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Covalent Binding of Reduced Metabolites of [$^{15}N_3$]TNT to Soil Organic Matter during a Bioremediation Process Analyzed by ^{15}N NMR Spectroscopy

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Evidence is presented for the covalent binding of biologically reduced metabolites of 2,4,6-$^{15}N_3$-trinitrotoluene (TNT) to different soil fractions (humic acids, fulvic acids, and humin) using liquid ^{15}N NMR spectroscopy. A silylation procedure was used to release soil organic matter from humin and whole soil for spectroscopic measurements. TNT-contaminated soil was spiked with 2,4,6-$^{15}N_3$-trinitrotoluene and ^{14}C-ring labeled TNT, before treatment in a soil slurry reactor. During the anaerobic/aerobic incubation the amount of radioactivity detected in the fulvic and humic acid fractions did not change significantly (11−16%), whereas the radioactivity bound to humin increased to 71%. The ^{15}N NMR spectra of the fulvic acid samples were dominated by a large peak that corresponded to aliphatic amines or ammonia. In the early stages of incubation, ^{15}N NMR analysis of the humic acids indicated bound azoxy compounds. The signals arising from nitro and azoxy groups disappeared with further anaerobic treatment. At the end of incubation, the NMR shifts showed that nitrogen was covalently bound to humic acid as substituted amines and amides. The NMR spectra of the silylated humin suggest formation of azoxy compounds and imine linkages. Bound metabolites possessing nitro groups were also detected. Primary amines formed during the anaerobic incubation disappeared during the aerobic treatment. Simultaneously, the amount of amides and tertiary amines increased. Nitro and azoxy groups of bound molecules were still present in humin at the end of the incubation period. Formation of azoxy compounds from partially reduced TNT followed by binding and further reduction appears to be an important mechanism for the immobilization of metabolites of TNT to soil.

Introduction

2,4,6-Trinitrotoluene (TNT) was the major explosive for charges and bombs during World Wars I and II (*1*). High concentrations of TNT and its congeners are still found in soil and groundwater at communities near former manufacturing sites. The toxicity and the mutagenic and carcinogenic potential of TNT, and particularly its congeners (*2*−*4*), require remediation measures for clean up of such sites.

The stability of TNT in the environment is attributed to the strong electron withdrawing character of the nitro groups and the electron deficiency of the π-ring system of nitroarenes (*5*). Thus, TNT becomes less susceptible to electrophilic attack by oxygenases. Initial reductive reactions of TNT have been widely described using cultures of fungi (*6*−*8*) and bacteria (*9*−*17*) under anaerobic as well as under aerobic conditions.

Recently, a biological treatment process was developed to clean up TNT-contaminated sites (*18*). The aim of the process was to induce the irreversible binding of reduced metabolites of TNT to soil organic matter by an anaerobic/aerobic treatment of a soil slurry. It was shown that some metabolites of TNT such as 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), and 2,4,6-triaminotoluene (TAT) strongly bind to humic substances and clay minerals (*19*). Using ^{14}C-labeled TNT, it was determined that up to 98% of the initial radioactivity was bound to soil during the treatment; extraction of this soil with various chemicals did not lead to a release of TNT or its metabolites (*20*).

To evaluate the stability of the bound residues, direct proof of the irreversible character of the binding is necessary. Three different kinds of interactions between TNT and its metabolites are possible: (i) physical sorption, (ii) sequestration, and (iii) covalent binding to soil organic matter. Only if TNT and its metabolites are bound through covalent linkages, are they considered to be an integral part of humus and thus do not represent a threat to the environment. Soil-bound metabolites of xenobiotics were evaluated in recent studies with aniline, anilazine, and cyprodinil using ^{13}C- or ^{15}N-enriched compounds (*21, 24, 29, 30*). Haider et al. (*22, 23*) analyzed soil-bound residues of the fungicide anilazine by a silylation procedure that allowed subsequent NMR spectroscopic analysis. Using silylation agents such as trimethylchlorosilane, silyl moieties substitute active (i.e., acidic) hydrogen atoms in various functional groups such as carboxy, hydroxy, amino, or thiol groups without further alteration of the carbon skeleton of the derivatized molecule. Apparently, the replacement of active hydrogen in the soil organic matter with silyl groups causes a disintegration of humic aggregates or micelles into smaller units and leads to a release of physically entrapped (sequestered) metabolites. In addition, silylation has proven to be capable of releasing significant amounts of organic substances from the humin fraction (which normally remains insoluble after alkali extraction). Since silylated humic materials dissolve in organic solvents, they can be analyzed by liquid NMR spectroscopy.

The low natural abundance of ^{15}N background signals and the use of ^{15}N-labeled xenobiotics makes liquid state ^{15}N NMR spectroscopy an attractive nondestructive method for structural characterization of bound residues. ^{15}N NMR spectroscopy has been effectively utilized before to investigate the coupling reactions of aniline to Suwannee River humic acids (*24*). The limitations of ^{15}N NMR are the large relaxation time (T_1) values of nonprotonated nitrogens that do not permit rapid pulse repetition rates and the negative nuclear Overhauser effect (NOE) observed with proton decoupling, which can cause negative peaks or loss of signal (*25*). These

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TABLE 1. Summary of Assignments of Chemical Shift Regions to ^{15}N Containing Functional Groups (*24, 25*)

δ (NH_3, liquid, 298 K)/ppm)	assignment
0−25	highly protonated amines[a]
15−70	primary amines[b]
70−100	secondary amines[a]
100−130	tertiary amines[a]
120−150	secondary/tertiary amides[a]
150−250	heterocyclic compounds: pyrrole like N[a]
275−350	azoxy compounds, imines[a]
360−370	nitro groups[b]

[a] Tentative. [b] As determined in the present publication; in DMSO-d_6.

drawbacks can be minimized (while still providing good sensitivity) with the use of inverse-gated decoupling (*24, 26, 27*) and moderate relaxation delays. ^{15}N chemical shift values of characteristic functional groups were obtained from labeled standards when possible, and in other cases the information was obtained from literature (Table 1).

In the present work, the silylation procedure was combined with liquid ^{15}N NMR spectroscopy and ^{14}C-radiocounting to evaluate the binding of reduced metabolites of ($^{15}N_3$-labeled and ^{14}C-labeled) TNT to soil organic matter. The results of the study should confirm the covalent and irreversible character of the binding and could elucidate mechanistic pathways for the chemical immobilization of metabolites of TNT in the soil.

