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Improved TNT detoxification by starch addition in a nitrogen-fixing *Methylophilus*-dominant aerobic microbial consortium

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HIGHLIGHTS

- A novel aerobic microbial consortium for complete TNT detoxification was developed.
- Co-addition of starch and target explosive enhanced aerobic explosive detoxification.
- Several novel putative TNT-degrading bacteria were identified by pyrosequencing.
- This is the first study to report the involvement of *Methylophilus* in enhancing aerobic TNT degradation.
- This is the first study to show evidence of the involvement of *N*-fixation in aerobic TNT degradation.

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ABSTRACT

In this study, a novel aerobic microbial consortium for the complete detoxification of 2,4,6-trinitrotoluene (TNT) was developed using starch as a slow-releasing carbon source under nitrogen-fixing conditions. Aerobic TNT biodegradation coupled with microbial growth was effectively stimulated by the co-addition of starch and TNT under nitrogen-fixing conditions. The addition of starch with TNT led to TNT mineralization via ring cleavage without accumulation of any toxic by-products, indicating improved TNT detoxification by the co-addition of starch and TNT. Pyrosequencing targeting the bacterial 16S rRNA gene suggested that *Methylophilus* and *Pseudoxanthomonas* population were significantly stimulated by the co-addition of starch and TNT and that the *Methylophilus* population became predominant in the consortium. Together with our previous study regarding starch-stimulated RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) degradation (Khan et al., J. Hazard. Mater. 287 (2015) 243–251), this work suggests that the co-addition of starch with a target explosive is an effective way to stimulate aerobic explosive degradation under nitrogen-fixing conditions for enhancing explosive detoxification.

1. Introduction

2,4,6-Trinitrotoluene (TNT) is one of the most widespread nitroaromatic explosives in the world [1]. TNT is toxic to many organisms, is known to be a potential human carcinogen, and is persistent in the environment [2]. Because TNT is recalcitrant to microbial degradation and has a relatively high solubility, the toxic TNT from manufacturing activities and military training operations reaches rapidly into surface and groundwater bodies [3]. To protect water resource from contamination by explosives, it is necessary to clean up the groundwater of contaminated sites. The use of microorganisms for TNT remediation is an attractive option from economic and sustainability standpoints [4].

Although, anaerobic microbial degradation of TNT has been reported [5], several aerobic TNT-degrading bacteria have been isolated and studied [6–8]. Aerobic microbial degradation of TNT, due to its faster degradation rate [9] and lower toxicity of metabolites [10], may have several advantages over anaerobic degradation. Aerobically, TNT can be degraded via two distinct pathways: (i) nitro moiety reduction and (ii) denitration [11]. Because nitrogen atoms of the nitro groups of TNT are highly oxidizable, the reduction of nitro groups by microbes is thermodynamically favorable [12]. The aerobic nitro moiety reduction of TNT via oxygen-insensitive cytoplasmic nitroreductases provides potentially toxic intermediates, such as hydroxylaminodinitrotoluenes (HADNTs), monoaminodinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs) [13,14]. Meanwhile, aerobic denitration via

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pentaerythritol tetranitrate (PETN) reductases, xenobiotic (XenB) reductases, or *N*-ethylmaleimide (NemA) reductases results in the formation of relatively less toxic intermediates, such as TNT hydride–Meisenheimer complexes and dinitrotoluenes (DNTs), and the subsequent release of nitrite [7,8]. Furthermore, the denitration pathway is more advantageous to microbes than the nitro-moiety reduction pathway because the decrease of the electrophilic nature of TNT through the formation of denitrated intermediates (such as dinitrotoluenes) leads to ring cleavage via dioxygenases, enhancing mineralization [11,15,16]. In microbial communities or mixed culture conditions, the co-presence of nitro-moiety reduction and denitration pathways has often been observed to enhance mineralization [15,17]. The use of microbial consortia is expected to provide more stable and efficient outcomes for bioremediation and more opportunities for complete mineralization of contaminants than the use of pure cultures (microbial isolates) because of the microbial diversity and synergetic activities that occur in the microbial communities [18–22]. However, the currently reported aerobic TNT-degrading microbial consortia or mixed community resources showed a relatively low degree of mineralization [17,23] or partial transformation of TNT to dead-end products, including azoxy and amino derivatives [24–26]. To improve the completeness of TNT detoxification, a novel aerobic TNT-degrading microbial consortium for TNT mineralization or its culturing method has yet to be developed.

To develop a microbial consortium for complete detoxification, it is important to ensure the co-presence of bacteria-degrading diverse toxicants and degradation intermediates. For pure cultures, simple carbon compounds such as glucose and succinate are typically used as external carbon sources. For mixed culture conditions, however, simple carbon sources may not be effective in stimulating the growth of a target group of degradative bacteria because simple carbon compounds are mainly consumed by copiotrophic non-degradative bacteria rather than by relatively slow-growing degraders [22,27,28]. To maintain the diversity and activity of microbial degradation in consortia or mixed cultures, it is practical and cost-effective to use slow-releasing complex carbon sources, such as molasses and starch [17,22,29,30]. In previous TNT degradation studies, the addition of molasses improved the rate of aerobic TNT degradation but not TNT detoxification, exhibiting the accumulation of potentially toxic TNT degradation intermediates [3,23,31–33]. Starch, a plant-produced carbohydrate polymer, is another alternative slow-releasing complex carbon source for stimulating aerobic biodegrading bacteria, particularly for rhizospheric bacteria [34]. Our previous study regarding starch-stimulated aerobic RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) degradation [24] suggested that the addition of starch may have a positive effect on aerobic explosive degradation and detoxification under nitrogen-fixing conditions. However, this hypothesis has yet to be examined for explosive contaminants other than RDX.

