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Reductive Transformation of Bound Trinitrophenyl Residues and Free TNT during a Bioremediation Process Analyzed by Immunoassay

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To follow the fate of bound metabolites of TNT in soil, a synthetic trinitrophenyl residue covalently linked to humic acids was used as model compound. A selective monoclonal antibody was able to detect chemical changes of the nitro groups of the bound residues. The general possibility of reductive transformations of nitro groups of bound molecules and the reductions rates should be determined. In comparison to the reduction of free TNT and its metabolites, the reductive transformation of the bound trinitrophenyl residue was delayed, and the transformation rate was considerably slower. Trinitrophenyl residues also could be detected by the immunoassay in humic acids extracted from TNT contaminated soil. The reductive transformation of these trinitrophenyl residues started after the reduction of free TNT. At the end of the treatment, small amounts of these residues were still detectable indicating that some of these structures were not completely reduced during the process. From present results one can conclude that the further reduction of nitro groups of bound metabolites requires a prolonged anaerobic treatment. Not only the monitoring of free nitroaromatic compounds is recommended during the bioremediation process but also the measurement of bound residues to determine the optimal conditions and duration of the treatment.

Introduction

2,4,6-Trinitrotoluene (TNT) was the major explosive produced during World War II: In Germany the production amounted to approximately 800 000 tons of TNT (*1*). Due to the biological persistence TNT and related components are still present at high concentrations at former production sites and can be found in the water supplies of the neighboring communities (*2*). The toxicity, the mutagenic, and carcinogenic potential of TNT and particularly its reduced metabolites (*3*) require remediation of these sites.

As an alternative to thermal processes of decontamination of soil, biological remediation techniques were considered. Since all attempts failed to establish a complete mineralization of TNT in soil, most biological treatment processes were directed toward an irreversible binding of reduced metabolites of TNT to soil organic matter. The difficulties to achieve mineralization of TNT can be explained by the high xenobiotic character of nitroaromatic and particularly polynitroaromatic compounds and the resistance of the highly oxidized trinitro-substituted aromatic ring against oxidative attack (*4*). In contrast, initial cometabolic reduction of the nitro groups of TNT has often been observed in cultures of bacteria (*5*−*14*) and fungi (*15*−*18*). The successive reduction of the nitro groups leads to an activation of the molecule toward electrophiles. While under aerobic conditions partially reduced metabolites such as ADNT and DANT were formed, complete reduction of all nitro groups can only be accomplished under strict anaerobic conditions (*19*). Triaminotoluene (TAT) or highly reactive intermediates such as hydroxylaminodinitrotoluenes strongly interact with soil components (*19*). If these partially reduced metabolites bind to soil, the resulting structures carry intact nitro groups. To minimize the chance that nitro- or aminoaromatic compounds were released from the soil, all three nitro groups should be reduced which is the precondition of multivalent binding. A reduction of nitro groups of covalently bound metabolites, however, cannot be affected by microbial cells but may be possible through soluble reduction equivalents that act as redox mediators (*20*). A general evidence that these nitro groups can be further transformed is still missing. Besides using NMR spectroscopic methods of [^{15}N]TNT (*21*), immunological techniques may also be used to recognize such transformations.

In this paper we investigated whether nitro groups of covalently bound and partially reduced metabolites are further transformed by a previously described anaerobic/aerobic treatment process (*22*). For this purpose, a synthetic conjugate of a 2,4,6-trinitrotoluene derivative with humic acids and real contaminated soil was treated. To detect reductive transformations of the nitro groups of covalently bound molecules during the bioremediation process, an optimized immunoassay which had been developed by Pfortner et al. *(23*−*25*) was used. The immunoassay was designed to detect only covalently bound trinitrophenyl residues. This system should simulate the transformation of TNT covalently bound to humic acids. Therefore, this new approach should allow to elucidate whether nitro groups of bound TNT in soil organic matter can be reduced.