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 using xanthine oxidase and NADH (*31*). 2,2′,6,6′-Tetranitro-4,4′-azoxytoluene (4,4′-AZOXY) and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-AZOXY) were obtained from Jochen Michels (Frankfurt, Germany). The uniformly [ring-^{14}C]TNT had a chemical and radiochemical purity >98% and was purchased from Chemsyn Science Laboratories (Lenexa, KS, U.S.A). The specific activity was 21.58 mCi/mmol (= 0.095 mCi/mg TNT). The purification of the [^{14}C]TNT was described earlier (*20*). The 2,4,6-trinitro-$^{15}N_3$-toluene ($^{15}N_3$-TNT) was synthesized by Michael Major (U.S. Army Center for Health Promotion, Aberdeen Proving Ground, MD). The purity was tested by HPLC and GC-MS. Deuterated methanol (99.8% D) and dimethyl-d_6 sulfoxide (99.9% D) were purchased from Cambridge Isotope Laboratories (Andover, MA). NaOD (1% v/v) solution was prepared by further dilution of 40% NaOD in D_2O (Isotec, Miamisburg, OH).

Organisms. The undefined anaerobic mixed culture used in the experiments was recently shown to reduce TNT to TAT cometabolically while growing on glucose (*19*). The culture conditions were described earlier (*19*). A second undefined mixed culture was able to degrade the fermentation products of glucose aerobically (*20*).

Experimental Setup of the Soil Slurry Experiment. The contaminated soil used in the present study was obtained from a former TNT production site at Hessisch Lichtenau-Hirschhagen near Kassel (Germany). The soil was contaminated with approximately 350 mg TNT/ kg dry soil and had the characteristics described earlier (*18*). Before use, the soil was air-dried and sieved through a 2 mm mesh.

The general experimental setup was described recently (*20*). Air-dried soil (600 g) was spiked with 4.0 g [$^{15}N_3$]TNT per kg of soil. The soil was filled into the reactor containing 1.2 l of 50 mM sodium/potassium phosphate buffer (pH 7.3). The soil slurry was mixed and spiked with [^{14}C]TNT (5.0×10^6 Bq). The temperature of the reactor was kept at 30 °C, and the pH was maintained at 7.3. At the beginning of the anaerobic phase, 20 mL of the mixed bacterial culture, 20 mL of mineral salts (*19*), and glucose (final concentration: 10 mM) was added to the soil slurry. The gas phase of the reactor system was replaced with N_2. Glucose (0.9 mM) was added daily during the anaerobic phase. The volatile phase was vented through three sorption vessels each containing 100 mL of 1 M NaOH to trap CO_2 and was then recirculated into the reactor. The duration of the anaerobic phase was 51 days. This was followed by an aerobic phase of 32 days. At the start of the aerobic phase, 20 mL of the mixed bacterial culture capable of degrading the fermentation products of glucose was added. During the aerobic phase air was introduced into the soil slurry system. The exhaust gas was passed through the same drying and trapping vessels as for the anaerobic phase. After completion of the aerobic treatment, the soil slurry was taken out of the reactor, air-dried, and then stored at 4 °C for further experiments.

Soil Sampling. Aliquots (3−4 mL) of soil slurry were taken at predetermined intervals from the sample port at the bottom of the bioreactor. The samples were centrifuged for 10 min at 2500*g* to separate the supernatant from the soil. The residual soil pellet of each sample was extracted twice for 1 h each, with 10 mL methanol, by shaking at 30 °C. Both methanolic extracts were combined and evaporated under vacuum at 30 °C to a volume of 2 mL. The extractable contaminants and metabolites present in the supernatant and in the methanol extracts were analyzed by high-performance liquid chromatography (HPLC). All residual soil pellets were dried at room temperature in order to relate the amount of sorbed and extractable contaminants to the dry mass.

For NMR analysis aliquots (2 × 30 mL) were taken from the reactor after 1 day, 4 days, 25 days, 51 days, and 83 days. The samples were frozen immediately and stored at −30 °C.

Analytical Methods. Concentrations of TNT, 2-HADNT/4-HADNT, 2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT were determined by reverse-phase HPLC described recently (*18*). 4,4′-AZOXY and 2,2′-AZOXY were separated under the same conditions with a mobile phase of 70/30 (v/v) methanol/water. Concentrations of TAT were determined by reverse-phase HPLC according to Daun et al. (*19*). All contaminants and their metabolites were characterized—in addition to their retention time—by their UV−visible spectra measured in situ with a diode array detector (UVD 340S, Gynkotek, Germering, Germany).

An aliquot (50 μL) of the supernatant or the methanolic soil extract was mixed with 10 mL of scintillation cocktail (rotiszint2200, Roth, Germany), and the radioactivity was determined with a β-scintillation counter (1414 Winspectral, Wallac, Finland). To determine radioactivity of the trapped $^{14}CO_2$ in the NaOH solution, an aliquot (50−200 μL) was mixed with scintillation cocktail and measured as described above. Determination of the total amount of radioactivity in the soil was conducted by combustion of three subsamples of 300 mg (dry weight) and subsequent measurement of the evolved $^{14}CO_2$ with an oxidizer (Biological Oxidizer OX 500, Harvey Instruments Inc., Buffalo, NY).

NMR Spectroscopy. The samples were analyzed on a Bruker AMX-2-500 spectrometer, operating at 50.678 MHz for ^{15}N. Samples were placed in 5 mm NMR tubes after dissolving in appropriate deuterated solvents. All samples were referenced versus an external standard of neat dimethylformamide (DMF), which has a known chemical shift

of 103.81 ppm (25). The chemical shifts are reported downfield of the resonance of liquid ammonia at 25 °C (taken to be 0 ppm). The pulse program used for the analysis of the fulvic acid, humic acid, and silylated humin samples was a 1-D sequence for ^{15}N with inverse-gated proton decoupling in order to enhance polarization transfer while suppressing the undesired NOE effects. The calibrated 90° high power transmitter pulse for ^{15}N was usually about 15 μs. The ^{1}H decoupler pulse widths were also calibrated (average pulse-width for a flip angle of 90° was 110 μs). A spectral width of 40 kHz provided a sufficient window for the observation of most signals. For the methanol extracts of soil (taken in the early stages of the treatment process), a larger spectral width (80 kHz) was employed in order to detect the presence of nitroso groups. The methanol extracts were also run using longer relaxation delays and without decoupling in order to eliminate NOE effects. The relaxation delay was longest for the methanol extracts (30−60 s); the fulvic and humic acids containing bound residues had delays of 15 s; and the viscous silylated humin extracts were pulsed every 6 s. The selection of the relaxation delay was made after a few experimental trials.

