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Effect of Nitrate on Biodegradation of Mononitrotoluenes (MNTs) by Several Pure Microbial Strains in Submerged Aerobic Cultures

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Abstract: Four microbial strains, isolated from a mixed culture, were used to study biodegradation of 2-nitrotoluene, 3-nitrotoluene, and 4-nitrotoluene individually and in a mixture. The strains were identified as *Pseudomonas putida* S7, *Comamonas testosteroni* Pb 50, *Rhodococcus* sp. Pa 50, and *Stenotrophomonas malthophilia* K3. The degradation studies were carried out with suspended cells in aqueous media under aerobic conditions and the results were compared with new extensive studies on biodegradation of mononitrotoluenes with a mixed culture isolated from explosives' contaminated soil from another site.
The results showed that (a) the degradation rate of MNTs by the individual strains were of the same order of magnitude as that by the Pardubice mixed culture, (b) the

presence of additional N-source positively affected the degradation rate for 3-NT and 4-NT, but adversely affected the degradation rate of 2-NT, (c) no incompletely oxidized dead-end intermediates were found under aerobic conditions, (d) all the cell lines were able to efficiently degrade all the MNTs, even when all were present together, and (e) the degradation rates of individual MNTs in a mixture were slightly lower than when just one NT was present. Substrate concentration affected degradation rates according to classical substrate inhibition.

Keywords: individual nitrotoluenes, mixture of nitrotoluenes, mixed culture, process kinetics

Introduction

Mononitrotoluenes (MNTs) are used as intermediates in production of explosives, dyes, pesticides, pharmaceuticals, and plastics [1-4]. These are present as pollutants in subsurface soil, and ground and surface waters at several sites. MNTs exhibit genotoxicity [5-8] and are characterized as hazardous substances. Several microorganisms possess ability to transform MNTs and even utilize them as sole sources of carbon, nitrogen, and energy [3, 9-12]. Microbial metabolism of MNTs involves both oxidative as well as reductive pathways. The oxidative metabolism is known to be catalyzed either by monooxygenases or by dioxygenases and, in both cases, the nitro group is released in the broth as nitrite. It has been suggested that the oxygenases involved in biodegradation of MNTs are similar to those involved in degradation of aromatics toluene and xylene. The reductive metabolism is catalyzed by dehydrogenases and releases the nitro group as ammonium ion in broth. Microorganisms capable of metabolizing MNTs have mostly been isolated from contaminated sites and all carry one or more of the above mentioned metabolic traits. Haigler and Spain [10] have observed that these traits are quite common in several microorganisms. Most of the MNT-degraders belong to the genera *Pseudomonas*, *Rhodococcus*, and *Mycobacterium*, although other genera are also known to possess capability to metabolize MNTs. In some microbes, more than one metabolic pathway for degradation of MNTs is active [11, 13]. In some cases it has been suggested that presence of nitrates could be harmful to cells.

In our laboratory, a mixed culture capable of degrading nitrotoluenes was isolated from contaminated soil from an old explosive production facility. Results of biodegradation of mono- and dinitrotoluenes using this mixed culture have been presented and published elsewhere [14-16]. Four pure culture strains were also isolated from biofilters being used to treat toluene/xylene, gasoline, and

nitrotoluenes at another explosives facility. This study deals with biodegradation of individual MNTs and their mixtures by the pure culture strains and the mixed culture. Specifically, the effect of addition of nitrate to the culture broth on biodegradation rates has been presented here.

Material and Methods

Microorganisms

Pure culture strains were isolated using enrichment culture techniques from samples collected from biofilters films being used at our Department. The enrichment techniques have been presented by Paca *et al.* [14]. Two strains, *Rhodococcus* sp. Pa 50 and *Comamonas testosteroni* Pb 50, were isolated from waste air biotricking filter treating toluene and xylene vapors. The source of strain *Pseudomonas putida* S7 was biofilter treating gasoline containing waste air and that of strain *Stenotrophomonas malthophilia* K3 was biofilter treating nitrotoluenes in aquous phase. Identification of the pure strains was conducted using methods described earlier [15]. The mixed culture was isolated from soil samples collected from the Synthesia explosives production facility in Pardubice, Czech Republic that has been in operation for the past eighty years. Paca *et al.* [14] have reported the enrichment procedures used for the isolation of the mixed culture. The cells were stored in deep freezer at -70 °C.

