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Substrate interactions between 4-nitrophenol and 4-nitrotoluene during biodegradation of their mixture

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ABSTRACT

Nitroaromatic compounds are toxic and rather recalcitrant pollutants of water and soil. Microbial degradation of the individual nitroaromatic compounds has already been well described in the literature. However, because several compounds often occur in the environment mutually influencing each other's degradation, further research into the biodegradation of their mixtures is still needed. We investigated the degradation of a mixture of 4-nitrophenol (4-NP) and 4-nitrotoluene (4-NT) by a mixed microbial culture immobilized in a continuously operated packed-bed reactor (PBR) and by free suspended cells in shake flasks. Each compound was at first degraded separately, then in a mixture, and their degradation characteristics were compared. When treated separately, 4-NP and 4-NT were degraded with efficiency over 99 and 95%, respectively. When in a mixture, 4-NP was still completely removed from the media but the 4-NT removal efficiency dropped by a half and remained about the same during the whole experiment regardless the increasing 4-NP concentration at the PBR inlet. The shake flask experiments corroborated our finding that 4-NP negatively influenced 4-NT degradation by competitive inhibition and also indirectly on the adaptation level.

Keywords: Biodegradation; 4-nitrophenol; 4-nitrotoluene; Waste water treatment

1. Introduction

Nitrophenols and nitrotoluenes are toxic and rather recalcitrant pollutants of water and soil. The main sources of pollution by these compounds are the manufacturing and use of explosives, pesticides, dyes, and pharmaceuticals. Although various biological [1,2] and physicochemical [3–5] strategies for their removal from the environment have been investigated, there are still some unresolved issues, such as the microbial degradation of multiple nitroaromatic compounds.

Microbial degradation is considered to be an efficient method for the removal of nitroaromatic compounds from the environment and has been thoroughly studied (see [6] for review). However, most of

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the published studies focus on a single compound metabolized by a single microbial strain. While these studies are beneficial for broadening the knowledge of particular metabolic pathways and existing microbial degraders, their applicability for *in situ* remediation under real-life environmental conditions has certain limitations. One of them is the fact that nitroaromatic compounds are often released into the environment in mixtures and can be further transformed by various environmental factors, thus giving rise to even more complex compound mixtures.

Yet, few reports deal with the biodegradation of nitroaromatic compounds in a mixture. So far, some of the reports indicate that nitroaromatic compounds, if treated simultaneously, can affect each other's degradation characteristics. An induction effect by a pathway intermediate was described by Iwaki et al. [7], who found that 4-nitrophenol, an intermediate in 2,4-dinitrophenol degradation pathway, induced 2,4-dinitrophenol degradation by *Burkholderia* sp. KU-46. Similarly, 2,4-dinitrophenol induced trinitrophenol degradation by *Rhodococcus opacus* HL PM1 [8]. Conversely, 2,4-dinitrotoluene competitively inhibited 2,6-dinitrotoluene degradation by a mixed microbial culture [9], and 2-nitrophenol and 4-nitrophenol inhibited 2,4-dinitrophenol degradation by *Rhodococcus* sp. RB1 [10]. Clearly, substrate interactions can play an important role in the bioremediation of sites contaminated by several nitroaromatic compounds, but further research into this field is still needed.

As a part of our ongoing research of nitrophenols and nitrotoluenes biodegradation [11–15], we here present a study on substrate interactions between 4-nitrophenol (4-NP) and 4-nitrotoluene (4-NT) during their degradation from simulated wastewater by a mixed microbial culture. These two compounds were chosen on account of their massive annual production (both are listed as high production chemicals by US EPA [16] with annual production exceeding 1 million pounds in the US) and similar applications, therefore being likely to appear together in the environment in significant quantities. The interactions between these two compounds are evaluated by comparing several parameters, such as the degradation rate and maximal removed organic loading of each compound treated separately and in a mixture.

