Environ. Sci. Technol. **1998**, *32,* 1956−1963

Biological Treatment of TNT-Contaminated Soil. 1. Anaerobic Cometabolic Reduction and Interaction of TNT and Metabolites with Soil Components

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The explosive 2,4,6-trinitrotoluene (TNT), found as a major contaminant at armament plants from the two world wars, is reduced by a variety of microorganisms when electron donors such as glucose are added. This study shows that the cometabolic reduction of TNT to 2,4,6-triaminotoluene by an undefined anaerobic consortium increased considerably with increasing TNT concentrations and decreased with decreasing concentrations and feeding rates of glucose. The interactions of TNT and its reduction products with montmorillonitic clay and humic acids were investigated in abiotic adsorption experiments and during the microbial reduction of TNT. The results indicate that reduction products of TNT particularly hydroxylaminodinitrotoluenes and 2,4,6-triaminotoluene bind irreversibly to soil components, which would prevent or prolong mineralization of the contaminants. Irreversible binding also hinders a further spread of the contaminants through soil or leaching into the groundwater.

Introduction

2,4,6-Trinitrotoluene (TNT), first synthesized by Willbrand in 1865, was for many years the major explosive for charges and bombs. TNT is produced by the stepwise nitration of toluene through nitric and sulfuric acid with mononitrotoluene and dinitrotoluene as intermediates. During World War II (*1*), Germany produced approximately 800 000 ton of TNT. Half a century after the end of TNT production, TNT and assoziated contaminants are still present at high concentrations and have migrated in the water supplies of neighboring communities (*2*). Due to the toxicity, the mutagenic, and the carcinogenic potential of TNT and particularly of its congeners (*3*), there is an urgent need for remediation techniques to clean up these sites.

As an alternative to incineration of TNT-contaminated soil, microbiologists have investigated the biodegradation of TNT. As yet, significant mineralization of TNT has not been demonstrated by defined bacterial strains. This can be explained by the rareness of nitroaromatic and particularly polynitroaromatic compounds in nature and the resistance of the highly oxidized trinitro substituted aromatic ring to oxidative microbial attack (*4*). In contrast, reduction of the nitro groups of TNT has often been observed in human beings (*5*, *6*), rats and rabbits (*6*), plants (*7*), and particularly in cultures of fungi (*8*−*11*) and bacteria (*12*−*21*). The ease with which TNT reacts with nucleophiles and reducing agents explains the large variety of secondary products found in soil and the pronounced misrouting observed in enrichment cultures provided with TNT as substrate or cosubstrate. Another mechanism of TNT transformation was reported by Vorbeck et al., who demonstrated a reducing enzyme system that is responsible for the addition of one or two hydride ions to the aromatic nucleus (*21*). Even in fungi containing lignin peroxidases the initial enzymatic reaction of TNT is the reduction of a nitro group. In contrast to the studies with defined bacterial strains, certain TNT mineralization has been shown with fungi (*9*−*11*, *22*) and in soil (*23*).

As in most biological reductions, the transformation of aromatic nitro groups into amino groups is considered to take place by the triple transfer of two reduction equivalents [2 H], thus leading to nitroso, hydroxylamino, and finally amino groups. Figure 1 (I) schematically shows the specific case of the reduction of an aromatic nitro group during the fermentation of glucose. Reduction equivalents from anaerobic glucose oxidation usually give rise to reduced fermentation products; however, when nitroaromatic compounds are present, some of these reduction equivalents are transferred to the aromatic nitro groups. According to Preuss et al. (*14*), complete reduction of all three nitro groups of TNT (Figure 1, II) can only be achieved under strict anaerobic conditions. And as well, all partially reduced nitroaromatic compounds that were generated by aerobic or semianaerobic transformations of TNT and also present at contaminated sites are converted into 2,4,6-triminotoluene (TAT) only under strict anaerobic conditions. Figure 1 (II) also shows 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-4,6-dinitrotoluene (2-HADNT/4-HADNT), 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (2-ADNT/4-ADNT), and 2,4-diamino-6-nitrotoluene (2,4-DANT), the major detectable intermediates of TNT reduction by the anaerobic consortium used in the present investigation.

To carry out efficient reductive transformation, microorganisms need an auxiliary substrate such as glucose. Following Criddle's definition of cometabolism (*24*), this reduction of TNT should be assigned as a cometabolic reduction. While a variety of compounds such as acetate (*17*, *18*), citrate (*17*), formate (*18*), glucose (*8*, *14*, *25*), gluconate (*10*), hydrogen (*12*), molasses (*17*), pyruvate plus sulfate (*14*), sucrose (*17*), succinate (*17*), and malate (*17*) have been used as substrates for the cometabolic reduction of TNT, little is known about the efficiency of the process as quantified by the ratio of TNT reduction and consumption of the auxiliary substrate/electron donor.

When cometabolic reduction of TNT takes place in soil or in a slurry of TNT-contaminated soil, TNT and all of its reduction products will interact with organic and inorganic soil components. These physical interactions may strongly influence the velocity of the biological reduction of TNT and its reduced products. While Pennington (*26*) and Xue et al. (*27*) have investigated the adsorption of TNT to a variety of soils or bentonite/sand mixtures, little is known about the sorption of the different reduction products, especially of TAT, to soil components.