Experimental Section

Chemicals. Highly pure 2,4,6-trinitrotoluene (TNT) was supplied by 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), 2,6-diamino-4-nitrotoluene (2,6-DANT), and 2,4,6-triaminotoluene (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 xanthine oxidase (Merck, Darmstadt, Germany) and NADH as described by Michels and Gottschalk (*18*). N-(2,4,6-trinitrophenyl)-4-aminobutyric acid (TNP−C4) was obtained from Research Organics (Cleveland, U.S.A.); peroxidase-labeled goat anti-mouse immunoglobulin class G

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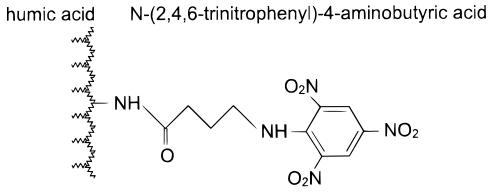

FIGURE 1. Structure of the synthetic 2,4,6-trinitrophenyl-4-aminobutyric acid/humic acids (TNP−C4−HA) conjugate.

(IgG) was from Sigma (Steinheim, Germany); gelatin, poly(acrylic acid), and methylated bovine serum albumin were obtained from Fluka (Neu-Ulm, Germany). The monoclonal TNT antibody was kindly provided by Strategic Diagnostics Inc. (Newark, DE). Buffers and the substrate solution were prepared as previously described *(24)*. All other chemicals were obtained from commercial sources.

Synthesis of the TNP−C4−HA Conjugate. The synthetic N-(2,4,6-trinitrophenyl)-4-aminobutyric acid/humic acids conjugate (designated as TNP−C4−HA conjugate, Figure 1) was synthesized according to an earlier published procedure *(23)*. N-(2,4,6-Trinitrophenyl)-4-aminobutyric acid (TNP−C4) was converted to the corresponding TNP−C4−N-hydroxysuccinimide-ester with dicyclohexylcarbodiimide and coupled to primary amino functions of humic acids previously isolated from a standard soil (see below) through extraction with diluted NaOH. Purification of the coupling product was achieved through gel chromatographic separation eliminating components with low molecular weight. The density of coupling groups in the humic acids was the same as reported in ref *23*.

Soils and Isolation of Humic Acids. The contaminated soil used in the slurry experiment was obtained from a former TNT production site at Stadtallendorf near Marburg (Germany). The soil had the following characteristics: total organic carbon, 2.5%; N(total), 0.1%; P(total), 0.09; density, 1.23 g/mL; clay-fraction (diameter < 20 μm), 20.8%. These were determined according to Lenke et al. (*22*). The soil was air-dried and sieved to a mesh of 2 mm. For detection of bound trinitrophenyl residues humic acids were extracted from the soil at different times during the anaerobic/aerobic treatment. Prior to humic acids extraction, the extractable nitroaromatic compounds were removed from the soil samples by exhaustive Soxhlet-extraction with methanol. Subsequently, humic acids were isolated with 0.1 M tetrasodium pyrophosphate as described previously *(23)*.

A standard soil (type 2.2) purchased from the Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer (Speyer, Germany) was used for the isolation of humic acids for synthesis of the TNP−C4−HA conjugate and soil-free reduction experiments. Samples from the experiment with the synthetic TNP−C4−HA conjugate were diluted to a humic acid concentration of 10 mg/L, and the immunological response was measured without any further treatment.

Humic acids for sorption experiments with HADNT were extracted from TNT contaminated soil (Hessisch-Lichtenau, Germany, *22*). The extraction procedure was described elsewhere (*26*).

Analytical Methods. **HPLC Analysis of Nitroaromatic Compounds.** The concentration of TNT, 2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT were determined by reverse-phase high performance liquid chromatography (HPLC) as described recently (*22*). Concentrations of TAT were determined by reversed-phase HPLC according to Daun et al. (*19*). Isomers of hydroxylaminodinitrotoluene were measured by reversed-phase HPLC with Lichrocart 125-4 column, filled with 5 μm particles of Lichrospher 100 RP-8 (Merck, Germany). A mobile phase of 40/60 (v/v) acetonitrile/phosphate buffer (15 mM, pH 7.3) was used.

