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Characterization of ^{15}N -TNT Residues After an Anaerobic/Aerobic Treatment of Soil/Molasses Mixtures by Solid-State ^{15}N NMR Spectroscopy. 2. Systematic Investigation of Whole Soil and Different Humic Fractions

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An anaerobic/aerobic composting experiment with ^{15}N -2,4,6-trinitrotoluene (TNT) spiked soil was performed to investigate the fate of the explosive under the applied conditions. For a qualitative description of TNT-residues formed during the composting process, bulk soil and different soil fractions were subjected to solid-state ^{15}N NMR spectroscopy. Major resonance signals could be detected in the chemical shift regions of five-ring heterocyclic nitrogen and in the area of aniline derivatives and primary amines. Distinct nitro peaks were found in the bulk samples and in the humic fractions obtained with a mild extraction procedure. This signal disappeared in the material extracted with a more drastic procedure. Quantitative investigations of the ^{15}N distribution in the composted material revealed that 33% of the stable nitrogen isotope was incorporated into the humic- and fulvic acid, and 23% was present in the humin. Furthermore 38.8% of the ^{15}N present in the composted material could be allotted to condensed TNT residues, whereas 1.9% are assigned to nitro functions and 15.2% to amino functions. In the investigation presented here a bioremediation method was simulated with ^{15}N -TNT spiked soil. The nonradioactive label allowed a qualitative and quantitative characterization of residues of the explosive. Our results give strong evidence for a stable incorporation of the nitroaromatics into the humic material of soils. However, further investigations will be necessary to prove a long-time stability of bound TNT residues and to assess toxicological effects of the treated soil.

Introduction

Soil and groundwater of most former TNT production and processing plants are contaminated with nitroaromatics and aromatic amines. At present, strong efforts are being made to develop bioremediation strategies to clean up these sites. Currently, incineration is the only remediation technique accepted by authorities. This technique is, however, very costly and destroys the soil biology and structure. Recent investigations concerning the biodegradability of TNT revealed that the molecule is very recalcitrant against microbial mineralization (1–3). On the other hand, it seems to be an ubiquitous ability of soil microorganisms to reduce the nitro groups of the explosive to amino groups (1, 3–5). This was the starting point for the development of different bioremediation strategies for TNT contaminated soil. Currently under investigation are different systems, i.e., soil composting (3, 4, 6–11), anaerobic slurry reactor treatments with and without a subsequent aerobic phase (2, 12, 13), and anaerobic/aerobic composting processes (14–18). The latter was first performed with an anaerobic phase during which the soil/supplement mixture was flooded with water (14, 15). The method was improved by compressing the material instead of flooding it (18). Considerable efforts were made to understand the biotransformation processes during the anaerobic/aerobic composting. Figure 1 depicts the present understanding of the system. Nitroso and hydroxylamino functions are intermediate products of this reaction (19). Azoxynitrotoluenes can be generated spontaneously by the condensation of these molecules. Remaining nitro groups can be reduced thereafter. Furthermore, a condensation of TNT reduction products is expected (16, 20, 21). As a result, different acetylated and formylated TNT metabolites are generated. For 4-*N*-acetylamino-2-amino-6-nitrotoluene it could be shown that the acetyl group is easily removed and 2,4-diamino-6-nitrotoluene (2,4-DANT) is formed (16).

It is generally well accepted that aromatic amines can bind covalently to soil organic matter, but only little is known about this humification process (Figure 1). Three different incorporation models are frequently discussed: formation of imine bonds, generation of secondary amines, and incorporation into heterocyclic ring systems (3, 22–28).