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S0013-936X(97)00949-8 CCC: $15.00
Published on Web 05/21/1998

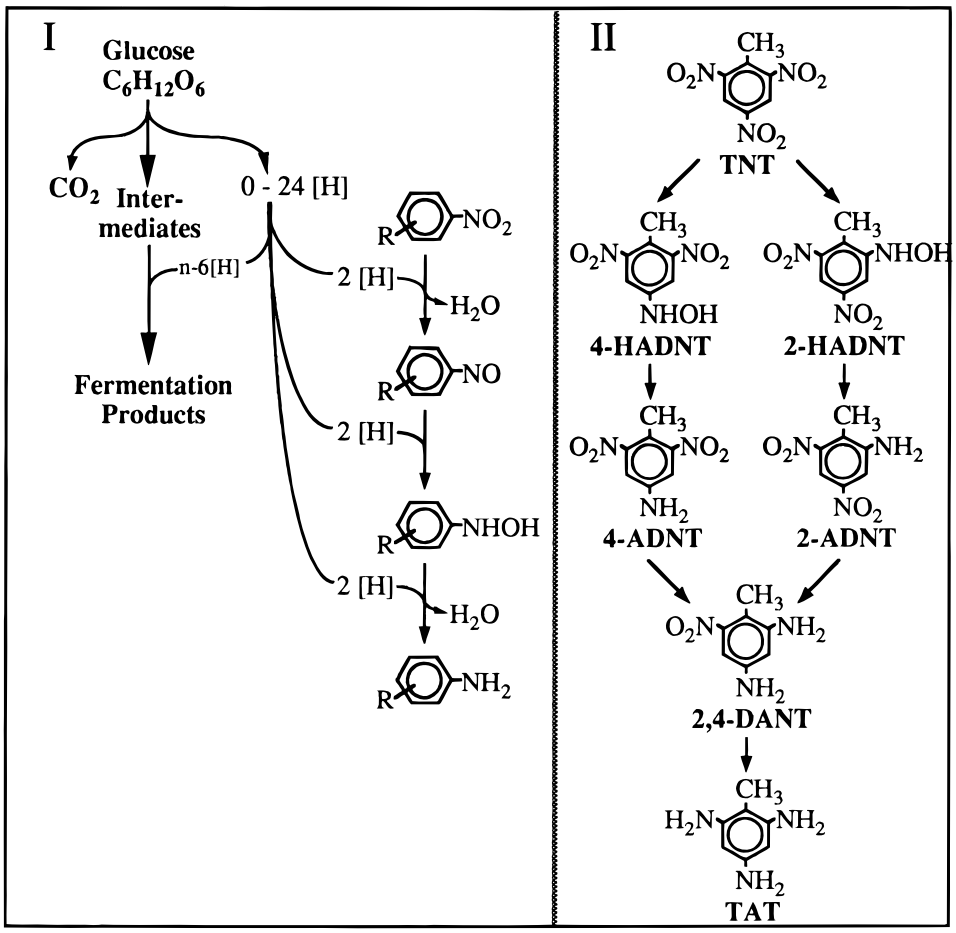

FIGURE 1. Cometabolic reduction of a nitro group during fermentation of glucose (I) and metabolites detected during the reduction of TNT (II).

In this study, we investigated the effiency of cometabolic TNT reduction during fermentation of glucose at different feeding rates of glucose. We also examined the interaction of TNT and its major reduction products with soil components in nonbiological adsorption experiments and during anaerobic microbial treatment of soil components artificially contaminated with TNT.

Experimental Section

Organisms and Culture Conditions. The mixed culture was collected from a continuous fixed bed culture grown with glucose (20 mM) in the presence of TNT (0.5 mM) that had originally been obtained from the sewage plant in Stuttgart-Büsnau (Germany; *28*). The culture was incubated in closed bottles at 30 °C on a rotary shaker at 150 rpm and was transferred once a week into fresh mineral medium (*29*) containing glucose (40 mM) and TNT (0.5 mM). The mineral medium was modified by adding a triple amount of $MgSO_4$.

Chemicals. Highly pure TNT was generously supplied by T. Rosendorfer (MBB Deutsche Aerospace, Schrobenhausen, Germany). 2-Amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), and 2,4,6-triaminotoluene trihydrochloride (TAT) were obtained from Promochem (Wesel, Germany). A mixture of 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene (2-HADNT/4-HADNT) was generated by enzymatic reduction of TNT with xanthin oxidase (Merck, Darmstadt, Germany) and NADH as described by Michels and Gottschalk (*11*). All other chemicals were obtained from commercial sources.

Soil Components. Montmorillonitic clay (Moosburger Ca−bentonite Süd-Chemie, München, Germany) and humic acids (Huminsäure Natriumsalz, Roth, Karlsruhe, Germany) were chosen to represent mineral and organic soil components with a high sorptive capacity.

Analytical Methods. Concentrations of TNT and its reduction products were determined by reversed-phase high-pressure liquid chromatography (HPLC) with Lichrocart 125-4 columns, filled with 5 μm particles of Lichrospher 100 RP-8 (Merck, Darmstadt, Germany). TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, and 2,4-DANT were analyzed in a mobile phase of 40/60 (v/v) acetonitrile/phosphate buffer (15 mM, pH 7.3) via UV absorption at 240 nm. The isomeric hydroxylaminodinitrotoluenes that coeluted were estimated with a 4-ADNT standard. The UV−visible spectra of the isomeric hydroxylaminodinitrotuolenes were very similar to those of the isomeric aminodinitrotoluenes. The isomeric aminodinitrotoluenes also coeluted, and their concentration was calculated with a 4-ADNT standard as they showed a similar absorption at 240 nm. TAT was eluted with a mobile phase of 1/99 (v/v) acetonitrile/phosphate buffer (15 mM, pH 7.3) and detected by its UV absorption at 220 nm. The optical density (OD) of cell suspensions was measured at 546 nm and was directly proportional to the dry cell mass C_X with a factor of 3.9 L/g (*30*). Glucose was measured enzymatically based on a coupled hexokinase/glucose-6-phosphate dehydrogenase reaction (D-glucose, Boehringer Mannheim, Mannheim, Germany).

Fed Batch Fermentation of Glucose in the Presence of TNT. Bioreactors were filled with a starting volume, $V(0)$, of 1.5−5 L of mineral medium with different initial concentra-

tions of glucose, $C_G(0)$, and of TNT, $C_C(0)$, and inoculated (1%, o.d.$_{546\ nm} = 3$) with the culture described above. At times $t_{F_{in}}$, further additions of concentrated substrate solution ($C_{G,in}$) was made every 2.4 h at an average feeding rate F_{in}. Samples (1 mL) were taken to measure optical density and, after centrifugation (10 min at 13000*g*), to measure nitroaromatic compounds and glucose in the supernatant. The variation of the fermentation volume due to different feeding rates and sampling was negligible.