The present work attempts to use starch in developing a novel aerobic TNT-degrading microbial consortium for improving TNT detoxification. To determine whether the addition of starch has a positive effect on aerobic TNT degradation and detoxification under nitrogen-fixing conditions, laboratory experiments were conducted to explore the effects of starch addition on the rate and intermediates of aerobic TNT degradation. In addition, pyrosequencing targeting the 16S rRNA genes in the starch-stimulated microbial consortium was performed to examine the effects of starch addition on the characteristics of microbial communities and to identify microbial populations potentially involved in aerobic TNT degradation and detoxification.

2. Materials and methods

2.1. Chemicals

The TNT (purity, 99%), 2-amino-4,6-dinitotoluene (2-ADNT) and 4-amino-2,6-dinitotoluene (4-ADNT) were obtained from AccuStandard, Inc. (New Haven, CT). The high performance liquid chromatography (HPLC)-grade acetone (for preparation of TNT stock solution), methanol, isopropyl alcohol, and acetonitrile (for HPLC mobile phase) were purchased from J.T. Baker (Phillipsburg, NJ). The soluble starch (extra pure) was purchased from Junsei Chemicals Co., Ltd. (Japan) and its physicochemical properties are given in Table S1. To prepare the aqueous growth 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 carried out using soil samples that were collected from a Darokdae military shooting range (Gyeonggi province, South Korea), under nitrogen-limiting conditions. Aerobic incubation experiments were carried out in 300 mL Erlenmeyer flasks containing 100 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), modified Wolfe's vitamin and mineral mixtures (each 10 mL/L), and 0.5 mL of 1 mM Na_2SeO_4 [22,35]. Starch (1.0 g/L, 2.5 g/L, and 5.0 g/L) was added as the sole carbon source. The treatment details are given in Table 1. The final pH was 7.1 ± 0.2. After autoclaving the prepared starch-amended flasks, the TNT was added with acetone as a carrier solvent. The solvent was completely evaporated by placing the flask on a clean bench for 2 h [22]. Approximately 10 mL of the prepared aqueous growth medium was taken from the prepared flask and then added to a clean Falcon tube containing approximately 5 g of soil from the TNT contaminated site. The tubes were shaken for one hour to extract the inoculum. After settling the bigger soil particles, extractant (containing inoculum) was added to the corresponding flask. Furthermore, killed controls (autoclaved) were prepared to assess the significance of any abiotic degradation of TNT. The control flasks were autoclaved after receiving the inoculum, and TNT was added at the end. The flasks were wrapped with aluminum foil to prevent any photochemical reactions of TNT. 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 was conducted using freshly prepared autoclaved aqueous growth medium containing TNT for selective culturing of the aerobic TNT-degrading consortia. The experiments were carried out in three replicates for each treatment. To assess the nitrogen fixation in the microbial culture, acetylene reduction assay (ARA) was carried out according to the procedure described by Khan et al. [22]. The nitrogenase activity (nmol per hour per 10^8 cells) was expressed as nmol of ethylene produced per hour per 10^8 cells. The quantitative real-time PCR was performed to determine the total number of cells.

2.3. Specific TNT degradation rates

The specific TNT degradation rates (R) were estimated using the following Eq. (1):

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

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

Table 1
Scheme of the microcosm experiments.

Experiment	Microcosm IDs	Initial TNT conc. (mM)	Starch conc. (g/L)
1st enrichment (with some soil particles)	No TNT-1	0.00	2.5
	S0.0-1	0.28	0.0
	S1.0-1	0.28	1.0
	S2.5-1	0.29	2.5
	S5.0-1	0.29	5.0
	Autoclaved-1	0.30	0.0
	S2.5-autoclaved-1	0.28	2.5
2nd enrichment (without soil)	No TNT-2	0.00	2.5
	S0.0-2	0.31	0.0
	S1.0-2	0.31	1.0
	S2.5-2	0.32	2.5
	S5.0-2	0.31	5.0
	Autoclaved-2	0.32	0.0
3rd enrichment (without soil)	No TNT-3	0.00	2.5
	S0.0-3	0.32	0.0
	S2.5-3	0.32	2.5
	Autoclaved-3	0.32	0.0

corresponding sampling times, and OD_{av} represents the average OD (optical density) values between t_n and t_{n-1}.

