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Simultaneous Biodegradation of 2,4-Dinitrotoluene and 2,6-Dinitrotoluene in an Aerobic Fluidized-Bed Biofilm Reactor

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Mixtures of 2,4- and 2,6-dinitrotoluene are produced in large quantities as precursors of polyurethane foams and the explosive 2,4,6-trinitrotoluene (TNT). The two isomers are widely distributed in contaminated groundwater and soil. Bacteria capable of growing with the individual isomers as the sole source of carbon, nitrogen, and energy have been isolated previously. However, attempts to degrade 2,4- and 2,6-DNT simultaneously have failed. We tested the hypothesis that a mixed culture could degrade an isomeric DNT mixture if the bacteria were grown in an aerobic biofilm at low substrate concentrations. Such conditions were achieved with a fluidized-bed biofilm reactor (FBBR). The reactor was fed aqueous solutions containing 2,4- (40 mg L^{-1}) and 2,6-DNT (10 mg L^{-1}). The feed flow rate was gradually increased to yield surface loading rates of 36−600 mg of DNT m^{-2} d^{-1}. Removal efficiencies higher than 98% for 2,4-DNT and 94% for 2,6-DNT were achieved at all loading rates. The nitrogen released from DNT was found quantitatively as nitrate that indicated the presence of nitrite-oxidizing bacteria. COD measurements of the reactor effluent confirmed complete mineralization of the DNT. The results demonstrate that mixtures of 2,4- and 2,6-DNT can be biodegraded successfully in an aerobic FBBR.

Introduction

Dinitrotoluenes are intermediates in the production of the explosive trinitrotoluene and precursors of toluene diisocyanate used in the manufacture of polyurethane foams (*1*). Typically, synthesis yields 76% 2,4-DNT, 19% 2,6-DNT, and small amounts of other isomers (*2*). The production of TNT required large amounts of water for the purification of the product. Therefore, ammunition plants were often located near large groundwater reservoirs (*3*). Improper disposal practices associated with TNT manufacturing have resulted in contamination of soils and waters with dinitrotoluenes. They are usually found in surface water and ground water in the area of former ammunition plants (*4*−*6*). Traces of DNT have been found in the rivers Elbe (*5*) and Rhine (*7*).

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2,4- and 2,6-DNT exhibit acute toxicity and low level carcinogenicity (*8*, *9*). The 14-day LC50 for guppy (*Poecilia reticulata*) of 2,4-DNT (69 μM, 12.5 mg L^{-1}) and of 2,6-DNT (98 μM, 18 mg L^{-1}) indicate aquatic toxicity at low concentrations (*10*). Both isomers are listed as U.S. EPA priority pollutants (*11*). EPA treatment standards are 0.32 mg L^{-1} for 2,4-DNT and 0.55 mg L^{-1} for 2,6-DNT (40 CFR, Section 268.48). Because of the hazards DNT and TNT along with their transformation products pose for drinking water supplies, large efforts have been undertaken mainly in the United States and in Germany to understand the fate of these compounds in the environment (*4*, *6*, *12*). Extensive research has focused on the microbial transformation of nitroaromatic compounds (*13*−*15*) and on how contaminated sites could be cleaned up using bioremediation (*16*). Removal of 2,4-DNT, 2,6-DNT, and 2,4,6-TNT by microbial cultures has been reported. However, whether the compounds were biodegraded or only transformed remains unclear because several unidentified metabolites were found along with aromatic amines (*13*). In water samples of the New River, downstream of Radford Army Ammunition Plant, VA, mixed microbial populations capable of degrading 2,4- and 2,6-DNT were detected (*17*). The authors isolated a pure culture able to degrade 2,4-DNT but not 2,6-DNT as the sole carbon source.

In our laboratory, bacterial strains capable of degrading 2,4-DNT were isolated and characterized, and a degradation pathway has been proposed (*18*−*20*). One of these strains [*Pseudomonas* sp. DNT (*18*)] successfully degraded 2,4-DNT in a fixed-bed bioreactor at a hydraulic residence time of 1 h (*21*). Recently, we isolated strains able to use 2,6-DNT as the sole growth substrate (*22*). However, 2,6-DNT at concentrations higher than 20 mg L^{-1} inhibited growth of both 2,4- and 2,6-DNT degrading strains. Therefore, the degradation of isomeric mixtures was not successful in batch cultures where initial DNT concentrations were high.