Drzyga et al. (17) investigated the humification of ^{14}C -TNT in the same anaerobic/aerobic composting process that was employed in this study. They showed that approximately 83% of the radioactivity was bound in humic fractions by this process. Sixty-two percent was incorporated in the humin fraction, 11.1% in the humic acid, and 9.5% in the fulvic acid. Other authors reported an immobilization of approximately 22–83% of ^{14}C -TNT by aerobic composting treatments (4, 6, 8). These findings support the assumption that TNT transformation products can bind to soil organic matter. Until today, N mass balance studies and a qualitative description of the TNT residues formed during the anaerobic/aerobic compost process are missing. The aim of the study presented here was to describe the chemical structures of immobilized residues of TNT formed during an anaerobic/aerobic

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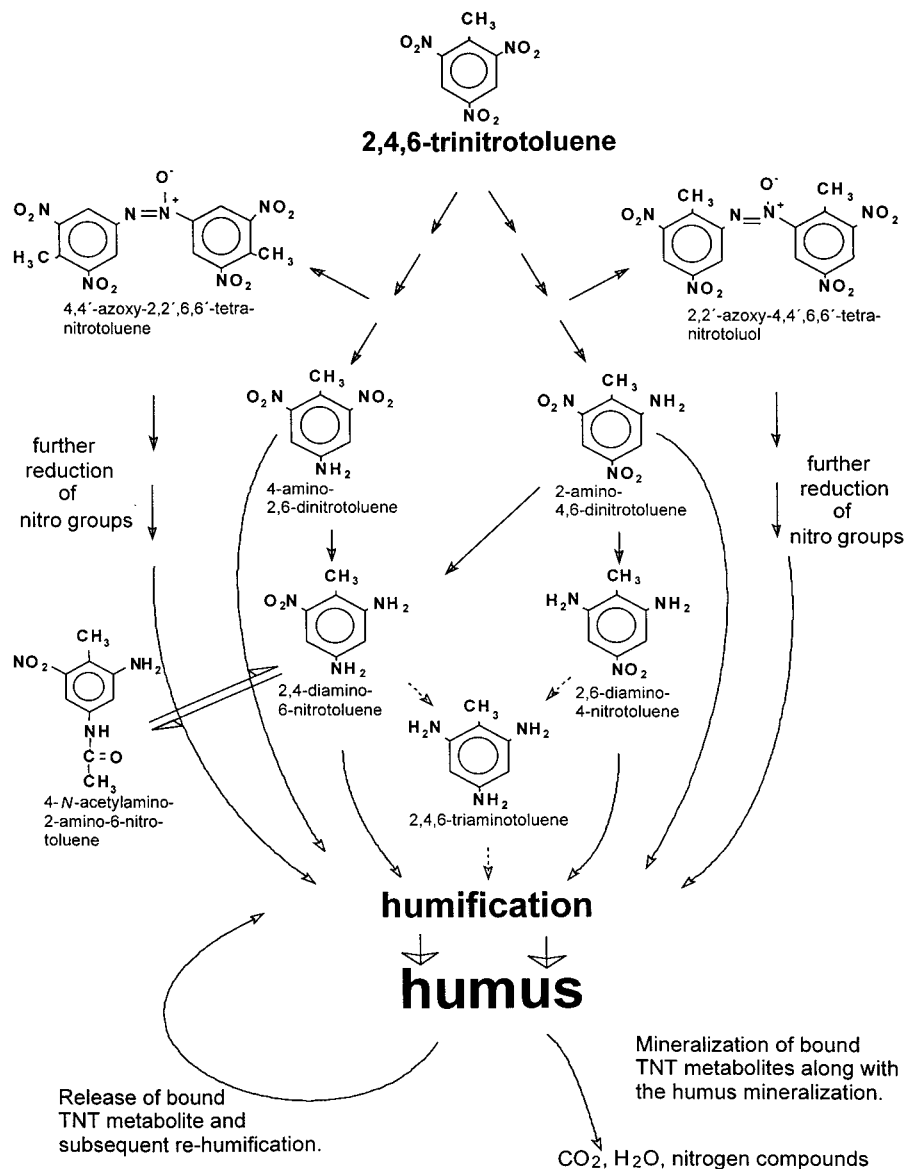


FIGURE 1. Transformation and humification of TNT in an anaerobic/aerobic composting system. The explosive is first transformed to aromatic amines. The nitro group is reduced via the formation of a nitroso- and hydroxylamino function. These intermediates can spontaneously form azoxyderivatives. Triaminotoluene is only generated at $E_h < -200$ mV and has never been identified in our experiments.

composting process by applying solid-state ^{15}N NMR spectroscopy. Furthermore, a mass balance of the applied ^{15}N -TNT was performed.

