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Aerobic biodegradation of 2,4-DNT and 2,6-DNT: Performance characteristics and biofilm composition changes in continuous packed-bed bioreactors

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ABSTRACT

This manuscript deals with continuous experiments for biodegradation of individual dinitrotoluenes by a defined mixed culture in packed-bed reactors (PBRs) containing either poraver or fire-clay as packing material. Removal efficiencies and volumetric biodegradation rates were measured as a function of the loading rate of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) under steady-state conditions. The poraver reactor showed higher removal efficiencies for both the DNTs. The removal efficiency for 2,4-DNT remained greater than 90% in the poraver reactor whereas it dropped steadily from 85 to 65% in the fire-clay reactor as the organic loading rates were increased from 19 to 60 mg L^{-1} day^{-1}. Similar trends were seen for the volumetric degradation rate as well. In both the reactors, 2,4-DNT degraded more effectively than 2,6-DNT. The microbial consortium was characterized both in the inoculum as well as in the operating PBR. Cell numbers per gram dry packing material were similar in the two reactors. However, there was a distinct difference in the nature of microorganisms that were found in the two packings. The fire-clay contained a larger number of cells that were not primary degraders of DNTs.

1. Introduction

2,4-DNT and 2,6-DNT are formed as intermediates in nitration of toluene during production of 2,4,6-trinitrotoluene (TNT). The DNTs are also intermediates in production of herbicides, dyes, and synthetic foams [1]. Extensive contaminations of soil and groundwater with DNTs are observed around their production and processing facilities. DNTs are US EPA priority pollutants [2] and discharges from their production facilities need to be controlled [3,4]. 2,4- and 2,6-DNT cause acute toxicity and are categorized as suspected carcinogens [5,6]. The reported 14-day LC_{50} for *Poecilia reticulata* (guppy) are 12.5 mg L^{-1} with 2,4-DNT and 18 mg L^{-1} with 2,6-DNT [7]. Permissible levels for 2,4- and 2,6-DNT for discharge streams are 0.32 and 0.55 mg L^{-1}, respectively (US wastewater treatment standards, 40 CFR, Section 268.48).

In the environment, the dinitrotoluenes are known to persist even though several microorganisms have been identified as possessing ability to degrade and even mineralize the individual DNTs [3,8]. As a result, ex situ biodegradation of DNT-contaminated soils and ground water has been pursued. Under appropriate operating conditions, the DNTs can serve as sole sources of carbon, nitrogen, and energy for the microorganisms [3,9], and even complete degradation of different nitrotoluenes when present together has been reported [10]. Several recent studies have shown that microorganisms demonstrate an ability to adapt to higher concentrations of DNTs [11,12]. For this reason, microorganisms isolated from local contaminated sites have been used in treatability studies.

In our laboratory, a mixed culture has been enriched from contaminated soil samples collected from a TNT production facility [13]. Pure cultures of bacterial strains capable of degrading nitrotoluenes were isolated from the mixed culture, and the ability of the pure strains and their defined mixed culture to degrade dinitrotoluenes individually and in a mixture was characterized [14]. In this paper, results of biodegradation of the individual dinitrotoluenes in continuously operated packed beds have been reported.

2. Materials and methods

2.1. Chemicals

2,4-Dinitrotoluene and 2,6-dinitrotoluene were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Other

Abbreviations: BSM, basal salt medium; CFM, colony forming microorganisms; DNT, dinitrotoluene; DOC, dissolved oxygen concentration; HRT, hydraulic retention time (day); OL, organic loading (mg L^{-1} day^{-1}); PBR, packed-bed reactor; RE, removal efficiency (%); SEM, scanning electron microscopy; STDEV, standard deviation; TNT, trinitrotoluene.

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Nomenclature

C_{in}	DNT concentration in influent medium (mg L^{-1})
C_{out}	DNT concentration in effluent medium (mg L^{-1})
F	flow rate of medium (L day^{-1})
n	number of data points
q_i	degradation rate of DNT (mg L^{-1} day^{-1})
V_L	reactor liquid volume (L)
x	data point
$\bar{x}$	arithmetic average

chemicals were from Lachema (Neratovice, Czech Republic). All the chemicals were analytical grade.

2.2. Microorganisms

Soil contaminated with a mixture of mononitrotoluenes, 2,4-DNT and 2,6-DNT was collected from a Czech Ammunition plant in Pardubice, Czech Republic, that has been in operation for 90 years. Originally a mixed culture was enriched from this contaminated soil by submerged cultivations. This suspension has been used for immobilization of expanded slate that served as the packing material of a packed-bed reactor (PBR). After 3 months of operation degrading a mixture of mono- and dinitrotoluenes, the biofilm was removed and the pure strains were isolated and identified as described in our earlier paper [14]. Eight Gram-negative (G^-) and one Gram-positive (G^+) bacterial strains were selected to make a defined mixed culture for the reactor inoculations in this study.

