2PO_4{\cdot}H_2O$ (0.6), KCl (0.1), the modified Wolfe's vitamin and mineral mixtures (each 10 mL/L), and 1 mL of 1 mM Na_2SeO_4 [40]. Starch (1.0 g/L, 2.5 g/L, and 5.0 g/L) was added as the sole carbon source in the main experiment of this study. The treatment details are given in Table S1. The final pH was 7.0 ± 0.3. After autoclaving the prepared starch-amended flasks, the RDX was added with acetone as a carrier solvent. The solvent was completely evaporated by placing the flask on a clean bench for 2 h. The sterile flasks (2-L) were wrapped with aluminum foil to prevent any photochemical reactions of the RDX. Each of the prepared flasks was inoculated with 10.0 ± 0.3 g of soil. In addition, sterile controls were prepared to evaluate the significance of any abiotic degradation of RDX. Microcosm flasks were continuously shaken on a rotary shaker (150 rpm) at $25.0 \pm 0.5\,^{\circ}C$ in the dark. After the 1st enrichment experiment, sub-culturing or serial dilution was conducted using freshly prepared autoclaved aqueous growth medium containing RDX for selective sub-culturing of the RDX-degrading consortia. The experiments were conducted in triplicate for each treatment. Among the three replicate microcosms, the one with the best RDX degradation was selected for sub-culturing and for estimating the degradation rates. The RDX concentrations that were used were low during the 1st enrichment (0.12–0.15 mM) and increased during the 2nd (0.18–0.19 mM) and 3rd (0.20–0.29 mM) enrichments before decreasing again during the 4th enrichment (0.09 mM).

To determine the optimal growth conditions of the microbial consortium with enhanced RDX biodegradability, a separate set of experiments was conducted. In this case, RDX biodegradation and microbial growth were monitored with different initial concentrations (2.5–5.0 g/L) for each of the three carbon sources (viz., glucose, acetate, and starch) and in the presence of different supplemental nitrogen sources (viz., $NaNO_2$, $NaNO_3$, and NH_4Cl at 0.5 g/L). Furthermore, the pH was varied from acidic to alkaline (i.e., 3.0–9.5), and different temperature levels were used (i.e., 4–35 °C).

2.3. Specific RDX degradation rates

The specific RDX degradation rates (R) were estimated using the following equation:

$$R = \frac{(C_n - C_{n-1})}{(t_n - t_{n-1})} \times \frac{1}{\mathrm{OD_{av}}}$$

where t_n and t_{n-1} represent two continuous sampling times, C_n and C_{n-1} represent the RDX concentrations in the microcosm at

the corresponding sampling time and OD_{av} represents the average OD values between t_n and t_{n-1}.

2.4. Acetylene reduction assay (ARA)

To assess the nitrogen fixation in the microbial culture, ARA was carried out according to the procedure described by Smibert and Krieg [41], with only slight modifications. Briefly, after two days of incubating MI in a 160-mL serum bottle with 100 mL of aqueous growth medium containing RDX, starch or both, acetylene was injected into the culture vessels through the stopper to achieve an acetylene content of 10% (vol/vol). During several incubation periods, 1-mL gas samples from the culture vessel were removed using a gas-tight 1-mL syringe (BD, Franklin Lakes, NJ) and gas-tight teflon valves with luer lock adapters. These samples were tested for the presence of ethylene by using gas chromatography (GC). The nitrogenase activity (nmol per hour per 10^8 cells) was expressed as nmol of ethylene produced per hour per 10^8 cells.

2.5. Analytical methods

The RDX was analyzed using HPLC (Agilent Technologies 1200 series, USA) at 230 nm according to US EPA method 8330 [32,42], with slight modifications. The samples were filtered using a sterilized PTFE membrane filter and were automatically injected into a SHISEIDO CAPCELL PAK C18 column (Shiseido, 5 μm particle size, 250 × 4.6 mm LD) at ambient temperature. A mobile phase consisting of 70% deionized water, 18% isopropanol, and 12% acetonitrile was used with a flow rate of 0.75 mL/min. Hexahydro-1-nitroso-2,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) peaks were detected in the anaerobic consortium (Khan et al., unpublished results). These peaks were not detected in this study when using the aerobic consortium.

Microbial growth was quantified by measuring the optical density (OD) at 600 nm [43] using a UV–vis spectrophotometer (Mecasys Co., Ltd., Korea). The dissolved oxygen content and pH were measured using a Thermo Scientific Orion 5 Star device (Thermo Fisher Scientific Inc., USA). The ammonia-N concentrations were measured using a DR/4000 UV spectrophotometer (HACH, USA) with a C-MAC Nessler Kit [44].

Nitrite, nitrate, and formate were analyzed by ion chromatography (IC) using a Dionex system consisting of an AS40 auto-sampler and an ICS-90 ion chromatograph (Dionex, Sunnyvale, CA) equipped with a conductivity detector. Samples (50 μL) were assayed for anions by using 3.5 mM sodium carbonate/1 mM sodium bicarbonate as the eluent using in an AS14 column (250 × 4 mm, Dionex) at a flow rate of 1.2 mL/min.

For ARA, the acetylene and ethylene contents were measured using a Hewlett–Packard 6890 GC equipped with a flame ionization detector (FID) and an HP-PLOT U column (10 m × 0.32 mm × 30 μm). Helium was used as the carrier gas with a flow rate of 0.7 mL/min. The oven temperature was maintained at 60 °C for 10 min, increased at a rate of 10 °C min^{-1} to 180 °C, and then held at 180 °C for 2 min.

2.6. Statistical analysis

The chemical and biological data were analyzed using the Statistical Package for the Social Sciences 16.0 (SPSS, Chicago, IL) for Windows [45]. A one-way analysis of variance was used to detect any significant differences ($P < 0.05$) between the controls and the treated microcosm samples.

2.7. DNA extraction, PCR amplification, and 454 titanium pyrosequencing

The total genomic DNA was extracted from the untreated soil samples and from the RDX-degrading microcosms (after three sequential transfers) using a Power Soil DNA Extraction Kit (MOBIO, CA, USA) according to the manufacturer's instructions. Bacterial 16S rRNA genes were PCR amplified using the V4-forward (5′-AYTGGGYDTAAAGNG-3′) and V5-reverse (5′-CCGTCAATTYYTTTRAGTTT-3′) primers [46]. Three different barcodes (AGAGAGAG for the initial soil, AGCAGCAG for S1.0, and AGCAGATG for S5.0) were linked with the primers and were used to distinguish each sample prior to sequencing at Macrogen. The PCR reaction was performed according to Lee et al. [47], with only slight modifications. Each PCR reaction contained 1 μL of template DNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 100 pM of each primer, and 1.25 U of Taq polymerase (Invitrogen, Inc., WI, USA) (for a total volume of 25 μL). Amplification occurred in a C1000TM Thermal Cycler (BIO-RAD, CA, USA) using the following conditions: (i) an initial denaturation step of 94 °C for 3 min, (ii) 25 cycles of denaturation, annealing and extension (94 °C for 1 min followed by 54 °C for 30 s, with an extension step at 72 °C for 2 min), and (iii) a final extension at 72 °C for 5 min.