Materials and Methods

Soil Treatment. For all experiments TNT-contaminated surface soil from the former ammunition plant "Tanne" near Clausthal-Cellerfeld, Lower Saxony, Germany was used. The soil is contaminated with about 4000 mg of TNT/kg of dry soil. A more detailed characterization of the soil can be found elsewhere (27). The TNT-contaminated soil was mixed in a 1:4 (w/w) ratio with soil, free of nitroaromatics. The soil obtained by this procedure was spiked with ^{15}N -TNT.

As bioreactors, 1 L vessels were used. The soil treatment was very similar to the one developed by the company PlambeckContraCon (Cuxhaven, Germany) and the University of Marburg, recently published by Winterberg et al. (18). Drzyzga et al. (17) simulated the same bioremediation technique in their work.

To buffer the compost, 3 g of CaCO_3 were added to 122 g of dry soil. Thereafter, this material was spiked with 2.5 g

of ^{15}N -TNT. To achieve a homogeneous distribution of the explosive without using solvents that may influence microbial activity, the ^{15}N -TNT was added to a small amount of soil, and the mixture was ground carefully in a mortar with a pestle. The amount of soil was continuously increased. When about 50 g of soil were in the mortar, the soil was transferred to a 1 L vessel and mixed with a spatula. The remaining soil was slowly added during this treatment. Thereafter, the soil was stored for 14 days at 4 °C. Then it was mixed with 31.25 g of molasses slivers, corresponding to a 80:20 (w/w) ratio, and filled in the bioreactor. The compost material was compressed, and the reactor was flushed for 5 min with nitrogen. Thereafter, it was closed with a rubber sealed lid. The mixture was incubated in the dark at 30 °C. Once a week, the incubate was mixed intensively with a spatula. Thereafter, it was again compressed and flushed with nitrogen. This treatment was referred to as anaerobic phase and lasted for 13 weeks. During a subsequent aerobic phase of 7 weeks, the mixture was stirred intensively every day to achieve a well aerated system.

To stimulate the biological activity, 0.6 g of glucose were added at week 11 and 1 g at week 12 and 18, respectively.

During the first 2 weeks the water content was adjusted to 30%. Thereafter, it was increased to about 40%. During the aerated phase it was again increased to 30%.

As a sterile control, soil was spiked with nonlabeled TNT and mixed in a 80:20 ratio (w/w) with molasses slivers. Fifty grams of this mixture were poisoned with 2 g of NaN₃. This sample was kept in the dark at 4 °C during the composting period. After the treatment the sterility was tested by a MPN method. No growth of bacteria or fungi was visible. Furthermore no significant TNT transformation and elimination occurred during the treatment of the sterile control.

Solvent Extraction of Soil Compost. To follow the time course of the nitroaromatics during the composting process, regularly withdrawn samples of the incubate were extracted for 30 min in an ultrasonic bath (Bondelin Sonorex Super, Berlin, Germany) with methanol (soil:solvent ratio 1:4 (g/mL)).

Prior to the isolation of humic substances, the remediated soil was dried at 30 °C, ground, and sieved (mesh size 2 mm). A sample of 50 g was obtained. It was extracted with acetonitrile in a soil-to-solvent ratio of 1:10 (g/mL). Acetonitrile was used because it was reported that humic compounds are not dissolvable in acetonitrile (30). This was of major importance because the solvent extracted compost was subjected to extraction of humic compounds after solvent residues were evaporated.

Extraction of Humic Compounds. The humic compounds were isolated according to Drzyzga et al. (17). The acetonitrile-extracted soil was suspended in 200 mL of 0.5 M NaOH, the suspension was flushed for 10 min with nitrogen, and thereafter it was shaken for 24 h on a horizontal shaker (This is referred to as mild extraction in the following). The suspension was then centrifuged (5000 rpm for 45 min). The supernatant was separated from the solid soil residues (referred to as humin) and was acidified with 37% HCl to pH < 1 to precipitate humic acids (HA). HA were separated by centrifugation (5000 rpm for 30 min). The isolated HA were redissolved in 0.5 M NaOH and precipitated again. Finally, the enriched HA was dissolved in 0.5 M NaOH. The supernatants, which were left after the acidification procedures, contained fulvic acids (FA).