2. Materials and methods

2.1. Medium

A modified version of the basal salt medium (BSM), described by Paca et al. [17], was used for the inoculum preparation and in all degradation experiments. A lower concentration of the main components was used to better fit the composition of the model low strength wastewater used in the experiments. The medium was composed of 0.5 g L^{-1} K_2HPO_4, 0.4 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgCl_2$, and 50 μL L^{-1} trace elements solution containing 5.0 g $FeSO_4 \times 7H_2O$, 5.0 g $ZnSO_4 \times 7H_2O$, 5.0 g $MnSO_4 \times H_2O$, 5.0 g $CuSO_4 \times 5H_2O$, 0.1 g $CoCl_2 \times 6H_2O$, 0.1 g $Na_2B_4O_7 \times 10H_2O$, and 0.1 g $Na_2MoO_4 \times 2H_2O$ per liter of distilled water. 4-NP and 4-NT were added to the medium in the form of concentrated solutions and served as the only sources of carbon, nitrogen, and energy for the microorganisms.

2.2. Microorganisms

A mixed microbial culture was used for the degradation experiments. It was taken from a long-term operated dinitrophenol degrading bioreactor used in our previous study [14], resuspended in the BSM medium, and cultivated in shake flasks in the presence of 4-NP and 4-NT. Thus induced culture was used as an inoculum for a packed-bed reactor. After 12 months of operation, a small portion of the biofilm formed on the reactor's packing was removed and resuspended in the BSM medium, and used for the shake flasks experiments. The microbial composition of the reactor inoculum, the biofilm formed within it after 12 months of operation, and the suspended culture at the end of the shake flask experiments is further discussed in Section 3. It was determined by standard biochemical and genetic methods described by Páca et al. [11] and Hudcova et al. [12], respectively.

2.3. Packed-bed reactor

A major part of the degradation experiments was carried out in a continuously operated packed-bed reactor (PBR) with the empty bed working volume of 735 mL, the effective working volume of 290 mL (the sum of aqueous phase and biofilm volumes within the working volume of the reactor), containing 480 g of expanded slate as a packing material, and operated at 30°C. The reactor scheme was shown in our previous work [13]. The BSM medium with dissolved 4-NP and/or 4-NT was fed to the bottom of the reactor concurrently with the air flow. The organic loading of the reactor was varied by changing the inlet concentration of 4-NP and 4-NT in the medium, while the medium flow rate was kept constant at 1.7 mL min^{-1}. The compounds were degraded separately and in a mixture with equal concentration of both compounds as shown in Fig. 1. Prior to each degradation test, the

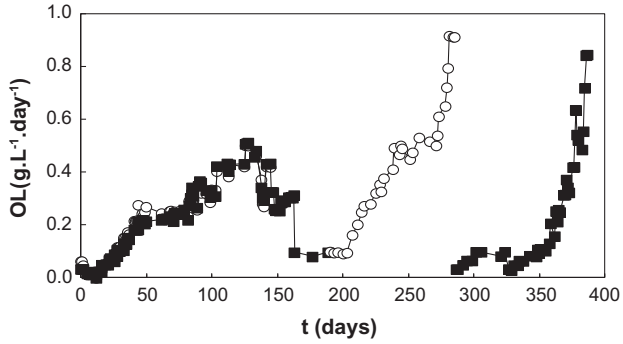

Fig. 1. PBR loading during 387 d of operation by varying the inlet concentration of 4-NP (○) and 4-NT (■) or their mixture.

microorganisms in the PBR were induced by recirculating the medium with the given compound(s) through the reactor until their concentration at the reactor outlet reached zero.