The solutions were stirred with magnetic stir bars. The temperature was kept at 30 °C, and pH was maintained at 7.0 by the addition of 10 M NaOH. The fermenters were sealed to prevent introduction of O_2, and a sampling device allowed analyses of the culture fluid without aeration. Redox potential was monitored in the batch fermentation with a platinum electrode connected with a Ag/AgCl reference system (Ingold Pt-4805-S7, Steinbach, Germany).

The efficiency of the cometabolic reduction is described by the ratio of TNT reduced to glucose consumed. Because glucose was added periodically throughout the fermentation, we had to define a certain time in the experiments to calculate this ratio. The time at which less than 5% of the initial TNT was found as TNT, HADNT, ADNT, or DANT is defined as $t_{5\%}$. At that time, 95% of the initial TNT has been reduced to TAT. The volumetric reduction rate R is defined as the amount of completely reduced TNT per time and volume:

$$R = 0.95C_C(0)/t_{5\%} \quad (1)$$

the amount of glucose consumed per volume, $\Delta C_G(t_{5\%})$:

$$C_G(t_{5\%}) = C_G(0) + C_{G,in}\, F_{in}\, t_{5\%}/V(0) - C_G(t_{5\%}) \quad (2)$$

and the cometabolic efficiency coefficient, $E_{C/G}$, as the ratio of completely reduced TNT to glucose consumed:

$$E_{C/G}(t_{5\%}) = 0.95C_{TNT}(0)/\Delta C_G(t_{5\%}) \quad (3)$$

Sorption of TNT and Metabolites to Montmorillonitic Clay. In a first series of experiments, 4.6 g (dry weight at 105 °C) of clay was suspended in 50 mL of aqueous solutions of TNT, 2-ADNT, and 2,4-DANT or 50 mL of phosphate-buffered (50 mM) solution of TAT (pH 7.4). In addition, a suspension of clay in 50 mM phosphate buffer was flushed with argon before a solution of TAT in phosphate buffer under argon was added to the suspension. All suspensions were incubated in 100-mL serum bottles and incubated on a rotary shaker in horizontal position at 30 °C at 100 rpm. The supernatant was analyzed via HPLC to measure the concentration of contaminants, C_C, in relation to their initial concentration, $C_C(0)$.

In a second series of experiments, 0.46 g (dry weight) of clay was suspended in 5 mL of aqueous solutions of TNT, 2-ADNT, 4-ADNT, and 2,4-DANT and incubated for 2 h in 10-mL test tubes on a rotary shaker in horizontal position at 30 °C at 100 rpm. After the adsorption period, the tubes were centrifuged for 20 min at 30 °C and 3200*g*; the supernatant was removed and analyzed. The clay pellet was suspended in 4−5 mL of freshwater and incubated for 2 h of desorption under the same conditions before determination of concentrations in the supernatant after desorption. For TAT, 2.7 g of clay was suspended in 30 mL of 50 mM phosphate buffer and sparged with argon before TAT solution was added under argon. After 2 h of adsorption, the concentration was measured in the supernatant of centrifuged samples before 30 mL of fresh buffer was added under argon. After 2 h of desorption, the supernatant of the centrifuged samples was analyzed. Additionally, after 2 h of adsorption of TAT, various desorption experiments were carried out with 30 mL of solvents including acetonitrile, methanol, ethanol, methanol/2 M HCl, 2 M HCl, 2 M NaOH or 2 M $CaCl_2$.

For all contaminants, the initial concentration and the concentrations after adsorption and desorption were used to calculate the amount sorbed per dry mass S_C after adsorption and desorption.

Cometabolic Reduction of TNT in the Presence of Montmorillontic Clay. Fed batch fermentations were performed in the presence of suspended montmorillonitic clay. A mass, m, of clay was added to a starting volume, $V(0)$, of TNT/glucose solution before the inoculum was added. A control vessel without clay was started simultaneously.

In the experiments with 3.3% (w/v) clay (m = 44.2 g dry weight, $V(0)$ = 1.33 L) or 10.3% (w/v) clay (m = 148 g dry weight, $V(0)$ = 1.43 L), the magnetic stirr bar was replaced with a helical ribbon impeller operated at 200 rpm. Samples of 3−10 mL of clay suspension were centrifuged for 20 min at 5000 rpm (approximately 3200*g*) to measure the supernatant concentrations of glucose, C_G, and of TNT and its metabolites, C_C. The remaining clay pellet was extracted twice for 1 h with methanol and dried at 105 °C to determine the amount of contaminants sorbed and extracted, $S_{C,ex}$, per dry mass.

Interaction of TNT and Metabolites with Humic Acids. Humic acids (2 g) were suspended in 100 mL of 50 mM phosphate buffer, and the pH was adjusted to 7.4. A 25-mL sample of the suspension was added to 100-mL serum bottles and sparged with argon before 25 mL of a solution of TNT, 2-HADNT/4-HADNT, 4-ADNT, 2,4-DANT, or TAT in phosphate buffer under argon was added with a final concentration of 1 g of humic acids/100 mL of suspension. NaN_3, which was shown to supress the biological nitro group reduction in a control experiment, was added to a final concentration of 0.2% (w/v). The bottles were incubated on a rotary shaker in horizontal position at 30 °C at 100 rpm.

Cometabolic Reduction of TNT in the Presence of Humic Acids. A mass, m, of humic acids was added to a starting volume, $V(0)$, of TNT/glucose solution before the inoculum was added. A control vessel without adsorbent was started simultaneously.

Samples containing 1% (w/v) humic acids (m = 13 g, $V(0)$ = 1.3 L) were centrifuged for 10 min at 13000*g* before the supernatant was analyzed to determine the concentrations of glucose, C_G, and of TNT and its metabolites, C_C. Even after centrifugation the bulk of the humic acids remained in the liquid phase and was not extractable from the pellet. At the end of the fermentation, samples (20 mL each) were made alkaline (pH 12) with NaOH or acidified (pH 1.5) with 10 M HCl and incubated at 30 °C on a rotary shaker. Aliquots taken after 1, 5, and 24 h were neutralized, centrifuged, and analyzed by HPLC. Additionally, one sample (30 mL) was mixed with 600 μL of H_3PO_4 to precipitate the humic acids. After 25 min of centrifugation at 3200*g*, the humic acids were extracted with 20 mL of 5 M NaOH/methanol (1:4 v/v) for 2 h at 90 °C. After neutralization, samples were taken after 1 and 2 h, centrifuged, and analyzed by HPLC.