In a continuous growth system, e.g., a chemostat, the substrate concentration in the culture liquid can be kept very low as compared to that in the feed (*23*). If the concentrations of 2,4- and 2,6-DNT can be kept at subtoxic levels, a mixed culture should be able to degrade 2,4- and 2,6-DNT simultaneously. Therefore, we set out to test whether water contaminated with a DNT mixture could be treated with a continuous process. Because of the low specific growth rates of the DNT-degrading organisms, a FBBR was chosen for this study. Cells growing in a biofilm are protected from system washout. Therefore, a large volumetric biomass concentration is retained in a FBBR. Sand was selected as the carrier material because it does not sorb DNT and thereby allows for a rigorous mass balance. A mixed enrichment culture selected for the simultaneous degradation of 2,4- and 2,6-DNT served as inoculum. DNT removal was quantified over a wide range of 2,4- and 2,6-DNT loading rates. The system survived shock loads and temporary starvation with little perturbation. The results clearly showed the applicability of a FBBR for large-scale treatment of DNT-contaminated water and provided the engineering parameters for a pilot-scale study at the Volunteer Army Ammunition Plant in Chattanooga, TN.

Materials and Methods

Chemicals. 2,4-Dinitrotoluene, 2,6-dinitrotoluene, 2,4-diaminotoluene, 2,6-diaminotoluene, 2-amino-4-nitrotoluene, 2-amino-6-nitrotoluene, and 4-amino-2-nitrotoluene were obtained from Aldrich (Milwaukee, WI).

Bacterial Culture. A mixed culture simultaneously degrading 2,4- and 2,6-DNT was obtained by chemostat

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TABLE 1. Biomass Concentration in FBBR[a]

	FBBR biofilm			FBBR effluent			
HRT (h)	COD (mg g^{-1})	protein (mg g^{-1})	ratio (protein COD^{-1})	COD (mg L^{-1})	protein (mg L^{-1})	ratio (protein COD^{-1})	shear loss rate[b] (h^{-1})
12.5	0.85 ± 0.14	0.18 ± 0.03	0.21	18 ± 2.0	6.5 ± 0.2	0.36	0.0034
6.3	0.95 ± 0.03	0.27 ± 0.02	0.29	17 ± 1.5	5.5 ± 0.1	0.32	0.0059
3.1	2.22 ± 0.11	0.65 ± 0.08	0.29	22 ± 1.2	4.3 ± 0.9	0.19	0.0064
1.5	3.11 ± 0.07	0.73 ± 0.09	0.24	17 ± 2.0	5.2 ± 0.1	0.31	0.0074
0.75	5.03 ± 0.77	1.57 ± 0.29	0.31	22 ± 2.0	6.3 ± 0.1	0.29	0.0118

[a] The biofilm was quantified as COD and protein per unit of sand. [b] The biofilm shear loss rate was estimated as follows: $b_s = (COD_{eff} \cdot F)/(COD_s M_s)$ where COD_{eff} is the effluent COD concentration (column 5), F is the feed flow rate, COD_s is the the biofilm COD concentration (column 2), and M_s is the mass of sand in the FBBR.

enrichment using a mineral salt medium (*24*) supplied with 2,4-DNT (81 mg L^{-1}), 2,6-DNT (18 mg L^{-1}), and 2,4,6-TNT (1 mg L^{-1}). TNT was included to ensure that only strains tolerating its presence at low concentrations would be selected. Cells were grown in a microcarrier spinner flask (Bellco, Vineland, NJ) with a culture volume of 250 mL equipped for chemostat operation. The dilution rate was increased from 0.005 to 0.05 h^{-1} over a period of 60 days. The inoculum to the chemostat included filtrate (0.8 μm) of a batch enrichment culture on 2,4- and 2,6-DNT containing contaminated soil collected from several Army ammunition plants and 2,4- and 2,6-DNT degrading strains previously isolated in our laboratory (*22*).

Fluidized-Bed Reactor. A 1.5 L volume water-jacketed fluidized-bed reactor vessel with an inner diameter of 5.2 cm (Bioengineering, Wald ZH, Switzerland) was filled with 0.74 kg of acid-washed Ottawa sand (diameter 0.425−0.595 mm). The estimated sand surface area was 3.3 m^2. The conical part at the bottom of the reactor was filled with 3 mm stainless steel balls to facilitate flow distribution. Temperature in the reactor was maintained at 20 °C. pH was controlled to 7 ± 0.1 by automatic addition of NaOH/KOH (1 M each) and phosphoric acid (10% v/v). Dissolved oxygen (DO) concentration at the top of the reactor bed was monitored using a Ingold DO electrode (Ingold, Wilmington, MA) and maintained higher than 4.5 mg L^{-1}. Aeration was provided using a peristaltic pump delivering air to the recirculation line. The recirculation flow through the bed was maintained with a centrifugal pump (March Pumpen GmbH, Germany) at 1.5−1.6 L min^{-1} as measured with an in-line flowmeter (Gilmont Instruments, Barrington, IL) resulting in approximately 40% bed expansion. Because of the low biofilm coverage (Table 1), no adjustment of the recirculation flow was necessary. The fluidized-bed reactor was operated at hydraulic retention times (HRT) of 12.5, 6.3, 3.1, 1.5, and 0.75 h in turn. For each HRT, steady state was assumed to be achieved when the concentrations of 2,4- and 2,6-DNT in the effluent varied less than 20% over a period of 3 days. Feed and recirculation lines were of stainless steel and glass. The feed was prepared in 150-L batches in stainless steel barrels and, unless stated otherwise, consisted of 2,4-DNT (40 mg L^{-1}), 2,6-DNT (10 mg L^{-1}), and H_3PO_4 (70 mg L^{-1}) in tap water. Nitrate and nitrite in the tap water were below the detection limit, and ammonia was below 0.1 mg L^{-1} as determined with a Model 95-12 ammonia electrode (Orion Research Inc., Boston, MA). The feed solution was delivered to the FBBR recirculation line with a peristaltic pump.

