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Aerobic degradation of 2,4-dinitrotoluene by individual bacterial strains and defined mixed population in submerged cultures

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ABSTRACT

The degradation efficiencies of isomeric mononitrotoluenes (2- and 4-NTs) and dinitrotoluenes (2,4-DNT and 2,6-DNT) by either individual bacterial strains (*Bacillus cereus* NDT4, *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, and *Achromobacter* sp. NDT3) or their mixture were compared in submerged batch cultivations. The mixed culture degraded 2,4-DNT nearly 50 times faster than any of the individual strains. The mixed culture also demonstrated significantly shorter lag periods in 2,4-DNT degradation, a lack of nitrite or organic intermediates accumulation in the liquid medium and the ability to degrade a broader spectrum of nitrotoluenes over a wider concentration range. The presence of both readily degradable 2-NT (or 4-NT) and poorly degradable 2,6-DNT in the medium negatively affected 2,4-DNT biodegradation. However, the mixed bacterial culture still effectively degraded 2,4-DNT with only slightly lower rates under these unfavorable conditions, thus showing potential for the remediation of 2,4-DNT contaminated sites.

1. Introduction

2- and 4-nitrotoluenes (NTs) and 2,4- and 2,6-dinitrotoluenes (DNTs) are by-products of chemical explosives manufacturing (e.g., precursors of TNT in the process of nitration). 2,4-DNT and 2,6-DNT are US EPA priority pollutants [1]. The nitro group is strongly electron-withdrawing; thus, nitroaromatic compounds are resistant to aerobic biodegradation, particularly those with multiple nitro groups [2]. Furthermore, 2,4-DNT is known to furnish very small biomass yields, apparently because several intermediates of its catabolism are well-known uncouplers of respiration and oxidative phosphorylation [3]. Underscoring inherent problems with 2,4-DNT biodegradation, the first bacterial strain capable of complete 2,4-DNT mineralization was reported only 20 years ago [3]. However, since then several aerobic bacteria have been found to possess specific oxygenases, which oxidize the dinitroaromatic ring with a concomitant release of nitrite [4–6]. Under anaerobic conditions DNTs were only reduced to corresponding diaminotoluenes [7]. By contrast, mononitrotoluenes are readily biodegradable by many bacterial strains, often even without prior adaptation [8,9].

When planning bioremediation treatments, the contributions of multiple factors have to be assessed separately. For example, the rates of microbial degradation of nitroaromatic compounds in wastewater are affected by both biological and environmental/physico-chemical parameters. Biological factors include the number and diversity of microorganisms present [6,10]. When single microbial strains are employed, this variable can be controlled upon inoculation. However, it remains under control only under aseptic conditions whereas for most real-world bioremediation scenarios microbial contamination is inevitable resulting in a gradual development of mixed cultures. Thus, mixed microbial cultures have been tested as an alternative to individual strains, particularly in contaminated soil treatment applications [2,6,8,11].

In our earlier short communication [12], we examined the rate and efficiency of 2,4-DNT degradation by several individual bacterial strains, isolated from the biofilm of a long-term operated packed bed reactor. The results were compared to those obtained with artificially constructed 3- and 5-bacterial strain consortia. These consortia exhibited much higher DNT degradation rates than any of the individual strains, although the ultimate removal efficiency for 2,4-DNT remained low at less than 50%. Thus, it remained inconclusive whether the increase of the substrate removal rate that resulted from mixing some of the individual strains was an artifact caused by their poor performance or whether the observed effects were, in part, due to prior biomass immobilization.

The current study provides the correct comparison of bacterial strains using cultures that were unaltered by long-term cultivation as immobilized cells and then preparing a well-defined mixed culture, under similar environmental conditions (submerged cul-

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Table 1
Kinetic parameters of 2,4-DNT degradation by the individual strains and mixed culture.

Microorganisms	RE (%)	q_s (mg L^{-1} day^{-1})[a]
Pseudomonas putida NDT1	100.0 ± 0.0	0.67 ± 0.01
Pseudomonas fluorescens NDT2	98.5 ± 0.2	0.66 ± 0.02
Bacillus cereus NDT4	31.8 ± 0.3	0.21 ± 0.02
Achromobacter sp. NDT3	47.1 ± 0.2	0.31 ± 0.03
The mixed culture	100 ± 0.0	35.04 ± 0.2

[a] C_0 (2,4-DNT) = 10 mg L^{-1}.

tures). A different enrichment culture was used to determine if the effect observed earlier was universal.

A second, related objective of this study was to examine how 2,4-DNT removal rates are influenced by environmental factors, such as pH, concentration of pollutants and the presence of other nitrotoluenes, which are by-products of toluene nitration [13]. These factors are known to affect 2,4-DNT bioavailability [14,15] and biodegradation [16,17]. Separation of microbiological and environmental factors is often a difficult task because the developing mixed cultures represent dynamic, complex and poorly defined microbial consortia. The use of both pure bacterial strains and their defined combination minimizes this problem, which is important for practical applications.

2. Materials and methods

2.1. Chemicals

2,4-Dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene and 4-nitrotoluene were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Other chemicals were from Lachema (Neratovice, Czech Republic). All chemicals were of analytical grade.

2.2. Microorganisms

Soil contaminated with a mixture of mononitrotoluenes, 2,4-DNT and 2,6-DNT, was collected from the Explosia Pardubice ammunition plant, Czech Republic (lat 50°3′47″N, lon 15°43′23″E). A soil sample was cultivated repeatably for 7 days (4 times). Then, the liquid phase was separated. The cells were detached from the soil using a laboratory vibrator followed by an ultrasound bath treatment. The liquid cell suspensions after soil sedimentation were collected and combined. The cells were separated by centrifugation and repeatably cultivated with DNTs and NTs as the sole carbon, nitrogen, and energy sources for four months. After 5 months of cultivation at pH 7.0, an enrichment culture was obtained. Three Gram-negative (*Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, and *Achromobacter* sp. NDT3) and one Gram-positive (*Bacillus cereus* NDT4) bacterial strains were isolated. The same strains were used in combination, to make a defined mixed culture (Table 1). All of these strains were shown to be primary 2,4-DNT and NT degraders.