After PCR amplification, the PCR products were purified once using gel electrophoresis/isolation and twice using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and the QIAquick PCR purification kit (Qiagen). Pyrosequencing of the amplicons was conducted by Macrogen Inc. (Seoul, South Korea) using a 454/Roche GS-FLX titanium instrument (Roche, NJ). Sequences that were shorter than 300 nucleotides or with an average Quality Score of less than 20 were removed through quality filters. The sequences were analyzed by following the modified protocol suggested by Schloss et al. [48] by using Mothur, and all filtering and clustering steps were performed according to the methods described by Van Doan et al. [49]. Sequences were classified using the Ribosomal Database Project (RDP)'s pyrosequencing pipeline [50]. We estimated the OTU richness, the Shannon index and the Shannon evenness [51,52].

In addition, a phylogenetic tree analysis was conducted to compare the obtained sequences with the existing GenBank 16S rRNA gene sequences by using BLAST: http://www.ncbi.nlm.nih.gov/BLAST/. The sequences were aligned using MUSCLE [53,54] and a phylogenetic tree was constructed using MEGA4 [55] with a neighbor-joining algorithm that employed a similarity matrix of pairwise comparisons with 1000 bootstrap replicates [54]. The representative nucleotide sequences obtained in this study (Table 3 and Fig. S6) were deposited in the GenBank under accession numbers KF577693–KF577717 and KF597050–KF597061.

2.8. Quantitative real-time PCR analysis

Real-time PCR analysis was performed using BIORAD iQ5 (BIO-RAD, CA, USA). The amplification reactions were carried out using a volume of 25 μL containing 12.5 μL iQ_ SYBR Green Supermix (BIO-RAD, CA, USA), 1 μL of each primer (10 mM), 10 ng of total DNA, and RNase-free water. The primer sets for amplifying the 16S rRNA and functional genes (for RDX degradation, nitrogen fixation and denitrification) are summarized in Table S2. All primers used in this study were published previously and have been extensively used. The qPCR thermocycling step conditions for 16S rRNA amplification were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 58 °C [56]. The qPCR conditions for the *narG* and *nirK* amplification were as the same as described by Van Doan et al. [49]. The *xplA* qPCR amplification conditions consisted of a single step at 95 °C for 12 min followed by 50

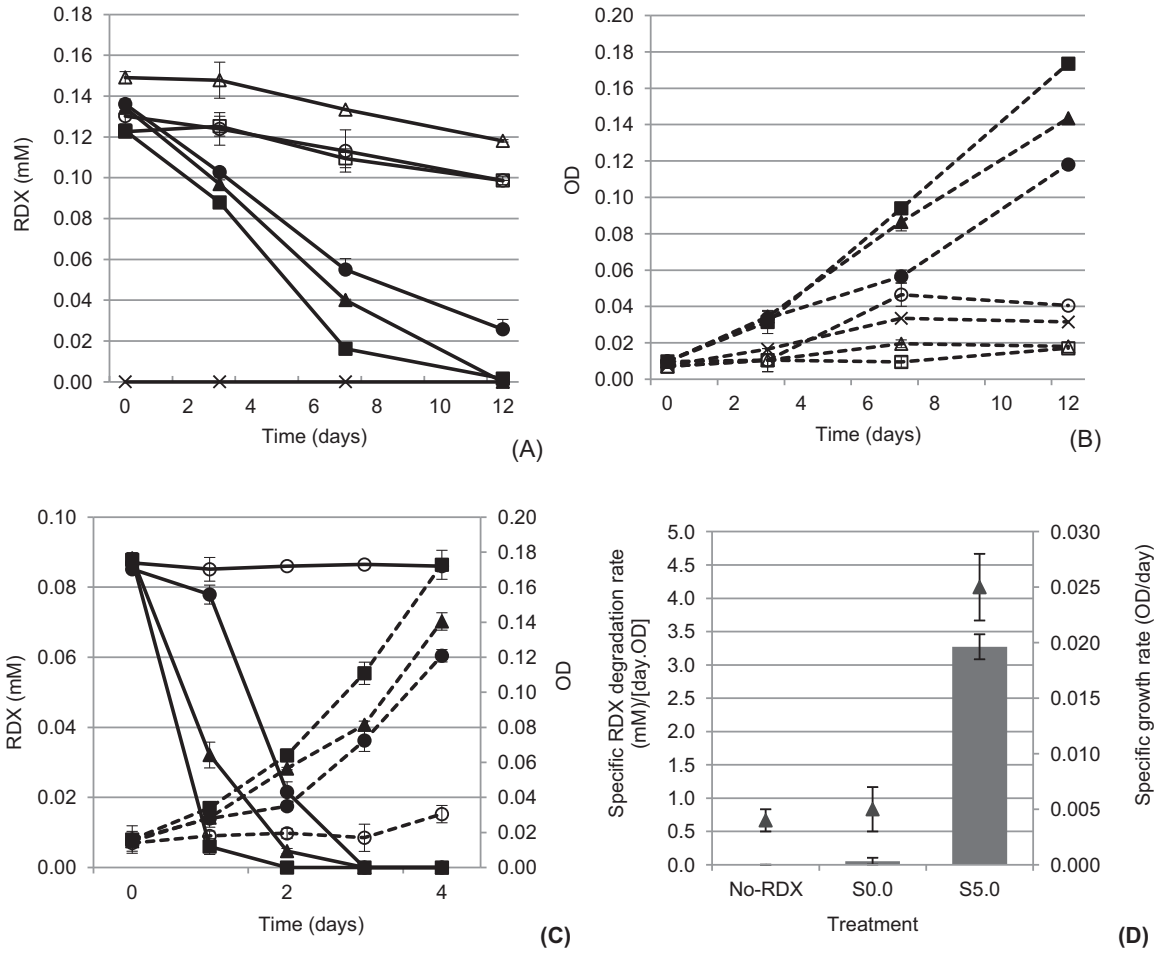

Fig. 1. Aerobic biodegradation of the RDX (—) and the microbial growth (–) during the 1st enrichment (A and B) and the 4th or final enrichment (C) of the MI consortium, and the specific RDX degradation rate (columns) and specific growth rate (triangle markers) in the MI consortium (D). The symbols indicate the control with no RDX (– × –), the autoclaved control with RDX (–□–), the autoclaved control with RDX and 2.5 g/L starch (–Δ–), RDX with no starch (–○–), the RDX with 1.0 g/L starch (–●–), the RDX with 2.5 g/L starch (–▲–), and the RDX with 5.0 g/L starch (–■–). Each error bar represents one standard deviation of three replicates. (No-RDX, the MI consortium without RDX but with 5.0 g/L of starch; S0.0, the MI consortium with RDX but without starch; S5.0, the MI consortium with RDX and with 5.0 g/L starch; OD, optical density at 600 nm).