DNT Analysis. Liquid samples (700 μL) were withdrawn from the top of the reactor and mixed with 300 μL of methanol in a microcentrifuge vial. Solids were removed by centrifugation at 14 000 rpm for 2 min, and the supernatant was analyzed on a Hewlett-Packard 1050 HPLC system equipped with a variable wavelength UV detector (Hewlett-Packard, Wilmington, DE). Separation of 2,4-DNT and 2,6-DNT was achieved on a Spherisorb Hexyl (C_6) column (Alltech, Deerfield, IL). A water:methanol mixture (70:30) was used as eluent at a flow rate of 1 mL min^{-1}. Compounds were detected by UV absorbance at 254 nm and quantified by comparison with external standards.

Transformation Products. 2-Amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2-amino-6-nitrotoluene, 2,4-diaminotoluene, and 2,6-diaminotoluene in the reactor liquid were analyzed on a Hewlett-Packard 1050 HPLC system equipped with a diode array detector. Separation was achieved on a Spherisorb Octyl (C8) column (Alltech, Deerfield, IL). Compounds were eluted in a linear gradient of water (60 to 40%) acidified with trifluoroacetic acid (0.1% v/v) and acetonitrile (40−60%) at a flow rate of 1 mL min^{-1}. Samples were prepared as described above for DNT analysis.

Nitrite and Nitrate Analysis. Samples were centrifuged at 14 000 rpm for 2 min, and the supernatant was analyzed with a Dionex DX-300 Series Chromatography System equipped with a Dionex AS11 column and CDM-2 conductivity detector (Dionex, Sunnyvale, CA). The eluent was 19 mM NaOH at a flow rate of 0.65 mL min^{-1}.

Biomass Determination. Biofilm concentration in the FBBR was measured as chemical oxygen demand (COD) or protein per mass of sand. Samples were removed from the bed and directly added to COD vials (HACH, Loveland, CO) with additional water to a final volume of 2 mL and measured along with standards according to manufacturer's procedure. No COD was detected in control samples containing clean sand. To determine the weight of the sand, the supernatant was discarded, and the sand was rinsed five times with deionized water, dried at 102 °C overnight, and weighed. For protein measurements, samples of bed material were brought to a final liquid volume of 1.0 mL with water, and 1 mL of BCA protein reagent was added (Pierce, Rockford, IL). To dissolve the biofilm, samples were vortexed and sonicated repeatedly during the 30-min incubation time at 30 °C. The liquid in the vials was centrifuged at 14 000 rpm for 2 min to precipitate quartz fines produced during sonication. The absorbance of the supernatant fluid was measured at 562 nm. The sand was weighed as described above. Effluent COD and protein concentrations were measured with HACH COD vials and the BCA protein assay.

FBBR Load-Shift Experiments. When the FBBR attained steady state, it was subjected to short-term (4-h) shifts in the applied surface loading by instantaneously increasing or decreasing the feed flow rate. After 4 h, the reactor was returned to steady-state flow rate for at least 8 h before the next transient load experiment.

Results and Discussion

Steady-State Performance. Figure 1 shows the flow rates, influent, and effluent concentrations of 2,4- and 2,6 DNT of the FBBR during 4 months of uninterrupted operation. Constant feed concentrations of 2,4-DNT (35.7 ± 3.3 mg L^{-1}) and 2,6-DNT (10 ± 0.4 mg L^{-1}) were maintained for the majority of the operation. Only two short episodes of unstable reactor performance occurred. During the period