2.4. Shake flask experiments

The experiments with free suspended cells were performed in 250-mL shaken Erlenmeyer flasks with 50 mL working volume and 0.6 initial optical density of the cell suspension measured at 500 nm by Spekol 11 spectrophotometer (Analytik Jena). The culture was cultivated with 20 mg L^{-1} of 4-NP, 4-NT or their mixture which were added to the medium three times a week. To assess the influence of culture adaptation on degradation rate, the culture was subjected to the following feeding scheme: 4-NP/4-NT mixture for 3 weeks, 4-NT for 21 weeks, and finally 4-NP for 3 weeks. The degradation experiments were performed after 3, 6, 13, 24, and 27 weeks of cultivation.

2.5. Analytical methods

4-NP and 4-NT concentrations in the samples of media taken at the inlet and outlet of the reactor and from the shake flasks was measured by reverse phase HPLC (System DeltaChrom, Watrex, Prague, CZ, with a Nucleosil 120-C18 column) with a diode array detector (Model UV 6000 LP, Thermo Separation Products Inc., San Jose, CA) at 265 nm. The mobile phase was composed of methanol and demineralized water in 1:1 ratio with the addition of 0.1% (v/v) phosphoric acid. From acquired data, following standard parameters (Eqs. (1)–(3)) were calculated:

$$\text{Removal efficiency (RE):} \quad \text{RE} = \left(1 - \frac{C_{out}}{C_{in}}\right) \times 100 \ (\%) \tag{1}$$

$$\text{Degradation rate } (q): \quad q = \frac{F \times (C_{in} - C_{out})}{V_L} \ (\text{g L}^{-1}\ \text{d}^{-1}) \tag{2}$$

$$\text{Organic loading (OL):} \quad \text{OL} = \frac{C_{in} \times F}{V_L} \ (\text{g L}^{-1}\ \text{d}^{-1}) \tag{3}$$

where C_{in} and C_{out} are concentrations of 4-NP and 4-NT in the medium at the inlet and outlet of the reactor (g L^{-1}); F is the liquid medium flow rate (L d^{-1}); and V_L is the reactor's empty bed working volume.

3. Results and discussion

3.1. PBR experiments

To assess the mutual effect of 4-NP and 4-NT on their degradation, both compounds were at first treated in the PBR separately, then in a mixture, and their degradation characteristics were compared. When treated separately, 4-NP and 4-NT were degraded with the efficiency over 99 and 93% up to the loadings of 0.72 and 0.64 g L^{-1} d^{-1}, respectively (Fig. 2(a)). When degraded from their mixture, 4-NP was still completely removed from the medium until the overall load removed from the reactor reached 0.69 g L^{-1} d^{-1}, a value close to the maximal eliminated load by 4-NP as the sole source of carbon, suggesting the saturation of the PBR's elimination capacity. On the other hand, the 4-NT removal efficiency in 4-NP presence dropped by half and remained about the same (44 ± 6%) during the whole experiment regardless the increasing loading by both substrates (Fig. 2(b)).

For the microorganisms in the PBR, 4-NP was evidently a preferable substrate. Not only the presence of 4-NP suppressed the 4-NT degradation, but even after the reactor's loading was switched back from mixed to 4-NT loading, a long adaptation period was required to restore the cells' ability to efficiently degrade 4-NT as after 20 d 4-NT removal efficiency increased only by 20% (Fig. 3). This could be explained by the fact that, despite their structural similarity, each compound was degraded by a different metabolic pathway [18] and that 4-NP was able to induce its degradation in the present microorganisms stronger than 4-NT.

The PBR proved an efficient tool for the removal of relatively high organic loadings of 4-NP and 4-NT if each compound was treated separately. Although both compounds were also degraded simultaneously to some extent, a considerable portion of 4-NT (36–51%) remained in the media at the PBR outlet.