NMR Analysis of the Methanol Extracts. The soil samples from 4 and 25 days were extracted twice with methanol to accumulate those portions of TNT and its metabolites that could be desorbed from the soil. After extraction, the methanol extracts were analyzed for radioactivity by liquid scintillation counting. The combined methanol extracts were evaporated to dryness under vacuum (5.2×10^{-6} bar) at 30 °C. The dry residue was redissolved in deuterated methanol and analyzed by ^{15}N NMR spectroscopy.

Soil Fractionation and Preparation of Fulvic Acids and Humic Acids for NMR Analysis. Prior to fractionation, all the soil samples for NMR analysis (see above) were freeze-dried. After methanol extraction of the samples from 4 and 25 days, the fulvic acid and humic acid fractions were obtained by extraction of the soil residues (10−20 g dry soil) with a solution of 300−600 mL of 0.5 M NaOH overnight under a blanket of N_2 gas. After centrifugation, the NaOH-extracted soil (designated as humin) was freeze-dried and saved for further extractions and silylation under nitrogen. The radioactivity present in the humin fraction was determined by combustion. The NaOH extract was acidified with 5 M HCl to a pH of 1.5. This was followed by the overnight precipitation of the humic acids at 4 °C. The precipitate was separated by centrifugation. The fulvic acid fraction remained in solution after the acid treatment. The radioactivity of both fractions was analyzed by liquid scintillation counting.

The solution of fulvic acids was freeze-dried and redissolved in deionized water. To remove divalent cations and paramagnetic heavy metal ions, the fulvic acid solutions were passed through a Dowex macroporous [H^+] ion-exchange resin, MSC-1 (Supelco, Bellefonte, PA). After freeze-drying of the cleaned and protonated fulvic acids, the solid residue was dissolved in methanol for precipitation of remaining salts. The insoluble salts were discarded, and the methanol solution of fulvic acid was evaporated to dryness under vacuum (5.2×10^{-6} bar) at 30 °C. The dry residue was redissolved in 0.6 mL of dimethyl-d_6 sulfoxide (99.9% D) and placed in 5 mm NMR tubes for ^{15}N NMR spectroscopy.

The precipitate of humic acid was redissolved in NaOH solution and dialyzed for 48 h against frequently changed deionized water using a membrane with a 6000−8000 D cutoff (Spectrum, Houston, TX). After freeze-drying, the purified humic acid was dissolved in 1% deuterated NaOD and analyzed by ^{15}N NMR spectroscopy.

Silylation and Preparation of Humin for NMR Analysis. Prior to silylation, the freeze-dried humin fraction was extracted two times with dilute HCl to remove paramagnetic heavy metals from the soil. The first extraction was carried out with a 0.25 M HCl solution and the second with a 0.5 M HCl solution. Then the soil was washed with deionized water. All extractions were carried out for 1 h at 25 °C. The radioactivity in the extracts was measured by liquid scintillation counting to calculate the release of radiolabeled compounds during this treatment. After freeze-drying, approximately 5 g of the dehydrated soil sample was placed in a 25 mL Nalgene centrifuge tube (Nalge Company, Rochester, NY) and mixed with 10 mL of dimethyl sulfoxide (DMSO), 1 mL of trimethylchlorosilane (TMSCl), and 50 μL of pyridine. Each of the solvents was freshly distilled and stored under nitrogen in the presence of molecular sieve prior to use. The centrifuge tube was capped and shaken horizontally at 25 °C for 12−16 h. After the incubation the reaction mixture was centrifuged; then, the DMSO/TMSCl supernatant was separated from the soil residue and analyzed for radioactivity. Approximately 5−7 mL of the DMSO/TMSCl extract was transferred into a round-bottom flask, and the solvent was evaporated under high vacuum (15×10^{-6} bar). The residue was redissolved in 0.5 mL of dimethyl-d_6 sulfoxide and transferred into 5 mm NMR tubes for ^{15}N NMR spectroscopy. The wet silylated soil residue was also dried, and the radioactivity of the dry soil was measured by combustion.

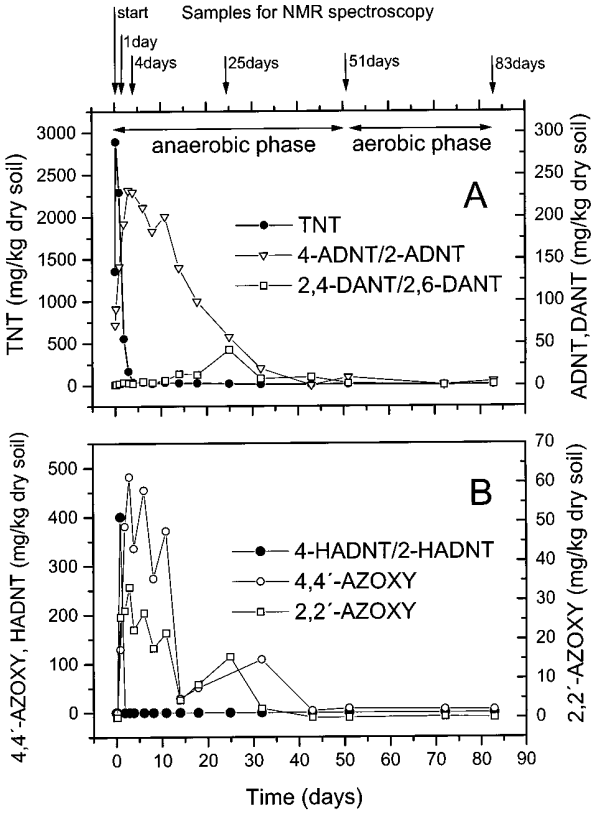

FIGURE 1. Reduction process of TNT and its metabolites during the anaerobic/aerobic treatment. Amounts of (A) TNT, aminodinitrotoluenes (ADNT), and diaminonitrotoluenes (DANT) and (B) 4,4′-AZOXY, 2,2′-AZOXY, and hydroxylaminodinitrotoluenes (HADNT) that were desorbed from the soil with methanol.