Media and culture conditions

Basal salt medium without nitrate (BSM w/o nitrate) consisted of 3.4 g KH_2PO_4, 4.3 g K_2HPO_4, 0.34 g $MgCl_2 \cdot 6H_2O$ and 1 mL trace element solution per liter of medium. BSM solution with nitrate (BSM) was prepared by adding 0.80 g L^{-1} KNO_3 in BSM w/o nitrate. The trace element solution contained 5 g L^{-1} $FeSO_4 \cdot 7H_2O$, 5 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 5 g L^{-1} $MnSO_4 \cdot H_2O$, 5 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.1 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.1 g L^{-1} $Na_2B_4O_7 \cdot 10H_2O$ and 0.1 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$. pH of medium was adjusted to 7.2 before autoclaving for 20 minutes at 120 °C. All the chemicals used in this work were of analytical grade and were obtained from Sigma-Aldrich.

All the degradation experiments were carried out in triplicate in 500 mL Erlenmeyer flasks with working volume of 100 mL at 26 °C in a rotary shaker at 120 min^{-1}. All the flasks were wrapped in aluminum foil to avoid any photocatalytic (abiotic) degradation. Sterile media in flasks were inoculated either with one of the pure strains or with the mixed culture after these were preincubated for 48 h. The started cell dry mass concentration in the inoculated flasks was estimated to be approximately 0.3 g DW L^{-1}.

Analytical Methods

Samples were collected from the flasks at regular intervals and analyzed for cell density and contaminants. For analyses, the samples were first centrifuged (HEREAUS, Kendro Laboratory Products, Germany) at 20000 min^{-1} for 10 minutes. Analyses were conducted by HPLC (System DeltaChrom, Watrex Pargue Ltd., Prague, Czech Republic) using a Watrex column (250 x 4 mm Nucleosil 120-5 C-18, Watrex Praha s.r.o., Czech Republic) and a mobile phase of methanol/water (50:50) at 30 °C and a flow rate of 1mL min^{-1} for separation of peaks. Peaks were detected by measuring absorbances at 230 nm and 238 nm using a diode array detector (Model UV 6000 LP, Thermo Separation Products Inc., San Jose, CA, USA). Under these conditions, retention times of 2-NT, 3-NT, and 4-NT were 22.2, 26.2, and 23.7 min, respectively.Calibration curves were prepared by measuring peak areas for different concentrations of pure nitrotoluenes in aqueous solutions and the calibration curves were used to determine concentrations in samples.

Results and Discussion

Biodegradation of Nitrotoluenes by pure strains:

The experimental data of NT concentrations when media were inoculated with pure culture strains are presented in Figures 1-4. These include data when only one nitrotoluene contaminant was present and when all the three nitrololuenes were present together in solution.The Figures show biodegradation information in BSM with as well as without nitrate. In all the cases, MNTs disappeared from media over a period of two weeks. In some cases, such as with 2-NT and 3-NT in BSM media inoculated with either *Comamonas testosteroni* Pb 50 or *Pseudomonas putida* S7, the nitrotoluenes disappeared from media within two to three days. In other cases, considerably more time was needed and small amounts of contaminants were still left in the medium at the end of two weeks. Analysis of samples suggested that no incompletely oxidized metabolites of degradation of MNTs accumulated in the broth.The effect of nitrate addition on disappearance patterns was somewhat dependent on the position of nitro group on the aromatic ring. In most cases, addition of nitrate to basal salt medium enhanced the rate of disappearance of 3-NT. The same appears to be true for 4-NT also, but the effect is not as pronounced as for 3-NT. Disappearance of 2-NT appears to be variably affected by the addition of nitrate to medium. For Pardubice mixed culture, Paca *et al.* [14] reported no effect of nitrate on biodegradation patterns of 2-NT and 4-NT although the effect of nitrate on 3-NT disappearance was dramatic and positive.