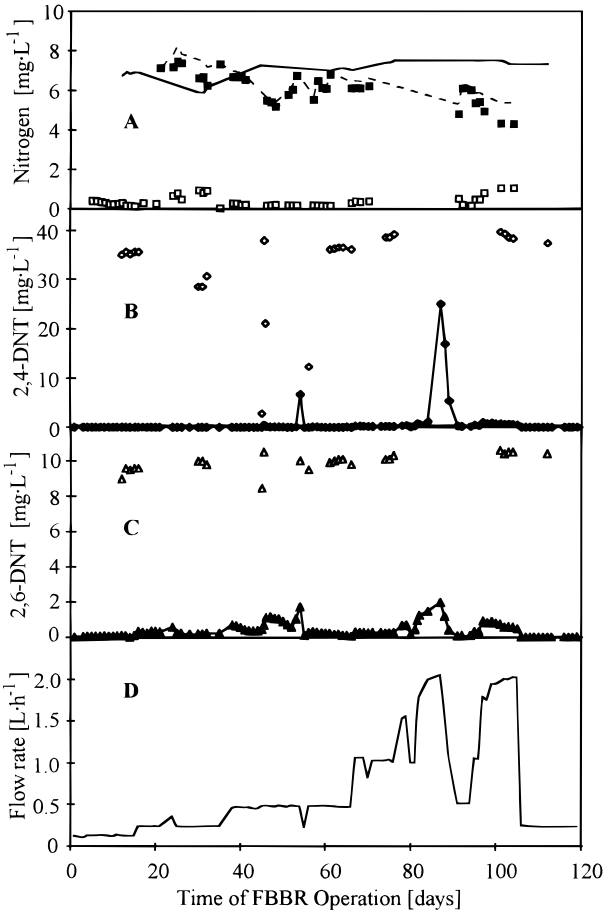

FIGURE 1. Time course of FBBR operation. (A) (□) effluent nitrite, (■) effluent nitrate, (—) DNT−nitrogen (estimated from feed concentrations), (---) recovered nitrite + nitrate. All concentrations of nitrogen compounds are given in mg(N) L^{-1}. (B) (◊) influent, (♦) effluent concentrations of 2,4-DNT. (C) (△) influent, (▲) effluent concentrations of 2,6-DNT. (D) (—) flow rate.

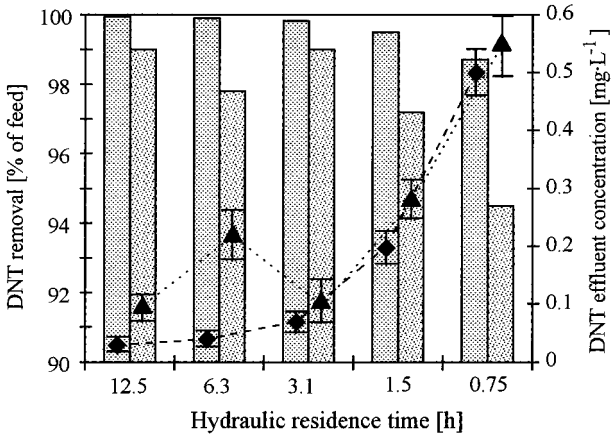

FIGURE 2. Summary of steady state FBBR performance in terms of percentage removal (bars) and effluent concentrations of (♦) 2,4-DNT and (▲) 2,6-DNT.

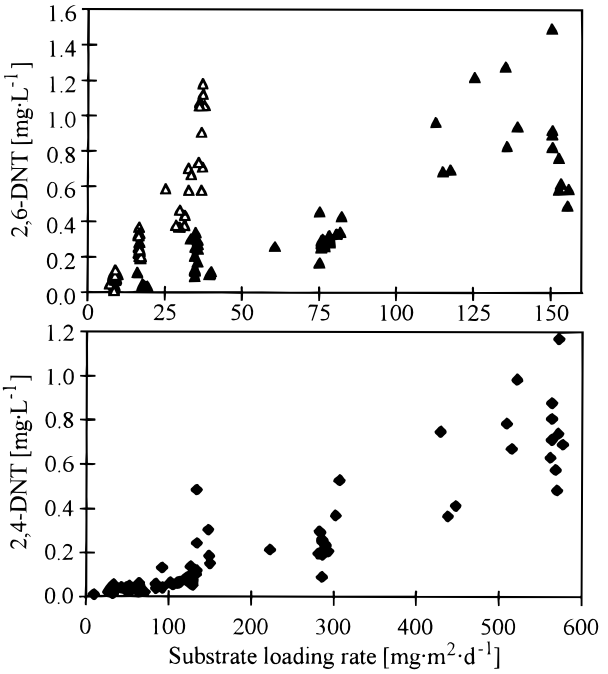

FIGURE 3. DNT effluent concentrations as a function of the applied surface loading rate. (♦), 2,4-DNT; (△), 2,6-DNT before day 53; (▲), 2,6-DNT after day 53. The loading rate scales are such that 2,4- and 2,6-DNT data points in the upper and lower panel correspond to the same time points.

from day 40 through day 57, significant microbial contamination of the feed reservoir resulted twice in lowered influent concentrations to the reactor. Therefore, the DNT medium was acidified to pH 4 with phosphoric acid to prevent growth in the reservoir and feeding lines for the remainder of the experiments. An accidental settling of the bed resulted in significant biofilm washout during resuspension on day 53. A second reactor upset occurred between day 78 and day 98. Flow rates were increased to 1500 mL h^{-1} on day 78, but on day 86 an interruption of aeration and recirculation caused the reactor bed to settle, which resulted in significant biomass washout upon resuspension. This biofilm loss caused temporary breakthrough of DNT (Figure 1). Subsequently, flow rates were decreased until the culture recovered. The performance usually recovered within 4 days after a biofilm loss occurred. Figure 2 summarizes the steady-state performance of the fluidized-bed reactor.