Immunoassay Procedure. Polystyrene microtiter plates were coated with 200 μL of a 0.05% solution of methylated bovine serum albumin in phosphate buffered saline (pH 7.6) overnight. The coated plates were washed three times, and 200 μL of a 10 mg/L humic acid sodium salt solution of samples or blank in 12 replicates was incubated for 1 h. Another washing step was performed, and 50 μL of water was added in six wells of each sample, while the remaining six wells of each sample were filled with 50 μL of a TNT solution (1 mg/L in water). Two hundred microliters of the monoclonal TNT antibody (0.74 mg/L) in phosphate buffered saline, containing 1% w/v poly(acrylic acid) and 0.2% w/v gelatin, was added to all wells and incubated for 15 min. After a further washing step, 200 μL of a 1:10 000 dilution of peroxidase-labeled goat anti-mouse IgG was added for 30 min. A final washing step was performed, and 200 μL of freshly prepared substrate solution was added. The enzymatic reaction was stopped after 25−30 min by adding 100 μL of 5% v/v H_2SO_4, and the absorbance at 450 nm was measured by a TR 400 MTP reader from SLT (Grödingen, Austria). The difference of the signals without and with competitive inhibition of the antibody with TNT resulted from highly specific antibody/antigen interactions and was used as parameter for the amount of covalently bound trinitrophenyl residue in the sample.

Calculation of the TNT Equivalents. A TNP−C4−HA standard conjugate, which was earlier characterized *(24)* in detail (coupling density: 14 μmol TNP−C4/g humic acids), was used for calibration of the immunoassay for the detection of trinitrophenyl residues which were bound to humic acids. Solutions of 10 mg/L TNP−C4 standard conjugate and HA, respectively, were prepared. These solutions were mixed in different ratios so that samples with different amounts of bound TNP−C4 but constant HA content resulted. After measurement in the described immunoassay, the determined signal differences showed a linear correlation with the TNP−C4 content of the samples. This calibration graph was used to calculate the molar content of bound TNT equivalents in the unknown samples.

Setup for Reduction Experiments. Cometabolic Reduction of TNT and TNP−C4−HA Conjugate in Solution. The experiments in the aqueous system were carried out in bottles (500 mL), which were closed with rubber stoppers. After autoclaving 180 mL of 50 mM sodium phosphate (pH = 7.3), crystals of TNT were added and dissolved by boiling for 3−5 h. After cooling, the gas phase was exchanged 4 times with argon. Before inoculation, a 2 mL mineral salts solution and a 2.2 mL glucose solution (2 M) were added anaerobically with a syringe (*19*). The experiments were started by adding 2.0 mL of a mixed culture of TNT reducing bacteria. All suspensions were incubated on a rotary shaker in horizontal position at 30 °C and 100 rpm. Samples were centrifuged for 10 min at 14 000 rpm (approximately 13000*g*) before the supernatants were analyzed by HPLC to measure the concentration of TNT and its reduction products. Three different experiments were performed: reduction of the TNP−C4−HA conjugate with a small amount of TNT (27 μM) added,—as a control—reduction of TNT in the presence of the same humic acids without TNP−C4, and reduction of TNT without humic acids and conjugate. Detailed incubation conditions are given in Table 1.

Reduction of TNT in Contaminated Soil. For the soil experiments, a slurry of 850 g of contaminated soil and 850 mL of sterile sodium phosphate buffer (*19*) was filled in a 1.3 l bioreactor. The soil slurry was mixed with a high-grade steel stirrer (150 rpm). The temperature of the bioreactor was kept at 30 °C, and the pH value was maintained at 7.0 by adding 10 M NaOH solution. The bioreactor was closed, and the gas exchange with the environment was minimized to maintain the established anaerobic conditions. The

TABLE 1: Conditions of Cometabolic Reduction of TNT in an Aqueous System in Presence/Absence of Humic Acids

	unit	experiment		
		TNT without humic acids	TNT with humic acids	TNT with humic acid conjugate[a]
vol (50 mM PP,[b] pH = 7.3)	mL	193	193	189
concnd TNT	mM	0.32	0.35	0.027
concnd humic acids	% (w/v)		0.98	
concnd conjugate[a]	% (w/v)			0.66
source of humic acids			soil type 2.2	soil type 2.2[a]
concnd glucose	mM	22	22	22
duration of the expt	h	200	200	850

[a] TNP−C4−HA conjugate. [b] Sodium phosphate buffer.

experiment was started by the addition of glucose (initial concentration 10 mM) and a mixed culture (*19*). Glucose was added at an average rate of 1.0 mmol glucose/d. Samples of 3−4 mL soil-slurry were taken from the top of the bioreactor by flushing with N_2. The samples were centrifuged for 10 min at 4000 rpm (approximately 2500*g*) to separate the supernatant from the soil. The remaining soil pellet of each sample was extracted two times for 1 h with methanol. Both methanol extracts were collected and vaporized at 30 °C. The concentration of TNT and its metabolites were measured in the supernatant and in the methanol extract by HPLC. The soil pellet was dried at room temperature to refer the amount of extractable TNT and reduction products to the dry mass.