An aliquot of approximately 3 g of the humin was withdrawn for NMR analyses. The remaining humin was suspended in 150 mL of 0.5 M NaOH and incubated for 3 h at 95 °C in a reflux unit (This is referred to as drastic extraction in the following). Thereafter, HA and FA were separated as described above.

The two humin fractions were dried at 30 °C. The two HA and the two FA fractions were lyophilized. The six dry samples were then dialyzed against bidistilled water (dialyzing membrane: Spectra/Por 6, MWCO 1000 dialysis tubings). After the dialysis the humin was dried at 30 °C, whereas the HA and FA fractions were lyophilized.

Analytical Procedures. **HPLC.** The solvent extracts were analyzed by HPLC/DAD for nitroaromatic compounds. For separation, a Nucleosil 120-3 C18 column (3 mm by 25 cm; CS-Chromatographie Service, Langerwehe, Germany) was used. The analytical method has previously been described in detail elsewhere (29).

Solid-State ¹⁵N NMR Spectroscopy. The solid-state ¹⁵N NMR spectra were obtained with a Bruker DMX 400 spectrometer at a resonance frequency of 40.55 MHz, with the cross polarization magic angle spinning technique. A commercial Bruker double air bearing probe with 7 mm o.d. rotors was used, applying a spinning speed of 5.5 kHz a contact time of 1 ms and a pulse delay of 300 ms. Between 60 000 to 400 000 single scans were accumulated. These parameters were used according to those previously optimized for humified TNT (31). The chemical shift scale was calibrated with neat glycine (−347.6 ppm) and is reported relative to nitromethane (= 0

TABLE 1. Extractable Amount of Nitroaromatics Before the Anaerobic/Aerobic Incubation (1), After the Anaerobic Incubation (2), and After the Anaerobic/Aerobic Incubation (3)^a

sample	mg/kg dry soil						
	TNT	4-ADNT	2-ADNT	2,4-DANT	2,6-DANT	4-N-AcANT	4,4'-Az
1	11 963	13	11	nd	nd	nd	nd
2	4981	3065	944	nd	nd	nd	59
3	195	1920	618	3	2	18	74

^a TNT, 2,4,6-trinitrotoluene; 4-ADNT, 4-amino-2,6-dinitrotoluene; 2-ADNT, 2-amino-4,6-dinitrotoluene; 2,4-DANT, 2,4-diamino-6-nitrotoluene; 2,6-DANT, 2,6-diamino-6-nitrotoluene; 4-N-AcANT, 4-N-acetyl-amino-2-amino-6-nitrotoluene; 4,4'-Az, 4,4'-azoxy-2,2',6,6'-tetranitrotoluene; nd = not detectable.

ppm). Using this scale, the chemical shift of liquid ammonia is reported at −381.9 ppm. A 90° ¹H-pulse width of 5.6 ms was used. The spectra were integrated by an integration routine supplied with the instrument software.

Pyrolysis Capillary Gas Chromatography-Atomic Emission Detection. The ¹⁵N/¹⁴N ratios of the soil, humin, HA, and FA were determined by pyrolysis capillary gas chromatography and atomic emission detection (AED). A Hewlett-Packard gas chromatograph (GC) (HP 6890) and AED (HP 5921A AED) was used. The injection temperature of the GC was set to 220 °C. Helium was used as carrying gas with a pressure of 15 psi. A HP-5 capillary column (30 m × 320 μm) was used for substance separation. The oven temperature was set to 200 °C. One minute after the beginning of the pyrolysis, the split was opened (flow rate 5 mL/min). The temperature of the transfer line and the cavity of the AED was set to 250 °C. H₂ (10 psi), O₂ (32 psi), and methane (16 psi) were used as reactant gases. Pyrolysis temperature was 800 °C; the pyrolysis time was 10 s. 2,4-DNT and ¹⁵N-TNT were used as external standards.

CHN-Analysis. The CHN-analysis was performed with a elemental analyzer Elementar vario EL (Elementar Analysensysteme GmbH, Hanau, Germany).

Calculation of ¹⁵N Amount. The distribution of ¹⁵N originating from ¹⁵N-TNT in soil, humin, HA, and FA was calculated from the ¹⁵N/¹⁴N ratios of the soil and its fractions, and their total N was determined by the CHN-analysis.