Results and Discussion

Cometabolic Reduction of [^{14}C]TNT/[^{15}N]TNT in Soil. The amounts of TNT, 2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT/2,6-DANT, and TAT were measured in the supernatant of the soil slurry (data not shown) and in the methanolic soil extracts during the anaerobic/aerobic treatment process (Figure 1 A).

TABLE 2. Distribution of Radioactivity in Different Fractions of the Soil during the Anaerobic/Aerobic Treatment Process

	% of applied radioactivity				
fraction	1 day[b]	4 days[b]	25 days[b]	51 days[b]	83 days[b]
methanol extract	102	75.9	63.9	17.4	1.1
NaOH extract	nd[a]	11.3	16.4	14.6	13.3
fulvic acids	nd[a]	5.5	8.3	8.9	6.2
humic acids	nd[a]	5.8	8.1	5.7	7.1
humin	nd[a]	19.5	25.9	64.6	71.0
HCl extract	nd[a]	1.4	1.1	1.8	3.4
silylated extract (DMSO)	nd[a]	10.7	15.2	30.6	44.2
soil residue after silylation	nd[a]	7.4	9.6	32.2	23.4
recovery	nd[a]	106.7	106.2	96.6	85.4

[a]Not determined: nd. [b] Incubation time.

First, the reduction of one nitro group occurs, leading to the formation of isomeric hydroxylaminodinitrotoluenes (maximum amount formed was approximately 400 mg/kg dry soil from the initial TNT concentration of 4000 mg/kg soil) after 1 day of anaerobic incubation (Figure 1B). Similar observations were also made in experiments that did not involve the use of soil (19). Further reduction led to the formation of isomeric aminodinitroluenes. The aminodinitroluenes were then reduced to 2,4-DANT.

TAT, which could not be detected in experiments with moderately contaminated soil (<500 mg TNT/kg dry soil) (19, 20), was observed in small amounts in the supernatant (0.3−0.4 mM). It was detectable after the disappearance of 2,4-DANT (35 days of the incubation) until the end of the anaerobic phase.

In contrast to former observations (20), after the formation of hydroxylaminodinitrotoluenes (1 day of incubation), significant amounts of 4,4′-AZOXY and 2,2′-AZOXY were measured in the methanol extract (Figure 1B). The maximum amount of 4,4′-AZOXY was 482 mg/kg dry soil after 3 days of incubation. The maximum concentration of 2,2′-AZOXY amounted to 33 mg/kg dry soil for the same sample. The major part of the azoxy compounds disappeared during anaerobic incubation; at the end of the anaerobic phase, less than 9 mg/kg dry soil of 2,2′-AZOXY was detected. However, two additional isomers of azoxy compounds could also be formed: 2,4′-dimethyl-3,3′,5,5′-tetranitro-ONN-azoxybenzene (2,4′-AZOXY) and 2′,4-dimethyl-3,3′,5,5′-tetranitro-ONN-azoxybenzene (2′,4-AZOXY) (28). In addition to 2,2′-AZOXY (retention time: 12.9 min) and 4,4′-AZOXY (retention time: 21.4 min), the HPLC analysis showed two signals of unidentified compounds at 14.3 min (peak A) and 16.6 min (peak B). The absorption maximum was 238.3 nm for peak A and 242.4 nm for peak B. These absorption maxima were similar to those of 2,2'-AZOXY (247.1 nm) and 4,4'-AZOXY (236.8 nm). This indicates that the 2,4′-AZOXY and 2′,4-AZOXY compounds may have been formed. During the treatment, the sum of the peak areas of these compounds was approximately half of the area of the 4,4'-AZOXY compound.

During incubation, the sum of extractable metabolites of TNT (2-HADNT/4-HADNT, 2-ADNT/4-ADNT, 2,4-DANT/2,6-DANT) decreased in comparison to the total amount of TNT that was extractable from the soil at the beginning of the experiment (Figure 1A). After 42 days of the anaerobic treatment, neither TNT nor its metabolites could be extracted from the soil.

Distribution of Radioactivity in Different Soil Fractions. To evaluate the distribution of reduced TNT metabolites within the soil fractions, the radioactivity was measured in fulvic acid, humic acid, and humin after methanol extraction (Table 2). The results showed that the amount of radioactivity found in the NaOH extract at different stages of the treatment was nearly the same (11.3% of the initial radioactivity at the beginning to 13.3% at the end of the incubation). Approximately half of this radioactivity was bound to fulvic acids and the other half to humic acids. Since the concentration of the radioactivity within these fractions remains relatively constant, it appears that there is a finite amount that can be incorporated into this portion of soil. The humin fraction then becomes available for interactions with the TNT metabolites. This was confirmed by the increasing amount of radioactivity bound to humin during prolonged incubation which reached a maximum at the end of the treatment (Table 2).

Before silylation, HCl wash of the humin released only small amounts of radioactivity (1.1%−3.4%). Upon silylation of the humin of the final stage (83 days), 44.2% of the total bound radioactivity was released into DMSO (Table 2). Upon silylation of the humin fraction at different stages of the experiment, about 45−65% of the radioactivity bound within humin could be released into DMSO in all cases. The amount of total radioactivity initially measured in humin increased from 19.5% (4 days) to 71.0% (83 days); correspondingly, the total radioactivity extracted from the soil by silylation of humin ranged from 10.7% (4 days) to 44.2% (83 days). For the samples of 4 and 25 days, only 7.4% and 9.6%, respectively, of the initial radioactivity remained in the soil after all extractions and silylation. The radioactivity remaining in the soil was 32.2% for the sample of 51 days and 23.4% for the sample of 83 days.

From the radioactivity results obtained for the silylation treatment, it can be concluded that the technique is an efficient method for the release of bound xenobiotics by derivatization and the subsequent dissolution of humin into an organic solvent (DMSO), thereby making this fraction available for liquid NMR spectroscopy.

^{15}N NMR Spectra of Reference Compounds. Reference compounds were measured in different solvents for comparison with ^{15}N NMR shifts of compounds from extracted samples. The NMR spectrum of TNT in methanol, obtained using a pulse relaxation delay of 10 s, showed two peaks: 361.8 ppm (4-NO_2) and 368.1 ppm (2,6-NO_2). The spectrum of 4-ADNT in methanol (obtained with a 15 s relaxation delay) also showed two peaks: 56.2 ppm (4-NH_2) and 371.2 ppm (2,6-NO_2). Peaks at 50.7 ppm (4-NH_2), 52.9 ppm (2-NH_2), and 377.5 ppm (6-NO_2) were seen in the spectrum of 2,4-DANT in methanol, also using a 15 s pulse delay.