2.4. Analytical methods

TNT and its degradation intermediates, including 2-ADNT and 4-ADNT, were analyzed using HPLC (high performance liquid chromatography [Agilent Technologies 1200 series, USA]) at 230 nm according to the US EPA Method 8330 [36] described by Khan et al. [22]. HPLC chromatograms showed the formation of two unknown metabolites (Fig. S1), which were identified as 2,4-dinitrotoluene (DNT) and diaminonitrotoluene (DANT) through liquid chromatography–mass spectrometry (LC–MS) analysis (Fig. S2). Nitrate and nitrite were analyzed by ion chromatography (IC) using a Dionex system consisting of an AS40 auto-sampler and an ICS-90 ion chromatograph (Dionex, Sunnyvale, California, U.S.A.) equipped with a conductivity detector, according to the procedure described by Khan et al. [22]. The starch conversion was not traced along with TNT degradation. Microbial growth was quantified by measuring the OD at 600 nm using a UV–vis spectrophotometer (Mecasys Co., Ltd., Korea). The OD values were linearly correlated with the copy number of the total bacterial 16S rRNA gene (Fig. S3). The dissolved oxygen content and pH were measured using a Thermo Scientific Orion 5 Star device (Thermo Fisher Scientific Inc., USA). For ARA, the acetylene and ethylene were analyzed 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), according to the procedure described by Khan et al. [22].

2.5. Statistical analysis

The biological and chemical data were analyzed using the Statistical Package for the Social Sciences 16.0 (SPSS, Chicago, IL) for Windows [37]. A one-way analysis of variance was applied to detect significant differences ($P < 0.05$) between the controls and the treated microcosm samples.

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

The total genomic DNA was extracted from the initial soil and from the three replicate TNT-degrading microcosms (from 3rd enrichment) using the 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 [38]. Four different barcodes (AGCAGAGC for the initial soil, AGCAGATG for No TNT-3, AGCAGCAG for S0.0–3, and AGCAGCTC for S2.5–3) were linked with the primers to facilitate sample discrimination. The PCR reaction was performed according to Khan et al. [22]. Following band excision, the PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The products were further purified using the QIAquick PCR purification kit (Qiagen) [22]. In order to reduce the bias from replicates and recover sufficient amount of purified amplicons, PCR products from replicates were combined before purification steps [39]. Pyrosequencing of the amplicons was performed by Macrogen (Seoul, South Korea) using a 454/Roche GS-FLX titanium instrument (Roche, NJ). The sequences shorter than 300 nucleotides and the sequences with an average quality score of less than 20 were removed through quality filters [40]. We analyzed the sequences by following the modified protocol suggested by Schloss et al. [41] using Mothur. All of the filtering and clustering steps were performed according to the methods described by Van Doan et al. [42]. The sequences were classified using the Ribosomal Database Project (RDP)'s pyrosequencing pipeline [43]. The Shannon index and the Shannon evenness were estimated [44]. Furthermore, a phylogenetic tree analysis was performed to compare the obtained sequences with the existing GenBank 16S rRNA gene sequences using BLAST: http://www.ncbi.nlm.nih.gov/BLAST/.The sequence alignment was performed using MUSCLE [45], and a phylogenetic tree was built using MEGA4 software program [46], according to the methods described by Khan et al. [22]. According to our previous study, the deviation in relative abundance estimation among technical replicates for sequencing is within 5% [47]. All sequences obtained in this study were deposited into the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP061266 (NCBI BioProject PRJNA289558). The representative nucleotide sequences were deposited in the GenBank under accession numbers KJ792753–KJ792772 and KP863893–KP863901.

2.7. Quantitative real-time PCR analysis

Real-time PCR amplification reactions were performed using BIORAD iQ5 (BIORAD, CA, USA). Amplification reactions were carried out in a volume of 25 μL and the reaction mixture contained 12.5 μL iQ_SYBR Green Supermix (BIORAD, CA, USA), 1 μL of each primer (10 mM), 10 ng of total DNA, and RNase-free water. The primer sets for amplification of bacterial 16S rRNA genes described elsewhere [48–50] were used in this work. The qPCR thermocycling

conditions for 16S rRNA amplification were described elsewhere [22].

3. Results

3.1. Effect of starch addition on aerobic biodegradation of TNT

In the 1st enrichment experiment, significant TNT disappearance was detected in the test microcosms (*solid symbols* in Fig. 1[A]) compared to the abiotic TNT losses observed in the autoclaved control (*blank squares* and *triangles*) ($P < 0.05$). This indicates that the TNT disappearance in the test microcosms was due to microbial degradation. After 11 days of incubation, the added TNT was completely removed in the microcosms amended with starch (*solid symbols*). Although soil microbes could degrade TNT without starch (*cross symbols*), the addition of starch enhanced microbial TNT degradation (*solid symbols*). Cell growth was also significantly stimulated by the addition of starch and further enhanced by the co-addition of starch and TNT (Fig. 1[B] and Fig. S4). Interestingly, the no-starch added microcosms with TNT (*solid circles*) showed a greater microbial growth than the starch-amended without TNT (*blank circles*) ($P < 0.05$), suggesting that the growth of TNT degraders on TNT itself. The results indicate that the addition of starch stimulated TNT degradation as well as microbial growth. Throughout the following sub-culture transfer experiments, consistent trends were observed under the soil-free aqueous medium condition (Fig. 1[C–F]).