An amplification of the 16S rRNA genes of strains NDT1, NDT2, NDT3 and NDT4 was performed using universal primers 16S_F1: 5′-AGA GTT TGA TCC TGG CTC AG-3′ [18] and 16S_R1530: 5′-AAG GAG GTG ATC CAG CCG CA-3′ [19]. Bacterial DNA was extracted by heating of one loop of bacterial cells suspended in 500 μL of sterile deionised water at 80 °C for 20 min. Then, the crude extract was centrifuged (Hetich MIKRO 220R, Germany) at 16,060 × *g* for 1 min and the supernatant was directly used as a template for PCRs. The PCR products were purified using the High Pure PCR product purification Kit (Roche Diagnostics, Basel, Switzerland).

The sequencing procedure was performed by Eurofins MWG Operons (Ebersberg, Germany). The sequence data were executed by the Czech Collection of Microorganism (CCM), Department of Experimental Biology, MU. Sequences obtained in this study were deposited in the GenBank database. The accession numbers for the 16S rRNA nucleoside sequences are: JF837513 (strain NDT1), JF837514 (strain NDT2), JF837515 (strain NDT3) and JF837516 (strain NDT4).

2.3. Medium and culture conditions

A basal salt medium (BSM), a 0.1 M [20] phosphate buffer of pH 7.0 with 1 mL of a trace element solution added per L (5.0 g $FeSO_4{\cdot}7H_2O$, 5.0 g $ZnSO_4{\cdot}7H_2O$, 5.0 g $MnSO_4{\cdot}H_2O$, 5.0 g $CuSO_4{\cdot}5H_2O$, 0.1 g $CoCl_2{\cdot}6H_2O$, 0.1 g $Na_2B_4O_7{\cdot}10H_2O$, and 0.1 g $Na_2MoO_4{\cdot}2H_2O$ per L), was used both for the preparation of inoculum and in batch degradation experiments. The initial pH of BSM was set at 7.0 before inoculation, except for the experiments focused on testing pH influence on nitrotoluenes degradation. In that case, the pH was set at a specific value within the range of 5.9–8.0 as described elsewhere [20]. The biomass was grown for two weeks on a growth substrate consisting of a mixture of 2- and 4-NTs in the presence of 2,4-DNT for adaptation. The cultivation temperature was 28 °C.

2.4. Measurements of microbial growth and substrate degradation rates

Preliminary growth tests of the individual bacterial strains with 2,4-DNT and also with 2- and 4-NTs were performed using a laboratory mini-well plate incubator (Bioscreen C® Microbiology Reader – Labsystems Oy, Helsinki, Finland). Growth curves of each individual bacterial strain were obtained with 10 mg L^{-1} of 2,4-DNT and within a 5.9–8.0 pH range. The wells were filled with 310 μL of the medium plus 20 μL of the stock solution of an appropriate substrate and then inoculated with 20 μL of an inoculum. The cells were cultivated under aerobic conditions, at 28 °C, and aerated via shaking for 30 s before and after each absorbance measurement. Absorbance values were read at 600 nm every 10 min. The obtained data were presented as the mean value of five measurements with variances shown as standard deviations. Data were collected over 36 h to generate growth curves. Due to a significant oxygen limitation inherent for mini-wells, these measurements were used only for fast preliminary screening.

Experiments assessing NT/DNT degradation rates and efficiencies were performed in aerobic batch cultures, i.e., submerged cultivations with freely suspended cells. These were conducted under controlled environmental conditions (at 28 °C), using Erlenmeyer flasks with a working volume of 100 mL on a rotary shaker, with both pure and mixed bacterial cultures. BSM was used as a mineral medium while one of the nitrotoluenes or their mixture was used as the sole carbon, energy and nitrogen source.

The batch degradation experiments were carried out with the same starting biomass concentration corresponding to the O.D. (600 nm) = 0.093 ± 0.003. The initial 2,4-DNT concentration was 10 mg L^{-1}. Throughout the duration of this experiment, 15 days, samples were taken once every three days and analyzed for 2,4-DNT. Each set of experiments was performed in triplicate.

2.5. Analytical methods

All samples were centrifuged at 24,600 × *g* for 10 min before analysis (Hereaus, Kendro Laboratory Products, Hanau, Germany). Analyses of samples containing nitrotoluenes and their metabolic intermediates were conducted by HPLC (System DeltaChrom, Watrex Prague Ltd., Prague, Czech Republic) using a 250 mm × 4 mm Nucleosil, 120-5 C18 column (Watrex, Watrex Prague Ltd., Prague, Czech Republic) in a temperature-controlled chamber at 30 °C. A mobile phase of methanol/water (50:50) was

used at a flow rate of 1.0 mL min^{-1}. Peaks were detected by measuring the absorbance at 230 and 268 nm with a diode array detector (Model UV 6000 LP, Thermo Separation Products, Inc., San Jose, CA, USA). The detection limit was 0.05 mg L^{-1}.

The concentrations of nitrate and nitrite were determined using the same HPLC system with a 125 mm × 4 mm IC Anion N 10 μm column and UV 6000 LP detector. A mobile phase of 10.0 mM $NaClO_4$ and 5.0 mM NaH_2PO_4 in demineralized water was used, at a flow rate of 0.5 mL min^{-1}. The NH_4^+ cations were detected using HPLC with a 125 mm × 4 mm UniversalCat 1-2b column and Shodex CD-5 detector (Showa Denko K.K., Kenzai, Japan). The mobile phase consisted of 5.0 mM citric acid and 1.0 mM dipicolinic acid in demineralized water and was applied at a flow rate of 0.5 mL min^{-1}. The detection limits were 0.1, 0.1 and 0.5 mg L^{-1} for nitrate, nitrite and ammonia, respectively.