The NMR analysis of 4-ADNT in DMSO (pulse relaxation delay of 5 s) gave the following ^{15}N signals: 66.1 ppm (4-NH_2) and 367.1 ppm (2,6-NO_2).

^{15}N NMR Spectra of the Methanol Extracts. The methanol extracts of the soil samples from 4 and 25 days were studied by NMR with inverse-gated decoupling of the protons and a ^{15}N pulse relaxation delay of 30 s (Figure 2 A to C). At an early stage (4 days; Figure 2A,B) the presence of partially reduced TNT (2-ADNT/4-ADNT and 2,4-DANT/2,6-DANT) is clearly deduced from the appearance of signals for aromatic amines (59−73 ppm) and from the slight alterations of the chemical shifts of the nitro groups of these compounds (375 ppm). Azoxy species (at 321 ppm) were also observed (Figure 2A,B). The small splitting of the peak at 321 ppm arises from ^{15}N-^{15}N coupling. Nitro groups of azoxy species showed a shift of 370 and 368 ppm. After 25 days of incubation only minor amounts of 2-ADNT/4-ADNT and 2,4-DANT/2,6-DANT could be desorbed from the soil by methanol as indicated by HPLC analysis (Figure 1A,B). Therefore, the NMR spectrum of this extract showed a significant decrease of the nitro resonance (375 ppm, Figure 2C). Since free 4,4′-AZOXY was still present in the soil (Figure 1 B), the peaks at 321 and 368 ppm/370 ppm were also seen in the 25 day extract (Figure 2C). A characteristic feature to be noted for free residues in

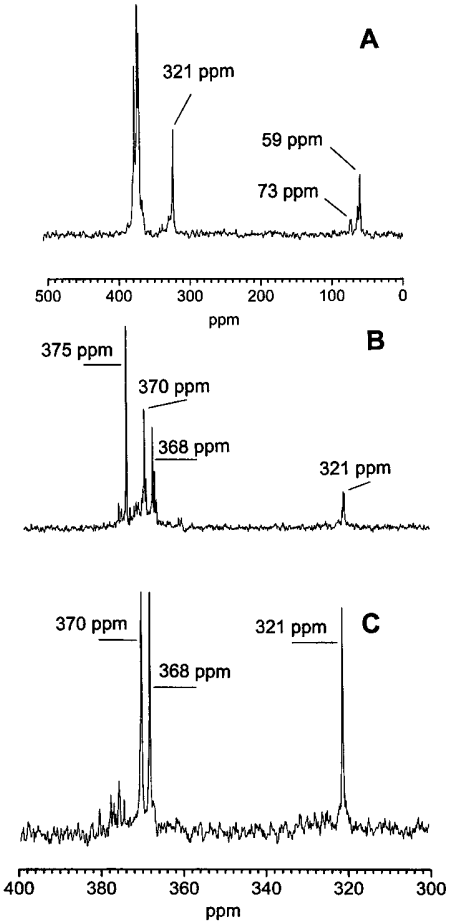

FIGURE 2. Inverse-gated decoupled ^{15}N NMR spectra of the methanolic soil extracts in methanol-d_4: (A) sample after 4 days (0−500 ppm), (B) sample after 4 days (300−400 ppm), and (C) sample after 25 days.

organic solvent is the sharp nature of all the peaks observed in the NMR spectra—all the signals are less than 0.5 ppm wide at half the peak height.

The presence of nitroso could not be ascertained even with longer relaxation intervals (60 s) and without proton decoupling. Perhaps even longer delays of 120 s are necessary for the observation of a signal from this functional group (that usually appears between 900 and 1000 ppm) because of the very long relaxation time (T_1) it would have in an organic solvent. It is quite likely that the nitroso, if present, would only exist as a transient species and would be quickly converted to a hydroxylamino moiety.

^{15}N Spectra of Extracted Fulvic Acids. Figure 3 shows the ^{15}N NMR spectrum of fulvic acids obtained in DMSO-d_6 with inverse-gated decoupling and a 15 s relaxation delay. The NMR spectra of fulvic acids of samples after 25 days (3136 scans), 51 days (2724 scans), and 83 days (Figure 3, 2465 scans), all showed the presence of one prominent peak that corresponded to a protonated aliphatic amino group. The NMR line width of this peak is also quite sharp, and a measurement of the "relative" T_1 (in the presence of dissolved oxygen) of this amine (51 day sample) versus the free amine of a standard urea sample indicated that the former had a slightly faster relaxation time (2.56 s versus 4.57 s). This implies that there exists some interaction of this amine with the fulvic acid that makes it relax a little faster than expected. Since the fulvic acids are moderately sized mobile molecules which upon protonation (via H^+ ion-exchange) dissolve readily in an organic solvent (DMSO), they exhibit rapid

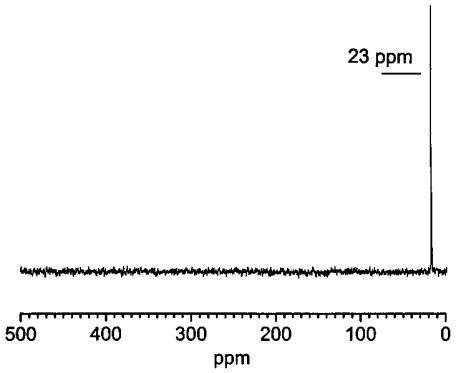

FIGURE 3. Inverse-gated decoupled ^{15}N NMR spectra (0−500 ppm) of fulvic acids in DMSO-d_6: sample after 83 days.

rotation because of the very low viscosity in solvent. Hence, the relaxation time of the amine would be unaffected by only the presence of fulvic acid. Ionic interactions with fulvic acid, however, are likely and could be the explanation for the short T_1 of this species. Also, the chemical shift value of 23 ppm in DMSO suggests a NH_3^+ species that arises either from an aliphatic amine or from dissolved ammonia that is solvated by H-bonding with DMSO—both of these possibilities do not preclude the formation of this species as a result of hydrolysis during the preliminary extraction of soil organic matter. When the fulvic acid sample (51 days) is dissolved in basic medium (1% NaOD), the H-bonding interactions no longer exist, and the same peak (originally observed in DMSO at 23 ppm) is seen to move to a shift of 0 ppm. This behavior strongly suggests that the peak corresponds to ammonia (which is formed by cleavage of amides as a result of pH influence). The presence of ammonia was verified by positive reaction of litmus paper to the gently heated vapors of the fulvic acid sample (83 days) in NaOD.