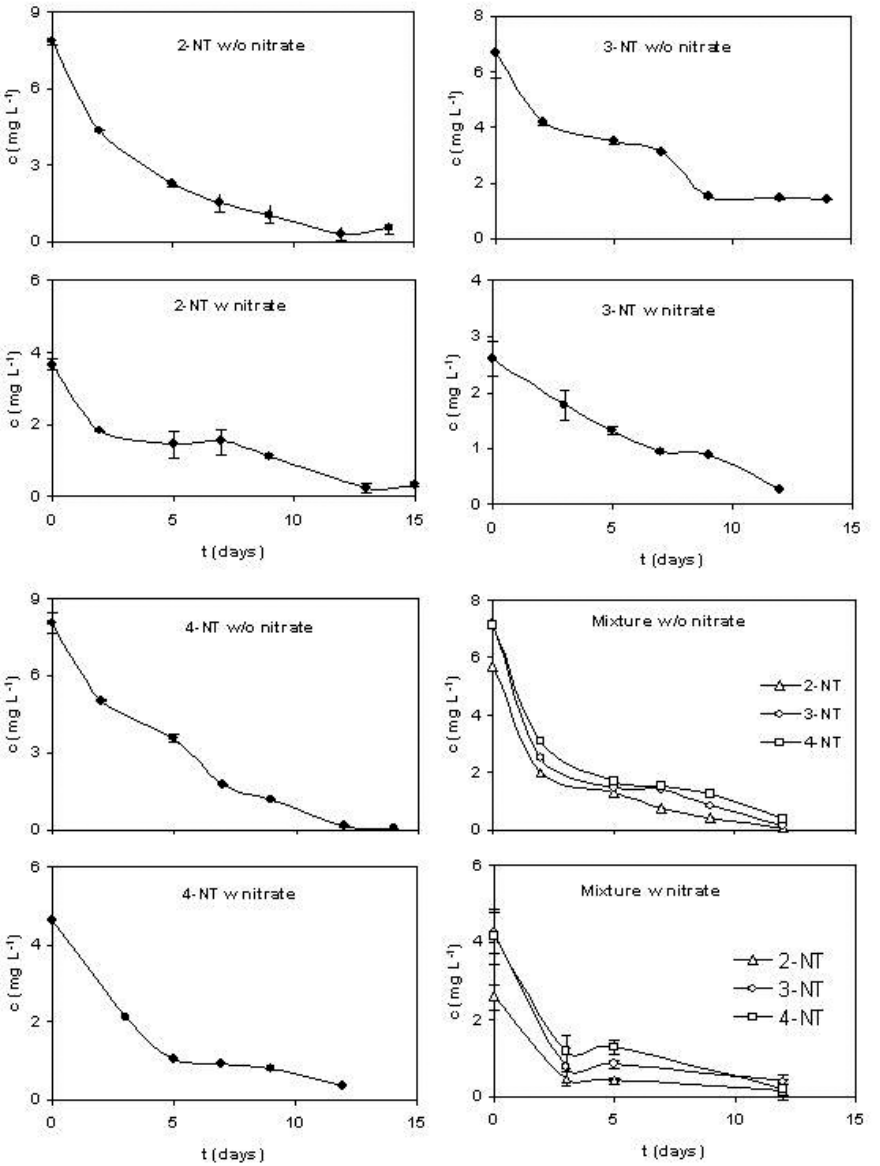

Figure 1. Degradation of nitrotoluenes by *Rhodococcus* sp. Pa 50 in BSM with and without nitrate.