The dependency of the effluent DNT concentration on the loading rate can best be seen when it is plotted as a function of the applied surface loading rates, *J*, i.e., the mass of DNT applied per unit of biofilm surface area per unit of time. The applied surface loading was calculated as

$$J = \frac{FS_0}{M_s(a/m)}$$

where F is the feed flow rate (L d^{-1}), S_0 is the DNT concentration in the influent (mg L^{-1}), M_s is the total mass of sand in the reactor (g), and *(a/m)* is the surface area per unit mass of sand (m^2 g^{-1}). 2,4-DNT effluent concentrations rose in an approximately linear fashion with increased applied surface loading rates (Figure 3). The data indicate that the FBBR was never operated in a true low load region, where increases in the load do not impact the effluent concentration (*25*). Steady-state effluent concentrations could be achieved with loading rates up to 500 mg m^{-2} d^{-1} of 2,4-DNT. Between day 50 and day 60, the 2,6-DNT effluent concentration dropped from 1.1 to 0.15 mg L^{-1} (Figure 1). After this adaptation process, removal of 2,6-DNT remained at a higher efficiency. The change in the performance of 2,6-DNT removal may have been due to the selection of bacteria able to degrade both isomers simultaneously. Bacteria isolated from the FBBR before day 53 were mostly able to degrade either 2,4- or 2,6-DNT, whereas toward the end of the operation, bacteria able to degrade both compounds became predominant (*26*).

TABLE 2. Effluent Pseudo-Steady-State Concentrations with Standard Deviations (±) of 2,4- and 2,6-DNT during the Flow-Shift Experiments for Each Steady-State HRT[a]

flow rate (mL h^{-1})	steady-state HRT (h)									
	12.5	6.3	3.1	1.5	0.75	12.5	6.3	3.1	1.5	0.75
	2,4-DNT effluent concentrations (μg L^{-1})					2,6-DNT effluent concentrations (μg L^{-1})				
27 ± 1	5 ± 1					19 ± 2				
58 ± 1	11 ± 1	6 ± 1				45 ± 2	43 ± 3			
128 ± 1	**37 ± 8**	17 ± 1				**101 ± 16**	98 ± 4			
238 ± 8	59 ± 4	**38 ± 4**	18 ± 1			209 ± 11	**218 ± 20**	82 ± 1		
483 ± 8	331 ± 21	116 ± 5	**71 ± 11**	79 ± 1	74 ± 1	no s-s	605 ± 11	**164 ± 61**	102 ± 3	104 ± 36
696 ± 9		289 ± 13	160 ± 2				no s-s	222 ± 2		
1010 ± 17			320 ± 2	**215 ± 20**	217 ± 4			219 ± 3	**279 ± 24**	271 ± 5
2029 ± 40				997 ± 56	**639 ± 97**				756 ± 23	**644 ± 12**
3000 ± 30				no s-s	1737 ± 23				no s-s	1543 ± 6
4000 ± 30					3940 ± 392					1813 ± 64

[a] The average effluent concentrations at the steady-state HRT prior to the load shifts are typed in bold face. The feed contained 2,4-DNT (35.7 ± 3.3 mg L^{-1}) and 2,6-DNT (10 ± 0.4 mg L^{-1}). The HRT is the ratio of the reactor liquid volume to the flow rate. Load-shifts that did not reach pseudo-steady-state are indicated by no s-s.

Biofilm Quantification. As measured by COD or protein, the biofilm increase from the first to the second steady state was small but became very apparent with the subsequent increases in the applied DNT load (Table 1). The protein: COD ratio of the biofilm was 0.27 ± 0.04 mg protein mg of $(COD)^{-1}$. The same ratio (0.29 ± 0.6) was found in the effluent liquid (Table 1). This indicates that the COD measured in the effluent derives from biomass sheared off the biofilm and not from substrate transformation products dissolved in the medium.

The relatively constant values of the COD_{eff} (19.3 ± 2.5 mg L^{-1}) and protein (5.6 ± 0.9 mg L^{-1}) concentrations in the effluent (Table 1) indicate that the balance between biofilm growth and shear off was stable at all steady states. Because the effluent COD derived mainly from biomass, the biofilm growth yield and the biofilm shear loss rate could be calculated. The yield (0.42 ± 0.06 mg of (COD) mg of $(DNT)^{-1}$], i.e., the effluent COD per DNT consumed, was constant at all HRTs. The shear loss rate (Table 1) increased about three times as compared to the 16-fold increase of the loading and dilution rate. This indicated that the higher loading rates resulted not only in an increased biofilm concentration but also in an increased bacterial growth and shearing rate.