2.3. DNT degradation medium

A modified basal salt medium (BSM) containing 0.2 g L^{-1} KH_2PO_4 and 1 mL trace element solution per liter was used for preparation of inoculum. The trace element solution contained 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. Initial pH of the medium was set to 7.2. For continuous experiments, the medium with DNTs was stored in black opaque plastic containers to prevent light-mediated biodegradation.

2.4. Reactor

Two jacketed packed-bed reactors, both of 50 mm internal diameter and 300 mm bed height, were used for continuous degradation studies (Fig. 1). The PBRs were packed either with poraver or with fire-clay. The physico-chemical properties of both the packing materials used are shown in Table 1. Volume of liquid in the reactors, V_L, was measured with wet packing material as follows: Each packing material was placed into the bioreactor. Inlet tube at the bottom of the bioreactor was shut tight and the bioreactor was filled with water up to the outlet tube. Then the bottom inflow tube was opened and the actual water volume was measured using graduated cylinder (±0.001 L). The liquid volume in the reactor was measured to be 420 mL for fire-clay and 352 mL for poraver, respectively.

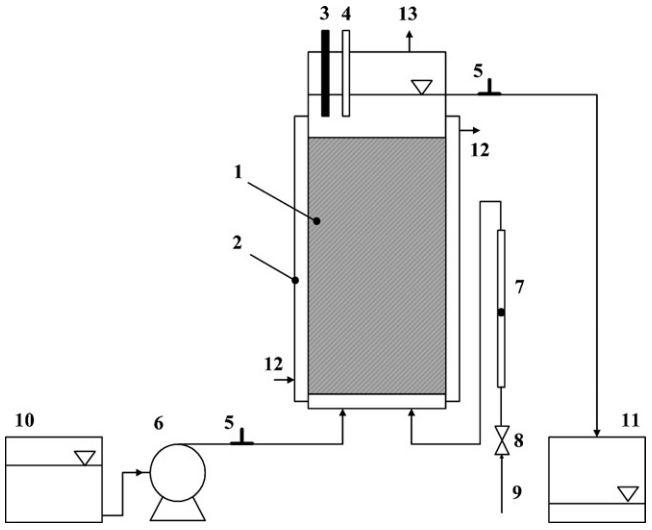

Fig. 1. Schematic of PBR. (1) Packing, (2) tempering jacket, (3) pH electrode, (4) combined electrode for DOC and temperature, (5) sampling port, (6) membrane pump, (7) rotameter, (8) needle valve, (9) air inlet (non-sterile), (10) contaminated water, (11) purified water, (12) in/outlet of tempering water, and (13) gas outlet.

Table 2
Bacteria in the inoculum

G^- bacteria	
Pseudomonas putida A1	Short rods
Pseudomonas veronii B1	Rods
Pseudomonas sp. C1	Rods
Chryseobacterium sp. D1	Rods
Stenotrophomonas maltophilia D2	Rods
Sphingobacterium multivorum	Rods
Sphingomonas sp. PCN 3	Rods
G^+ bacteria	
Paenibacillus glucanolyticus D1/B	Rods

Aluminum foil was wrapped over the jacket to prevent light-mediated biodegradation of DNTs. For the same reason, all the tubing to transport medium to the reactor was also made of opaque silicon rubber. The mixed culture was immobilized on the packing materials by circulating the cells suspended in BSM without any carbon and energy source through the reactors for 24 h. The inoculum composition is shown in Table 2. Both reactors were operated in cocurrent up-flow mode with aqueous phase containing the contaminants and air at 30 °C. pH of the medium at column inlet was 7.2. Concentrations of the individual DNTs in the feed stream to the packed-bed reactor were 8, 15, 25, 35 and 45 mg L^{-1} and these

Table 1
Physico-chemical properties of packing materials used in bioreactors

Packing material	Porosity	Bulk density (kg m^{-3})	Particle size (mm)	pH of extract	Average representation of major components
Poraver	0.385	188	4–8	9.5	SiO_2 ~ 69% Na_2O ~ 16% CaO ~ 10% MgO ~ 2% Al_2O_3 ~ 2%
Fire-clay	0.437	979	4–8	7	SiO_2 ~ 54% Al_2O_3 ~ 38% Fe_2O_3 ~ 3%

were increased in the order presented above. The dissolved oxygen concentration (DOC) in PBR effluent was maintained at >4 mg L^{-1} by manipulating airflow rate to the reactor. The hydraulic retention time was maintained at 10 h.