No other peaks could be detected in the ^{15}N fulvic acid spectra partly due to the ion-exchange cleanup procedure and subsequent methanol extraction, which together caused significant losses (>50%) in radioactivity. Also, the pulse repetition rate utilized in the NMR sequence for fulvic acid samples did not permit complete relaxation (and hence detection) of nonprotonated species in mobile organic solvent. The spin−lattice relaxation time (T_1) of the NO_2 groups is quite large (>30 s) because of the very low solvent viscosity of these particular samples in DMSO, and hence, it is quite likely that this signal was lost due to the rapid pulse repetition rate of 15 s. The amine groups (with much smaller T_1 values) can be observed under these conditions. For the 83 day-old sample, the NMR analysis was repeated using a delay of 30 s. This spectrum clearly showed azoxy resonances that emerge well above the baseline (spectrum not shown). It is likely that longer relaxation delays of 60−120 s will allow the detection of all ^{15}N functional groups present; however, the large amounts of NMR time required to achieve good signal-to-noise discouraged this possibility.

The control sample of fulvic acids, obtained from the identical soil (Hessisch-Lichtenau) without addition of ^{15}N-labeled TNT and subjected to the same pretreatment, showed no peaks in the NMR spectrum after the acquisition of 3056 scans using the same conditions.

^{15}N NMR Spectra of Extracted Humic Acids. The ^{15}N NMR spectra were obtained for humic acids of samples taken after 4 days (5780 scans; Figure 4 A), 25 days (2916 scans; Figure 4B), 51 days (4508 scans; Figure 4C), and 83 days (2627 scans; Figure 4D); all of which were dissolved in 1% NaOD. The early stage of the experiment (4 days; Figure 4A) showed that initial binding to humic acid first occurred via ionic interactions of azoxy compounds (300−310 ppm). Nitro groups (374

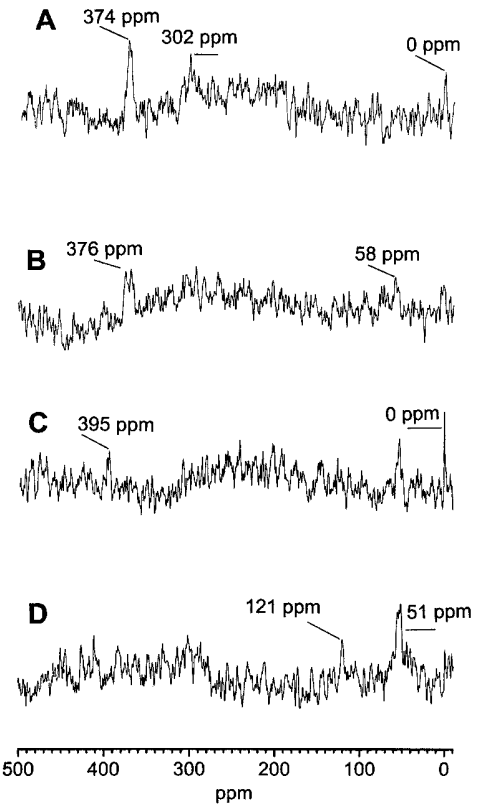

FIGURE 4. Inverse-gated decoupled ^{15}N NMR spectra (−10 to 500 ppm) of humic acids in 1% (v/v) NaOD: (A) sample after 4 days, (B) sample after 25 days, (C) sample after 51 days, and (D) sample after 83 days.

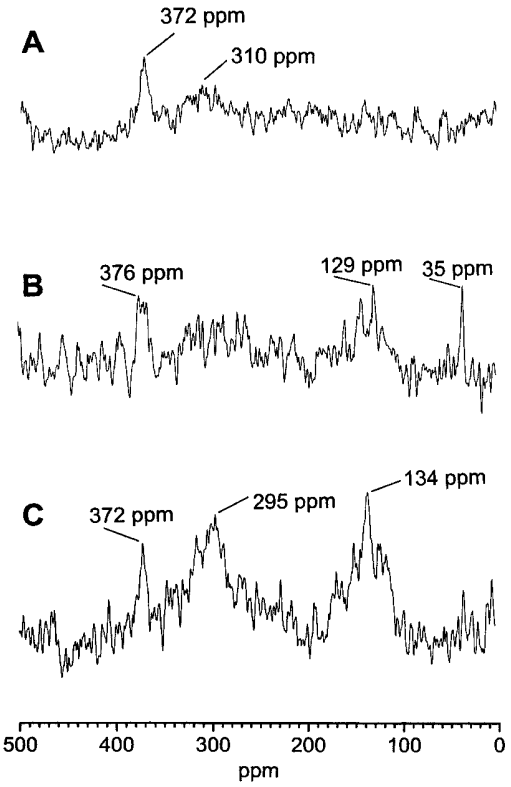

FIGURE 5. Inverse-gated decoupled ^{15}N NMR spectra (0−500 ppm) of silylated humin in DMSO-d_6: (A) sample after 25 days, (B) sample after 51 days, and (C) sample after 83 days.

ppm) were also observed. The small sharp signal at 0 ppm indicates the presence of an aliphatic amine like ammonia that is formed by the high pH in strongly basic solvent, as was seen earlier for the protonated amine in fulvic acid. In the case of humic acid samples in alkali, a small amount of ammonia could be trapped and sequestered within humic micelles/cages.

As a result of the anaerobic treatment (Figure 4B,C) there is a dramatic reduction of the nitro peaks and an increase in the intensity of the peak corresponding to aromatic amines of bound molecules (peak at 58 ppm in spectra of samples taken after 25 and 51 days). Also, there is disappearance of the azoxy signals (centered about 300 ppm in Figure 4A for the sample after 4 days) and small changes in the chemical shifts of the nitro groups due to reductive changes in the overall molecule, 374 ppm (Figure 4A) to 395 ppm (sample after 51 days, Figure 4C). The peak at 0 ppm, attributed to protonated aliphatic amine or ammonia, is also seen for the 25 and 51 day-old samples (strongly reducing conditions). After aerobic treatment (83 days), this peak is no longer seen. This can be explained by the reactive nature of such a species in the presence of oxygen. During the aerobic treatment, there is an increase in the peak seen at about 50 ppm, indicating an increased amount of amino groups, and a clear peak at 121 ppm that could arise from the formation of tertiary amines or amides (Figure 4D). This chemical shift region also includes anilinoquinones and pyrrole-like N—some of which could be formed due to increasing humic-like character of the bound residue, effectively promoted within the humic acid fraction.