Load-Shift Experiments. The short-term shift experiments allowed measurement of the effluent concentrations over a wide range of loading rates at constant biofilm concentration. During the 4-h shift experiments, the biofilm did not grow significantly. One set of load-shift experiments is illustrated in Figure 4. For all load-shift experiments, the pseudo-steady-state concentrations are listed in Table 2 along with the corresponding steady-state data. The response in effluent concentrations was very rapid, and pseudo-steady-states were typically obtained within 1 h after shift-up and in less than 10 min after shift-downs. The FBBR was able to respond immediately to doublings of the applied loading rate. However, shifts to three times the steady-state flow rate usually resulted in accumulation of 2,6-DNT. For each compound, moving from the left to the right through Table 2 at a given flow rate, lower effluent concentrations are measured. This reflects the increased biofilm concentrations (Table 1) and removal capacities at higher steady-state loading rates.

Flow Interruption Experiments. Biological treatment systems often experience discontinuous loads of the compounds to be degraded. Hence, optimal performance requires that the culture retains the ability to degrade the desired compound even when it is temporarily absent from the waste stream. In liquid culture, 2,4- and 2,6-DNT-degrading bacteria exhibit considerable lag periods prior to growth. Therefore, we tested during the 6-h HRT steady state whether the FBBR culture would retain the ability to degrade DNT after feed interruption. After a flow stop of 4 h, transient accumulation of 2,4- and 2,6-DNT was not observed when the flow was resumed (Figure 5). Similar results were observed for flow interruptions of 1 and 16 h. Hence, the culture retained the ability to immediately resume DNT degradation for at least 16 h.

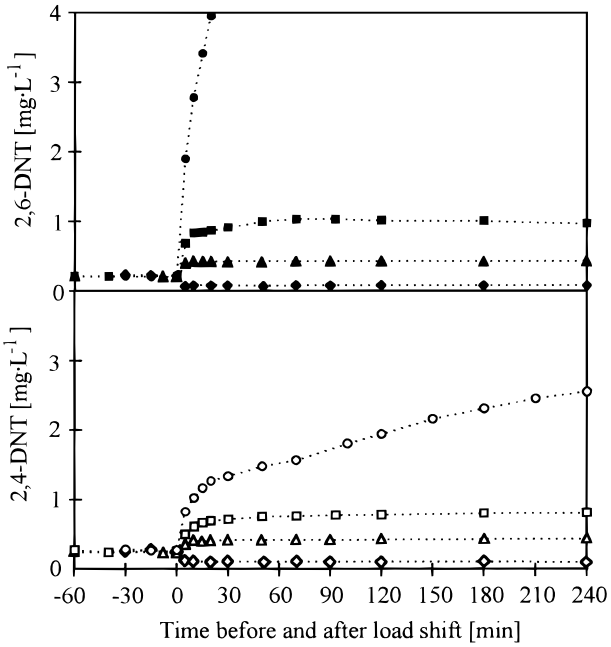

FIGURE 4. Effluent concentrations of 2,4- and 2,6-DNT during load shift experiments for the 1.5-h HRT steady state. The steady-state flow rate was 1.0 L h^{-1}. Shift flow rates: (○, ●) 3.0; (□, ■) 2.0 ; (△, ▲), 1.5; (♦, ◇) 0.48 L h^{-1}.

Effect of Feed Concentration on FBBR Performance. Biofilm reactor theory predicts that the effluent substrate concentration is determined by the surface loading rate and not by the substrate concentration in the reactor influent (*25*). In order to test whether DNT degradation in the FBBR was stable at low feed concentrations and high flow rates, we compared DNT effluent and reactor biofilm concentrations at a constant surface loading rate applied either at a long HRT (6 h) and a high DNT feed concentration (40 mg L^{-1} 2,4-DNT and 10 mg L^{-1} 2,6-DNT) or at a short HRT (0.6 h) and a low DNT feed concentration (4 mg L^{-1} 2,4-DNT and

TABLE 3. Comparison of FBBR Performance at a Constant Loading Rate Applied as Low Flow, High Concentration and High Flow, Low Concentration

	influent concn (mg L^{-1})		effluent concn (μg L^{-1})		Biofilm [mg (g^{-1} of sand)]	
HRT (h)	2.4-DNT	2,6-DNT	2,4-DNT	2,6-DNT	COD	protein
6.0	37.3 ± 0.7	10.0 ± 0.5	45 ± 7	40 ± 5	2.3 ± 0.3	0.47 ± 0.04
0.61	3.9 ± 0.1	1.06 ± 0.01	47 ± 6	43 ± 3	2.4 ± 0.4	0.51 ± 0.04