Results and Discussion

Transformation and Elimination of TNT. To ensure an acceptable signal-to-noise ratio of the solid-state ¹⁵N NMR spectra, the soils were spiked with 5–10 times higher amounts of ¹⁵N-TNT than the amount of TNT that is present in soils which are usually remediated with the anaerobic/aerobic composting process (12, 14). For this reason, rather high amounts of TNT and transformation products were extractable after the soil treatment was finished (Table 1). Most interestingly, besides well-known transformation products, two acetylated metabolites were generated during the composting process. 4-N-Acetylamino-2-amino-6-nitrotoluene (4-N-AcANT) was already described in several reports as a biotransformation product of TNT (16, 20, 21). 4-N-Acetyl-amino-2,6-dinitrotoluene was found for the first time in this investigation. The identification was confirmed by the retention time of a reference standard and comparative measurements by means of UV-vis and solution NMR. The importance of these acetylated metabolites is unknown at this time. An acetylation of dinitrotoluene has also been described previously (33). Furthermore, the generation of 4-N-AcANT by a *Serratia* strain was observed (unpublished data). This transformation might be part of a detoxification mechanism as known for anilines (34). In general, it seems

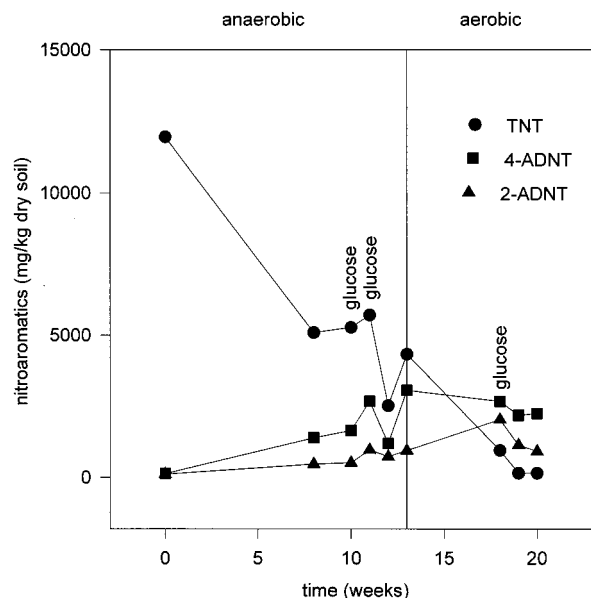


FIGURE 2. Time course of the transformation and elimination of TNT and metabolites in an anaerobic/aerobic composting experiment as they were extracted with acetonitrile. It is indicated when glucose was added.

that acetylated metabolites of nitroaromatics can be formed by a number of microorganisms and therefore are most likely present in certain states of bioremediation processes. At present, only little information about the toxicity of these compounds is available. Recently, we investigated the impact of acetylaminodinitrotoluenes on human monocytes (35). It was shown that the metabolites were less toxic than TNT and had a similar effect as ADNT. Further investigations are necessary to evaluate the importance of these metabolites.

A time course of the decrease of TNT that can be extracted with acetonitrile from the bulk soil and the formation of aminodinitrotoluenes (ADNT) during the anaerobic/aerobic treatment is shown in Figure 2. After the anaerobic phase more than 50% of the initial amount of TNT disappeared. The subsequent aerobic phase caused a further considerable decrease of the amount of TNT and only a negligible reduction of the extractable amount of ADNT. The addition of glucose at the 17th week had no considerable influence on the degradation of the nitroaromatics. For this reason the experiment was stopped at this time. Previous investigations with ^{14}C -TNT (17) showed that the decrease of the explosive was not caused by a complete mineralization of the explosive. Furthermore, it could be established that TNT residues, which were not extractable from the compost material, remained in a very stable manner in the soil.