In general, the specific rate of TNT degradation was increased as the starch concentration increased (Fig. 2), confirming the positive effect of starch addition on TNT degradation activity. The specific rates of TNT degradation reached plateau levels when starch concentrations were equal to or higher than 2.5 g/L starch in the 1st and 2nd enrichment experiments. After the third sub-culturing experiment, the specific rates of aerobic TNT degradation increased by several fold (Fig. 2), indicating a successful enrichment of TNT-degrading consortia. The TNT-degrading microbial consortia obtained from the final (the 3rd) sub-culturing experiments were named MIK. The (specific) rate of aerobic TNT degradation by the MIK consortium grown on starch (2.5 g/L) was greater ($P < 0.05$) than the rate for most of the previously known aerobic TNT-degrading bacterial isolates belonging to a variety of genus groups (*Pseudomonas*, *Methylobacterium*, and *Rhizobium*) [12,51,52] and was comparable to the rate for the strongest aerobic TNT degraders (*Stenotrophomonas* sp. OK-5 [33,53] and *Pseudomonas putida* HK-6 [31]) (Table 2).

Approximately six major TNT degradation intermediates, including nitrite, nitrate, and DNT (probably produced via denitration pathway [6,25,54]), DANT, 2-ADNT and 4-ADNT (produced via nitro group reduction pathways [15,31]), were detected in all the TNT-degrading microcosms (Fig. 3). In the starch amended microcosms, the production of 2-ADNT, 4-ADNT, DNT, and DANT occurred together with the TNT degradation, and in turn, their amounts were gradually decreased to undetectable levels with longer incubation times after the 3rd enrichment (Fig. 3). Meanwhile, in the no-starch amended microcosm experiments, these intermediates continuously accumulated, and their further degradation remained low; their higher amounts were detected even after 20 days of incubation in the 3rd enrichment. A similar trend of intermediate accumulation in no-starch amended microcosms was observed in the 1st and 2nd enrichment experiments (Fig. S5). This suggests that the bacteria might have needed starch to activate some specific enzyme for further detoxification of 2-ADNT and 4-ADNT or to reduce their toxicity. All degradation intermediates (with the exception of nitrite and nitrate) were non-detectable by 20 days incubation in the 2.5 g/L starch amended microcosms (S2.5–3) of the 3rd enrichment (Fig. 3), suggesting complete detoxification of TNT. The results supported improvement of TNT detoxification by starch addition.

3.2. Characterization of microbial communities

The microbial community analysis using pyrosequencing targeting of the 16S rRNA gene showed that sub-culturing with starch or TNT amendment had a reducing effect on alpha-diversity in the microbial consortia (Table S2). Taxonomic supervised dendrogram analysis at the phylum and genus levels showed that the microbial communities from the TNT-amended microcosms (i.e., S0.0–3 and S2.5–3) were different from those from the no-TNT treated microcosms (i.e., initial soil or No TNT-3) (Fig. S6[A and B]). This finding was confirmed by the results of Principle Component Analysis (PCA) (Fig. S6[C and D]). The beta-diversity results indicate that TNT had a more influential effect on community shift than starch.

The major bacterial population responding to starch alone (No TNT), TNT alone (S0.0–3), and the co-addition of starch and TNT (S2.5–3) were identified at the genus level (Table 3). The identified bacterial populations were found to belong to alpha-, beta-, and gamma-subclasses of Proteobacteria. In the TNT-amended microcosms without (S0.0–3) and with starch (S2.5–3), the *Methylophilus* population (TCS1–5) belonging to beta-subclass of Proteobacteria were predominant among the identified major bacterial members in Table 3. Other abundant groups in the S2.5–3 and S0.0–3 include *Pseudoxanthomonas* (TCS6–10), *Brevundimonas* (TCS11–12), *Pseudomonas* (TCS13–17), *Dokdonella* (TCS18–19), and *Bordetella* (TCS22–23). While in the No TNT-3 microcosm the *Hydrogenophaga* population (TCS28–29) were predominant, followed by *Pseudoxanthomonas* and *Pseudomonas* population. Only *Methylophilus* and *Pseudoxanthomonas* population were significantly stimulated by the co-addition of TNT and starch accounted for more than 53% of the final consortium, and their relative abundance correlated with TNT degradation activity. These findings suggest that the serial culturing with starch together with TNT was effective in selectively enriching microbes growing on TNT.

Most of the previously characterized aerobic TNT-degrading bacterial isolates belong to gamma-Proteobacteria as shown in Fig. 4 (clades III and IV). The *Methylophilus*, *Dokdonella*, *Bordetella*, *Brevundimonas*, and *Methyloversatilis* (TCS24–25) population were found to be phylogenetically distant (more than 5% dissimilarity) from the previously known aerobic TNT-degrading isolates. This suggests that these are mostly novel TNT-degrading bacteria. The *Pseudomonas* population are phylogenetically close to the previously known TNT-degrading isolates (clade IV). Among them, TCS16 is close to known the *Pseudomonas aeruginosa*-containing denitration pathway [55], whereas TCS15 and TCS17 are close to the *Pseudomonas putida*/*fluorescens*-containing nitroreductase pathways [8,14]. The *Pseudoxanthomonas* population were found in clade III. They are phylogenetically close to the known TNT-degrading *Escherichia coli* and *Klebsiella* isolates-containing nitroreductase pathway [56,57].