Standard US EPA Method 8270 was used to extract nitrotoluenes and their biodegradation intermediates from the aqueous phase, with dichloromethane as the extraction solvent. GC/MS measurements were performed on an Agilent 6890N GC (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a program temperature vaporizer (PTV) and CombiPal G6500 autoinjector (CTC Analytics AG, Zwingen, Switzerland), coupled with a 5973 MSD (Agilent Technologies, Inc., Santa Clara, CA, USA). MS with electron impact ionization (EI, 70 eV) was operated in the full scan and SIM modes. The fast US EPA 8270 semivolatiles' analysis method (with the 6890N/5975 inert GC/MSD) was used for the qualitative determination of the extracted compounds only.

The density of free suspended cells in the medium was monitored by measuring the absorbance at 600 nm using a Specol 11 (Carl Zeiss, Jena, Germany) spectrophotometer. An oxygen probe (Type CSOT44LL, INSA Corp., Prague, Czech Republic) was used to measure the dissolved oxygen concentration (DOC); the actual values of DOC were recorded daily. pH of the medium was measured using a standard MFD 77 pH meter (INSA Corp., Prague, Czech Republic).

2.6. Calculations

The degradation rate, removal efficiency, and standard deviation for the treatment of nitrotoluenes were calculated as follows.

Degradation rate (q_s):

$$q_s = \frac{C_0 - C}{\Delta t} (\text{mg L}^{-1}\,\text{day}^{-1}) \quad (1)$$

Removal efficiency (RE) determined after 15 days of cultivation:

$$\text{RE} = \left(1 - \frac{C}{C_0}\right) \cdot 100\,(\%) \quad (2)$$

Statistics (standard deviation):

$$\text{STDEV} = \sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}} \quad (3)$$

where C_0 and C (both in mg L^{-1}) are the initial and final pertinent substrate concentrations for the allotted time of the batch process, Δt; $\bar{x}$ is the sample mean and n is the sample size.

3. Results and discussion

3.1. Effect of pH on the biodegradation characteristics

Fig. 1 shows the pH profiles for maximum biomass growth of all four strains. The results demonstrated that all of the strains used were able to grow on each of the mononitrotoluenes and 2,4-DNT as sole C-, N- and energy sources. However, when grown on 2,4-DNT, the optical density, OD_{600}, increased only 1.5–2.5 times compared to the initial value (0.093 ± 0.003). The optimal pH range was found

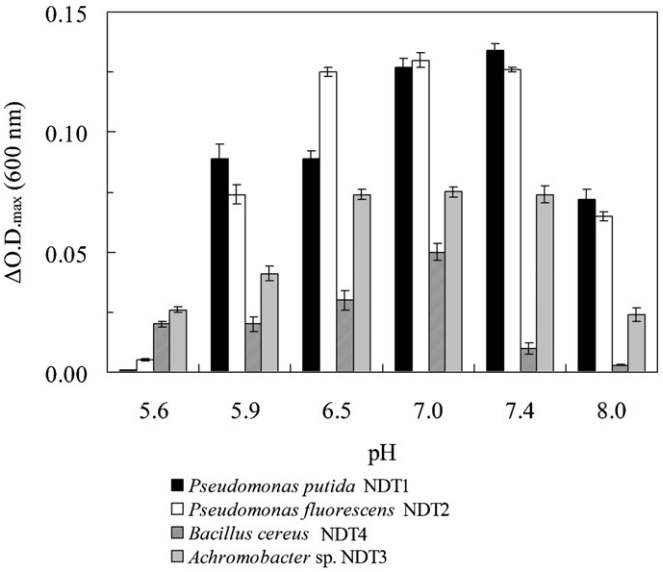

Fig. 1. Influence of the pH on the growth of bacterial strains on 2,4-DNT.

to be between 6.5 and 7.4. These results corroborate an earlier reported the inhibition of 2,4-DNT biodegradation by *Pseudomonas* at pH < 6 [21,22].

A similar pH profile was observed in the experiments with submerged cultivations. The effect of pH on the 2,4-DNT removal rate and efficiency is shown in Fig. 2. A complete removal of 2,4-DNT from the medium was observed within a pH range of 6.4–7.8 for both the individual *Pseudomonas* strains (the most efficient degrader) and the mixed culture. Therefore, pH = 7.0 was selected for all subsequent experiments.

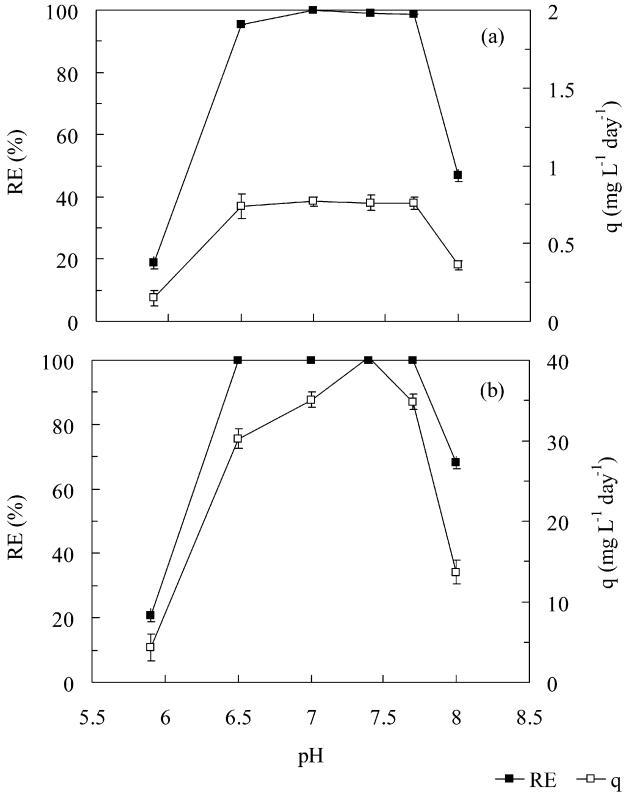

Fig. 2. Influence of the initial pH value on the biodegradation of 2,4-DNT using *Pseudomonas putida* NDT1 (a) and the mixed culture (b).