Solid-State ^{15}N NMR Investigations of Soil and Soil Fractions. Figure 3 shows the solid-state CPMAS ^{15}N NMR spectra of the bulk compost material after the anaerobic phase and the complete anaerobic/aerobic treatment. A most tentative assignment of chemical shift regions to certain chemical structures is compiled in Table 2. A significant signal at -8 ppm is visible in both spectra of the soil. This peak derives from nitro groups of TNT and its reduction products. The high contribution of this signal to the total signal intensity of the spectra correlates well with the analyses of the solvent extracts of these samples (Table 1). After the anaerobic phase, approximately 5000 mg of TNT and more than 600 mg of ADNT per kg of dry soil were extractable. Analyses of solvent extracts of anaerobic/aerobic treated material revealed that about 200 mg of TNT and 2500 mg of ADNT per kg of dry soil were extractable (Table 1). Signals marked with an asterisk are spinning sidebands and occur due to insufficient removal

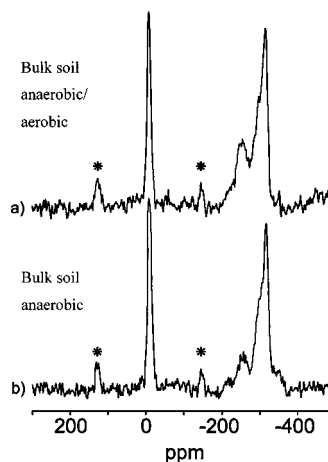


FIGURE 3. Solid-state ^{15}N NMR spectra of bulk soil: (a) anaerobic/aerobic treated soil, 271 730 scans and (b) anaerobic treated soil, 363 599 scans. Asterisks indicate spinning sidebands.

of the chemical shift anisotropy by spinning of the sample at 5.5 kHz. Considering that free TNT is in the sample and that such compounds show saturation effects in a solid-state ^{15}N NMR spectra obtained with the applied parameters, an underestimation of the relative contribution of nitro groups to the total ^{15}N of the sample is possible (31).

A considerable signal intensity is observed in the chemical shift region between -165 and -350 ppm. The signals in the chemical shift region between -270 and -350 ppm indicate the presence of amine nitrogen of aniline derivatives (32). A sharp peak at -320 ppm is present in both spectra and is indicative for aromatic amines without electron withdrawing substitution such as nitro groups. The presence of nitro groups, thus, will shift the aromatic amine signal to lower fields and explains the signal intensity of the downfield shoulder of the peak at -320 ppm. This intensity, therefore, is best explained with aromatic amines such as aminodinitrotoluenes (ADNT) or to a lower extent diaminonitrotoluenes (DANT) which were generated from ^{15}N -TNT during the composting process. Other possible structures are phenoxazines, anilinoquinones, hydrazines, and aminodiphenylamines (27). The assumption that soil composting leads to condensation reactions of TNT transformation products, probably with organic constituents of the soil, is further supported by the appearance of an additional intense resonance line in the chemical shift region between -165 and -270 ppm. In this region, resonance lines of nitrogen in heterocyclic structures such as pyrroles, imidazoles, indoles, quinolones, and carbazoles are expected (30). Furthermore, enaminones and amides appear in this region. The relative signal intensity in the region between -165 and -270 ppm of the solid-state ^{15}N NMR spectrum of the anaerobic/aerobic treated soil was twice as high as the one of the anaerobic treated soil (Table 2). It can be concluded from this result that the aerobic phase enhanced reactions leading to the formation of heterocyclic and/condensed TNT transformation products. The first corresponds to the multistep binding reaction proposed for the binding of 2,4-dichloraniline (DCA) toward humic compounds (26). According to this theory, DCA first reacts with quinoidal groups on humic acid compounds. Subsequent addition and oxidation reactions finally lead to the formation of heterocyclic structures.

Figure 4 shows the solid-state ^{15}N NMR spectra of the humic fractions obtained after a mild extraction. In all spectra, signals of nitro functions (-8 ppm) are present. This can be explained in two ways. First, it is possible that the solvent extraction prior to the humic substance fractionation was not exhaustive. Second, partially reduced TNT products may