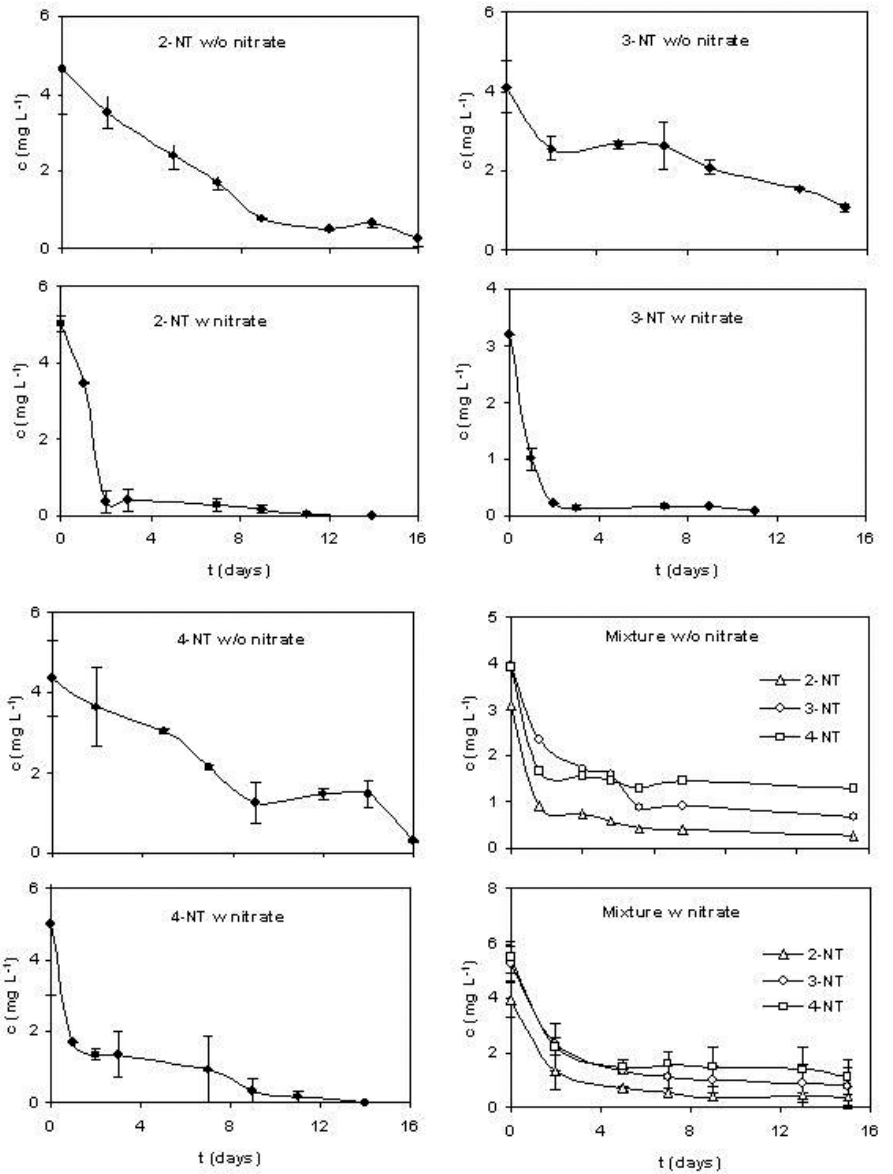

Figure 2. Degradation of nitrotoluenes by *Comamonas testosteroni* Pb 50 in BSM with and without nitrate.