4. Discussion

In the present study, we attempted to use the co-addition of starch and TNT to develop microbial consortia for complete detoxification of TNT. For this, we explored the effects of the co-addition of starch and TNT on the rate and intermediates of aerobic TNT degradation and on microbial community characteristics. A nitrogen-fixing TNT-degrading microbial consortium was successfully developed for complete TNT detoxification by the co-addition of starch and TNT.

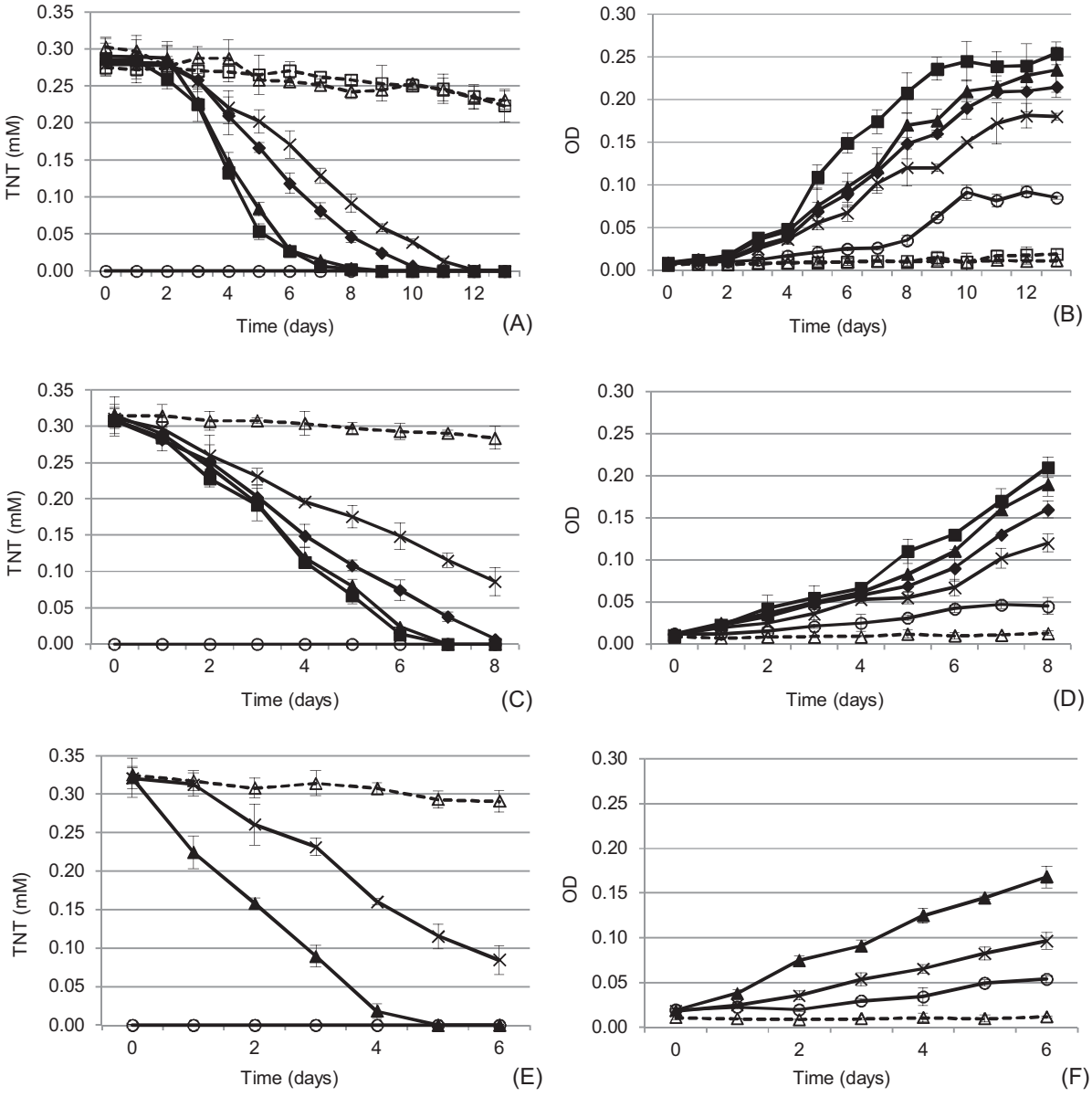

Fig. 1. Disappearances of TNT and microbial growth during the 1st (A, B), 2nd (C, D), and 3rd (E, F) enrichment experiments The symbols indicate the control with 2.5 g/L starch in the absence of TNT (—○—), the autoclaved control with TNT in the absence of starch (—Δ—), the autoclaved control with TNT in the presence of 2.5 g/L starch (—□—), the microcosm with TNT in the absence of starch (—×—), the microcosm with TNT in the presence of 1.0 g/L starch (—♦—), the microcosm with TNT in the presence of 2.5 g/L starch (—▲—), and the microcosm with TNT in the presence of 5.0 g/L starch (—■—). Each error bar represents one standard deviation of the three replicates. (OD, optical density at 600 nm).