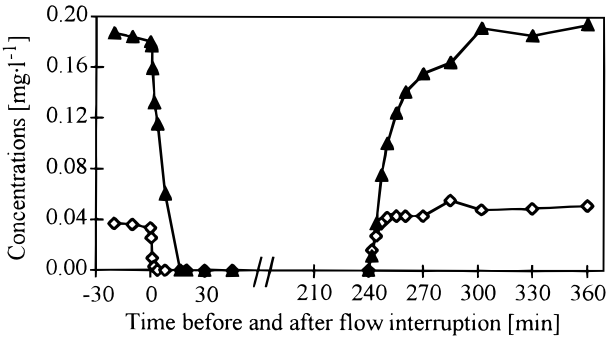

FIGURE 5. Concentrations of (◊) 2,4-DNT and (▲) 2,6-DNT during a 4-h flow interruption at a HRT of 6 h. The flow was stopped at time 0 and resumed at 240 min.

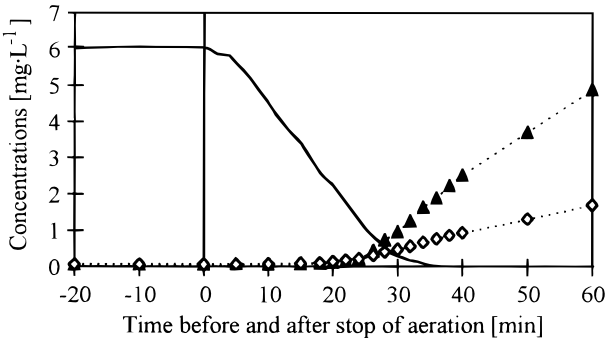

FIGURE 6. Effluent concentrations of (◊) 2,4-DNT and (▲) 2,6-DNT during oxygen depletion (—) at a HRT of 3.1 h.

1 mg L^{-1} 2,6-DNT). Both operations yielded identical surface loading rates of approximately 68 mg m^{-2} d^{-1} 2,4-DNT and 19 mg m^{-2} d^{-1} 2,6-DNT. First the 6-h HRT, steady state was established and then the feed was switched to the low DNT concentrations, and the flow rate was increased 10 times. The biofilm concentration after 3 days and the DNT effluent concentrations after 3, 4, and 5 days remained unchanged (Table 3). Hence, the FBBR is able to successfully degrade DNT in contaminated water at both low and high influent concentrations.

Oxygen Requirement. The oxygen requirement was measured at an HRT of 3.1 h. The aeration was decreased stepwise until incomplete DNT removal was observed in the reactor. At each step the oxygen concentration was allowed to adjust until 30 min of steady state was observed prior to sampling the reactor for DNT concentration measurement. The measurements were repeated during subsequent stepwise increases in the aeration. From 7.8 down to 3.8 mg L^{-1} dissolved oxygen (DO) concentration, the effluent concentrations of 2,4- and 2,6-DNT remained constant at 63 ± 7 and 61 ± 7 μg L^{-1}, respectively. Below 3.8 mg L^{-1} DO, DNT concentrations started to increase and reached 166 ± 10 and 369 ± 21 μg L^{-1}, respectively, at a DO concentration of 0.5 mg L^{-1}. Consequently, the dissolved oxygen concentration should be maintained above 1 mg L^{-1} for satisfactory aerobic degradation of DNT at this loading rate.

When the air supply to the FBBR was interrupted, DNT accumulated as soon as the dissolved oxygen concentration was less than 2 mg L^{-1} (Figure 6). From the dissolved oxygen concentration in the influent (7.8 ± 0.6 mg L^{-1}) and the linear part of the depletion curve, the oxygen consumption rate was estimated to be 15.4 ± 1.8 mg L^{-1} h^{-1} (0.48 ± 0.06 mM h^{-1}) from triplicate experiments. The combined consumption rate of 2,4- and 2,6-DNT was 14.3 ± 0.7 mg L^{-1} h^{-1} (0.079 ± 0.004 mM h^{-1}). Hence the culture requires 1.07 ± 0.3 mg of O_2 (mg of DNT)$^{-1}$, which corresponds to 6.1 ± 0.8 mol of oxygen/mol of DNT.

Nitrogen Balance. The effluent concentrations of the examined nitrogen species are illustrated in Figure 1. The majority of the effluent nitrogen was nitrate-N, although in the pathways of oxidative 2,4-DNT degradation, the nitro groups are liberated as nitrite (*18*, *19*, *22*). The results indicate that nitrite-oxidizing bacteria were present in the bioreactor. This is not surprising since oxidation of nitrite to nitrate by *Nitrobacter agilis* has been found in airlift bioreactors (*27*) and oxidation of ammonium and nitrite to nitrate has been reported in fluidized sand bed reactors (*28*).