Sorption and Desorption of Hydroxylaminodinitrotoluene to Humic Acids. The experiments were carried out in closed bottles (125 mL) filled with 20 mL of 50 mM sodium phosphate buffer (pH = 7.3) and 0.75% (w/v) humic acids. Humic acids were extracted from TNT contaminated soil (Hessisch-Lichtenau). To obtain anaerobic conditions the gas phase was replaced by argon. A solution of the isomers of HADNT in methanol (0.5 mL; final concentration: 36 μM) was added anaerobically. Incubation was carried out on a rotary shaker in horizontal position at 30 °C and 100 rpm. While avoiding an excess of O_2, aliquots were taken and immediately analyzed by HPLC. For desorption experiments humic acids were precipitated with HCl (10 M) and centrifuged. The precipitate was extracted with 5 mL of each different solvent (methanol; NaOH, 0.5 M; sodium phosphate buffer, 50 mM, pH = 7.3; acetonitrile) 1 h at 30 °C on a rotary shaker. After centrifugation the extracts were analyzed by HPLC for HADNT and other reduced TNT metabolites. The desorption experiments were carried out in the presence and absence of O_2.

Results and Discussion

Reduction of Free TNT and TNP−C4−HA Conjugate. The cometabolic reduction of TNT during a fed batch fermentation of glucose in the presence of the TNP−C4−HA conjugate is shown in Figure 2 A. The conditions of the fermentation experiment are listed in Table 1. TNT was reduced to DANT with isomeric HADNT and ADNT as intermediates. TAT was only detected in the control experiment without the TNP−C4−HA conjugate or humic acids (data not shown). The presence of the TNP−C4−HA conjugate caused a significant decrease of the total amount of TNT and its metabolites in solution (Figure 2A), particularly during the initial reduction step (TNT to HADNT) and the last reduction step (DANT to TAT). According to previous sorption experiments with TNT and its metabolites (*19*), it can be concluded that hydroxylaminodinitrotoluene and TAT strongly interact with the humic acid part of the TNP−C4−HA conjugate. Similar observations were also made during reduction experiments with the same humic acids without TNP−C4 (data not shown). Obviously, the modification of humic acids with TNP−C4 did not change their ability to interact with certain reduced metabolites of TNT.

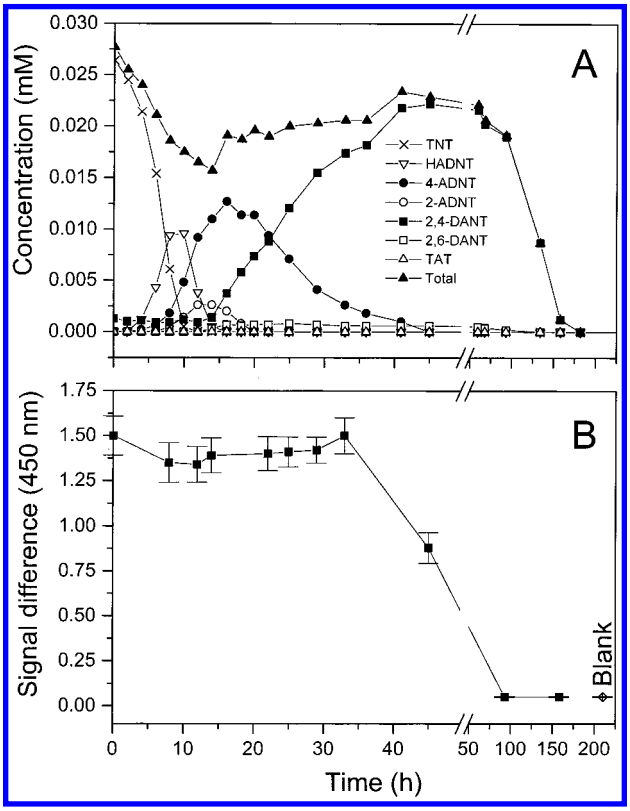

FIGURE 2. Cometabolic reduction of TNT in the presence of the TNP−C4−HA conjugate. (A) TNT and its metabolites analyzed by HPLC (starting concentration 0.027 mM) and (B) immunoassay of extracted TNP−C4−HA conjugate. The signal difference correlates with the amount of bound trinitrophenyl residues measured by immunoassay. The signal difference of the immunoassay of humic acids without TNP−C4 conjugate is also given (blank).