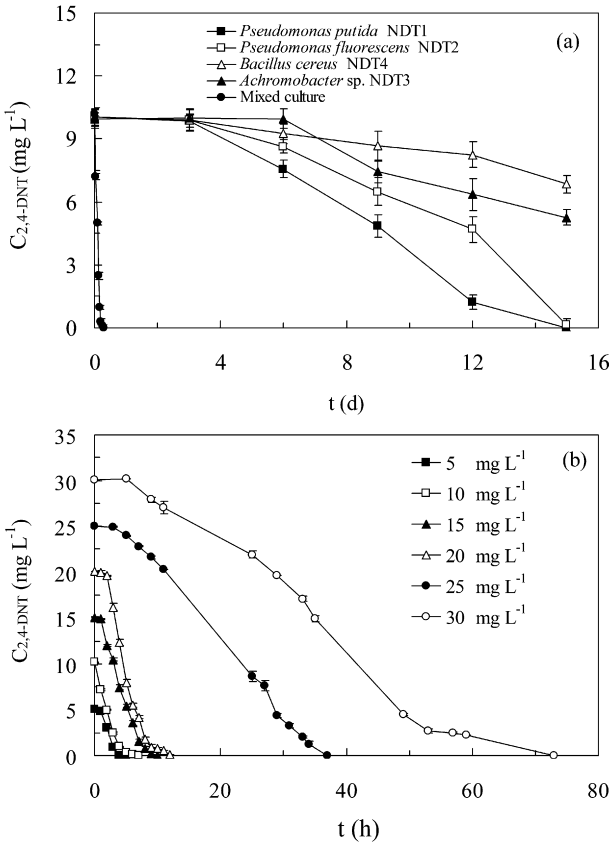

Fig. 3. Lag phases during the degradation of 2,4-DNT by the individual strains and by the mixed culture (a) and effect of the initial 2,4-DNT concentration on the length of the lag-phase with the mixed culture (b). Note that the two graphs are presented with different time scales.

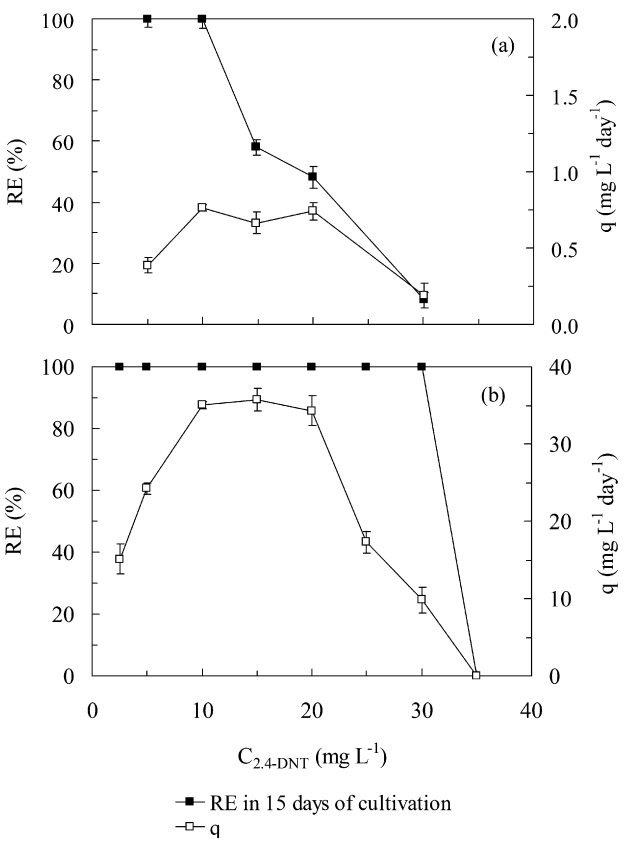

Fig. 4. Influence of the initial concentration of 2,4-DNT on its biodegradation using *Pseudomonas putida* NDT1 (a) and the mixed culture (b).

3.2. 2,4-DNT biodegradation rates by the mixed culture and its components, individual strains

Fig. 3 shows a comparison of 2,4-DNT removal (10 mg L^{-1}) by all four individual strains to that by their mixture. The numerical values of biodegradation parameters are listed in Table 1. The 2,4-DNT uptake rate by *B. cereus* NDT4 or *Achromobacter* sp. NDT3 was less than 50% of the rates shown by the *Pseudomonas* strains. The *Bacillus* and *Achromobacter* strains did not complete the substrate degradation in 15 days. Furthermore, *B. cereus* NDT4 did not exhibit complete 2,4-DNT mineralization as shown below in Section 3.4. By contrast, *P. putida* NDT1 and *P. fluorescens* NDT2 turned out to be relatively efficient, yielding 0.19 mg L^{-1} residual concentration of 2,4-DNT in 15 days of cultivation. This value is below the limit set by the US EPA, 0.32 mg L^{-1} [23].

Notably, the 2,4-DNT removal rate by the mixed culture was nearly 50 times higher than that of *P. putida* NDT1, the most efficient 2,4-DNT degrader of all four individual strains comprising this mixture (Table 1). One specific feature of 2,4-DNT degradation by the mixed culture was the lack of a lag phase, i.e., delay in substrate degradation at the start of the run, as opposed to any of the pure strains (Fig. 3a). Lag periods were observed for the mixed culture only at much higher 2,4-DNT concentrations (Fig. 3b) being significantly shorter than those of the individual strains at similar substrate concentrations (1–2 h vs. several days). This feature demonstrates that a synergism developed between the strains of the mixed culture leading to faster adaptation to this toxic substrate.