2.5. Analytical methods

All samples were centrifuged at 24,600 × *g* for 10 min before analysis (HEREAUS, Kendro Laboratory Products, Germany). Sample analyses were conducted by HPLC (System DeltaChrom, Watrex Prague Ltd., Prague, Czech Republic) using 250 mm × 4 mm Nucleosil, 120-5 C18 column (WATREX, Watrex Praha, s.r.o. Czech Republic) in temperature-controlled chamber at 30 °C and a mobile phase of methanol/water (50:50) at a flow rate of 1 mL min^{-1}. Peaks were detected by measuring absorbance at 230 and 268 nm with a diode array detector (Model UV 6000 LP, Thermo Separation Products Inc., San Jose, CA, USA). Under these conditions, retention times of 2,4-DNT and 2,6-DNT were 16 and 18 min, respectively.

pH of the medium was measured continuously in the outflow from the PBRs using pH meter, model MFD 77 (INSA Corp., Czech Republic). Actual values of pH were stored in MFD 77 each hour and downloaded to computer once a week using INSACom software.

An oxygen probe (Type CSOT44LL, INSA Corp.) was used to measure dissolved oxygen concentration in the effluent from PBR. Actual values of DOC were stored each hour and downloaded to computer once each week.

2.6. Microbial analyses

Experiments for the degradation of dinitrotoluenes in the reactors lasted 8 months. On day 81 of these degradation tests, packing and biofilm samples were collected from a depth of 5 cm from the top of PBRs for estimation of cell numbers and the scanning electron microscopy (SEM). The same procedure was repeated after 8 months of the reactors performance for identification of the strains present in the biofilm. The cells of biofilms, immobilized on the packing material, were suspended in BSM by vigorous mixing before any microbial analysis. Identification of the individual strains in biofilm was performed using biochemical tests (Gram-staining and morphology of colonies and free cells; hydrolysis of gelatin, esculine, ONPG, DNA, amylum, Tween 80, casein and lecithin; production of acid from glucose, cellobiose, xylose, mannitol, lactose, fructose and inositol; reduction of nitrate and nitrite; growth in presence of 6.5% NaCl, growth at 37 °C; tests of enzymes: urease, arginine, dihydrolase, catalase, hemolysis). Fungi were identified by their morphological characteristics by the shape of their fructification organs when grown on the Rose-Bengal Chloramphenicol agar base CM 549 with selective supplement SR 78.

Cultivations were carried out on the following selective solid agar media in order to determine the total cell numbers, the number of eukaryotic cells, the Pseudomonads, and the primary DNT degraders present in biofilm: Standard Plate Count agar CM 0463, Rose-Bengal Chloramphenicol agar base CM 549 with selective supplement SR 78, Pseudomonas agar base CM 559 with C–N supplement SR 102, and BSM agar [Agar Bacteriological (#1) LP0011 plus salts] (all from Oxoid Ltd., England). Individual cell colonies on the selective solid agar media were counted as colony forming microorganisms (CFM).

2.7. Electron microscopy

Samples of the packing material with the biofilm were used for scanning electron microscopy. Each sample was critical-point-dried using the following procedure: fixation in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 4 h at 4 °C, dehydration in a graded series of 50% ethanol solution, 70% ethanol solution, 96% ethanol solution and 100% ethanol, followed by drying in a desiccator. Samples were coated with gold and observed under electron microscope (Hitachi S 4700).

2.8. Calculations

The degradation rate q_i of DNTs in the packed-bed reactor:

$$q_i = \frac{F(C_{in} - C_{out})}{V_L} \,(\text{mg L}^{-1}\,\text{day}^{-1})$$

The removal efficiencies (RE) of individual DNTs:

$$\text{RE} = \left(1 - \frac{C_{out}}{C_{in}}\right) \cdot 100\,(\%)$$

Hydraulic retention time (HRT):

$$\text{HRT} = \frac{V_L}{F}\,(\text{day})$$

Organic load (OL):

$$\text{OL} = \frac{C_{in} \cdot F}{V_L}\,(\text{mg L}^{-1}\,\text{day}^{-1})$$

where q_i is the degradation rate of the individual DNT; C_{in} is the DNT concentration in the medium influent (mg L^{-1}); C_{out} is the DNT concentration in the medium effluent (mg L^{-1}); F is the flow rate of the medium (L day^{-1}); V_L is the reactor liquid volume (L). Statistics (standard deviation):

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

where $\bar{x}$ is the sample mean and n is the sample size.