cycles at 95 °C for 20 s, and 72 °C for 30 s [57]. The qPCR conditions for *nifH* amplification were 5 min of initial denaturation at 95 °C, followed by 40 cycles of 15 s of denaturation at 95 °C, 25 s of an annealing step at 53 °C, 45 s of elongation at 72 °C and final prolongation for 5 min at 72 °C [58]. After each qPCR run, a melting curve analysis was carried out to confirm the presence of the desired amplicon.

3. Results and discussion

3.1. Effects of co-addition of starch and RDX on aerobic RDX degradation under nitrogen limiting conditions

In the nitrogen-limiting microcosm experiments with the explosive-contaminated soil from the military shooting site (solid symbols in Fig. 1A), the co-addition of starch and RDX resulted in significant RDX disappearances and microbial growth relative to the corresponding observations in the no-starch (in presence of RDX) control or no-RDX (in the presence of starch) control microcosms ($P < 0.05$). This result indicates that aerobic RDX degradation by indigenous soil microbes was stimulated in the co-presence of starch and RDX under nitrogen limiting condition. Throughout the following sub-culture transfer experiments, the starch-utilizing aerobic RDX degrading microbes were further enriched under the nitrogen limiting aqueous medium condition (Fig. S1[A–B] and Fig. 1C). After the second sub-culturing experiment, the specific rate of aerobic RDX degradation increased by approximately 3–6-fold compared with those from the initial microcosm experiment with the soil (Fig. S2). The starch-stimulated RDX-degrading microbial consortia obtained from the final (the 4th) sub-culturing experiments with different starch concentrations were named as MI. In the MI-5.0 consortium (starch 5.0 g/L), the addition of starch in the absence of RDX resulted in little microbial growth, and the addition of RDX in the absence of starch resulted in little RDX degradation. These results confirmed the necessity of the co-addition of starch and RDX for stimulating microbial degradation and growth in the final enrichment of the aqueous medium.

During the 4th sub-culturing experiment with 5.0 g/L starch (MI-5.0), nitrite, nitrate, ammonia, and formate were observed (Fig. S3C). Furthermore, the same intermediates were detected in the 4th sub-culturing experiments with lower starch concentrations (1.0 g/L and 2.5 g/L [MI-1.0 and MI-2.5, respectively]). The detected compounds include potential intermediates of aerobic RDX degradation via the bacterial XplA/B pathway and indicate that ring cleavage occurred [10,59]. The production of nitrite in the microcosm supports the occurrence of a denitration mechanism [10,59]. An unidentified HPLC peak (suspected to be NDAB) with a

Table 1
Comparison of the aerobic RDX biodegradation rates from previous studies and this work.

Microbial isolates/consortia	Carbon substrate (g/L)	RDX (mM)	Degradation rate (mM/day)	Specific degradation rate [(mM)/(day × OD)][a]	References
Gordonia sp. strain KTR9	Glucose (0.9) + succinate (0.6)	0.135	0.090	1.150–1.350	Indest et al. [21]
Gordonia sp. strain KTR9	Succinate (2.36)	0.280	0.841	0.701	Zhu et al. [22]
Gordonia strain YY1	Glucose (0.5) + sodium citrate (0.5)	0.045	0.009	0.045–0.050	Ronen et al. [13]
MI consortium	Starch (5.0)	0.085	0.085	0.816–0.960	This study
MI consortium	Glucose (5.0)	0.072	0.072	0.609–0.665	This study
MI consortium	Acetate (5.0)	0.017	0.017	0.430–0.604	This study
Pseudomonas sp. HK-6	Succinate (0.6)	0.045	0.002	0.012–0.013	Chang et al. [65]
Rhodococcus sp. 11Y	Succinate (1.0)	0.019	0.019	*NA*	Fuller et al. [12]
Rhodococcus sp. DN22	Succinate (1.0)	0.019	0.019	*NA*	Fuller et al. [12]
Rhodococcus sp. T9N	Glucose (0.94)	0.130	0.032	0.130–0.140	Bernstein et al. [31]
Rhodococcus strain YH1	Cyclohexanone (0.95)	0.126	0.042	0.042–0.050	Nejidat et al. [9]
Stenotrophomonas maltophilia PB1	Glucose (0.9) + succinate (0.6) + glycerol (0.9)	0.250	0.040	*NA*	Binks et al. [64]

NA: not avaiable.
[a] Values are estimated using the degraded amounts of RDX over a specific time period and the final OD (optical density) value of microbial growth.

Table 2
Bacterial alpha diversity results at the genus level.

Sample	Filtered sequence	OTU richness*	Shannon index	Evenness	$Inv_{simpson}$	Coverage (%)
Initial[a]	6831	1124 ± 10	8.60 ± 0.03	0.99 ± 0.00	3958 ± 125.9	20 ± 1.5
MI-1.0[b]	1625	367 ± 5	5.49 ± 0.01	0.89 ± 0.01	130.5 ± 1.7	85 ± 0.8
MI-5.0[c]	1265	329 ± 3	5.29 ± 0.02	0.88 ± 0.00	107.8 ± 2.5	86 ± 0.9

*The size of the subsamples was 1265.
[a] Initial: the soil initially used for the 1st enrichment.
[b] MI-1.0: MI consortium with RDX and with 1.0 g/L starch.
[c] MI-5.0: MI consortium with RDX and with 5.0 g/L starch.

retention time of 3.9-min was observed from the first enrichment microcosm experiment. However, this unidentified peak was not detected in the following sub-culturing microcosm experiments (Fig. S4). This result may be explained by the rapid transformation of RDX and its intermediates to stable end products. Unlike the detection of aerobic degradation intermediates, no nitroso-derivatives of RDX (MNX, DNX, and TNX), which are potential intermediates of anaerobic RDX degradation via the bacterial XplA/B pathway, were detected. The absence of the aforementioned toxic metabolites in this study is advantageous because NDAB and all of the nitroso-derivatives of RDX are known to be toxic to living organisms [15,60,61] and the accumulation of these metabolites in the system can interfere with further biodegradation of the parent compound [62]. Furthermore, the GC/MS analysis showed the presence of nitrous oxide and ammonia in the liquid-phase of the culture in the RDX-degrading microcosms (data not shown). This finding indicates the further reduction of nitrite to nitrous oxide and ammonia [14,59]. In addition, real time PCR analysis (Fig. S7) indicated the presence of *narG* and *nirK* in the RDX-degrading microcosms which further supported the gaseous release of nitrogen via denitrification.