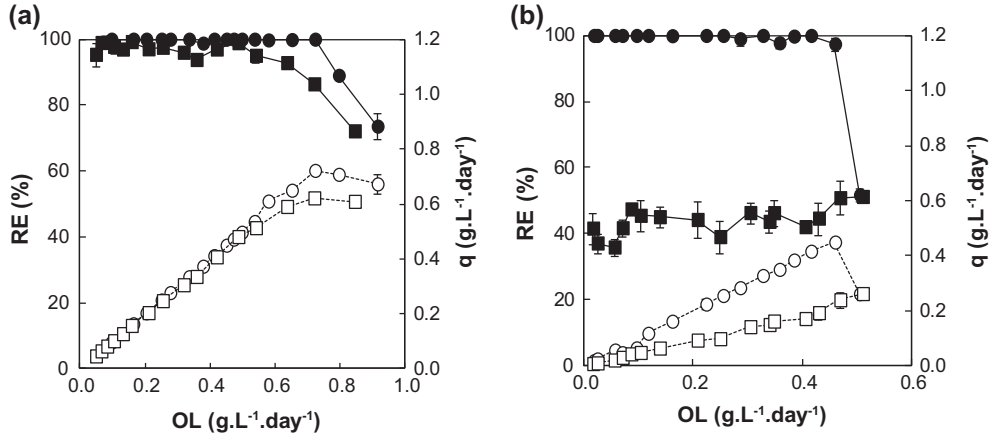

Fig. 2. Degradation of 4-NP and 4-NT in the PBR as single substrates (a) and as a mixture (b): (●) 4-NP removal efficiency, (■) 4-NT removal efficiency, (○) 4-NP degradation rate and (□) 4-NT degradation rate plotted as a function of organic loading (OL) by each compound.

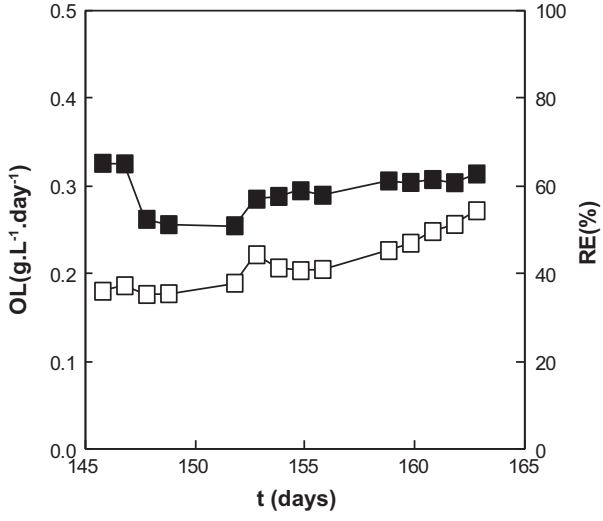

Fig. 3. Degradation of 4-NT after switching from mixed loading by 4-NP and 4-NT to loading by 4-NT: (■) 4-NT loading and (□) 4-NT removal efficiency.

Thus, for the complete detoxification of wastewater polluted by both 4-NP and 4-NT, a more efficient remediation technique would be needed.

3.2. Shake flask experiments

To gain a better insight into the interaction between 4-NP and 4-NT during their degradation, a series of shake flask experiments were performed with a mixed microbial culture isolated from the PBR at the end of 4-NT loading stage. The culture was cultivated for 3 weeks in the presence of either 4-NP/4-NT mixture or 4-NT and its ability to utilize both substrates was measured (Fig. 4). These results corroborated our finding that 4-NP was more easily degradable substrate and its presence negatively influenced 4-NT degradation. In all of the performed experiments, 4-NT degradation rate was distinctively lower than that of 4-NP. The highest 4-NT degradation rate was achieved when it was degraded as the only substrate and by 4-NT grown cells (Fig. 4(a)). If it was degraded from a mixture with 4-NP (Fig. 4) and/or by cells previously adapted to 4-NP and 4-NT (Fig. 4(b)), its degradation rate decreased by 60 and 40%, respectively. These results are in good agreement with the data obtained from the PBR experiments, where 4-NT degradation rate decreased in the presence of 4-NP by 50%.