The binding of partially reduced metabolites such as HADNT to humic acids leads to molecules which still carry two unreduced nitro groups. Further and finally complete reduction to amino groups is the precondition of additional linking to soil organic matter. For safety and maximum stability of the immobilized pollutants covalent bindings should if possible involve all three N-functions of the original nitro groups of TNT. These cross linkages may increase the stability of the bound residues so that no low molecular weight nitro- or aminoaromatic compounds would be released from the soil even during long-term incubation or natural turnover of organic matter. As a precondition of this multiple cross-linking between reduced TNT metabolites and the humic substances, it is necessary that the reduction of nitro groups continues even if nitroaromatic metabolites are already covalently bound to humic material (Figure 5, reaction 3).

The trinitrophenyl moiety of the synthetic TNP−C4−HA conjugate (Figure 1) can serve as a model of a bound residue and be detected by an immunoassay with a highly specific monoclonal antibody. Chemical changes of the bound trinitrophenyl moiety (i.e. further reduction of nitro groups and covalent binding to humic acids) will lead to structures, which are not recognized by the monoclonal antibody and the signal of the immunoassay should thus decrease (*25*, *27*). To correlate the decrease of the immunoassay signal with

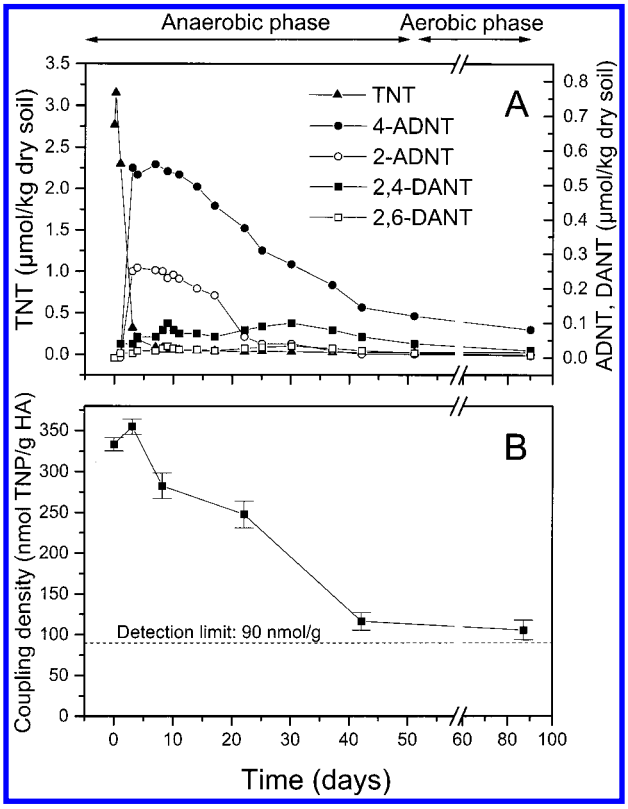

FIGURE 3. Reduction of TNT and its metabolites during the anaerobic/aerobic treatment of TNT contaminated soil in a soil slurry. (A) Amounts of TNT and its metabolites which were desorbed from the soil with methanol. (B) Amounts of trinitrophenyl residues measured by immunoassay from extracted humic acids of the treated soil expressed as nmol TNP equivalents per kg dry soil.