Table 2
Comparison of the aerobic TNT biodegradation rates from previous studies and this work.

Microbial isolates/ consortia	Carbon source (g/L)	TNT (mM)	Degradation rate (mM/day)	Specific degradation rate [(mM)/(day OD)][a]	References
Clavibacterium agropyri	Glucose+acetate + citrate (2.4)	0.42	0.060	*NA*	[21]
Consortium-AM06	Reducing sugar (3.7)	0.44	0.032	*NA*	[3]
Methylobacterium sp. strain BJ001	Fructose (5.0)	0.11	0.012	0.01	[4]
Pseudomonas aeruginosa TP6	Glucose (10)	0.48	0.022	0.05	[12]
Pseudomonas putida HK-6	Molasses (0.4)	0.44	0.110	0.55	[31]
Pseudomonas putida TP1	Glucose (10)	0.48	0.022	0.04	[12]
Rhizobium trifolii T10	Glucose + glutamate (2.8)	0.18	0.036	0.39	[50]
Sphingomonas sanguinis	Glucose + acetate + citrate (2.4)	0.44	0.063	*NA*	[21]
Stenotrophomonas maltophilia OK-5	Sucrose (0.2)	0.20	0.033	*NA*	[52]
Stenotrophomonas sp. OK-5	Molasses (0.4)	0.44	0.110	0.84	[33]
MIK	Starch (2.5)	0.32	0.064	0.45	This study

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

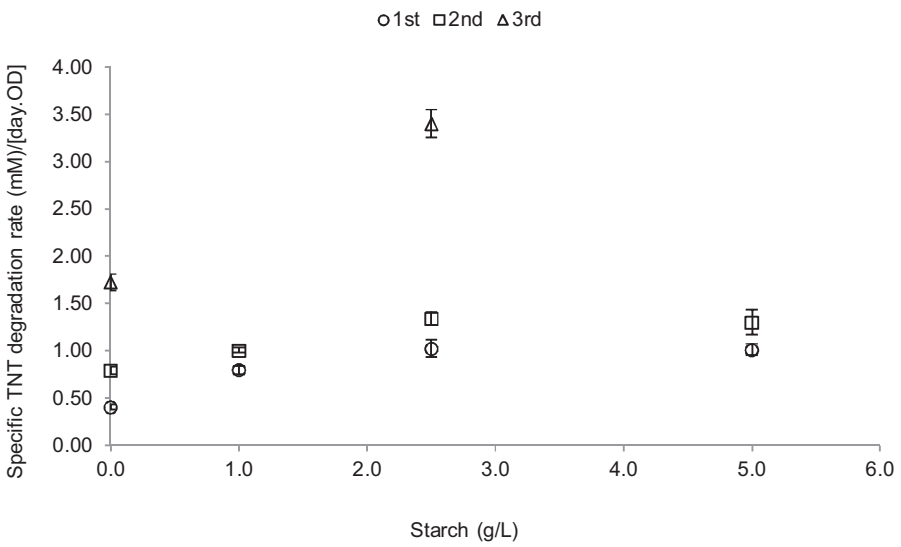

Fig. 2. Specific rate of TNT degradation in response to different starch concentrations during the 1st (○), 2nd (□), and 3rd (Δ) enrichment experiments. Each error bar represents one standard deviation of the three replicates. (OD, optical density at 600 nm).