The NMR line widths suggest strong (covalent) binding interactions between the metabolites and humic acid. The linebroadening factor of 60 Hz applied during processing of the raw data would widen each peak in these spectra by approximately 1−2 ppm. The line widths observed for these peaks (except for the resonance at 0 ppm) are between 8 and 15 ppm, clearly indicating that they are neither free nor sequestered within humic acid—either of these possibilities would have resulted in sharper line widths (1−5 ppm), similar to those seen for the methanol extracts. Thus, the characteristic broad nature of these resonances suggests that nitrogen is strongly incorporated within humic acid, indicative of covalent binding. The 0 ppm resonance, seen only under anaerobic conditions, has a relatively narrow line width, which suggests that the protonated amine responsible for this signal has free rotation or has only ionic interactions with humic acid. The line width of this peak lies between 1 and 3 ppm in all cases.

The spectrum of the humic acid obtained from the soil without addition of ^{15}N-labeled TNT did not show any characteristic signals as were detected above.

^{15}N NMR Spectra of Silylated Humin. Figure 5 shows the ^{15}N NMR spectra of the silylated humin after 25 days (8508 scans; Figure 5A), 51 days (11 608 scans; Figure 5B), and 83 days (11 464 scans; Figure 5C), obtained using DMSO-d_6 as the lock solvent and a pulse repetition rate of 6 s.

At an early stage of the incubation (4 days, 13 364 scans) the NMR spectrum resembled that of the control, implying that there was no NMR-detectable binding at this stage (spectrum not shown). Despite the very large number of acquisitions, no clear signals are observed—the low sensitivity of the nitro group and the small amount of bound radioactivity present after 4 days are contributing factors for the poor detection limits in this case.

The spectrum of the 25 day sample showed a large broad peak about 360−380 ppm that corresponds to the nitro region, and another very broad resonance centered about 310 ppm, which is representative of azoxy species (Figure 5A). The nitro group is also seen in the spectrum after 51 days (Figure

5B). Additional resonances corresponding to hydroxylamines, substituted amines, and amides (120−160 ppm) are seen in this spectrum. A prominent sharp peak corresponding to aliphatic amine is also seen at 35 ppm. The presence of this peak suggests cleavage of some of the amide present during the alkali extraction to isolate the humic acids. The aerobic phase caused a significant reduction of this amine moiety (83 days; Figure 5C). Also, large broad resonances arise in the region between 270 and 320 ppm, which can be attributed to imines arising from incorporation within humic matter and also to azoxy groups that materialize again due to incorporation of 6% additional nitroaromatic free residue into humin during the aerobic process (see radioactivity data, Table 2). The continued presence of nitro functionalities within humin supports this explanation. The broad group of signals ranging from 120 to 170 ppm also has increased in the spectrum in Figure 5C (83 days), implying an increase in tertiary amine groups and amides due to increased binding within humin in the presence of oxygen.

The high viscosity of the silylated humin samples contributed to reduced spin−lattice (T_1) relaxation times, enabling a faster pulse repetition rate (6 s) compared to that utilized for samples of humic acid (15 s). In general, the extraction of silylated soil matter into DMSO is seen to broaden ^{13}C NMR background signals considerably (data not shown)—the broad nature of such ^{13}C resonances can be partly attributed to the wide range of structural features present in humic substances (26) and also due to the strong solvent interactions of DMSO with the humin fragments. In comparison, no background ^{15}N signals are usually observed in liquid-state spectra of humic acids (24) nor are any seen in the spectrum of the control of silylated humin. However, the ^{15}N signals arising from the xenobiotics within humin (in DMSO-d_6) do exhibit broadened NMR line widths primarily as a result of covalent interactions; thus, each broadened ^{15}N resonance is reflective of strong binding of this ^{15}N-labeled moiety to a range of environments (of the ^{13}C-background) that are similar in structure. Residues that are unattached to these humic environments exhibit no broadening effects (Figure 6 A). The silylation of the humin would have released any sequestered residues (21) by fragmentation. The presence of such species would be detected as sharp peaks, unless these too are strongly bound to humin.

The NMR spectrum of silylated humin from the soil without addition of ^{15}N-labeled TNT showed no resonances that were significantly above baseline noise level after 12 696 scans.

^{15}N NMR Spectra of Silylated Whole Soil. A spectrum of the silylated whole soil of the sample taken after 83 days (Figure 6C) was obtained in order to compare the results with those obtained for the individual soil fractions. The spectrum seen here for silylated whole soil is very representative of the collective features observed for fulvic acid, humic acid and silylated humin. The picture of various characteristic nitrogen resonances that are observed included a sharp peak at 33 ppm, attributed to an aliphatic amine or ammonia. This peak corresponds to the peak at 23 ppm seen in fulvic acids. (The upfield shift of 10 ppm in fulvic acid is a result of the [H^+] ion-exchange treatment of fulvic acid). Aromatic amines of covalently bound molecules (47, 67 ppm), amides, tertiary amines (110−140 ppm), azoxy, imine (290−320 ppm), and nitro groups (365−375 ppm) were also observed.

To compare the NMR line widths in the absence of binding, the whole soil of the sample taken after 1 day was directly silylated and also analyzed by NMR spectroscopy (Figure 6A,B). The spectrum shows two large sharp resonances that correspond to the peaks for the ortho nitro groups (at 368 ppm) and the para nitro group (at 362 ppm) of labeled TNT (Figure 6B). Very small peaks at 321, 327, 371, and 373 ppm are seen to be emerging—these correspond to the partially reduced azoxynitrotoluene and aminonitrotoluene metabolites (Figure 2B). Most of the species observed in this spectrum (Figure 6A,B) are extractable by methanol. Also, the presence of paramagnetic substances that may not have been eliminated prior to NMR analysis does not seem to affect the line widths noticeably. The results clearly indicate that in the absence of covalent binding the line widths of the ^{15}N signals are narrow.