3. Results and discussion

The ability of the mixed culture to degrade 2,4-dinitrotoluene and 2,6-dinitrotoluene was investigated in continuous degradation experiments in PBRs. In each experiment, five feed concentrations of 2,4-dinitrotoluene and 2,6-dinitrotoluene (8, 15, 25, 35 and 45 mg L^{-1}) were used. The concentrations were increased gradually from the lowest to the highest while the hydraulic retention time was kept constant (0.42 day or 10 h). Under the operating conditions, the feed concentrations corresponded to organic loading rates (OL) of approximately 19, 36, 60, 84 and 108 mg L^{-1} day^{-1}, respectively.

Control experiments in uninoculated packed beds wrapped in aluminum foil showed no significant adsorption of DNTs on the packed-bed particles and on the column wall.

3.1. Start-up of PBRs

All the packed-bed reactors were loaded with cells by circulating cells suspended in the basal salt medium (without any carbon and energy source) for 24 h. During the start-up period, solutions containing 8 mg L^{-1} DNT (2,4- or 2,6-) were pumped through the beds in single-pass mode and concentrations of DNTs were monitored in the outlet stream. For the reactor packed with poraver, complete degradation (removal efficiency RE > 97%) of 2,4-DNT was observed after 11 days of acclimation. On the other hand, the packed bed using fire-clay as the packing material achieved

a removal efficiency of only 78% even after 20 days of acclimation. During all these periods, trace amounts of metabolites 2-amino-4-nitrotoluene and 2,4-diaminotoluene were observed in the outlet stream, and no other metabolite was detected. Since 2,4-diaminotoluene is thought to be a "dead-end" metabolite of anaerobic biotransformation of 2,4-DNT, either the biofilms had only few anaerobic pockets or the bacteria present in the consortium were able to transform this metabolite, as discovered by Freedman et al. [15]. For columns fed with 2,4-DNT, steady state was achieved after 20 days of acclimation; feed concentration was then gradually increased.

With PBRs receiving a solution of 8 mg L^{-1} 2,6-DNT, steady-state operation was achieved after 11 days of acclimation and the removal efficiencies achieved were 96% for the column packed with poraver and 75% for fire-clay packed column. Thus the start-up period for fire-clay PBR receiving 2,6-dinitrotoluene was about 10 days shorter than when the fire-clay PBR was fed with 2,4-dinitrotoluene. It is unclear why it was so, but it could potentially be due to differences in adaptation response to dinitrotoluenes by the cells in biofilm (induced enzymes such as oxygenases, dioxygenases, and nitroreductases). Again, only trace quantities of 2-amino-6-nitrotoluene were detected at the reactor outlet.

Bradley and Chapelle [16] and Funk et al. [17] found that complete nitrotoluene degradation occurs under a combination of aerobic and anaerobic conditions. Such conditions could possibly exist in the packed-bed reactors used in this study. Reductive degradation of nitrotoluenes occurs via formation of amino derivates (2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,4-diaminotoluene, 2-amino-6-nitrotoluene, 6-amino-2-nitrotoluene and 2,6-diaminotoluene). In our experiments, these reductive intermediates were not detected. As indicated earlier, potential reasons for the absence of the reductive intermediates can be either due to lack of their formation or due to their degradation in the upper regions of the reactor bed.

3.2. Loading characteristics

After the acclimation phases described above, the feed concentration of each DNT was gradually increased over the next 3 months from 8 to 45 mg L^{-1}. The hydraulic retention time was kept constant at 10 h.

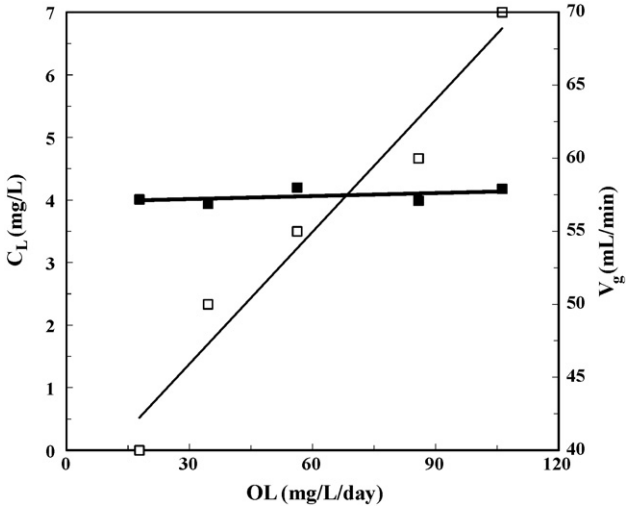

Fig. 2. Effluent stream dissolved oxygen concentration and air flow rates in steady-state poraver reactor fed with 2,4-DNT; (□) V_g, (■) C_L.