Results and Discussion

Cometabolic Reduction of TNT during Fed Batch Fermentation of Glucose. Figure 2 shows a representative fermentation experiment. Conditions are listed in experiment C, Table 1. Initially optical density of the bacteria increased exponentially with glucose consumption. After 14 h, glucose concentration decreased close to zero despite continued additions of glucose, and the optical density remained more or less constant. TNT was metabolized simultaneously with glucose consumption; an initial concentration $C_C(0)$ = 0.523 mM was completely converted within 13 h. The redox

TABLE 1. Influence of Initial TNT Concentration and Glucose Feeding Strategy on Reduction of TNT in a Series of Fed Batch Fermentations (A–E)

		experiments				
	unit	A	B	C	D	E
$C_C(0)$	mmol of TNT/L	0.052	0.140	0.523	0.454	0.395
$C_G(0)$	mmol of glucose/L	21.6	21.6	21.6	51.5	5.85
$t_{F_{in}}$	h	12	12	12	0	9; 79
$C_{G,in}F_{in}/V(0)$	mmol of glucose L^{-1}	0.290	0.252	0.248	0.868	0.054; 0.105
$t_{5\%}$	h	60	78	130	87	220
R	μmol of TNT L^{-1}	0.83	1.71	3.82	4.96	1.71
ΔC_G	mmol of glucose/L	35.5	38.2	50.9	127	24.4
$E_{C/G}$	mmol of TNT/mol of glucose	1.4	3.48	9.76	3.39	15.4

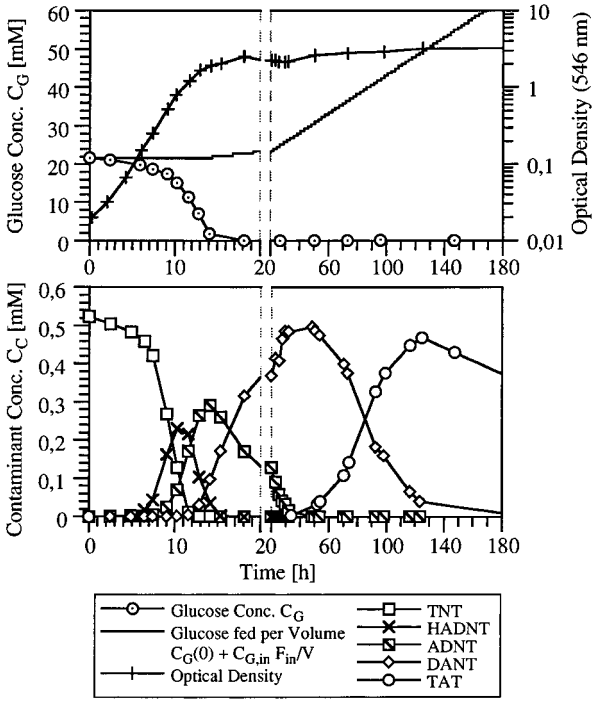

FIGURE 2. Reduction of TNT during fed batch fermentation of glucose.

potential dropped below E_h −200 mV within the initial 12 h and then stayed at this level until the end of the experiment. Reduction products accumulated transiently to different maximum concentrations: 2-HADNT/4-HADNT after 10 h to approximately 44% of $C_C(0)$, 2-ADNT/4-ADNT after 14 h to 55% of $C_C(0)$, 2,4-DANT after 48 h to 95% of $C_C(0)$, and TAT after 125 h to 90% of $C_C(0)$. Other metabolites were not detected during the cometabolic reduction of TNT, suggesting that nitroso intermediates, aminohydroxylaminonitrotoluenes, and diaminohydroxylaminotoluenes were reduced faster than they were generated. The overall rate-limiting step of the reduction to TAT seems to be the reduction of 2,4-DANT. This is in contrast to an earlier observation that the reduction of 2,4-diamino-6-hydroxylaminotoluene to TAT was the rate-limiting enzymatic reaction in a defined sulfate-reducing culture (*14*).

The $t_{5\%}$ for the initial TNT was 130 h, which gave a volumetric reduction rate, R, of 3.82 μmol of TNT L^{-1} h^{-1}. During this time period, $\Delta C_G = 50.9$ mmol of glucose/L was consumed to result in a cometabolic yield of $E_{C/G} = 9.76$ mmol of TNT/mol of glucose.

In experiments with the same glucose feeding strategy but different TNT concentrations (experiments A and B in Table 1), a lower initial TNT concentration led to lower volumetric reduction rates and lower cometabolic yield coefficients. In contrast, when the initial TNT concentration remained the same but glucose was added at different concentration (experiments D and E in Table 1), a smaller initial glucose concentration and a lower glucose feeding rate led to lower volumetric reduction rates but higher cometabolic yield coefficients. As shown by Daun (*30*), these observations are clearly not consistent with the mathematical models for cometabolic transformations proposed by Criddle (*24*).

When glucose is fermented and used as an electron donor for TNT, reduction intermediates of glucolysis are the natural electron acceptors and thus give rise to fermentation products such as ethanol or acetate (*31*, *32*). The increase of the cometabolic yield coefficient with increasing initial TNT concentrations and decreasing initial concentrations and feeding rates of glucose can be explained by a competition for reduction equivalents between artificial acceptors such as TNT and its metabolites and natural acceptors such as the intermediates of glycolysis (Figure 1I). In this system of natural and artificial acceptors of reduction equivalents, a decrease of glucose will lead to less fermentation products while an increase in TNT will increase artificial acceptor concentrations and lead to higher yields of cometabolic reduction products of TNT.