3.3. Influence of the initial substrate concentration on 2,4-DNT degradation

Fig. 4 displays plots of q and RE vs. initial 2,4-DNT concentration, obtained using the data of Fig. 3. For the most efficient of the strains used, *P. putida* NDT1, 2,4-DNT was removed quantitatively up to its initial concentration of 10 mg L^{-1} (Fig. 4a). A further increase in the initial concentration up to $C_{2,4\text{-DNT}}$ = 20 mg L^{-1} caused a significant drop in removal efficiency, even though the degradation rate (q) remained at its maximum value of 0.75 mg L^{-1} day^{-1}. Exceeding the threshold concentration of 20 mg L^{-1} resulted in a decline of both the RE and degradation rate of 2,4-DNT.

As in the experiments with a fixed 2,4-DNT concentration described in the previous section, the mixed culture showed a nearly 50 times higher degradation rate than the most efficient of the individual strains within the same optimal initial concentration range of 10–20 mg L^{-1} of 2,4-DNT. Even at slightly higher $C_{2,4\text{-DNT}}$ concentrations, the observed inhibition was not detrimental, yielding an RE of 100% in 15 days of cultivation up to an initial substrate concentration of 30 mg L^{-1} (Fig. 4b), i.e., three times greater than the breakthrough substrate concentration observed for *P. putida* NDT1 and any other individual strain used in this study.

Such a drastic enhancement of the 2,4-DNT degradation by mixing the four bacterial strains into one culture warranted further studies to find its cause, i.e., an investigation of intermediate metabolite production addressed in the next section.

3.4. Intermediates of 2,4-DNT aerobic degradation

Liquid chromatography analyses conducted in this study showed that any intermediates of 2,4-DNT cell catabolism accumulated only when the biodegradation experiments were performed

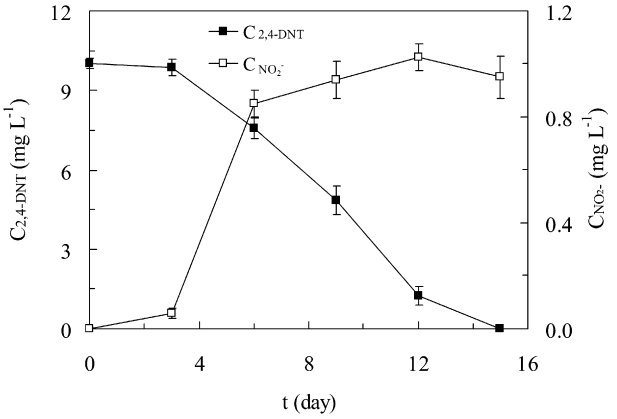

Fig. 5. Accumulation of nitrite ions during 2,4-DNT degradation by *Pseudomonas putida* NDT1.

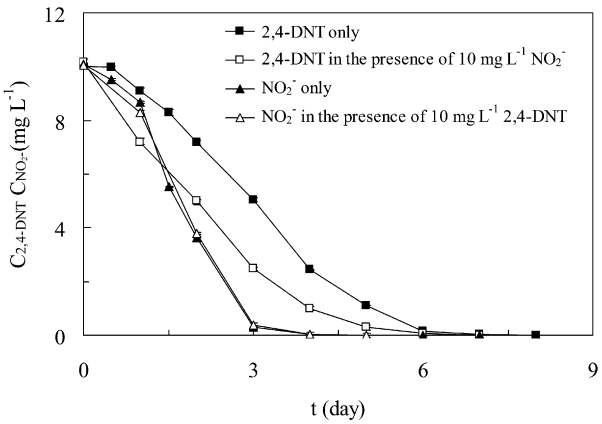

Fig. 6. Mixed culture uptake of 2,4-DNT in the presence/absence of NO_2^- and vice versa.

with the pure strain of *B. cereus* NDT4. Two different intermediates derived from 2,4-DNT were detected in the medium. By comparing their UV–vis absorption spectra obtained using a diode array detector with those of the pure standards of potential intermediates, these two chemicals were identified as 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. The chromatographic retention times of these two intermediates matched those of the standards, thus confirming their identification.

This identification was verified using GC–MS, while matching the fragmentation patterns of the observed metabolites with those provided in a standard MS library. Evidently, these metabolites were formed due to the conversion of the nitro group to the amino group as a result of a reductive metabolic pathway. The maximum accumulation of both intermediates was below 1 mg L^{-1} after 6 h of running a batch process. No such intermediates were detected while using the mixed culture. Apparently, these by-products of *B. cereus* NDT4 metabolism were consumed by the other microbial constituents. Consequently, an investigation was conducted of the accumulation of nitrite ions, which is critical for functioning of these other members of the microbial consortium, see the next section.

Accumulation of nitro group reduction intermediate products is common in TNT bioremediation [24–26]. Moteleb et al. [26] reported the formation of several reduced species, with 2,4,6-triaminotoluene as the final product. Accumulation of nitrophenolic or nitroamine intermediates was shown to inhibit the 2,4-DNT biodegradation [3]. This is significant because some of these chemicals are even more toxic than 2,4-DNT itself due to their well-known uncoupling effect on oxidative phosphorylation.

3.5. Accumulation of nitrite ions during 2,4-DNT degradation

2,4-DNT biodegradation is known to result in the release of nitrite ions [3]. Sometimes, this release can be used as an indicator of DNT removal [21]. But this feature is of concern due to the nitrite's high toxicity. The EPA drinking water limit for nitrite is 10 mg L^{-1} [27]. Fig. 5 shows data on nitrite accumulation during the 2,4-DNT removal by its most efficient degrader, *P. putida* NDT1. The observed nitrite concentrations were nearly 10-fold lower than the expected stoichiometric concentrations. Most, if not all, of the nitrite accumulation occurred right at the start of 2,4-DNT degradation, immediately following the lag phase. Neither nitrate nor NH_4^+ ions were detected.