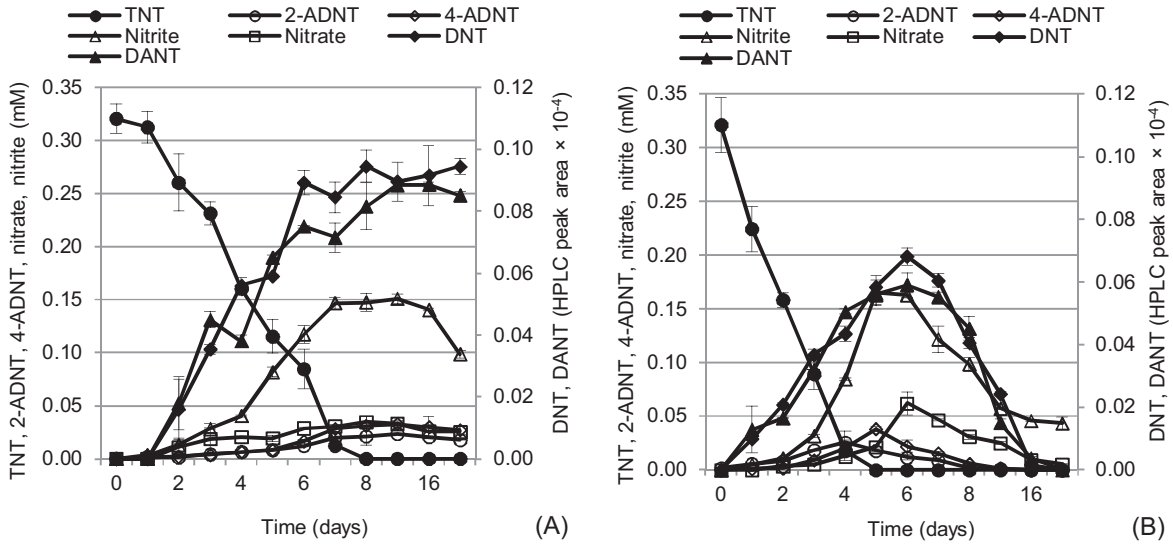

Fig. 3. Aerobic biodegradation of the TNT and the formation of metabolites in the microcosm with TNT in the absence of starch (A) and in the microcosm with TNT in the presence of 2.5 g/L starch (B) during the 3rd enrichment experiments. The 2-ADNT, 4-ADNT, DNT, and DANT indicate 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, dinitrotoluene, and diaminonitrotoluene, respectively. Each error bar represents one standard deviation of the three replicates.

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

OTU ID	Accession number	Phylum/class	Genera	Initial soil[a]	No TNT-3[b]	S0.0–3[c]	S2.5–3[d]
TCS1–TCS5	KJ792753–KJ792757	Proteobacteria/Beta	*Methylophilus*	0.00	0.04	24.3	42.4
TCS6–TCS10	KJ792758–KJ792762	Proteobacteria/Gamma	*Pseudoxanthomonas*	1.36	5.64	2.22	11.1
TCS11–TCS12	KJ792763–KJ792764	Proteobacteria/Alpha	*Brevundimonas*	0.00	0.82	10.7	7.05
TCS13–TCS17	KJ792765–KJ792769	Proteobacteria/Gamma	*Pseudomonas*	1.70	3.87	6.74	5.13
TCS18–TCS19	KJ792770–KJ792771	Proteobacteria/Gamma	*Dokdonella*	0.17	0.58	2.83	4.27
TCS22–TCS23	KP863894–KP863895	Proteobacteria/Beta	*Bordetella*	0.00	0.04	0.48	3.23
TCS24–TCS25	KP863896–KP863897	Proteobacteria/Beta	*Methyloversatilis*	0.34	0.04	2.51	2.77
TCS26–TCS27	KP863898–KP863899	Proteobacteria/Gamma	*Stenotrophomonas*	0.17	0.39	0.73	1.08
TCS20–TCS21	KJ792772– KP863893	Proteobacteria/Alpha	*Rhizobium*	0.00	0.90	1.12	1.06
TCS28–TCS29	KP863900–KP863901	Proteobacteria/Beta	*Hydrogenophaga*	0.00	18.6	0.83	0.67
Specific TNT degradation rate (mM)/[day OD]				0.11	NA	1.73	3.40

NA: not available.
[a] Initial soil: the soil initially used for the 1st enrichment.
[b] No TNT-3: the microcosm with 2.5 g/L starch in the absence of TNT.
[c] S0.0–3: the microcosm with TNT in the absence of starch.
[d] S2.5–3: the microcosm with TNT in the presence of 2.5 g/L starch.