To better understand the effect of substrate change and the ensuing culture adaptation on the degradation rate, the degradation experiments were then repeated after 10 and 21 weeks of cultivation in the presence of 4-NT and then again after 3 weeks of cultivation in the presence of 4-NP. The changes in degradation rates during 27 weeks of cultivation are summarized in Fig. 5. 4-NP was degraded faster than 4-NT by cells adapted to 4-NP, 4-NP/4-NT mixture, and even by cells shortly adapted to 4-NT, by which it was degraded without a visible lag phase (Fig. 4(b)).

The culture also showed much better adaptability to 4-NP than to 4-NT. After the switch from 4-NT feed to 4-NP feed, 4-NP quickly induced its degradation and its degradation rate increased 12 times in only 3 weeks, whereas 4-NT degradation rate increased only 1.7 times in the same time after the feed was switched from 4-NP/4-NT mixture to 4-NT (Fig. 5). It was only after 10 weeks of cultivation with 4-NT as the only substrate that the culture degraded 4-NT faster than 4-NP.

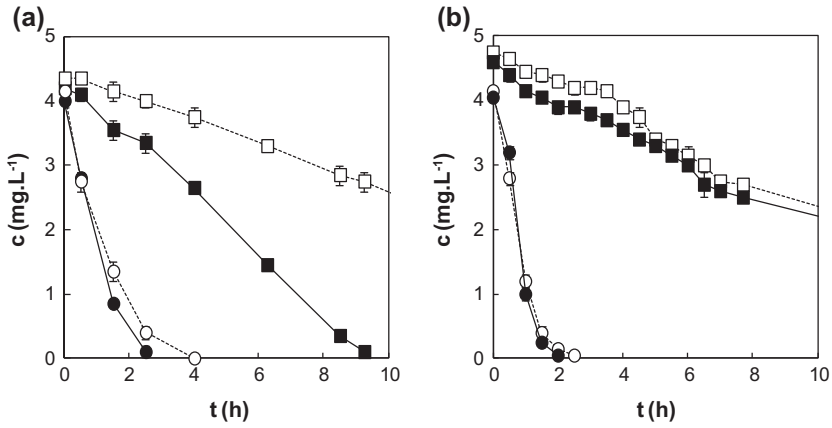

Fig. 4. Degradation of 4-NP and 4-NT in shake flasks by a microbial culture after 3 weeks of adaptation to (a) 4-NT, (b) 4-NP and 4-NT mixture: (●) 4-NP degradation as a single substrate, (■) 4-NT degradation as a single substrate, (○) 4-NP degradation from a mixture with **4-**NT and (□) 4-NT degradation from a mixture with 4-NP.

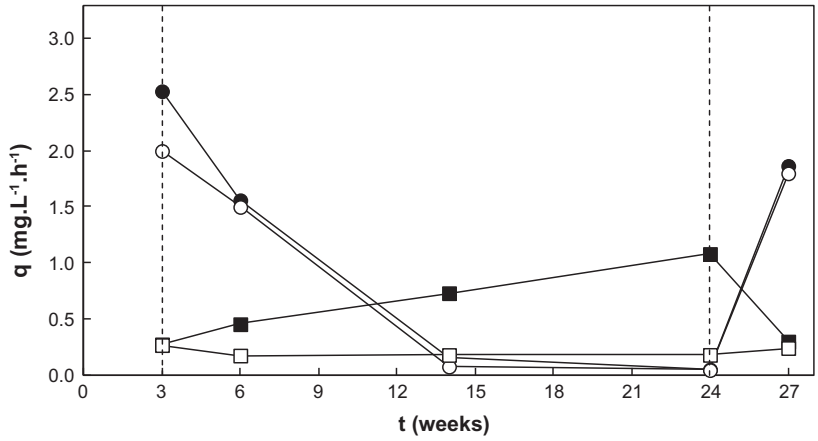

Fig. 5. Rate of 4-NP and 4-NT degradation in shake flasks by a microbial culture at various stages of adaptation: (●) 4-NP degraded as a single substrate, (■) 4-NT degraded as a single substrate, (○) 4-NP degraded from a mixture with 4-NT and (□) 4-NT degraded from a mixture with 4-NP.