Competitive effects between substrates and cosubstrates as electron donors have been investigated by Ely et al. (*33*). The 10-fold increase of the cometabolic yield coefficient, which we observed in our experiments ($E_{C/G}$ in experiments A and E in Table 1), is of high practical importance as it allows the remediation of contaminated soils using significantly less glucose as an auxiliary substrate.

Sorption of TNT and Metabolites to Montmorillonitic Clay. The kinetics of the adsorption of TNT, 2-ADNT, 2,4-DANT, and TAT by montmorillonitic clay are shown in Figure 3. The concentrations of TNT, 2-ADNT, and 2,4-DANT in the solution decreased within minutes from the initial concentration, $C_C(0)$, to a value that was constant for the next 5 h so that an equilibrium status seemed to be reached. In the long run (1000 h), however, the substances disappeared from the solution, and the disappearance could not be explained by the formation of reduction products due to some biological or chemical activity of the suspension.

Results of the experiments with 2 h of adsorption of TNT, 2-ADNT, 4-ADNT, and DANT by montmorillonitic clay followed by 2 h of desorption are shown in Figure 4. Adsorption data were fitted to linear adsorption isotherms $S_C = k_C C_C$. The slopes of the isotherms were determined by the method of the smallest sum of squares as 6.22 L/kg dry weight (TNT), 5.26 L/kg (2-ADNT), 1.77 L/kg (4-ADNT), and 1.80 L/kg (DANT). In the double logarithmic plot of the values in Figure 5 the isotherms are transformed into $\log S_C = \log k_C + \log C_C$ so that all linear isotherms have a slope of 1.

In the case of TNT, 2-ADNT, and 4-ADNT the desorption isotherms came very close to the adsorption isotherms, which indicates a fully reversible adsorption, while in the case of DANT there was an obvious hysteresis between adsorption

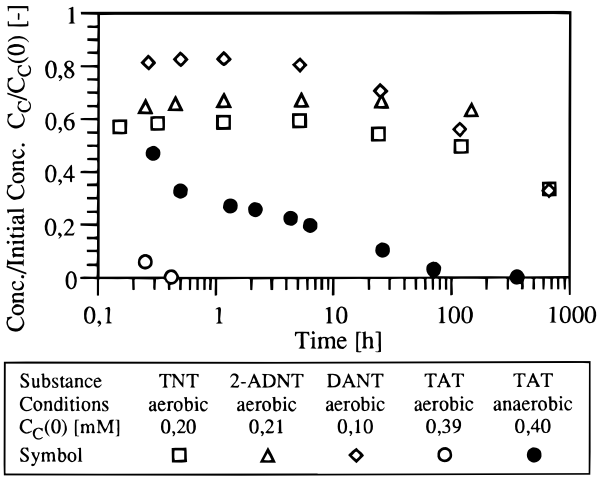

FIGURE 3. Kinetics of the adsorption of TNT and metabolites by montmorillonitic clay.

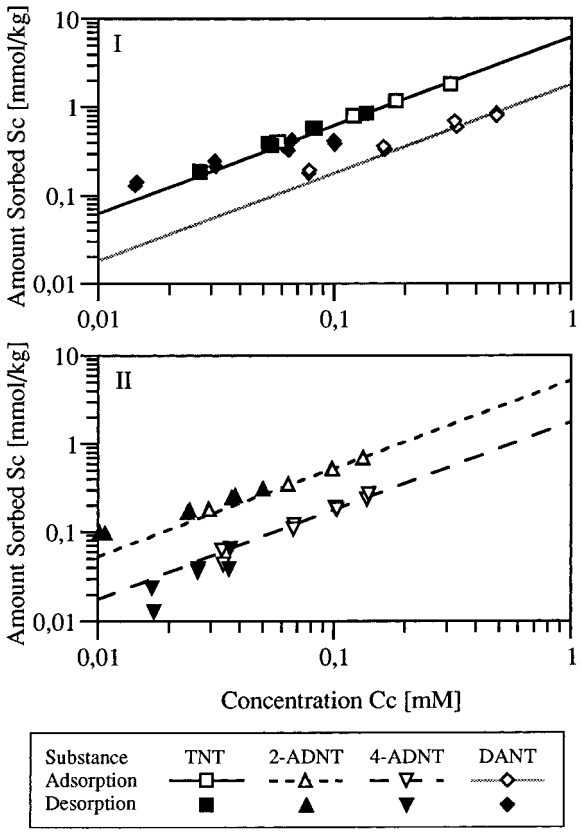

FIGURE 4. Isotherms of the adsorption and desorption of TNT and metabolites at montmorillonitic clay.

and desorption isotherm, indicating only partially reversible adsorption. Interestingly, TNT and 2-ADNT showed higher adsorption than 4-ADNT and 2,4-DANT, which might be due to a higher polarity of the latter compounds, an indication that hydrophobic interactions predominate. Haderlein and Schwarzenbach have investigated the adsorption of nitroaromatic compounds with mineral surfaces in greater detail and have differentiated between hydrophobic and specific interactions (*34*). The specific adsorption of nitroaromatic compounds was interpreted by the formation of coplanar electron donor−acceptor complexes between oxygen ligands at the external siloxane surface of kaolinite and the π system of the nitroaromatic compound. Especially, nitroaromatic compounds with several nitro groups seem to adsorb specifically and reversibly to natural clay minerals. In the