The most likely explanation for a lower than expected nitrite accumulation is its conversion into a gaseous form of nitrogen, either N_2 or one of the nitrogen oxides, which was observed in earlier studies. Voets et al. [28] reported that nearly 70% of the nitrogen content of some nitrite-containing wastewater could be reduced by aerobic denitrification. Vangnai and Klein [29] isolated several species of *Pseudomonas*, which were capable of reducing nitrite, but not nitrate, to nitrogen gas under aerobic conditions. *Pseudomonas stutzeri* SU2, which was isolated from the activated sludge of a sequencing batch reactor treating piggery wastewater, rapidly reduced nitrate to N_2 under aerobic conditions without nitrite accumulation [30].

It is of note that N_2 was previously shown to be the main product of aerobic denitrification at pH > 7 whereas NO became the predominant product at pH < 6 [31]. In the experiments reported here, this variable was not significant because the initial pH was set at 7.0 and, due to the presence of a phosphate buffer, only a slight drop in pH from its initial value was observed (ΔpH < 0.1).

The incorporation of nitrite-originated nitrogen into biomolecules when 2,4-DNT was used as the sole nitrogen source could also contribute to a lower nitrite release into the aqueous medium [32]. However, this pathway of nitrite utilization appears to be less significant because no detectable biomass accumulation was observed in any of the experiments conducted in this study. Another alternate explanation, that nitrite accumulates within the cells, is also unlikely due to the high toxicity of nitrite. To gain further insights into the use of nitrite by the mixed culture, several 2,4-DNT biodegradation experiments were conducted with and without an artificially elevated nitrite concentration.

3.6. 2,4-DNT and nitrite uptake by the mixed culture: a mutual influence

Fig. 6 shows the uptake profiles of two nitrogen sources, 2,4-DNT and nitrite, by the mixed culture. In contrast to *P. putida* NDT1 as an individual 2,4-DNT degrader, the defined mixed culture showed no nitrite accumulation (Fig. 6). Conversely, significant aerobic denitrification, i.e., nitrite removal activity, was observed (~2.5 mg L^{-1} h^{-1}), both in the presence and absence of 2,4-DNT. Denitrification in the mixed culture appears to be performed by *Achromobacter* sp. NDT3 and *B. cereus* NDT4 because these two strains exhibited this activity during microbial identification tests. *Achromobacter* bacteria are often characterized by expressing nitrite reductase [33].

The presence of additional nitrite led to only a slight increase of the *initial* 2,4-DNT degradation rate (Fig. 6). Conversely, the average 2,4-DNT removal rate, *q*, by nitrite was slightly reduced in the presence of large nitrite concentrations (Table 2). This feature suggested that nitrogen was not a limiting nutrient.

The slight enhancement of the initial 2,4-DNT degradation rate by nitrite corroborates our hypothesis that nitrite is partially used

Table 2
Comparison of removal rates of nitrite, NO_2^-, and 2,4-DNT as single substrates and in mixtures.

$C_0^{NO_2}/C_0^{2,4\text{-DNT}}$ (mg L^{-1})	0/10	5/10	10/10	15/10	5/0	10/0	15/0
q^{NO_2} (mg L^{-1} h^{-1})[a]	N/A[a]	1.3	2.0	1.9	1.7	2.1	2.5
$q^{2,4\text{-DNT}}$ (mg L^{-1} h^{-1})[a]	1.5	1.3	1.2	1.1	N/A[b]	N/A[b]	N/A[b]

[a] The rates were calculated from the entire corresponding substrate uptake curves, e.g., Fig. 6, starting immediately with the substrate addition.
[b] Not applicable.

by the cells as an oxidant, i.e., an acceptor of electrons, even under aerobic conditions. The nitrite removal rate did not correlate with the 2,4-DNT concentration (Table 2) which suggests that the denitrification activity was not induced by the addition of 2,4-DNT as an external reducing agent. Amor et al. [34] and Zhang et al. [35] showed that aromatic compounds inhibit the natural process of soil nitrification resulting from ammonia oxidation. The use of nitrite by cells for aromatics' (e.g., 2,4-DNT) oxidation observed in this study may explain this effect.

The lack of nitrite and other metabolite accumulation for the mixed culture, as opposed to individual *Pseudomonas* degraders, illustrates the synergism of its components. While the poor degraders, *Achromobacter* and *Bacillus*, removed nitrite, *Pseudomonas* degraded the organic metabolite accumulated by *B. cereus* NDT4. However, the increase of denitrification rate, *per se*, cannot entirely explain the observed nearly 50-fold increase of 2,4-DNT biodegradation rate by the mixed culture because the accumulation of nitrite did not inhibit the 2,4-DNT removal by *Pseudomonas* (cf. Fig. 5).

Apparently, reasons for a greater efficiency of the mixed culture are more subtle and may involve toxicological and/or inhibitory effects. To explore these effects, other substrate–substrate interactions, within the nitrotoluene family, were investigated using both the mixed culture and its most efficient individual component, *P. putida* NDT1.

3.7. The mutual influence of mono- and dinitrotoluenes on the biodegradation of each other

The impacts of mono- and dinitrotoluenes on each other's biodegradation by the most efficient individual strain, *P. putida* NDT1, and by the mixed culture are shown in Fig. 7. For both the individual strains and mixed culture, the biodegradation rate of isomeric mononitrotoluenes (2- and 4-NTs) was not affected by the presence of 2,4-DNT (not shown). By contrast, the 2,4-DNT biodegradation rate as a single substrate was markedly reduced in the presence of either 2-NT or 4-NT. This influence was expected since NTs are much more readily biodegradable than 2,4-DNT, thus leading to a shift of catabolic competition in favor of mononitrotoluenes.

However, while 2,4-DNT removal by *P. putida* NDT1 was completely inhibited by the addition of 5 mg L^{-1} mononitrotoluene (Fig. 7a), the mixed culture under similar conditions continued to remove this substrate, though with a threefold reduced rate (Fig. 7b).