Starch-stimulated aerobic RDX degradation in the MI-5.0 consortium was optimal at mesophilic temperatures (15–35 °C) and in near-neutral pH (pH 7.0–8.0) conditions (Fig. S5[B–C]). In addition, aerobic RDX degradation was investigated in response to simpler carbohydrate compounds (acetate and glucose, respectively) and their degradation rates were compared with the results from the addition of starch (Fig. S5A). Regardless of the different concentrations applied (2.5–5.0 g/L), the addition of starch resulted in greater degradation rates than the addition of acetate or glucose. This stimulatory effect could occur because the starch is a plant-produced carbohydrate, which could have a direct and positive influence on the growth and activity of the soil rhizosphere bacteria [39,63] and enhance RDX degradation. The (specific) rate of aerobic RDX degradation by the MI consortium grown on starch (5.0 g/L) was greater ($P < 0.01$) than most of the

Table 3
Relative abundances of the major genera (%) that were detected in this study.

OTU ID	Accession number	Phylum/Class	Genera	Initial[a]	MI-1.0[b]	MI-5.0[c]
ECS1–ECS10	KF577693–KF577702	Proteobacteria/Alpha	*Rhizobium*	0.16	9.35	28.2
ECS26–ECS27	KF597050–KF597051	Proteobacteria/Beta	*Rhizobacter*	0.00	0.12	10.9
ECS30–ECS31	KF597054–KF597055	Bacteroidetes/Sphingobacteriia	*Terrimonas*	0.21	4.8	10.9
ECS28–ECS29	KF597052–KF597053	Bacteroidetes/Cytophagia	*Dyadobacter*	0.015	5.17	5.22
ECS11–ECS15,ECS21–ECS25	KF577703–KF577707,KF577712–KF577717	Proteobacteria/Beta	*Hydrogenophaga*	0.00	8.49	3.72
ECS32–ECS33	KF597056–KF597057	Bacteroidetes/Sphingobacteriia	*Mucilaginibacter*	0.00	2.58	2.45
ECS34–ECS35	KF597058–KF597059	Bacteroidetes/Sphingobacteriia	*Ferruginibacter*	0.35	7.14	0.32
ECS16–ECS20	KF577708–KF577712	Proteobacteria/Alpha	*Brevundimonas*	0.12	10.5	0.08
ECS36–ECS37	KF597060–KF597061	Bacteroidetes/Sphingobacteriia	*Hydrotalea*	0.02	7.38	0.00
Specific RDX degradation rate (mM)/[day × OD]				0.00	2.35	3.27
xplA/16S gene copy ratio (10^{-5})				0.18	1.80	14.7

[a] Initial: the soil initially used for the 1st enrichment.
[b] MI-1.0: MI consortium with RDX and with 1.0 g/L starch.
[c] MI-5.0: MI consortium with RDX and with 5.0 g/L starch.

Table 4
The ratios of the fixed and biomass nitrogen species to the nitrogen from the degraded RDX in the 4th or final enrichment after 2 days of incubation.

Treatment	$\triangle NO_2 - N/\triangle RDX - N$	$\triangle NO_3 - N/\triangle RDX - N$	$\triangle NH_3 - N/\triangle RDX - N$	$\triangle$Biomass – N/$\triangle$RDX – N	Total N/$\triangle$RDX – N
S1.0	0.14	0.06	0.06	0.96	1.22
S2.5	0.02	0.08	0.14	1.59	1.83
S5.0	0.02	0.08	0.17	1.71	1.98

previously known aerobic RDX-degrading bacterial isolates belonging to a variety of genus groups (*Pseudomonas*, *Rhodococcus*, *Gordonia* and *Stenotrophomonas*) [9,12,31,64,65] and was comparable to the previously reported results for *Gordonia* sp. KTR9 [21,22] (Table 1). In addition, this trend was also true when the MI consortium was grown on glucose or acetate. These results indicate that the starch-stimulated MI consortium can effectively degrade RDX by using diverse carbon sources. However, RDX degradation is stimulated more effectively by the chemically complex sugar-polymer (starch) growth substrate than by simpler carbohydrate compounds (acetate and glucose).

RDX and its degradation intermediates can migrate through the soil and cause groundwater contamination due to the absence of suitable microbes or very slow degradative activities. Treating soil with a slow-releasing carbon compound, such as molasses and starch, can prevent contaminant migration by enhancing its degradation by microbes [37]. Starch is a readily available slow-releasing complex carbon source and relatively inexpensive [38]. In order to make the use of starch practical for the bioremediation, further studies should be done on bioremediation of RDX contaminated soil by irrigating the soil with different doses of starch (1.0–2.5 g/L) using MI consortium alone or in combination with plants.

3.2. Microbial community characteristics of the MI consortia

The microbial community pyrosequencing analysis targeting the 16S rRNA gene showed that the alpha-diversity values (OTU richness, Shannon Index, Evenness, and $Inv_{simpson}$) of the MI consortia with starch (at 1.0 and 5.0 g/L) were lower ($P < 0.01$) during enrichment relative to the initial soil sample (Table 2). In the MI-5.0 consortium that exhibited the greatest RDX degradation rate, the *Rhizobium* genus members were dominant (28.2% of the total), followed by *Rhizobacter* and *Terrimonas* (10.9% of the total each) (Table 3). In the MI-1.0 consortium, the *Brevundimonas* and *Rhizobium* members were dominant (10.5% and 9.4% of the total, respectively).

A phylogenetic analysis was conducted to examine the relationships of the predominant microcosm genera using previously characterized aerobic RDX-degrading bacterial isolates (Fig. S6). The 16S rRNA sequences of the *Rhizobium* (ECS1-ECS10) and *Brevundimonas* (ECS16-ECS20) members, for which the relative abundances significantly increased during enrichment, belonged to Alpharoteobacteria. The *Rhizobium*, *Brevundimonas* and the other dominant MI members were phylogenetically distant (more than 5% dissimilarity) from the known aerobic RDX-degrading bacterial isolates. The phylogenetic distances of these members suggested that they are either novel RDX degraders or non-RDX degraders. Their relative abundances increased as the specific RDX degradation rate increased (i.e., initial soil < starch 1.0 g/L [MI-1.0] < starch 5.0 g/L [MI-5.0]) (Table 3, $P < 0.01$). This result suggests that they were involved in the degradation of RDX by the consortia. The *Rhizobium* belongs to *Rhizobiales*, a group of nitrogen fixing bacteria. Members of *Rhizobiales*, such as *Agrobacterium*, *Bradyrhizobium* and *Sinorhizobium* are known to have capabilities to degrade a variety of contaminants [66,67].