DNT was the sole source of nitrogen for bacterial growth. This allowed us to compare the total effluent nitrite and nitrate-N to the DNT-N, assuming stoichiometric release of all nitro groups. During the 12.5, 6.3, and 3.1 h HRT operations more than 90% of the nitrogen originating from DNT was recovered as nitrite or nitrate (Figure 1). Because cell synthesis of the bacteria growing in the biofilm consumed some of the nitrogen, it can be concluded that the nitrogen was stoichiometrically released from DNT.

The effluent N was significantly lower than the stoichiometric value only at the highest two applied loading rates. This may have been due to a higher nitrogen requirement of the FBBR culture. The biofilm shear loss rate (Table 1) indicated that the culture was growing faster at the shorter HRTs. Typically the required C:N ratio for bacterial growth decreases with increasing growth rate. For example, carbon-limited growth of *Klebsiella pneumoniae* required a C:N ratio of 10.6 g g^{-1} at a specific growth rate of 0.05 h^{-1} whereas at 0.8 h^{-1} this ratio is 5.4 (*29*).

Oxygen Balance. The fraction of the growth substrate that was not incorporated into biomass was oxidized to CO_2 and must have consumed an equivalent amount of O_2. The theoretical oxygen demand required for complete mineralization of DNT can be estimated from the following equations.

$$C_7H_6N_2O_4 + 8O_2 \rightarrow 7CO_2 + 2H_2O + 2NO_2^- + 2H^+$$

In the FBBR, however, nitrite is subsequently oxidized to nitrate.

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$

Hence, 9 mol of oxygen is required for complete mineralization of 1 mol of DNT, which corresponds to 1.58 mg of O_2 (mg of DNT)$^{-1}$. Adding the COD of the biomass formed [0.42 ± 0.06 mg of COD (mg of DNT)$^{-1}$, see above] to the oxygen consumed by the FBBR [1.07 ± 0.03 mg of O_2 (mg of DNT)$^{-1}$, see above] yields 1.49 ± 0.07 mg of O_2 (mg of DNT)$^{-1}$. This value is approximately the same as the 1.58 mg of O_2 (mg of DNT)$^{-1}$ that is theoretically required for total mineralization of DNT. This closed mass balance on the oxygen demand

provides further evidence for the complete degradation of DNT in the FBBR.

Transformation Products. Under both aerobic and anaerobic conditions, microbial transformation of nitroaromatic compounds often yields reduction products (*30*, *31*). For example in a continuous culture with ethanol as primary substrate, 2,4-DNT was anaerobically transformed to 2,4-diaminotoluene via 2-amino-4-nitrotoluene and 4-amino-2−nitrotoluene (*32*). In a biofilm, cell lysis products could serve as electron donors for nitro group reduction. Therefore, we tested whether the expected reduction intermediates of 2,4- and 2,6-DNT, i.e., 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,4-diaminotoluene, 2-amino-6-nitrotoluene, and 2,6-diaminotoluene, could be found in the effluent of the fluidized bed reactor. An effluent sample from day 82 was analyzed by HPLC. The concentration of all expected reduction products was below the detection limit of approximately 10 μg L^{-1}.

Test with Authentic Groundwater. When it became clear that 2,4- and 2,6-DNT could be degraded simultaneously in tap water, a 50-gal barrel of contaminated groundwater was obtained from Volunteer Army Ammunition Plant (Chattanooga, TN). It contained 2,4-DNT (2.1 mg L^{-1}), 2,6-DNT (1.6 mg L^{-1}), 2-nitrotoluene (2.5 mg L^{-1}), and 4-nitrotoluene (1.7 mg L^{-1}). This water was stored at 4 °C for 2 months until it was fed to the FBBR at an HRT of 6 h under identical conditions as above. Initially, 2- and 4-nitrotoluene were not degraded satisfactorily because the FBBR culture was only adapted to 2,4- and 2,6-DNT. Nevertheless, after 1 week, 2,4-DNT, 2,6-DNT, 2-nitrotoluene, and 4-nitrotoluene were degraded at 98, 76, 83, and 86%, respectively. Subsequently, the HRT was reduced to 3 h, and the above compounds were degraded at 99.6, 62, 98, and 99.5%, respectively, after 10 days although steady state was not yet achieved. No addition of nutrients was necessary for the operation of the FBBR with authentic groundwater. However, small amounts of phosphoric acid were added by the pH control system.

In summary, the bench-scale FBBR effectively removed 2,4- and 2,6-DNT at all hydraulic residence times. Breakthrough of DNT was only observed as a consequence of mechanical failures that caused biofilm loss. More stable performance might be achieved using a carrier material allowing internal colonization since bed settlement would no longer cause major biofilm loss. Additionally, the ability to handle shock loads could be improved by using a carrier material able to sorb dinitrotoluene as suggested by results on the degradation of BTX (benzenes, toluenes, and xylenes) in an FBBR (*33*). The results clearly demonstrate that aerobic microorganims can degrade 2,4- and 2,6-DNT simultaneously if their concentration can be kept below inhibitory levels.

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