3.8. Effect of the 2,6-DNT presence on 2,4-DNT degradation by the mixed culture

In preliminary screening tests, 2,6-DNT was shown to sustain the growth of the defined mixed culture as the sole carbon, energy and nitrogen source, although its removal rate was low (0.03 mg L^{-1} day^{-1}). 2,6-DNT was not degraded by the mixed culture in the presence of 2,4-DNT. Yet, even the most efficient individual 2,4-DNT degrader, *P. putida* NDT1, was not able to degrade 2,6-DNT at all as a pure strain, neither as a single substrate nor in the mixture with 2,4-DNT.

The influence of an isomeric dinitrotoluene, 2,6-DNT, on the rate of 2,4-DNT biodegradation by the mixed culture is shown in Fig. 8 for two different 2,6-DNT concentrations and two different 2,4-DNT starting concentrations. The observed negative effect of 2,6-DNT on 2,4-DNT degradation cannot be explained by catabolic competition because 2,6-DNT is less biodegradable. Perhaps, this effect is due to a higher toxicity of 2,6-DNT to bacterial cells, which could be ameliorated by the presence of 2,4-DNT, a growth substrate.

Evidence confirming this hypothesis was obtained while varying the ratio of two dinitrotoluenes (Fig. 8). When 2,4-DNT was present in a larger amount (10 mg L^{-1}) (Fig. 8a), the eventual complete degradation of 2,4-DNT was observed, although with a twofold lower rate. By contrast, when 2,4-DNT was present in a lower concentration, 5 mg L^{-1} (Fig. 8b), its removal efficiency consistently decreased under increased 2,6-DNT concentrations, with a concomitant decline in 2,4-DNT removal rates (fourfold compared to

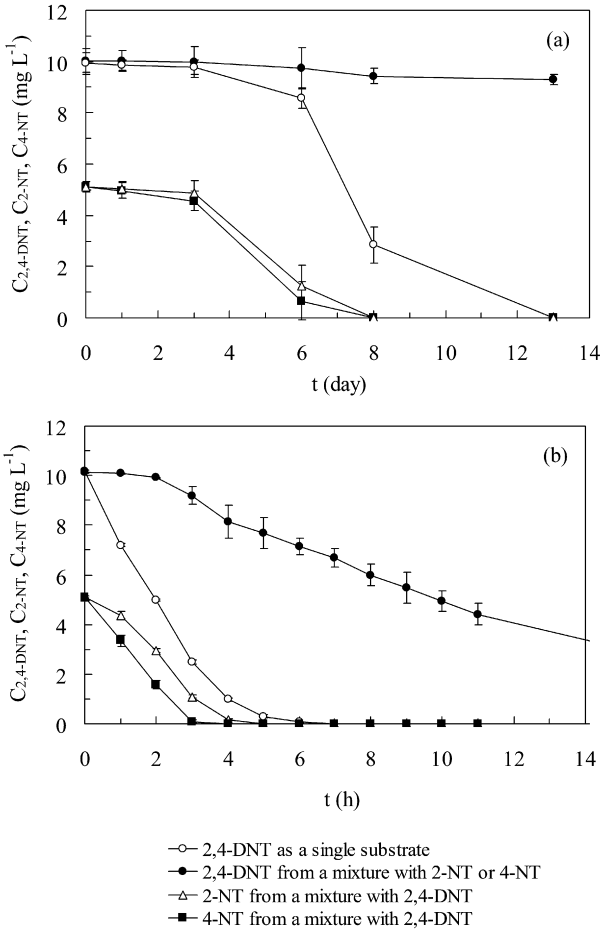

Fig. 7. Uptake of 2,4-DNT, 4-NT and 2-NT from the mixtures of 2,4-DNT with either 4-NT or 2-NT using *Pseudomonas putida* NDT1 (a) and the mixed culture (b).

Table 3
2,4-DNT degradation rates reported in literature.

Microorganism(s)	q_{max}[a] (mg L^{-1} day^{-1})	Refs.
Pseudomonas sp.	328.0	[3]
Burkholderia sp.	20.3 mg m^{-2} day^{-1}[b]	[37]
Sinorhizobium meliloti USDA 1936 pJS1	10.0	[38]
Alcaligenes sp. JS867 (oxygen limited)	4.8	[39]
Pseudomonas aeruginosa (incomplete mineralization due to a reductive metabolism, even under aerobic conditions)	182.4	[36]
Pseudomonas fluorescens	11.5	[40]
Consortium 1		
Variovorax paradoxus VM685	15.2	[6]
Pseudomonas marginalis VM683		
Consortium 2		
Pseudomonas sp. VM908		
P. aeruginosa VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905	28.8	[6]
P. viridiflava VM907		
Mixed culture *Pardubice 06*		
Pseudomonas putida NDT1		
Pseudomonas fluorescens NDT2	40	Present study
Bacillus cereus NDT4		
Achromobacter sp. NDT3		
Pseudomonas putida NDT1 (the most efficient 2,4-DNT degraders among those present in the mixed culture)	0.75	Present study

[a] The rates, q_{max}, are provided for optimum pH if pertinent.
[b] The value for a continuous flow sand column bioreactor [35].

that with no 2,6-DNT). In addition, the cells were no longer able to remove 2,4-DNT completely unlike the previous case (Fig. 8a).

Note that the common environmental contamination by 2,6-DNT is much lower than that by 2,4-DNT because the latter, unlike the former, is the main toluene dinitration product. Thus, under realistic conditions, when the 2,6-DNT concentration does not exceed that of 2,4-DNT, the 2,4-DNT removal by the mixed culture is still efficient (Fig. 8a).