In addition, the contributions of the XplA pathway to aerobic RDX degradation in the consortium were evaluated by quantifying the *xplA* gene copy numbers with the bacterial 16S rRNA gene copy numbers (Fig S7). The *xplA*/16S (16S-rRNA) gene copy ratios were less than 0.001, while the copy ratios generally increased with the RDX degradation activity (Table 3). By assuming that one bacterium has three copies of 16S rRNA gene [68], the *xplA*/16S ratio results indicated that only a small portion of the microbes in the consortia (<3%) contained the *xplA* gene. This finding suggests that RDX degradation pathway(s) other than XplA could be responsible for the observed RDX degradation by the consortia.

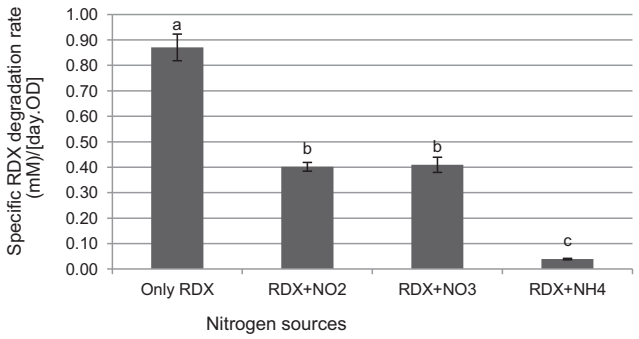

Fig. 2. Specific rate of RDX degradation by the MI consortium in response to the different supplemental nitrogen sources (i.e., $NaNO_2$, $NaNO_3$, and NH_4Cl, each 0.5 g/L). Each error bar represents one standard deviation of the three replicates. Different lower-case letters in the figure indicate significantly different values ($P < 0.05$).

3.3. Correlation of aerobic RDX degradation with nitrogen fixation

Furthermore, the MI-5.0 was subjected to RDX biodegradation in the presence of fixed nitrogen sources at high concentrations (i.e., nitrite, nitrate, and ammonium) (Fig. 2). The addition of fixed nitrogen sources resulted in repressing the RDX degradation activities compared to those in the nitrogen limiting conditions ($P < 0.05$). The addition of ammonium resulted in nearly zero RDX degradation activity, while the addition of nitrate or nitrite reduced the RDX degradation activity by approximately 50%. This finding suggested that the consortium will preferentially use fixed nitrogen sources over RDX as a nitrogen source [8,31,64]. Furthermore, these results agree with those of previous studies [8,64]. For example, Coleman et al. [8] showed that the presence of ammonium delayed the onset and significantly reduced the extent of RDX degradation (at 2 mM of ammonium) because of the preferential use of ammonium as a nitrogen source. Later, Wani and Davis [69] observed that low levels of inorganic nitrogen, such as nitrate (0.1 g/L) temporarily reduced the RDX degradation rate, while high nitrate levels (0.5 g/L) completely halted the biotransformation of RDX until the nitrate was denitrified. Another possible explanation for the reduced RDX degradation rate is the inhibition of the expression of the RDX degrading gene *xplA* by ammonium, nitrite, and nitrate [9,22,70,71]. However, it is possible that the repression of the *xplA* gene expression will not occur under environmental conditions because the environmental concentrations of nitrogen are normally much lower [22] than the concentrations that were applied in this study (i.e., 0.5 g/L).

The amount of total nitrogen ($\triangle NO_2 - N + \triangle NO_3 - N + \triangle NH_4 - N + \triangle$Biomass – N) was greater than the amount of nitrogen that resulted from RDX degradation (i.e., $\triangle$RDX – N) (Table 4). This result

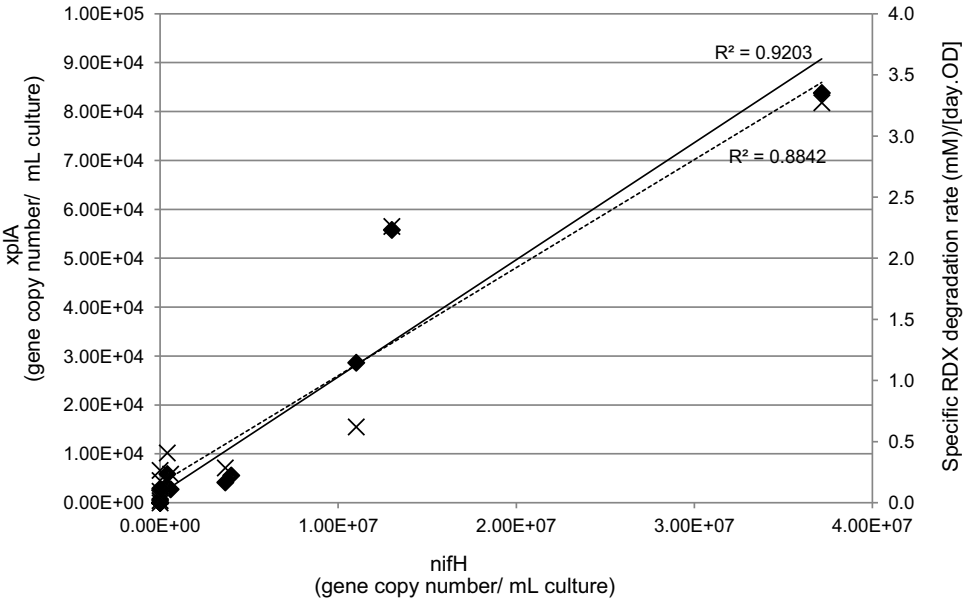

Fig. 3. Linear correlation of the copy number of the nitrogen fixing *nifH* gene with the copy number of the RDX degradative *xplA* gene (—) or with the RDX degradation rate (–).

Table 5
Nitrogenase activity results for MI-5.0.

Treatment	Nitrogenase activity (nmol of ethylene per hour per 10^8 cells)
RDX without starch	0.00 ± 0.00
RDX with 5.0 g/L starch	3.16 ± 0.58
No RDX with 5.0 g/L starch	0.00 ± 0.00

prompted us to speculate regarding the existence of a nitrogen source other than RDX. The results of the nitrogenase activity assay in MI-5.0 (Table 5) showed that nitrogen fixation is a mechanism that could provide an additional fixed nitrogen source with the degraded RDX in the system. The ratio of total N per △RDX – N (Table 4) generally increased (i.e., 1.22, 1.83 and 1.98, respectively) as the RDX degradation rate increased (S1.0 < S2.5 < S5.0) (Fig. S2), suggesting a positive correlation between RDX degradation and nitrogen fixation. This possibility was supported by a positive linear correlation between the RDX degradation gene (*xplA*) and nitrogen fixation gene (*nifH*) copy numbers that were quantified by real-time PCR (R^2 = 0.9203 [Fig. 3]). In addition, to determine if nitrate and nitrite reduction were involved with RDX degradation, the denitrification genes (*narG* and *nirK*) were quantified (Fig. S7). However, these genes showed much weaker correlations with the *xplA* gene copy numbers than the nitrogen fixation gene (*nifH*).