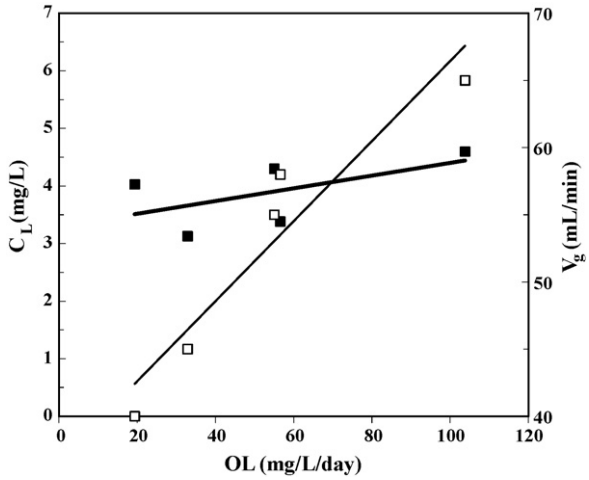

Fig. 3. Effluent stream dissolved oxygen concentration and air flow rates in steady-state fire-clay reactor fed with 2,4-DNT; (□) V_g, (■) C_L.

More or less stable dissolved oxygen concentration in the outlet stream could be achieved by controlling the air flow rate with a simultaneous adjustment of medium flow rate (to keep the hydraulic retention time constant). For both the poraver and fire-clay packed reactors, the volumetric flow rate of air required to maintain a dissolved oxygen concentration above 4 mg L^{-1} in the liquid stream at reactor exit have been plotted against the loading rate in Figs. 2 and 3. The fire-clay reactors had a similar trend behavior with respect to air demand in order to maintain dissolved oxygen concentration above 4 mg L^{-1}. Clearly the cells have higher oxygen demand with increasing inlet concentration of dinitrotoluenes. Theoretical estimates suggest that 1.58 mg O_2 is needed for complete oxidation of 1 mg of DNT including formation of nitrate for all the nitrogen in the compound. This corresponds to a maximum oxygen demand of 101.5 mg $O_2{}^{-1}$ L^{-1} day^{-1} in the poraver PBR, which should be easily met by oxygen transfer from air in the PBRs. In our studies, however, no nitrate was observed in any of the exit streams from packed-bed reactors. This could be due to facultative anaerobes such as *Pseudomonas* strains (identified in our biofilms, see Tables 3 and 4) using nitrate for respiration or other microorganisms using nitrate simply as nitrogen source.

Lendenman et al. [10] tested the degradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in a fluidized bed bioreactor. They maintained dissolved oxygen concentration at 4.5 mg L^{-1}. Dinitrotoluenes served as the only carbon, energy and nitrogen sources (as in our study). With the organic load ranging from 36 to 600 mg L^{-1} day^{-1} and with retention times ranging from

Table 3
Identification of the strains in the biofilm from fire-clay reactor after 8 months of continuous operation

G^- bacteria	
Sphingomonas sp.	Rods
Chryseobacterium indologenes untyp.	Rods
Pseudomonas sp.	Rods
G^+ bacteria	
Bacillus cereus	Rods
1× unidentified	Rods
1× unidentified	Rods
Fungi	
Cryptococcus humicolus	Yeast
Pichia guilliermondii	Yeast
Haplosporangium	
Stachybotris	

Table 4
Identification of the strains in the biofilm from poraver reactor after 8 months of continuous operation

G⁻ bacteria	
1× unidentified	Rods
Brevundimonas sp.	Rods
Achromobacter xylosoxidans ssp. *denitrificans*	Rods
Pseudomonas aeruginosa	Rods
G⁺ bacterium	
Bacillus cereus	Rods

0.75 to 12.5 h, they achieved removal efficiencies of 98% (2,4-dinitrotoluene) and 94% (2,6-dinitrotoluene). They also did not find any transformation of DNTs to the reduction metabolites.

pH values of the outlet stream depended very little on the type of packing; pH did not seem to possess any correlation with the rate of biodegradation or with the DNT.

3.2.1. Loading with 2,4-DNT

Removal efficiencies and degradation rates of 2,4-DNT in the poraver and the fire-clay PBRs are shown in Figs. 4 and 5, respectively, as a function of organic loading rate (OL). For the poraver-packed reactor (Fig. 4), the degradation rate steadily increased to 64 mg L^{-1} day^{-1} as the organic loading rate was increased to 108 mg L^{-1} day^{-1}. The removal efficiency remained in excess of 97% up to loading rates of 60 mg L^{-1} day^{-1} and then it gradually decreased to 60% at loading rate of 108 mg L^{-1} day^{-1}.