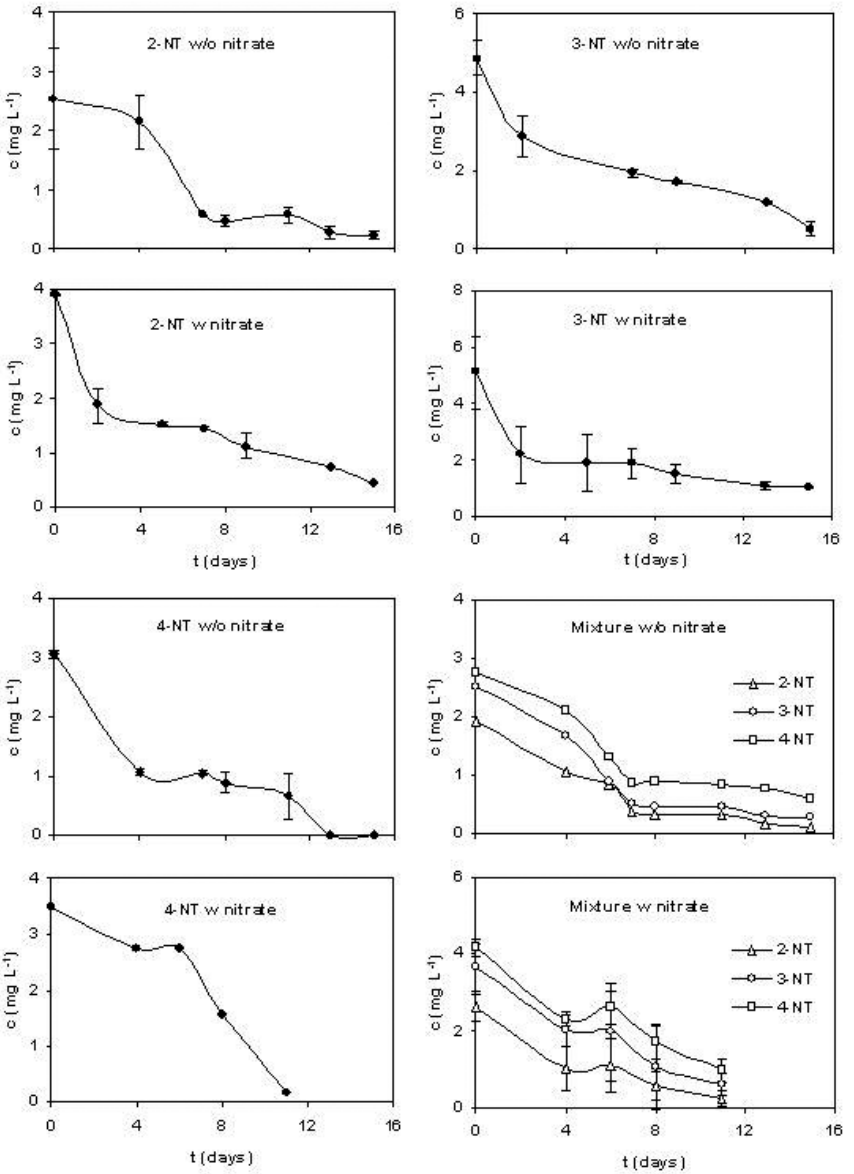

Figure 3. Degradation of nitrotoluenes by *Stenotrophomonas malthophilia* K3 in BSM with and without nitrate.