The majority of soils contaminated with explosives are nitrogen-deficient; thus, an additional nitrogen source will probably be required for their remediation [36]. Although high levels of other nitrogen sources, such as ammonium, reduced RDX degradation [22], the addition of low levels of supplemental nitrogen is often required to support the initial growth of bacteria that degrade explosives [36] or to enhance RDX degradation [13,72]. Bacterial nitrogen fixation could serve as a possible nitrogen assimilation mechanism when no fixed nitrogen is available. In addition, nitrogen fixation may enhance RDX degradation by providing sufficient nitrogen to support bacterial growth in nitrogen-limiting environments. The nitrogen fixing ability, which was observed for the RDX-degrading MI consortium, may provide sufficient nitrogen for supporting microbial growth in nitrogen-deficient environments and may be an excellent candidate for the rhizoremediation (a plant-microbe-based remediation approach) of RDX-contaminated environments. In rhizoremediation, plants support microbial growth and increase the metabolic activities of rhizosphere bacteria by providing oxygen and root exudates, while bacteria stimulate plant growth by fixing atmospheric nitrogen, secreting phytohormones and by providing defense against pathogens [73,74]. Thus, the RDX-degrading and nitrogen fixing activity of the *Rhizobium*-dominant MI consortium may have important implications for the bioremediation of nitrogen limiting RDX-contaminated sites. Furthermore, due to the beneficial characteristics of rhizosphere bacteria (present in the consortium), their use in large scale rhizoremediation operation will be safe and ecologically advantageous [75].

4. Conclusions

In this study, MI a consortium that can biodegrade RDX was successfully enriched through sub-culturing with the co-addition of starch and RDX. The developed consortium was capable of degrading RDX aerobically to form non-toxic and easily biodegradable intermediates. Here, we provided the first experimental evidence that nitrogen fixation is involved in aerobic RDX degradation, which has important implications for rhizoremediation and the bioremediation of nitrogen-deficient RDX-contaminated environments. We believe that the novel consortium developed in this study could be used for the in situ/ex situ bioremediation of RDX polluted environments.

Acknowledgments

This study was supported by the Korea Ministry of Environment via the GAIA project. In addition, the authors would like to acknowledge Jaejin Lee, Jangho Lee, Tuan Van Doan, and Keunje Yoo for their assistance with analyzing the molecular biological data.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.01.058.