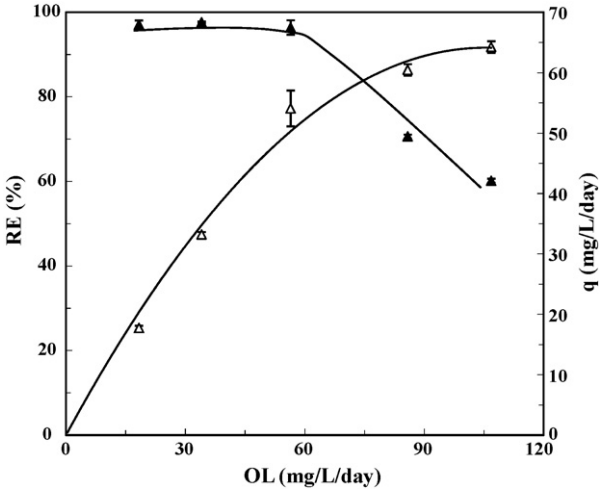

Fig. 4. Performance characteristics for 2,4-DNT in the poraver reactor. Error bars are standard deviations ($n = 3$); (△) q (2,4-DNT), (▲) RE (2,4-DNT).

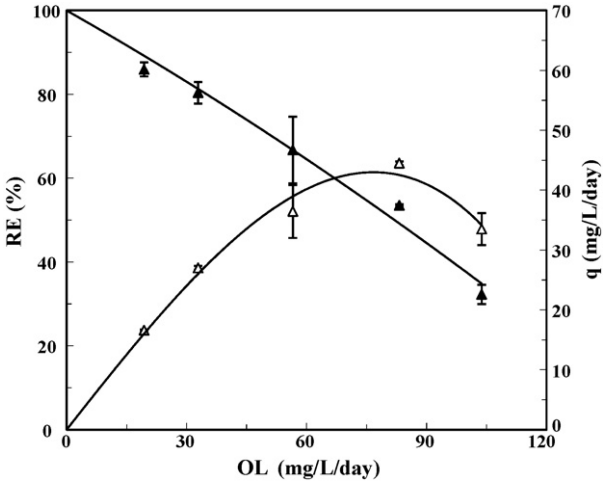

Fig. 5. Performance characteristics for 2,4-DNT in the fire-clay reactor. Error bars are standard deviations ($n = 3$); (△) q (2,4-DNT), (▲) RE (2,4-DNT).

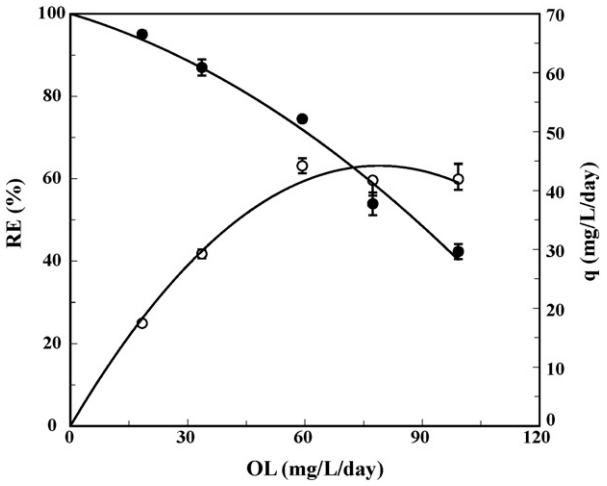

Fig. 6. Performance characteristics for 2,6-DNT in the poraver reactor. Error bars are standard deviations ($n = 3$); (○) q (2,6-DNT), (●) RE (2,6-DNT).

The fire-clay PBR (Fig. 5) was not as efficient as the poraver PBR; the removal efficiency in it was below 90% even at the smallest loading rate studied and it dropped steadily to 32% at the highest loading rate. The highest degradation rate in the poraver PBR was 45 mg L^{-1} day^{-1} at the loading rate of 84 mg L^{-1} day^{-1} (corresponding to the inlet concentration of 35 mg L^{-1}). The degradation rate dropped with further increase in loading rate.