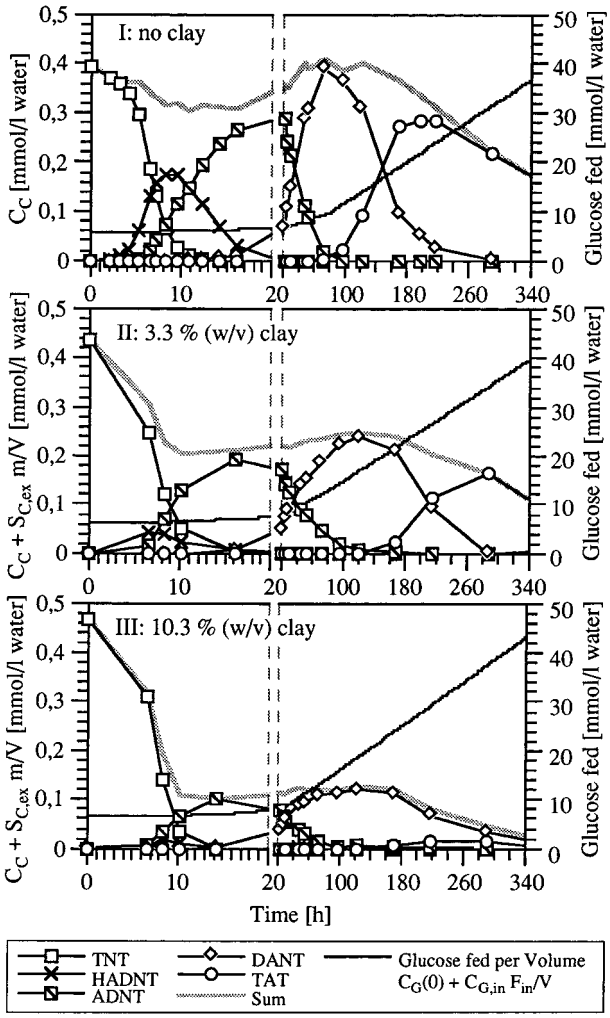

FIGURE 5. Reduction of TNT during fed batch fermentation of glucose in the presence of montmorillonitic clay.

case of TNT, it was recently shown that the reduction of an electron-withdrawing nitro group to an electron-donating amino group leads to a decrease in the k_d values (*35*). Additionally, a higher adsorption of 2-ADNT as compared to 4-ADNT was observed, which was explained by the ortho effect of the methyl group. Similar adsorption effects were observed in the present study.

In contrast to TNT and the aminonitrotoluenes, TAT showed a totally different behavior. TAT was completely removed from the solution within 25 min under aerobic conditions and within 360 h under anaerobic conditions. Control experiments without montmorillonitic clay under anaerobic or aerobic conditions showed that TAT was stable under the experimental conditions.

The sorption of TAT to montmorillonitic clay was completely irreversible. TAT could neither be extracted with different organic solvents nor could it be released by alkaline or acidic hydrolysis or by methanol/HCl mixtures.

Cometabolic Reduction of TNT during Fed Batch Fermentation of Glucose in the Presence of Montmorillontic Clay. Fermentation experiments in the presence of montmorillonitic clay were performed to investigate the adsorption of the reduction products of TNT, including the nitroso and hydroxylamino metabolites. Attention was paid to the sum of dissolved and extractable metabolites during the reduction of TNT. The results are shown in Figure 5.

In a control experiment without clay (Figure 5I), 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT

accumulated temporarily to maximum concentrations of 45%, 73%, 98%, and 71% of the initial TNT concentration ($C_C(0) = 0.4$ mM). In the presence of 3.3% (w/v) clay (Figure 5II), the amount of dissolved (C_C) and extracted ($S_{C,ex}$) 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT per fluid volume reached temporary maxima of 11%, 48%, 60%, and 41% of the initial TNT concentration ($C_C(0) = 0.4$ mM). During the fermentation with 10.3% (w/v) clay (Figure 5III), the amount of dissolved and extractable 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT per fluid volume reached temporary maxima of 4.5%, 25%, 30%, and 3.5% of the initial TNT concentration ($C_C(0) = 0.4$ mM).

The sum of the concentrations of TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT in the control experiment was, except for a temporary decrease within the first 20 h, almost constant for 140 h until it finally decreased with the formation of TAT. The drop below the 100% line during the early phase of reduction was probably due to the lack of analytical standards for the pure isomers of the hydroxylaminodinitrotoluenes. In the presence of 3.3% (w/v) clay, the total amount of dissolved and extracted TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT per fluid volume decreased from 110% (0.48 mM) of the initial TNT concentration (0.44 mM) within 10 h to 50−60% and remained at this level until it decreased after 170 h with the formation of TAT. After 386 h, the total concentration of all of the reduction products was below 2% of the initial TNT concentration. In the presence of 10.3% (w/v) clay, the total amount of dissolved and extracted TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT per fluid volume decreased from 117% (0.51 mM) of the initial TNT concentration (0.44 mM) within 10 h to 30% and remained at this level until another decrease after 170 h correlated with the formation of TAT. After 386 h, the concentrations of all of the reduction products were below the detection limit. In both experiments with clay, the initial sum of concentrations was greater than the calculated concentration of TNT (see Figure 5II,III) due to a non-representative high fraction of fine clay particles in our solid-phase samples.

The early rapid decrease of TNT and its metabolites in the presence of clay indicates that early metabolites of TNT must interact strongly with the solid phase. Figure 5 shows that in the presence of clay the concentration of the hydroxylaminodinitrotoluenes in the aqueous phase was very low, indicating that these metabolites may be candidates for irreversible binding. After the initial sorption, the sum was constant until it decreased further with the generation of TAT. These effects were more pronounced with increasing clay in the system and can only be explained by sorption of both hydroxylaminodinitrotoluenes and TAT. The assumption that hydroxylaminodinitrotoluenes are also good candidates for irreversible binding was confirmed with a sorption experiment. The concentrations of hydroxylaminodinitrotoluenes (0.03 mM) decreased very rapidly in the presence of montmorillonitic clay (4.6 g in 50 mL of phophate buffer) under anaerobic conditions and were no longer detected after 15 min of incubation. A control experiment without montmorillonitic clay showed that the hydroxylaminodinitrotoluenes were stable under the experimental conditions.

Interaction of TNT and Metabolites with Humic Acids. To investigate the interaction of TNT and its reduction products with soil organic matter, experiments with humic acids were carried out. Kinetics of the interaction of TNT, 4-ADNT, 2,4-DANT, and TAT with humic acids are shown in Figure 6. Obviously, binding increased with the number of amino groups present on the aromatic ring. As observed with montmorillonitic clay, interaction precipitously increased with TAT, which was not detectable after 24 h.