References

[1] J. Clausen, J. Robb, D. Curry, N. Korte, A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA, Environ. Pollut. 129 (2004) 13–21.
[2] J. Pichtel, Distribution and fate of military explosives and propellants in soil: a review, Appl. Environ. Soil Sci. 2012 (2012) 33.
[3] USEPA, 2006 Edition of the Drinking Water Standards and Health Advisories, EPA 822-R-06-13 Office of Water, Washington, DC, 2006.
[4] P. Andeer, D.A. Stahl, L. Lillis, S.E. Strand, Identification of microbial populations assimilating nitrogen from RDX in munitions contaminated military training range soils by high sensitivity stable isotope probing, Environ. Sci. Technol. 47 (2013) 10356–10363.
[5] C.L. Kitts, D.P. Cunningham, P.J. Unkefer, Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family Enterobacteriaceae from nitramine explosive-contaminated soil, Appl. Environ. Microbiol. 60 (1994) 4608–4611.
[6] N.G. McCormick, J.H. Cornell, A.M. Kaplan, Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine, Appl. Environ. Microbiol. 42 (1981) 817–823.
[7] D.M. Young, P.J. Unkefer, K.L. Ogden, Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a prospective consortium and its most effective isolate *Serratia marcescens*, Biotechnol. Bioeng. 53 (1997) 515–522.
[8] N.V. Coleman, D.R. Nelson, T. Duxbury, Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22, Soil Biol. Biochem. 30 (1998) 1159–1167.
[9] A. Nejidat, L. Kafka, Y. Tekoah, Z. Ronen, Effect of organic and inorganic nitrogenous compounds on RDX degradation and cytochrome P-450 expression in *Rhodococcus* strain YH1, Biodegradation 19 (2008) 313–320.
[10] H.M. Seth-Smith, S.J. Rosser, A. Basran, E.R. Travis, E.R. Dabbs, S. Nicklin, N.C. Bruce, Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus* rhodochrous, Appl. Environ. Microbiol. 68 (2002) 4764–4771.
[11] K.T. Thompson, F.H. Crocker, H.L. Fredrickson, Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp, Appl. Environ. Microbiol. 71 (2005) 8265–8272.
[12] M.E. Fuller, N. Perreault, J. Hawari, Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains, Lett. Appl. Microbiol. 51 (2010) 313–318.
[13] Z. Ronen, Y. Yanovich, R. Goldin, E. Adar, Metabolism of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in a contaminated vadose zone, Chemosphere 73 (2008) 1492–1498.
[14] M.I. Khan, J.J. Lee Park, Microbial degradation and toxicity of hexahydro-1,3,5-trinitro-1,3,5-triazine, J. Microbiol. Biotechnol. 22 (2012) 1311–1323.
[15] B. Zhang, S.B. Cox, S.T. McMurry, G.P. Jackson, T.A. Anderson, Effect of two major *N*-nitroso hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites on earthworm reproductive success, Environ. Pollut. 153 (2008) 658–667.
[16] R.G. Jackson, E.L. Rylott, D. Fournier, J. Hawari, N.C. Bruce, Exploring the biochemical properties and remediation applications of the unusual explosive-degrading P450 system XplA/B, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 16822–16827.
[17] P.F. Andeer, D.A. Stahl, N.C. Bruce, S.E. Strand, Lateral transfer of genes for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation, Appl. Environ. Microbiol. 75 (2009) 3258–3262.
[18] M.E. Fuller, K. McClay, J. Hawari, L. Paquet, T.E. Malone, B.G. Fox, R.J. Steffan, Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB, Appl. Microbiol. Biotechnol. 84 (2009) 535–544.
[19] C.L. Kitts, C.E. Green, R.A. Otley, M.A. Alvarez, P.J. Unkefer, Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6,-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), Can. J. Microbiol. 46 (2000) 278–282.
[20] H. Roh, C.P. Yu, M.E. Fuller, K.H. Chu, Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading microorganisms via 15N-stable isotope probing, Environ. Sci. Technol. 43 (2009) 2505–2511.
[21] K.J. Indest, D.E. Hancock, C.M. Jung, J.O. Eberly, W.W. Mohn, L.D. Eltis, F.H. Crocker, Role of nitrogen limitation in transformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by *Gordonia* sp. strain KTR9, Appl. Environ. Microbiol. 79 (2013) 1746–1750.
[22] S.H.J. Zhu Reuther, J.F.H. Liu Crocker, K.J. Indest, L.D. Eltis, W.W. Mohn, The essential role of nitrogen limitation in expression of xplA and degradation of hexahydro-1,3, 5-trinitro-1,3,5-triazine (RDX) in *Gordonia* sp. strain KTR9, Appl. Microbiol. Biotechnol. 99 (2015) 459–467.
[23] A. Lorenz, E.L. Rylott, S.E. Strand, N.C. Bruce, Towards engineering degradation of the explosive pollutant hexahydro-1,3,5-trinitro-1,3,5-triazine in the rhizosphere, FEMS Microbiol. Lett. 340 (2013) 49–54.
[24] A.M. Cupples, RDX degrading microbial communities and the prediction of microorganisms responsible for RDX bioremediation, Int. Biodeterior. Biodegrad. 85 (2013) 260–270.
[25] T. Hudcova, M. Halecky, E. Kozliak, M. Stiborova, J. Paca, Aerobic degradation of 2,4-dinitrotoluene by individual bacterial strains and defined mixed population in submerged cultures, J. Hazard. Mater. 192 (2011) 605–613.
[26] N.S. Panikov, M.V. Sizova, D. Ros, C. Christodoulatos, W. Balas, S. Nicolich, Biodegradation kinetics of the nitramine explosive CL-20 in soil and microbial cultures, Biodegradation 18 (2007) 317–332.
[27] S. Shahitha, Studies on atrazine mineralization by a consortium of bacteria isolated from sugarcane field soil, J. Appl. Pharm. Sci. 2 (2012) 113–223.
[28] D. Zhao, C. Liu, L. Liu, Y. Zhang, Q. Liu, W.-M. Wu, Selection of functional consortium for crude oil-contaminated soil remediation, Int. Biodeterior. Biodegrad. 65 (2011) 1244–1248.
[29] A.N. Rhodes, R.R. Fulthorpe, J.M. Tiedje, Probing the functional diversity of global pristine soil communities with 3-chlorobenzoate reveals that communities of generalists dominate catabolic transformation, Appl. Environ. Microbiol. 79 (2013) 6932–6940.
[30] M.I. Khan, J. Lee, J. Park, A toxicological review on potential microbial degradation intermediates of 2,4,6-trinitrotoluene, and its implications in bioremediation, KSCE J. Civil Eng. 17 (2013) 1223–1231.
[31] A. Bernstein, E. Adar, A. Nejidat, Z. Ronen, Isolation and characterization of RDX-degrading *Rhodococcus* species from a contaminated aquifer, Biodegradation 22 (2011) 997–1005.
[32] S. Sagi-Ben Moshe, O. Dahan, N. Weisbrod, A. Bernstein, E. Adar, Z. Ronen, Biodegradation of explosives mixture in soil under different water-content conditions, J. Hazard. Mater. 203–204 (2012) 333–340.
[33] D.B. Ringelberg, C.M. Reynolds, M.E. Walsh, T.F. Jenkins, RDX loss in a surface soil under saturated and well drained conditions, J. Environ. Qual. 32 (2003) 1244–1249.
[34] Y.J. Liu, S.J. Liu, H.L. Drake, M.A. Horn, Consumers of 4-chloro-2-methylphenoxyacetic acid from agricultural soil and drilosphere harbor cadA, r/sdpA, and tfdA-like gene encoding oxygenases, FEMS Microbiol. Ecol. 86 (2013) 114–129.
[35] A.M. Semenov, A.H.C. van Bruggen, V.V. Zelenev, Moving waves of bacterial populations and total organic carbon along roots of wheat, Microb. Ecol. 37 (1999) 116–128.
[36] S.B. Funk, D.J. Roberts, D.L. Crawford, R.L. Crawford, Initial-phase optimization for bioremediation of munition compound-contaminated soils, Appl. Environ. Microbiol. 59 (1993) 2171–2177.
[37] K.M. Lamichhane, R.W. Babcock Jr., S.J. Turnbull, S. Schenck, Molasses enhanced phyto and bioremediation treatability study of explosives contaminated Hawaiian soils, J. Hazard. Mater. 243 (2012) 334–339.
[38] S. Waisner, L. Hansen, H. Fredrickson, C. Nestler, M. Zappi, S. Banerji, R. Bajpai, Biodegradation of RDX within soil–water slurries using a combination of differing redox incubation conditions, J. Hazard. Mater. 95 (2002) 91–106.
[39] A.N. Oliveira, L.A. Oliveira, J.S. Andrade, A.F. Chagas Júnior, Rhizobia amylase production using various starchy substances as carbon substrates, Braz. J. Microbiol. 38 (2007) 208–216.
[40] M.J. Kwon, K.T. Finneran, Microbially mediated biodegradation of hexahydro-1,3,5-trinitro-1,3,5- triazine by extracellular electron shuttling compounds, Appl. Environ. Microbiol. 72 (2006) 5933–5941.
[41] R.M. Smibert, N.R. Krieg, Phenotypic characterization, in: P. Gerhardt, R.G.E. Murray, W.A. Wood, N.R. Krieg (Eds.), Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, D.C, 1994, pp. 607–654.
[42] USEPA, Nitroaromatics and Nitramines by HPLC. Second Update SW-846 Method 8330, Office of Solid Waste and Emergency Response, Washington, DC, 1994.
[43] J.J. Parnell, V.J. Denef, J. Park, T. Tsoi, J.M. Tiedje, Environmentally relevant parameters affecting PCB degradation: carbon source- and growth phase-mitigated effects of the expression of the biphenyl pathway and associated genes in *Burkholderia xenovorans* LB400, Biodegradation 21 (2010) 147–156.
[44] H. Song, B.H. Jeon, C.M. Chon, Y. Kim, I.H. Nam, F.W. Schwartz, D.W. Cho, The effect of granular ferric hydroxide amendment on the reduction of nitrate in groundwater by zero-valent iron, Chemosphere 93 (2013) 2767–2773.
[45] D. George, P. Mallery, SPSS for Windows Step by Step: a Simple Guide and Reference, 16.0 Update, 9th ed., Allyn & Bacon, Boston, 2009.
[46] S.E. Morales, W.E. Holben, Empirical testing of 16S rRNA gene PCR primer pairs reveals variance in target specificity and efficacy not suggested by in silico analysis, Appl. Environ. Microbiol. 75 (2009) 2677–2683.
[47] J. Lee, T.K. Lee, F.E. Loffler, J. Park, Characterization of microbial community structure and population dynamics of tetrachloroethene-dechlorinating tidal mudflat communities, Biodegradation 22 (2011) 687–698.
[48] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol. 75 (2009) 7537–7541.
[49] T. Van Doan, T.K. Lee, S.K. Shukla, J.M. Tiedje, J. Park, Increased nitrous oxide accumulation by bioelectrochemical denitrification under autotrophic conditions: kinetics and expression of denitrification pathway genes, Water Res. 47 (2013) 7087–7097.
[50] J.R. Cole, Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, J.M. Tiedje, The Ribosomal Database Project: improved alignments and new tools for rRNA analysis, Nucleic Acids Res. 37 (2009) D141–145.
[51] L. Jost, Entropy and diversity, Oikos 113 (2006) 363–375.