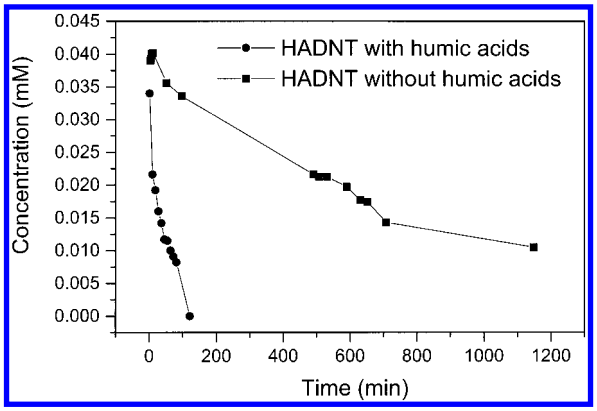

FIGURE 4. Kinetic of interaction of isomers of hydroxylaminodinitrotoluene with humic acids under anaerobic conditions. A control experiment is run without humic acids under the same conditions.

the reduction of the nitro groups of TNP−C4−HA conjugate a small amount of free TNT was added to the solution (Table 1). In contrast to the fast reduction of free TNT and its metabolites (Figure 2A), the immunoassay signal was nearly constant during the initial 35 h (Figure 2B). Therefore, it can be concluded that only minor or no changes of the bound trinitrophenyl moiety took place during this time period. The decrease of the signal differences after 35 h indicates that the covalently bound trinitrophenyl residue was chemically converted to structures which were not bound any more by the monoclonal antibody. Under the experimental conditions, it is likely that further reduction of nitro groups increases the reactivity of the groups toward organic soil components. This may lead to an extensive cross-linking to humic acids. However, it cannot completely be ruled out that other chemical alterations might have occurred in the nitroaromatic compound which have lowered the residual cross-reactivity. Due to the stability of the amid bond of the TNP−C4−HA conjugate, the hydrolysis and release of TNP residues can be excluded under the conditions used in the experiment (*23*).

Remarkably, a significant decrease of the immunoassay signal occurred only after most of the soluble TNT is converted to the DANT isomers. As expected, the tranformation of nitro groups of molecules which are bound to organic matter proceeds much slower than the reduction of free nitroaromatic compounds. Obviously, the bound molecules were less accessible to microorganisms or reducing species in the medium. Moreover, substructures of the humic matrix in the surrounding of the bound molecules could decrease the potential of reduction of the nitro groups which also lowers the reaction rate of the nitro groups of the covalently bound molecules, when compared with those of free nitroaromatic molecules. Nevertheless, the data from this experiments may indicate that covalently bound nitroaromatic residues were subject to further reductive transformation. The humic acids without TNP−C4 conjugate which was used as control experiment showed no immunological signal during the entire treatment process. This clearly showed that—even with high concentrations of TNT—the binding of reduced TNT to humic acid did not generate structures which are similar to the TNP conjugate. This observation supports the assumption that the N-functions are mainly responsible for the binding. To check whether these results are transferable to samples of humic acids from TNT contaminated soil, a biological anaerobic/aerobic treatment of soil samples from a contaminated site was carried out.

Bound Trinitrophenyl Residues during Reductive Treatment of Contaminated Soil. The soil used for this experiment was from a former TNT production plant in Stadtallendorf near Marburg (Germany), and earlier examinations had shown a strong signal in the immunoassay indicating bound trinitrophenyl residues (*24*). These residues could originate from reactions of partially oxidized methyl groups with soil organic matter. The formation of such nitrobenzoic acids has been described earlier by several authors during incubation of TNT with bacterial cultures (*28*) and in contaminated soils (*29*−*34*). As intermediates of the oxidation of the methyl group highly reactive aldehyde groups could be formed and bind covalently to soil organic matter. 2,4,6-Trinitrobenzaldehyde was identified as an intermediate of a photocatalytic degradation of TNT (*35*) and was found in soil of contaminated sites (*30*).

During the anaerobic/aerobic treatment of soil from Stadtallendorf the concentrations of TNT, ADNT, and DANT were measured in the supernatant of the slurry (data not shown) and in methanol soil extracts (Figure 3A). The data were very similar to those of experiments with contaminated soil from Hessisch-Lichtenau during an anaerobic/aerobic treatment (*22*). Figure 3A shows the succession of the reduction of TNT to the isomers of ADNT and DANT. Isomers of hydroxylaminodinitrotoluene or TAT could not be detected in the supernatant or in the methanol soil extracts. This verifies again the reactivity of these intermediates toward humic components of the soil.