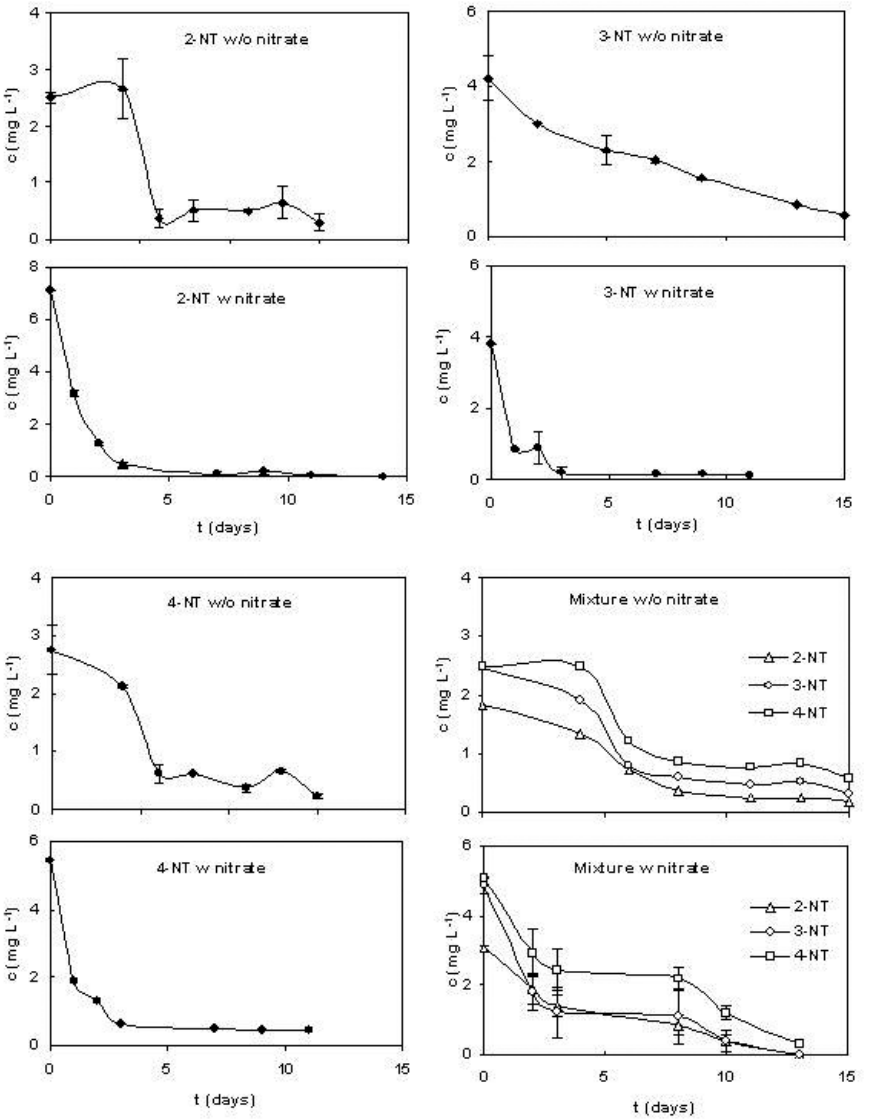

Figure 4. Biodegradation of nitrotoluenes by *Pseudomonas putida* S7 with and without nitrate.

It is known that methyl group is a strong ortho/para director of substitutions on the aromatic ring. As a result, nitration of toluene by nitric acid in presence of sulfuric acid results in preferential formation of 2-NT followed by 4-NT in a ratio

of 60:40 with little 3-NT production. During degradation reactions also, one may expect a strongest tendency for substitutions at ortho-position followed by para and the least tendency at the meta-position. Meta substitutions on benzene ring result in more difficult to biodegrade [17]. This would also be consistent with the strongly-leaving tendency of nitro group during nucleophilic substitutions on the aromatic ring. However, the microorganisms used in this study showed little tendency to prefer one MNT over the other. This is most clear in the experiments where all the three MNTs were present together in the medium. All the MNTs disappeared from the media simultaneously. The same observation was also made with the biodegradation of nitrotoluenes by the mixed culture isolated from Pardubice facility [14, 15]. It appears that ability to biodegrade mononitrotoluenes is quite common and exposure of the cells to aromatic compounds results in their acclimatization to nitroaromatics as well.

A quantitative way of analyzing the experimental data presented in Figures 1-4 is to look at the rate constants for disappearance of chemicals under different conditions.