[52] A.E. Magurran, Measuring biological diversity, Afr. J. Aquat. Sci. 29 (2004) 285–286.
[53] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (2004) 1792–1797.
[54] T.K. Lee, T. Van Doan, K. Yoo, S. Choi, C. Kim, J. Park, Discovery of commonly existing anode biofilm microbes in two different wastewater treatment MFCs using FLX titanium pyrosequencing, Appl. Microbiol. Biotechnol. 87 (2010) 2335–2343.
[55] S. Kumar, M. Nei, J. Dudley, K. Tamura, MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences, Briefings Bioinf. 9 (2008) 299–306.
[56] K.M. Ritalahti, B.K. Amos, Y. Sung, Q. Wu, S.S. Koenigsberg, F.E. Loffler, Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains, Appl. Environ. Microbiol. 72 (2006) 2765–2774.
[57] K.J. Indest, F.H. Crocker, R. Athow, A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene, J. Microbiol. Methods 68 (2007) 267–274.
[58] R. Brankatschk, N. Bodenhausen, J. Zeyer, H. Burgmann, Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples, Appl. Environ. Microbiol. 78 (2012) 4481–4489.
[59] H. Annamaria, D. Manno, S.E. Strand, N.C. Bruce, J. Hawari, Biodegradation of RDX and MNX with *Rhodococcus* sp. strain DN22: new insights into the degradation pathway, Environ. Sci. Technol. 44 (2010) 9330–9336.
[60] D. Fournier, A. Halasz, J. Spain, R.J. Spanggord, J.C. Bottaro, J. Hawari, Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 70 (2004) 1123–1128.
[61] S.A. Meyer, A.J. Marchand, J.L. Hight, G.H. Roberts, L.B. Escalon, L.S. Inouye, D.K. MacMillan, Up-and-down procedure (UDP) determinations of acute oral toxicity of nitroso degradation products of hexahydro-1,3, 5-trinitro-1,3,5-triazine (RDX), J. Appl. Toxicol. 25 (2005) 427–434.
[62] D.L. Kaplan, Biological degradation of explosives and chemical agents, Curr. Opin. Biotechnol. 3 (1992) 253–260.
[63] O. Inceoglu, J.F. Salles, L. van Overbeek, J.D. van Elsas, Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields, Appl. Environ. Microbiol. 76 (2010) 3675–3684.
[64] P.R. Binks, S. Nicklin, N.C. Bruce, Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1, Appl. Environ. Microbiol. 61 (1995) 1318–1322.
[65] H.W. Chang, H.Y. Kahng, S.I. Kim, J.W. Chun, K.H. Oh, Characterization of Pseudomonas sp. HK-6 cells responding to explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), Appl. Microbiol. Biotechnol. 65 (2004) 323–329.
[66] W. Kitagawa, S. Takami, K. Miyauchi, E. Masai, Y. Kamagata, J.M. Tiedje, M. Fukuda, Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment, J. Bacteriol. 184 (2002) 509–518.
[67] M.B. Leigh, V.H. Pellizari, O. Uhlik, R. Sutka, J. Rodrigues, N.E. Ostrom, J. Zhou, J.M. Tiedje, Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs), ISME J. 1 (2007) 134–148.
[68] J.A. Klappenbach, P.R. Saxman, J.R. Cole, T.M. Schmidt, rrndb: the ribosomal RNA operon copy number database, Nucleic Acids Res. 29 (2001) 181–184.
[69] A. Wani, J. Davis, Biologically mediated reductive transformation of ordnance related compounds by mixed squifer culture using acetate as the sole carbon source: laboratory treatability studies for field demonstration, Pract. Period. Hazard. Toxic Radioact. Waste Manage. 10 (2006) 86–93.
[70] C.S. Chong, D.K. Sabir, A. Lorenz, C. Bontemps, P. Andeer, D.A. Stahl, S.E. Strand, E.L. Rylott, N.C. Bruce, Analysis of the xplA/B containing gene cluster involved in the bacterial degradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine, Appl. Environ. Microbiol. 80 (2014) 6601–6610.
[71] K.J. Indest, C.M. Jung, H.P. Chen, D. Hancock, C. Florizone, L.D. Eltis, F.H. Crocker, Functional characterization of pGKT2 a 182-kilobase plasmid containing the xplAB genes, which are involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* sp. strain KTR9, Appl. Environ. Microbiol. 76 (2010) 6329–6337.
[72] M.C. Morley, S.N. Shammas, G.E. Speitel, Biodegradation of RDX and HMX mixtures: batch screening experiments and sequencing batch reactors, Environ. Eng. Sci. 19 (2002) 237–250.
[73] S.K. Soni, R. Singh, M. Singh, A. Awasthi, K. Wasnik, A. Kalra, Pretreatment of Cr(VI)-amended soil with chromate-reducing rhizobacteria decreases plant toxicity and increases the yield of *Pisum sativum*, Arch. Environ. Contam. Toxicol. 66 (2014) 616–627.
[74] G.-P. Yessica, A. Alejandro, F.-C. Ronald, A.J. José, M.-R. Esperanza, C.-S.J. Samuel, M.-L.M. Remedios, E. Ormeño-Orrillo, Tolerance, growth and degradation of phenanthrene and benzo[a]pyrene by *Rhizobium tropici* CIAT 899 in liquid culture medium, Appl. Soil Ecol. 63 (2013) 105–111.
[75] M. Labidi, D. Ahmad, A. Halasz, J. Hawari, Biotransformation and partial mineralization of the explosive 2,4,6-trinitrotoluene (TNT) by rhizobia, Can. J. Microbiol. 47 (2001) 559–566.