Regardless the culture adaptation, 4-NT degradation was competitively inhibited by 4-NP presence, whereas 4-NP degradation did not seem to be directly affected by 4-NT as only minor differences in 4-NP degradation rate were observed when it was degraded separately or in the mixture (Figs. 4 and 5). However, the negative effect of 4-NP on 4-NT degradation cannot be ascribed solely to the competitive inhibition, as it occurred also indirectly on the adaptation level. If the culture was cultivated in the presence of either 4-NP or 4-NP/4-NT mixture prior to the experiment, 4-NT degradation was negatively affected even if 4-NP itself was not present in the reaction mixture. In fact, the rate of 4-NT degradation by such culture was as low as in the presence of 4-NP (Fig. 5).

3.3. Microbial analysis

Significant changes in the microbial composition were observed during the PBR operation (Table 1). Five bacterial species were identified in the mixed microbial culture isolated from DNPs degrading reactor which was used as the inoculum for the reactor used in this study. After 12 months of the reactor operation, the microbial composition within the reactor significantly changed, only one bacterium found in

Table 1
Microorganisms identified in PBR inoculum and biofilm after 12 months of operation

Reactor inoculum	Microbial composition in the reactor after 12 months of operation	Shake flask culture after 24 weeks of cultivation
Bacillus cereus	*Bacillus* sp.	*Bacillus toyonensis*
Achromobacter sp.	*Pseudomonas* sp.	*Pseudomonas nitrireducens*
Rhodococcus sp.	*Arthrobacter* sp.	*Rhodococcus wratislaviensis*
Ralstonia paucula	*Alicycliphilus* sp.	*Sphingobium yanoikuyae*
Chryseobacterium gleum		Unidentified bacterium

the inoculum (*Bacillus*) was still present in the biofilm. The observed shift in the microbial composition could be explained by the selective pressure of the different substrates—nitrophenols at the beginning and nitrotoluene at the end of the reactor operation.

The mixed microbial consortium isolated from the reactor biofilm after 12 month of operation was used in the shake flask experiments. After 24 weeks of cultivation, two bacterial genera from the biofilm sample (*Bacillus*, *Pseudomonas*) and two from the reactor inoculum (*Bacillus*, *Rhodococcus*) were detected in the shake flask culture. Two different species of the genus *Bacillus* were identified in the reactor inoculum (*Bacillus cereus*) and in the shake flask culture (*Bacillus toyonensis*). However, the letter belongs to the *B. cereus* group and was only recently classified as a separate species [19]. Therefore, it is probable that it was in fact the same bacterium surviving in the culture throughout the whole experiment.

4. Conclusion

Our results have shown that the presence of 4-NP negatively influenced 4-NT degradation by a mixed microbial culture. When 4-NT was degraded from a mixture with 4-NP and/or by cells previously adapted to this mixture, its degradation was suppressed in favor of 4-NP. Consequently, in the batch experiments, the complete 4-NT removal from the mixture was significantly prolonged, and in the continuously operated PBR, 4-NT removal efficiency did not exceed 55%, even at the lowest loading of the mixture. However, when 4-NT was degraded by 4-NT grown cells as the only carbon source, it was readily degraded at a rate comparable to that of 4-NP. Thus, assuming that the PBR removes all 4-NP from the mixture, it might be possible to degrade the rest of the 4-NT remaining in the medium in a separate serially connected reactor adapted solely to 4-NT. The use of such a two-stage PBR for the treatment of nitrophenol and nitrotoluene mixtures will be the focus of our future work.

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List of symbols

C_{in}, C_{out} — concentrations at the inlet, outlet of the reactor
F — liquid medium flow rate
OL — organic loading
q — degradation rate
RE — removal efficiency
V_L — reactor's empty bed working volume

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