Table 1. First order rate constants for degradation of nitrotoluenes when present alone and when present as a mixture

	First order rate constant for NT-biodegradation (1/day)							
	Pa 50		Pb 50		K3		S7	
	w/o nitrate	w nitrate	w/o nitrate	w nitrate	w/o nitrate	w nitrate	w/o nitrate	w nitrate
2-NT	0.23	0.17	0.41	0.99	0.17	0.15	0.15	0.51
2-NT in mixture	0.33	0.27	0.25	0.29	0.20	0.21	0.17	0.19
3-NT	0.13	0.16	0.08	1.11	0.13	0.12	0.13	0.25
3-NT in mixture	0.26	0.24	0.16	0.22	0.12	0.15	0.12	0.25
4-NT	0.32	0.22	0.12	0.37	0.15	0.08	0.16	0.34
4-NT in mixture	0.26	0.27	0.15	0.18	0.15	0.12	0.13	0.18

Assuming negligible increase in cell mass, specific consumption rates of contaminants were calculated using a first order degradation pattern.

$$\frac{dS}{dt} = -k\,S \qquad (1)$$

where: S is concentration of contaminant in the medium and k is first order rate constant for biodegradation. Values of the rate constant were calculated from semi-log plots of concentration *vs* time using data shown in Figures 1-4 and these constants are presented in Table 1. These rate constants confirm the general visual observations made earlier. However, the differences in behavior of different strains become more obvious here. It appears that strains *Stenotrophomonas malthophilia* K3 and *Comamonas testosteroni* Pb 50 are considerably less sensitive to the addition of nitrate than the other two strains. The large degradation rate constants in some cases appear to be a result of sharp drop in concentration for a critical sample. Disregarding these values, presence of other MNTs appears to slightly slowdown the rate of disappearance of individual MNTs.

Effect of nitrate on biodegradation of MNTs by the Pardubice mixed culture:

As indicated above, there appeared to be some qualitative differences in the effect of nitrate addition on biodegradation rates of MNTs. Hence it was decided to perform a detailed study of the effect of nitrate addition on biodegradation of MNTs by the Pardubice mixed culture. In this case, MNT concentrations were varied over a wide range (0-300 g L^{-1}) and initial rates of disappearance of individual MNTs were measured in basal media with and without nitrate.

These data have been plotted in Figure 5 as discrete points. In all the cases, a classical substrate-inhibition behavior was observed. The effect of nitrate addition on rates of degradation is also obvious. Nitrate addition enhances the rate of biodegradation of 2-NT and but it suppresses the degradation rates of 3- and 4-NT. Simple Haldane type of substrate inhibition kinetics did not adequately describe all the patterns in Figure 5. Hence, a combined Hill-Haldane expression of the following type was utilized:

$$Q_S = Q_{S,max}\,\frac{S^n}{K_S + S^n + \dfrac{S^m}{K_I}} \qquad (2)$$

where: Q_S (mg L^{-1} day^{-1}) is the rate of degradation at MNT concentration S (mg L^{-1}), $Q_{S,max}$ is the theoretical maximum rate of degradation (mg L^{-1} day^{-1}), K_S is the saturation constant [(mg L^{-1})^n], K_I is the substrate inhibition constant

[(mg L^{-1})$^{m-n}$], and n and m are constants. The constants were obtained by fitting the expression into the experimental data using non-linear parameter estimation procedures available in 'Polymath' numerical analysis software. The constants are presented in Table 2 and the predictions using the expression are plotted in Figure 5 as solid lines. Obviously, the expression could be made to fit the experimental data very well.