3.2.2. Loading with 2,6-DNT

The performance characteristics of 2,6-DNT-fed PBRs are shown in Figs. 6 and 7. In these experiments, the inlet concentration was increased from 8 to 15 mg L^{-1} already after 10 days of start-up as a steady state was achieved sooner than in reactors fed with 2,4-DNT. The maximum degradation rates of 2,6-DNT were smaller than those of 2,4-DNT in the similar reactors. In the poraver reactor, the degradation rate of 2,6-DNT increased with loading rate to 44.2 mg L^{-1} day^{-1} at a loading rate of 60 mg L^{-1} day^{-1} correspond-

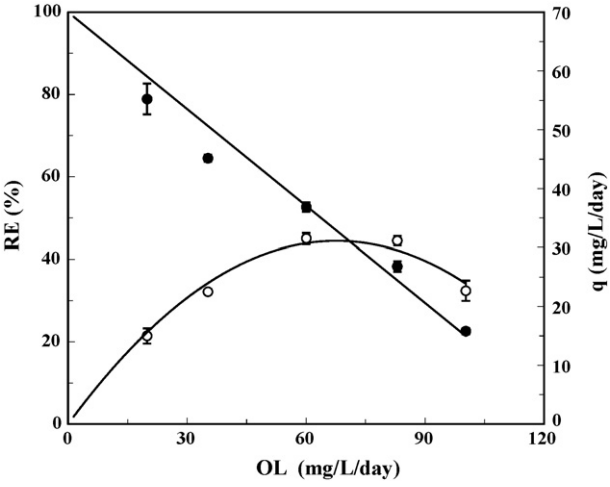

Fig. 7. Performance characteristics for 2,6-DNT in the fire-clay reactor. Error bars are standard deviations ($n = 3$); (○) q (2,6-DNT), (●) RE (2,6-DNT).

Table 5
Comparison of the maximum degradation rates and other performance/operational characteristics in the two reactors

Packing	Poraver	Fire-clay
2,4-DNT		
Acclimation period (days)	11	20
Degradation rate ($mg\,L^{-1}\,day^{-1}$)	64.2 ± 1.2	44.5 ± 0.3
RE (%)	60	52
Loading rate ($mg\,L^{-1}\,day^{-1}$)	108	84
2,6-DNT		
Acclimation period (days)	11	11
Degradation rate ($mg\,L^{-1}\,day^{-1}$)	44.2 ± 1.6	31.5 ± 1.5
RE (%)	74	52
Loading rate ($mg\,L^{-1}\,day^{-1}$)	60	60–84

ing to feed concentration of $35\,mg\,L^{-1}$ (Fig. 6). On the other hand, a maximum degradation rate of only $31.5\,mg\,L^{-1}\,day^{-1}$ could be achieved in the fire-clay reactor (Fig. 7).

The maximum degradation rates of DNTs and the corresponding performance and operational characteristics for the two packing materials used are presented in Table 5. For 2,4-dinitrotoluene, removal efficiencies remained in excess of 97% with poraver packing at loading rates up to $60\,mg\,L^{-1}\,day^{-1}$. For 2,6-DNT, removal efficiencies above 90% were seen only at the lowest volumetric loading rates. The same general behavior was observed for the fire-clay packing. The populations that evolved in the two PBRs exhibited inhibition from both the substrates although it is also clear that 2,4-DNT was not as strongly toxic as 2,6-DNT.

3.3. Microscopic observations

Electron micrographs of the biofilm collected from the packing materials on day 81 are shown in Figs. 8 and 9. The bacterial cells appear to be trapped in some extracellular polymeric material. The fire-clay and poraver packing materials differ in their external surfaces as can be seen by the crevices present in Fig. 8 for fire-clay. In spite of almost 3 months of the biofilter operation, particle surfaces were still not completely and homogenously covered by biofilm.

3.4. Microbial analyses

Microbial make-up represents an important biological characterization of biofilms. This was done by identifying the individual strains and estimation of cell numbers in biofilm samples collected from the two reactors. Several techniques have been used by researchers for the estimation of cell numbers and their identification in microbial films [18,19]. In our experiments we used classical and the most common techniques (see Section 2). The results have been presented in Tables 2–4 and 6. The total number of cultivable cells in day-81 samples was slightly higher in biofilms from fire-clay reactor than in the poraver biofilm. The same was also true for the total count of degraders and Pseudomonads. Fungal cells were present in the fire-clay biofilm, but none were found in biofilm from poraver reactor (even at 10^2 dilution). The absence of eukaryotic cells in the poraver could be due to uncultivable species or they simply were not present in biofilm.

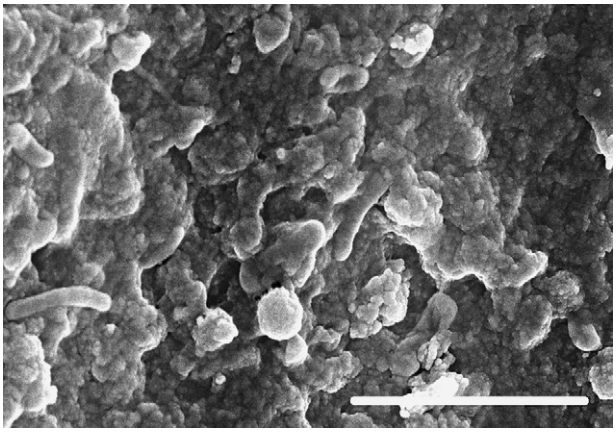

Fig. 8. SEM of the biofilm surface from the fire-clay reactor on day 81. Bar is 5.00 μm.