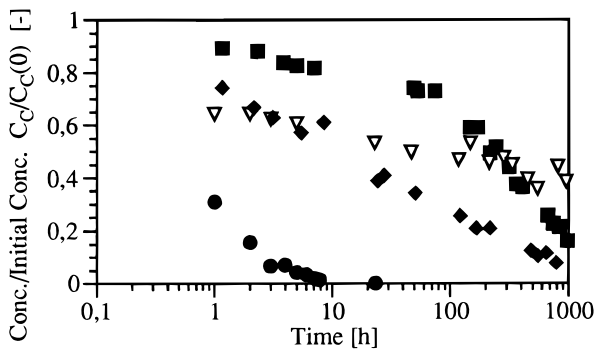

FIGURE 6. Kinetics of the interaction of TNT and metabolites with humic acids.

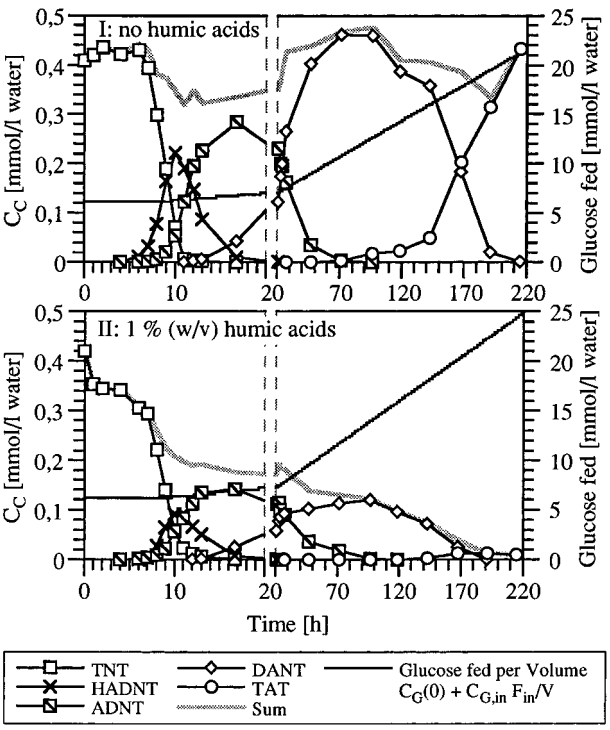

FIGURE 7. Reduction of TNT during fed batch fermentation of glucose in the presence of humic acids.

The strong interaction of hydroxylaminodinitrotoluenes with clay suggested that these compounds might also interact strongly with humic acids. This assumption was confirmed by adsorption experiments with 1 or 2% humic acids under anaerobic conditions. In both experiments, the concentration of the hydroxylaminodinitrotoluenes rapidly decreased. Hydroxylaminodinitrotoluenes were no longer detectable after 30 min in the presence of 1% humic acids and after 20 min in the presence of 2% humic acids. A control experiment without humic acids showed that the hydroxylaminodinitrotoluenes were stable under the experimental conditions.

Cometabolic Reduction of TNT in the Presence of Humic Acids. Cometabolic reduction of TNT with glucose as auxiliary substrate was also carried out under fed batch conditions. Interaction of TNT and its reduction products was studied with humic acids as a model for soil organic material. The results are shown in Figure 7.

In a control experiment without humic acids (Figure 7I) 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT accumulated temporarily at maximum concentrations corresponding to 53%, 68%, 110%, and 104% (after 214 h) of the initial TNT concentration ($C_C(0) = 0.42$ mM). Because an extraction of dissolved humic acids in the samples was highly impractical, the unbound metabolites are reflected in Figure 7II, which shows the experiment containing 1% (w/v) humic acids. Under these conditions the maximum concentration of accumulated metabolites were considerably lower corresponding to 22% (2-HADNT/4-HADNT), 26% (2-ADNT/4-ADNT), 29% (2,4-DANT), and 3.1% (TAT). As in the experiments with clay, the sum of dissolved metabolites decreased rapidly during the initial phase of the reduction of TNT. The sum of the concentrations of TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT fell to 50% of the initial TNT concentration within the first 10 h in the presence of humic acids after which time it decreased steadily at a constant rate. After 290 h all reduction products had disappeared, whereas the total concentration of TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT, and TAT in the control experiment was almost constant for 220 h. Deviations of the 100% line were due to the lack of analytical standards for the pure isomers of the hydroxylaminodinitrotoluenes.

Once complete disappearance of TNT and its reduction products had occurred, they could not be desorbed by alkaline or acidic hydrolysis nor by methanolic saponification. This indicates irreversible binding of the reduction products of TNT to humic substances and again both TAT and hydroxylaminodinitrotoluenes must be considered as reactive species for chemisorption to humic material.

Irreversible Binding of the Metabolites of TNT with Regard to the Microbial Degradation of TNT in Soil. The results of experiments with 10.3% (w/v) montmorillonitic clay and with 1% (w/v) humic acids showed quite similar removal of TNT and metabolites from solution, so that the humic acids seem to have a larger specific binding capacity for these compounds than the montmorillonitic clay. The specific interactions of TNT and its reduction products with these two soil components may however be of a very different nature. While interactions of nitroaromatic or reduced nitroaromatic compounds with minerals can be primarily explained by hydrophobic interaction or by the formation of coplanar electron-acceptor complexes as described previously (*34*, *35*), Parris (*36*) has dicussed several possibilities for a covalent binding between reduced nitroaromatics and humic substances. In the case of reduced TNT, and especially of TAT, it must be assumed that the binding occurs through nucleophilic addition of the amino group to the carbonyl functions or to C−C double bonds of quinoid structures of humic substances.

In our anaerobic treatment of artificially contaminated clay and humic acids, these sorptive interactions led to a complete removal of TNT and its metabolites from the solution. Metabolites of TAT that were found in control experiments were not detected in the presence of clay or humic acids. Our studies indicate that an irreversible binding of microbial reduction products of TNT with soil components is inevitable. On one hand, the metabolites become unavailable for further microbial degradation and mineralization. On the other hand, cometabolic reduction of TNT and related compounds opens a new possibility for immobilization of toxic compounds. The question of whether transformation and biologically induced immobilization of the contaminants might serve as a realistic remediation alternative to the unrealistic goal of mineralization is discussed in the companion paper (*32*).