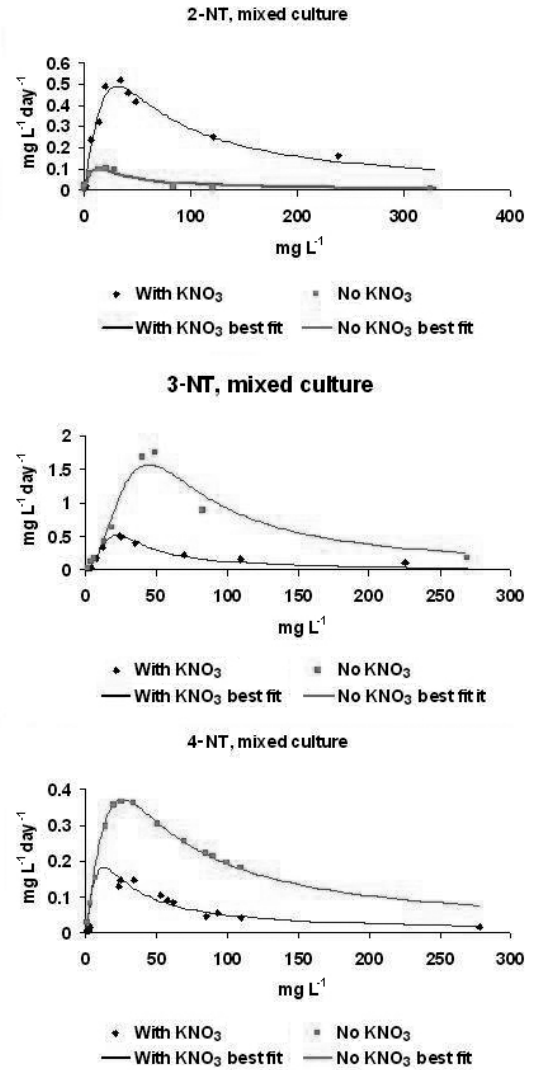

Figure 5. Effect of NT concentrations on their biodegradation rates by the Pardubice mixed culture.

Table 2. Model parameters for biodegradation of nitrotoluenes by mixed culture

		$Q_{S,max}$	K_S	K_I	n	m
		($mg\ L^{-1}day^{-1}$)	$(mg\ L^{-1})^n$	$(mg\ L^{-1})^{m-n}$	-	-
2-NT	w/o nitrate	0.87	48.2	3.48	1	2
	w nitrate	4.75	135.5	7.1	1	2
3-NT	w/o nitrate	18.9	2230	34.5	1.6	3
	w nitrate	6.6	774	12	1.6	3
4-NT	w/o nitrate	3.7	117	5.8		2
	w nitrate	1.8	57	2.8	11	2

The effect of nitrate addition on degradation rates were incorporated in the model by estimating new parameters ($Q_{S,max}$, K_S, and K_I). Following the lead of mixed inhibition, it was decided that all of these parameters will be the same function of nitrate concentration. Since only one nitrate concentration was used, the nitrate dependency reduces to a constant factor by which all of these parameters change. In other words,

$$\frac{(Q_{S,max})_{with\ nitrate}}{(Q_{S,max})_{without\ nitrate}} = \frac{(K_S)_{with\ nitrate}}{(K_S)_{without\ nitrate}} = \frac{(K_I)_{with\ nitrate}}{(K_I)_{without\ nitrate}} =$$

$$= \text{function of nitrate concentration} = \text{constant } \alpha$$

As a result, a single parameter was estimated to fit the expression with parameters derived from BSM without nitrate data to BSM with nitrate data for each MNT. The modified parameters are presented in Table 2 and the predicted values are presented in Figure 5. Again, a single parameter estimation approach worked well for 3-NT and 4-NT, but not for 2-NT. Hence, an independent three parameter estimation was conducted for 2-NT data with nitrate. The parameters are listed in Table 2 and the predictions are shown in Figure 5. No comparison of these parameter values with those in literature was possible because none have been found in published literature. For low substrate concentrations, the rate expression (2) with parameters in Table 2 would translate into degradation rates of MNTs that are comparable to the degradation rates suggested by equation (1) with rate constant in Table 1. This suggests that microorganisms from different sources had similar metabolic rates and patterns.

Conclusions

Microorganisms from two different sources and having different histories of exposure to aromatic/nitroaromatic compounds were found in this work to possess similar capability to aerobically biodegrade mononitrotoluenes. This was to be expected since oxygenases belonging to *Tol* plasmid are expected to participate in the transformation reactions. The microorganisms were able to degrade all the MNTs with almost equal ease. When all the MNTs were present in the solution together, simultaneous biodegradation of all the MNTs was observed. Effect of nitrate addition to the medium on degradation rates of MNTs appeared to depend on both the microorganisms as well as on the location of nitro group on the aromatic ring. The findings, as a part of the Joint Project, can help in field experiments.

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