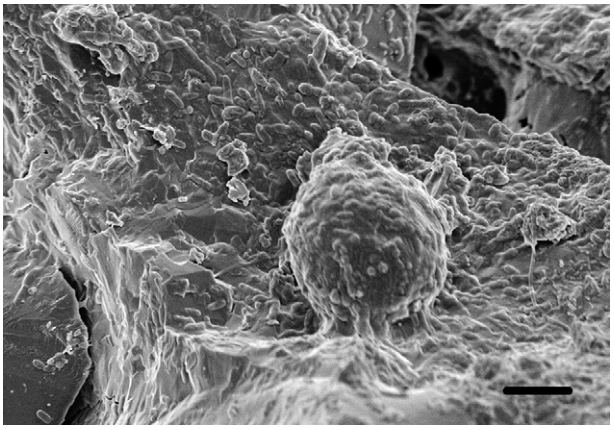

Fig. 9. SEM of the biofilm surface from the poraver reactor on day 81. Bar is 10.00 μm.

Details of microbial species present in the packing materials after 8 months of continuous reactor operation are presented in Tables 3 and 4. Two yeasts and two fungi were found in the fire-clay bioreactor after 8 months of operation, but none of them were primary degraders. Apparently, these species were able to tolerate the presence of nitrotoluenes and utilized some nitrotoluene metabolites produced by other bacterial species.

A comparison of populations in Tables 3 and 4 with those in Table 2 shows a large difference between the initial population and those after 8 months of the bioreactor operation. In fire-clay bioreactor only three species from the original inoculum were identified after 8 months and two G^+ species were not identified at all.

Species similar to those found here have been utilized by others for biodegradation of nitrophenols. Zablotowicz et al. [20] used *Sphingomonas* UG 30 for 2,4-dinitrophenol degradation. Leung et al. [21] used the same strain for 4-nitrophenol degradation. *Pseudomonas* sp. has also been used for 2,4-dinitrotoluene degradation by Spanggord et al. [8] and by Rhys-Williams et al. [22] for 4-nitrotoluene degradation. All these experiments were carried out under aerobic conditions.

In spite of a slightly lower value of the total cell number in the poraver reactor compared to the fire-clay reactor (Table 6), degradation rates of both 2,4-DNT and 2,6-DNT were higher in the poraver reactor (Table 5). An explanation follows from the differences in biofilm compositions in the two reactors after 8 months of operation. *Pseudomonas aeruginosa* is an active DNT degrader [23] but the other three identified bacteria (Table 4) have not been mentioned in published literature as DNT degraders yet. In addition, no fungal

Table 6
Analyses of the immobilized mixed cultures in biofilm samples collected on day 81

	CFM ($\#\,g^{-1}$ packing dry)	
	Fire-clay	Poraver
Total cell number	$(120.0 \pm 8.5) \times 10^6$	$(94.0 \pm 3.3) \times 10^6$
Degraders	$(97.0 \pm 2.6) \times 10^6$	$(68.0 \pm 8.6) \times 10^6$
Pseudomonas	$(46.0 \pm 5.4) \times 10^6$	$(36.0 \pm 5.4) \times 10^6$
Fungi	$(22.0 \pm 5.4) \times 10^5$	0

cells were detected in the poraver reactor. This can be due to more alkaline properties of this packing material (cf. Table 1).

Our results support the findings of Nishino et al. [24] that bacterial species capable of growth on a mixture of 2,4- and 2,6-DNT can be obtained by long-term selection in bioreactors that were fed mixtures of these substrates as the sole C-, N-, energy source [25].

4. Conclusions

Packed-bed reactors can be used for continuous aerobic biodegradation of 2,4-DNT and 2,6-DNT in liquid streams. A well adapted mixed culture, isolated from sites contaminated with nitroaromatics, was used successfully to inoculate packed-bed bioreactors for biodegradation of nitrotoluenes and dinitrotoluene concentrations up to 45 mg L^{-1}. Dinitrotoluenes can be satisfactorily degraded for a long period in the columns having fluid retention time of 10 h. We found that poraver is a better packing material for dinitrotoluene biodegradation in comparison to fire-clay. Using a mixed microbial culture as the catalyst, 2,4-DNT could be degraded more easily than 2,6-DNT.

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