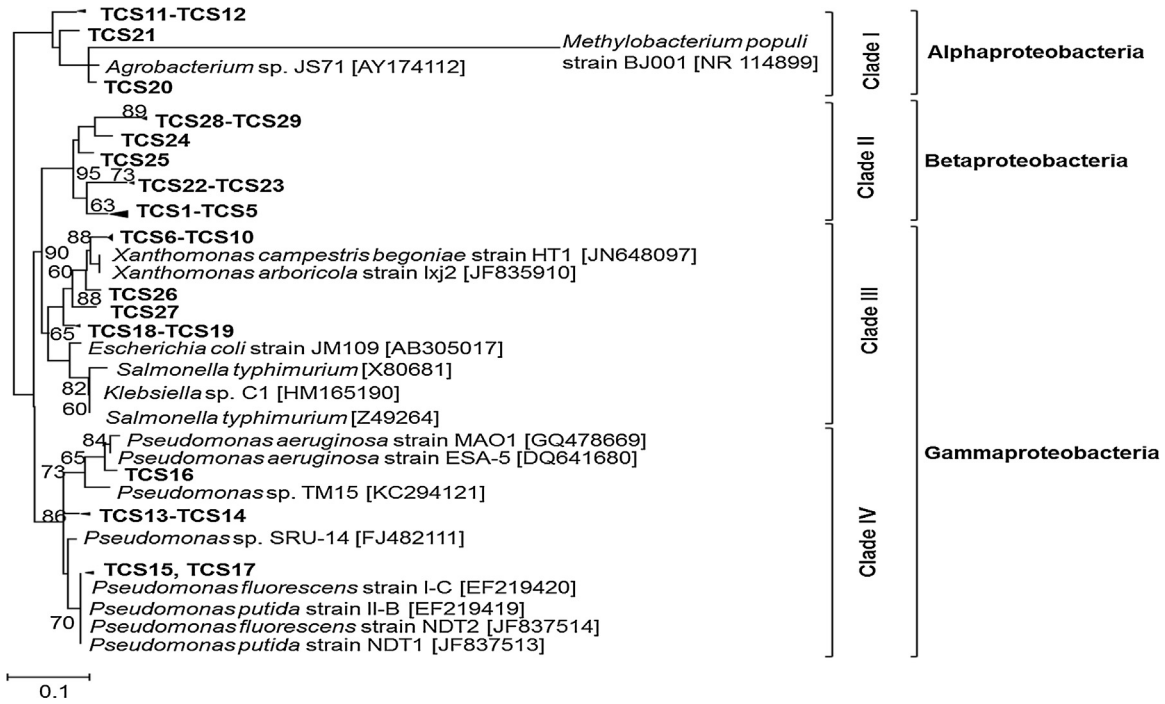

Fig. 4. Phylogenetic tree illustrating the distribution of the 16S rRNA sequences (the average size of the aligned and trimmed sequences was 419 bp), which were obtained by pyrosequencing, and their relationship with known aerobic TNT-degrading bacterial isolates. The numbers beside the nodes indicate the bootstrap values from the bootstrapping test (1000 replicates). The scale bar represents a 16S rRNA sequence with a divergence of 10%.

The co-addition of starch and TNT to the culture resulted in the complete detoxification of TNT via the mineralization of degradation products. This phenomenon may have been attributed to the bacterial populations stimulated by the co-addition of starch and TNT (mainly *Methylophilus* and *Pseudoxanthomonas*) because the significant stimulation of their growth by the co-addition of starch and TNT (Table 3) was correlated with the improved TNT mineralization (Fig. 3). A possible explanation for the improved TNT detoxification is that TNT mineralization may have been enhanced via ring-cleavage dioxygenase activities [58], which further enhances the degradation of TNT transformation products such as DNTs [11,15,16,59]. This speculation may be supported by the literature reports that *Methylophilus* sp. OH31 carries NADH:flavin oxidoreductases (WP_024929264), nitroreductases (drgA [WP_024929913]) and toluene 1,2-dioxygenases (Rieske protein [WP_024930049]) and that *Pseudoxanthomonas* spadix BD-a59 contains a variety of degradative enzymes, such as nitroreductases, oxidoreductases, dehydrogenases, monooxygenases and catechol-2,3-dioxygenases [60]. These suggest that the TNT detoxification in the consortium might have been improved, probably via nitroreductases (upstream pathway) and ring-cleavage (downstream pathway) mainly in the starch-TNT stimulated potential TNT degraders.

RDX-degrading and TNT-degrading bacteria are often phylogenetically close to plant-growth-promoting rhizosphere bacteria [34,61], leading to a speculation that nitrogen-fixing rhizosphere bacteria could be efficient explosives-degraders. Indeed, the developed TNT-degrading consortium by the stimulation with starch and TNT exhibited nitrogen-fixing activity (1.09 ± 0.36 nmol of ethylene per hour per 10^8 cells), which may be sufficient for supporting microbial growth in nitrogen-limiting environments [22]. In rhizoremediation (a plant-microbe-based remediation approach), plants enhance the metabolic activities of rhizosphere bacteria by supplying oxygen and root exudates, whereas bacteria stimulate plant growth by fixing nitrogen, secreting phytohormones, and providing defense means against plant pathogens [26,62,63]. Thus, the aerobic TNT-degrading and nitrogen-fixing starch-utilizing bacteria in the MIK consortium may be a useful microbial resource for the bioremediation of nitrogen-deficient TNT-contaminated sites. The predominant population in the MIK consortium (*Methylophilus*) is an aerobic methanol-oxidizing rhizosphere bacterium [61,64] with capability to degrade a variety of nitrogen-containing contaminants, including methylamine, tetramethylammonium, and formamide [65]. Although, until now, *Methylophilus* has not been reported for explosive degradation, our findings suggest that *Methylophilus* could be a potential candidate for explosive detoxification in a nitrogen-deficient environment or rhizosphere. To our knowledge, no *Methylophilus* isolates are known to be nitrogen fixers. Further studies are required to investigate whether the starch-stimulated TNT-degrading *Methylophilus* populations are novel nitrogen fixers or the fixed nitrogen source for potentially non-nitrogen-fixing TNT degraders (*Methylophilus*) was supplied by other nitrogen-fixing populations (for example, *Pseudomonas* [66]) in the consortium.

In summary, a *Methylophilus*-dominant aerobic TNT-degrading consortium was successfully enriched through sub-culturing with the co-addition of starch and TNT under nitrogen-fixing conditions, and its TNT detoxification could be improved by the starch-TNT co-stimulated consortium. The novel TNT-degrading consortium developed in this study may be a useful microbial resource for bioremediation of TNT-polluted environments under nitrogen limited conditions. A similar finding was reported in our previous work on aerobic RDX degradation under nitrogen-fixing conditions [22]. Taken together with the RDX study, the findings of this work suggest that the co-addition of starch with target explosive is an effective way to stimulate aerobic explosive degradation under nitrogen-fixing conditions to enhance explosive detoxification.

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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.2015.08.032.

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