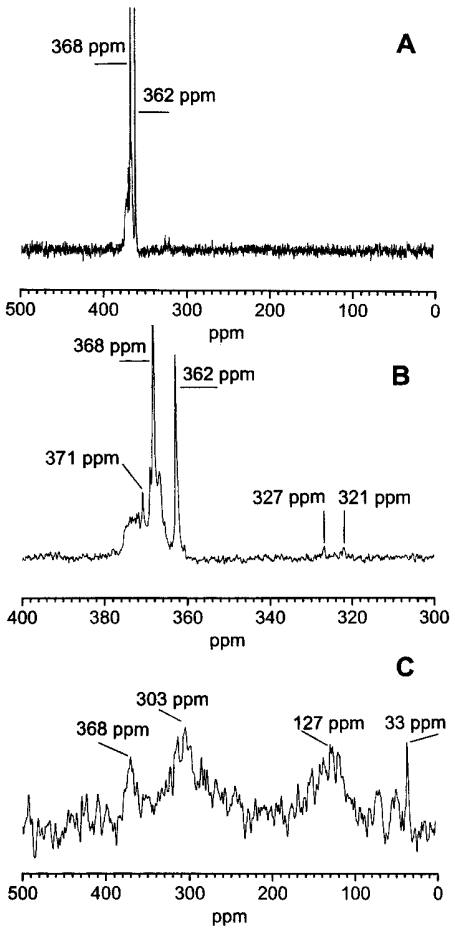

FIGURE 6. Inverse-gated decoupled ^{15}N NMR spectra of silylated whole soil in DMSO-d_6: (A) sample after 1 day (0−500 ppm), (B) sample after 1 day (300−400 ppm), and (C) sample after 83 days (0−500 ppm).

Elucidation of Pathways for Binding of Reduced TNT Metabolites to Soil Fractions. The elucidation of mechanisms involved in the binding processes is limited by the fact that NMR chemical shifts obtained for bound species in real soil systems cannot be readily compared with standards since it is not easy to mimic experimental conditions. This implies that unambiguous characterizations cannot be made; hence, the interpretations of the NMR signals must be accompanied by careful evaluation of all likely possibilities of species that share similar chemical shifts.

In fulvic acid, only mobile aliphatic amines were detected which did not undergo much visible change (by NMR) during the entire incubation. The presence of this species can be attributed to the cleavage of amides as a result of hydrolysis (during the extraction and isolation process for humic and fulvic acids), which leads to the release of ammonia. For the fulvic acid samples, the NMR pulse relaxation delay was insufficient for the observation of nonprotonated nitrogen functional groups. The fulvic acid results are hence not used for the elucidation of a pathway for binding.

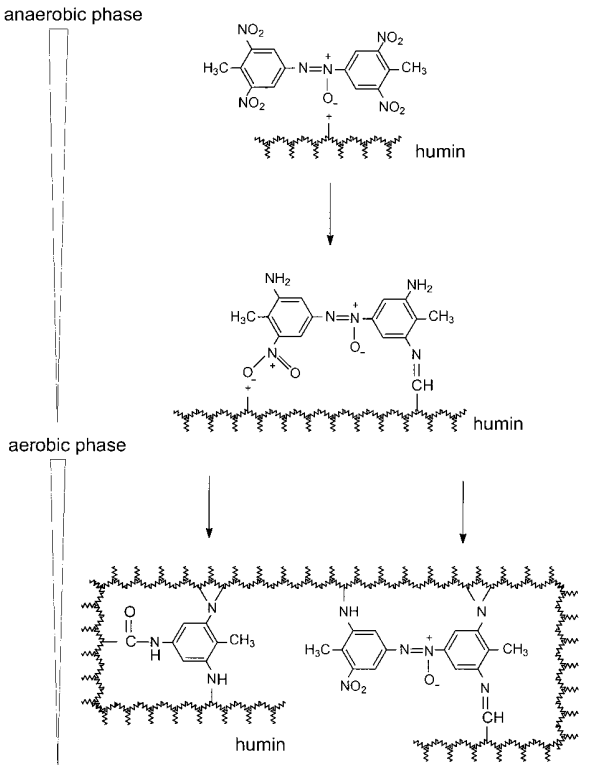

FIGURE 7. Proposed pathway of binding of reduced metabolites of TNT to humin during the anaerobic/aerobic treatment process.

The humic acid fraction, because of the presence of cagelike structures and its macromolecular nature, presented numerous reaction sites for the binding of reduced metabolites that were initially transformed by bacteria and reductive species (e.g. Fe^{2+}) in the soil. The presence of nitro groups and azoxy species (that were determined in the sample taken after 4 days of incubation) are not observable in humic acid at the end of the bioremediation. The anaerobic treatment indicates the formation of aromatic amines (Figure 4B,C). The further reaction and incorporation of aromatic amines within humic acid led to formation of tertiary amines and amides. It appears that some of the amide is cleaved during extraction as a result of hydrolysis, resulting in some amine and also a small amount of ammonia that remains sequestered or trapped within humic acid.

The bulk of xenobiotic residue was steadily incorporated into humin as incubation progressed, and the limited experimental period did not allow for complete transformation of all the nitro groups present in humin. The isolation of hydroxylamino compounds by HPLC and the presence of free and bound residues of azoxy species indicate that one pathway for reduction of TNT proceeds via condensation of the reactive hydroxylamine, followed by adsorption and subsequent integration of this species in soil organic matter (Figure 7). This appears to be an important pathway for the immobilization of TNT.

The radioactivity data suggests that reactions within humin progress rapidly after the organic sites within humic acid and fulvic acid are used up (25 days). In humin, nitro groups were first detected by NMR spectroscopy only after 25 days of incubation. Even though there was a continuous increase of radioactivity in the humin fraction, the NMR spectra indicated no corresponding accumulation of the nitro functionality. The amount of nitro groups present was seen to remain more or less constant (365−385 ppm, Figure 5), and a continuous increase of transformation products was observed, keeping the mass balance. This implies that an increase in the incubation period would very likely have resulted in the disappearance of all the nitro groups within the humin fraction. Aliphatic amines (35 ppm) which are seen only toward the end of the anaerobic phase along with amides (51 days, Figure 5B) are primarily the result of hydrolysis of the latter during the alkali extraction to separate humic acids from humin; after the aerobic phase the aliphatic amines are considerably decreased (83 days, Figure 5C). They are likely to be converted to imines (300−320 ppm) as a result of incorporation into humin.

Future work that includes a study of selected individual standards of likely metabolites that are subjected to experimental treatments and subsequent analysis by ^{15}N NMR spectroscopy would provide valuable insight into detailed mechanistic pathways.

Acknowledgments

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