Acknowledgments

We gratefully acknowledge Frank Desiere, Daniela Jackisch, Ute Sieglen, and Barbara Wagener for performing some of the experiments. We thank Shirley Nishino for critical reading of the manuscript. This research was made possible by a DECHEMA and Volkswagen scholarship and by research grants from the Federal Ministry of Education and Research (BMBF) for projects with the Hessische Industriemüll GmbH—Bereich Altlastensanierung (HIM-ASG) and Umweltschutz Nord GmbH & Co.

Literature Cited

(1) Preuss, J.; Haas, R.; Koss, G. *Geogr. Rundschau* **1988**, *40* (6), 31−38.
(2) Haas, R.; von Löw, E. *Forum Städte-Hyg.* **1986**, *37*, 33−43.
(3) Rickert, D. E., Ed. *Toxicity of Nitroaromatic compounds;* Hemisphere: Washington, DC, 1985.
(4) Rieger, P.-G.; Knackmuss, H.-J. Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related compounds in contaminated soil. In *Biodegradation of nitroaromatic compounds*; Spain, J., Ed.; Plenum Press: New York, 1995; pp 1−18.
(5) Ahlborg, G.; Einistö, P.; Sorsa, M. *Br. J. Ind. Med.* **1988**, *45*, 353−358.
(6) Yinon, J.; Hwang, D.-G. *J. Chromatogr.* **1987**, *394*, 253−257.
(7) Görge, E.; Brandt, S.; Werner, D. *Environ. Sci. Pollut. Res. Int.* **1994**, *1*, 229−233.
(8) Parrish, F. W. *Appl. Environ. Microbiol.* **1977**, *34*, 232−233.
(9) Stahl, J. D.; Aust, S. D. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 477−482.
(10) Bumpus, J. A.; Tatarko, M. *Curr. Microbiol.* **1994**, *28*, 185−190.
(11) Michels, J.; Gottschalk, G. *Appl. Environ. Microbiol.* **1994**, *60*, 187−194.
(12) McCormick, N. G.; Feeherry, F. E.; Levinson, H. S. *Appl. Environ. Microbiol.* **1976**, *31*, 949−958.
(13) Naumova, R. P.; Selivanovskaya, S. Y.; Cherepneva, I. E. *Appl. Biochem. Microbiol.* **1989**, *24*, 409−413.
(14) Preuss, A.; Fimpel, J.; Diekert, G. *Arch. Microbiol.* **1993**, *159*, 345−353.
(15) Funk, S. B.; Roberts, D. J.; Crawford, D. L.; Crawford, R. L. *Appl. Environ. Microbiol.* **1993**, *59*, 2171−2177.
(16) Boopathy, R.; Kulpa, C. F.; Wilson, M. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 270−275.
(17) Boopathy, R. B.; Manning, J.; Montemagno; C.; Kulpa, C. *Curr. Microbiol.* **1994**, *28*, 131−137.
(18) Boopathy, R.; Kulpa, C. F. *Can. J. Microbiol.* **1994**, *40*, 273−278.
(19) Fiorella, P. D.; Spain, J. C. *Appl. Environ. Microbiol.* **1997**, *63*, 2007−2015.
(20) Khan, T. A.; Bhadra, R.; Hughes, J. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 198−203.
(21) Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J. C.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1998**, *64*, 246−252.
(22) Scheibner, K.; Hofrichter, M.; Herre, A.; Michels, M.; Fritsche, W. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 452−457.
(23) Widrig, D. L.; Bootpathy, R.; Manning, J. F. *Environ. Toxicol. Chem.* **1997**, *16*, 1141−1148.
(24) Criddle, C. S. *Biotechnol. Bioeng.* **1993**, *41*, 1048−1056.
(25) Won, W. D.; Heckly, R. J.; Glover, D. J.; Hoffsommer, J. C. *Appl. Environ. Microbiol.* **1974**, *27*, 513−516.
(26) Pennington, J. C.; Patrick, W. H., Jr. *J. Environ. Qual.* **1990**, *19*, 559−567.
(27) Xue, S. K.; Iskandar, I. K.; Selim H. M. *Soil Sci.* **1995**, *160*, 317−327.
(28) Knackmuss, H.-J.; Dickel, O.; Ecker, S.; Haug, W. Entwicklung und Etablierung eines zweistufigen Verfahrens zum mikrobiellen Abbau von Trinitrotoluol, RDX und HMX. Studie des Instituts für Mikrobiologie der Universität Stuttgart, 1991.
(29) Dorn, E.; Hellwig, M.; Reinecke, W.; Knackmuss, H.-J. *Arch. Microbiol.* **1974**, *99*, 61−70.
(30) Daun, G. Entwicklung und Anwendung eines Verfahrens zur biologischen Sanierung TNT-kontaminierter Böden; Fortschr.-Ber. VDI Reihe 15, No. 171, VDI−Verlag: Düsseldorf, 1997.

(31) Desiere, F. Optimierung der anaeroben Reduktion von TNT für die biologische Behandlung TNT-kontaminierter Böden. Diplomarbeit, Universität Stuttgart, 1996.
(32) Lenke, H.; Warrelmann, J.; Daun, G.; Hund, K.; Sieglen, U.; Walter, U.; Knackmuss, H.-J. *Environ. Sci. Technol.* **1998**, *32*, 1964−1971.
(33) Ely, R. L.; Williamson, K. J.; Guenther, R. B.; Hyman, M. R.; Arp, D. J. *Biotechnol. Bioeng.* **1995**, *46*, 218−231.
(34) Haderlein, S.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1993**, *27*, 316−26.
(35) Haderlein, S.; Weissmahr, K. W.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1996**, *30*, 612−622.
(36) Parris, G. E. *Residue Rev.* **1980**, *76*, 1−30.

Received for review October 30, 1997. Revised manuscript received March 23, 1998. Accepted April 2, 1998.

ES970949U