The immunoassay signals of the covalently bound trinitrophenyl residues (Figure 3B), measured after extraction of humic acids, declined only slowly during the first reaction period (0−7 days of incubation) and decreased significantly after the ADNTs concentrations had reached their maxima. During further anaerobic and aerobic treatment (after 42 days) the immunoassay signal decreased very slowly. After the end of the treatment process, small amounts of bound trinitrophenyl residues were still detectable.

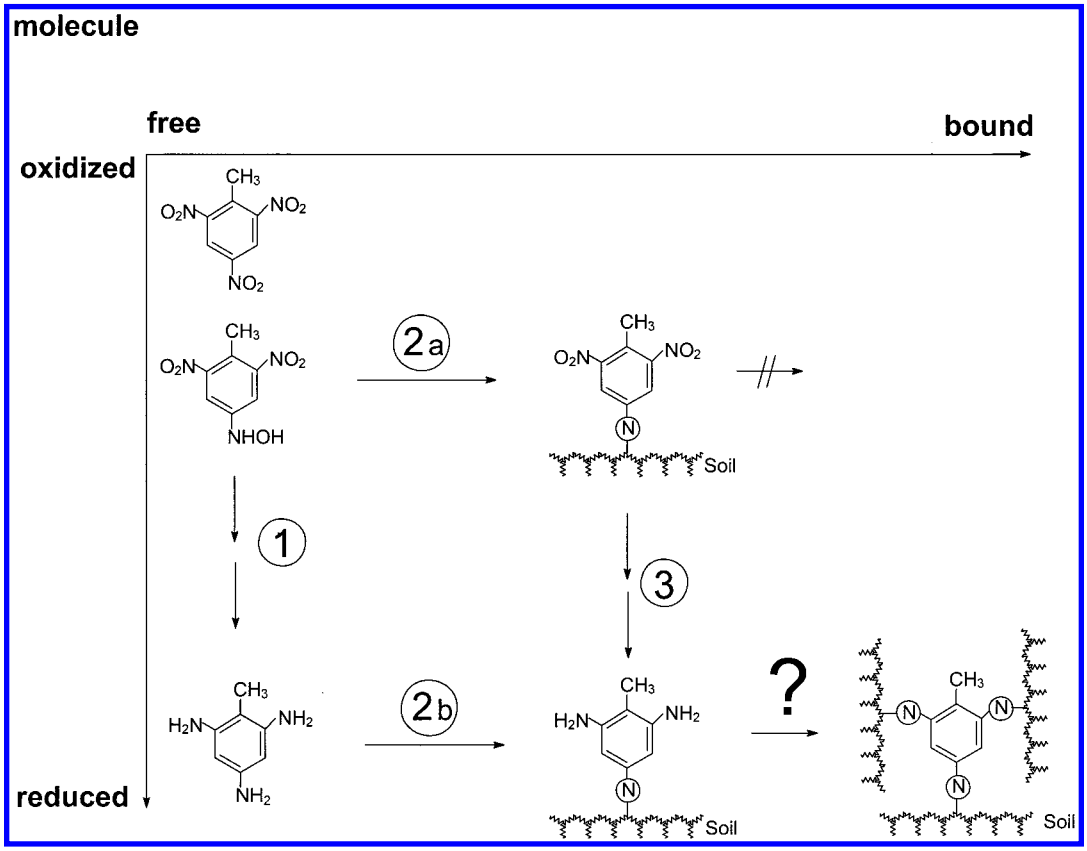

FIGURE 5. Potential reactions of reduced metabolites of TNT with soil. Reduction of nitro groups of free molecules (reaction 1), binding of partially reduced (reaction 2a) and completely reduced metabolites (reaction 2b) to soil organic matter, and further reduction of nitro groups of bound molecules (reaction 3). For multivalent binding at least two nitro groups should be reduced.

Immunoassay signal data obtained with the bioremediation experiment of a TNT contaminated soil may indicate that covalently bound trinitrophenyl residues were present. They were further transformed during the biologically mediated reduction. In contrast to the reduction of the corresponding free TNT and metabolites, such transformation of covalently bound trinitrophenyl residues was considerably slower. In addition, it started later than the reduction of the corresponding molecules in solution which were preferentially reduced.