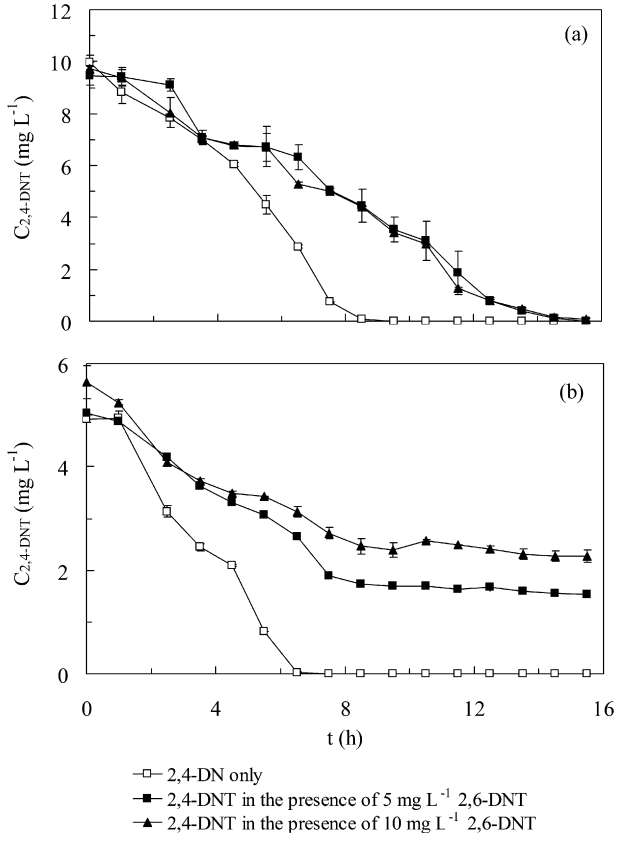

Fig. 8. Mixed culture uptake of 2,4-DNT as an individual substrate (□), and from mixtures with 5 mg L^{-1} (■) and 10 mg L^{-1} 2,6-DNT (▲) at starting 2,4-DNT concentrations of 10 mg L^{-1} (a) and 5 mg L^{-1} (b).

3.9. Comparison of the 2,4-DNT removal rates reported in literature and in this study

The values of 2,4-DNT aerobic removal rates observed previously for submerged suspended cultivations (calculation based on the raw data provided in the corresponding sources) are shown in Table 3 along with the rates obtained in previous studies. The reported 2,4-DNT degradation rates were within a range of 4–30 mg L^{-1} day^{-1}. Spanggord et al. [3] reported a higher value; however, based on the information presented in that paper, the cell count or optical density increased nearly 50-fold as a result of 2,4-DNT degradation, compared to just a twofold increase obtained in this study (cf. Fig. 1). Evidently, an external nitrogen source (as either yeast extract or ammonium) was present in the medium thus causing significant growth. The other high value of the 2,4-DNT biodegradation rate, obtained by Noguera and Freedman [36], can be explained by the fact that the strain used in that study did not completely mineralize 2,4-DNT. This rate was due to the substrate biotransformation rather than biodegradation.

The rates obtained in the current study while using individual strains were lower than those reported in literature, in contrast with the mixed culture. This trend is in agreement with literature. A microbial consortium that only *collectively* mineralized 2,4-DNT was reported earlier by Snellinx et al. [6]. However contrary to the results of Snellinx et al. [6], in our current study each bacterial strain in the mixture was capable of degrading 2,4-DNT. Yet, their use as a consortium resulted in the achievement of one of the highest 2,4-DNT degradation rates as the sole carbon, energy and nitrogen source among those reported in literature (Table 3). As our studies of substrate–substrate interactions have shown, this effect may be due to overcoming substrate toxicity and/or inhibitory effects.

Exchange of intermediates (as suggested by Snellinx et al. [6]) and/or release of anti-toxicity agents into the medium may contribute to the observed synergism. However, a simple "division of labor" between the components of the mixture demonstrated in this study (e.g., efficient denitrification performed by some strains while the others remove partially oxidized intermediates) may also contribute to the observed enhancement of substrate degradation.

The 2,4-DNT removal rates by the mixed culture observed in this study are similar in magnitude to those observed by Paca et al. in our prior work [12]. A similar enhancement of performance was then observed upon the mixing of several individual strains isolated from a biofilm, despite the fact that these

strains, as well as the original enrichment culture, were completely different from those used in this study and exhibited rather different features (e.g., consistent <100% 2,4-DNT removal). Thus, the observed effect is not specific to one particular microbial consortium and appears to be inherent for biodegradation of this substrate.

The use of single pure strains in microbiological processes is, in general, preferred because this is often the only way to obtain repeatable results. In addition, the use of single strains with well-defined properties is warranted from the standpoint of toxicology. However, this study shows that the use of mixed cultures may provide significant benefits when dealing with poorly biodegradable and toxic substrates, such as dinitrotoluenes and their mixtures with mononitrotoluenes. Even if a practical application is based on the use of a single strain, development of synergistic mixed cultures upon adaptation may result in an eventual increase in 2,4-DNT degradation rates.

4. Conclusions

A defined mixed culture of four individual strains degraded 2,4-DNT with a 50 times higher rate than any of its individual components. The mixed culture exhibited a broader optimum pH range with shorter, if any, lag periods during 2,4-DNT degradation. In addition, the mixed culture, unlike the individual strains, showed no nitrite or organic intermediates' accumulation in the liquid medium, while degrading a broader spectrum of nitrotoluenes (e.g., including 2,6-DNT as the sole energy, carbon and nitrogen source) within a wider concentration range.

The presence of readily degradable mononitrotoluenes (2-NT and 4-NT) in the medium caused a negative effect on the 2,4-DNT degradation rate due to a preference for a readily biodegradable substrate by cells. The presence of a much less degradable isomeric dinitrotoluene (2,6-DNT) in the medium also had a negative effect on 2,4-DNT degradation, apparently due to a toxic effect of 2,6-DNT on the bacterial cells. Yet, even under these unfavorable conditions, the mixed bacterial culture was shown to be an effective biocatalyst for removal of 2,4-DNT. Using mixed cultures may thus be recommended for treating aqueous environments contaminated with this chemical either as a single substrate or in mixtures containing other nitrotoluenes.

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