Coupling of HADNT to Humic Acids. To show the significance of binding of partially reduced metabolites of TNT to humic acids sorption experiments were carried out. Figure 4 shows the kinetics of sorption of isomeric HADNT to humic acids which were extracted from TNT contaminated soil. The concentration of HADNT rapidly decreased, and after 120 min all HADNT was no longer detectable. These results are similar to observations of sorption of HADNT to commercial humic acids from brown coal (*19*). The rate of sorption of HADNT to humic acids is much higher than that observed with TNT, 4-ADNT, DANT, and TAT (*19*). These observations suggest that the reaction of HADNT with organic compounds is an important mechanism for initial binding and immobilization of reduced metabolites of TNT (Figure 5, reaction 2a). Extractions with different solvents, both under anaerobic and aerobic conditions, did not release HADNTs or related compounds (ADNT, DANT). This confirmed the irreversible character of the binding of HADNT to humic acids. These HADNT humic acids coupling products did not interact with the trinitrophenyl monoclonal antibody which confirmed its highly specific character.

Significance for Bioremediation of TNT Contaminated Soils. Since extensive mineralization of TNT in soils cannot be accomplished by the available bioremediation techniques, at present all biological treatment processes aim at an irreversible binding of transformed metabolites of TNT to soil components. The fate of the contaminants during biological treatment and the analysis of the remediated soil is mostly monitored by the disappearance of free TNT and its metabolites and by using radiolabeled [^{14}C]TNT. These methods, however, do not allow for following the fate of partially reduced metabolites which are covalently and irreversibly bound to the soil. Especially the extent of reduction of nitro groups and the mode of incorporation of the residues into soil organic matter were not analyzed. At present only NMR spectroscopy (^{13}C or ^{15}N) or immunological tests can detect bound molecules and determine the degree of reduction and the status of immobilization at the end of a treatment process. The present results show that in addition to the fast disappearance of extractable TNT and its metabolites their complete and irreversible incorporation into the soil matrix must be demonstrated.

As shown here by the immunoassay, the detected trinitrophenyl residues are present in contaminated soils only in minor amounts. Although bound residues generated by chemisorption of HADNT and probably other reduced metabolites to humic acids are not detected with the monoclonal antibody used in the present immunoassay *(27)*, it is likely that the rate and the extent of reduction of any covalently bound structure (e.g. TNT bound by an oxidized methyl group or reduced nitro group) is slow.

In Figure 5, a hypothetical reaction scheme for reduction and binding of TNT and its metabolites during the anaerobic/aerobic treatment process is shown. Three major reactions must be considered. (i) Reduction of nitro groups of free molecules (reaction 1): This reaction is well documented in the literature *(4, 19)*. (ii) Binding of reactive reduced intermediates to soil organic matter (reaction 2a + 2b): Both

partially reduced metabolites (HADNT; reaction 2a) and completely reduced metabolites (TAT; reaction 2b) were shown to be highly reactive in the present of humic acids *(19)*. (iii) Further slow reduction of the bound residues (reaction 3) which was pointed out in the present work.

The rates of the three reactions may be very different. Obviously, the reactive HADNT intermediates interact very fast with humic acids and generate nonextractable residues. The slow and delayed transformation of the bound trinitrophenyl residues, as observed by the immunoassay, indicate that the bound HADNT is reduced much slower than the free molecules (reaction 1 versus reaction 3). It must be assumed that multivalent binding of metabolites of TNT is limited by the extent of reduction of nitro groups of the bound molecules. Therefore, the anaerobic phase of the biological treatment process and the reduction of the nitro groups may be crucial. For progressive cross-linking of the contaminants the anaerobic phase has to be continued even though free components are no longer detectable in organic soil extracts. This is in accord with ^{15}N NMR measurements which show that unreduced nitro groups of bound molecules were still found at the end of the anaerobic treatment process (*21*).

Thus, the immunoassay for nitroaromatic residues bound to humic acids can be a suitable tool for monitoring the progress of bioremediation of TNT contaminated soil. Moreover, this test system allows for following structural changes of bound residues during the treatment. Through combination of this newly developed technique with established methods for the detection of bound residues (i.e. ^{14}C labeling, ^{15}N NMR spectroscopy) additional information about the formation and long-term stability of bound residues become available. If an appropriate antibody for the detection of bound dinitrotolyl residues becomes available, it should be possible to follow the formation and fate of bound ADNT residues which are suspected to be key metabolites during bioremediation under composting (e.g. aerobic rather than anaerobic) conditions.

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