TABLE 2. Tentative Assignment of Chemical Shift Regions to ^{15}N Functional Groups (27, 30) and Relative Intensity Distribution in the Solid-State ^{15}N NMR Spectra of Unextracted Soil and Different Humic Fractions of Anaerobic/Aerobic Composted ^{15}N TNT Spiked Soil

chemical shift region (ppm)	assignment	relative intensity (%)							
		anaerobic treated bulk soil	aerobic/ anaerobic treated bulk soil	humins after mild extraction	HA after mild extraction	FA after mild extraction	humins after drastic extraction	HA after drastic extraction	FA after drastic extraction
50 to -25	nitrate, nitrite, nitro groups	31	26	12	12	14	0	2	0
-165 to -270	imidazoles, indoles, pyrroles, carbazoles, quinolones, anilides, amides, enamines	12	24	32	40	52	28	48	67
-270 to -310	nitroanilines, anilinohydro-quinones, phenoxazines, hydrazines	26	27	35	30	24	29	28	21
-310 to -350	aniline, phenylamines	31	23	21	18	10	43	22	12

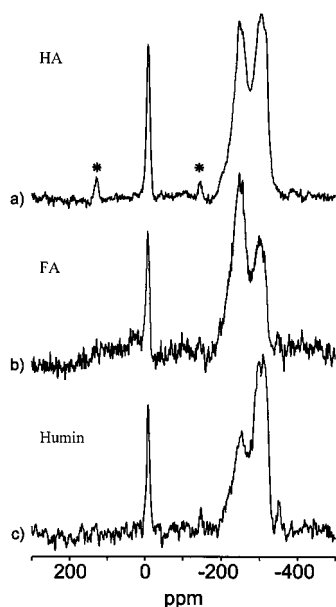


FIGURE 4. Solid-state ^{15}N NMR spectra of different soil fractions isolated from anaerobic/aerobic composted ^{15}N -TNT enriched soil. HA and FA were isolated from acetonitrile extracted soil by shaking the soil for 24 h with 0.5 M NaOH (second experiment, mild extraction): (a) HA, 61 148 scans, (b) FA, 200 711 scans, and (c) humins, 352 226 scans.

have coupled to humic substances, so that still free nitro groups remained. Further investigations are needed to clarify these questions.

Beside the peak at -8 ppm, signals were observed in the region between -165 and -350 ppm, as was the case for the bulk soils. The relative intensity between -270 and -350 ppm of the humins and HA and the bulk soil are very similar, whereas the relative intensity in the region from -165 to -270 ppm is the highest in HA (Table 2). Most strikingly, the FA spectrum shows a considerably different intensity distribution as compared to humins and HA. In this fraction most of the relative intensity is observed in the chemical shift region between -165 and -270 ppm, indicating a high portion of heterocyclic and/or condensed ^{15}N -TNT residues.

Figure 5 compiles the solid-state ^{15}N NMR spectra of the humic fractions obtained after additional drastic extraction of the humins after the mild extraction step. In comparison to the spectra shown in Figure 4, the relative intensity distribution of the signals in the different chemical shift regions remained unchanged with the exception that the signal at -8 ppm disappeared (Table 2). Only in the case of

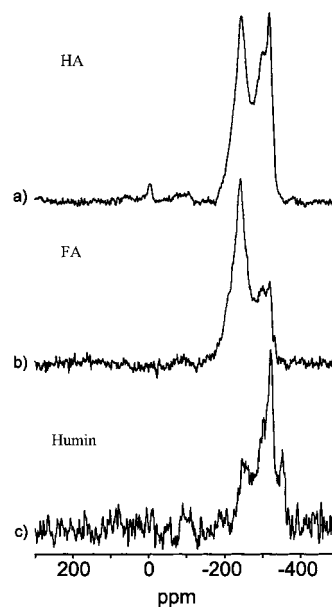


FIGURE 5. Solid-state ^{15}N NMR spectra of different soil fractions isolated from anaerobic/aerobic composted ^{15}N -TNT enriched soil, previously extracted with acetonitrile and 0.5 M NaOH. HA and FA were isolated by incubation of the soil for 3 h at 95 °C (second experiment, drastic extraction): (a) HA, 26 450 scans, (b) FA, 308 023 scans, and (c) humins, 775 797 scans.

the HA, a minor signal was detected in the chemical shift region of nitro groups. The lack of signals in the chemical shift region of nitro groups can be explained in two ways. On one hand, the first extraction procedure with NaOH might have removed all remaining, mobile TNT and partly reduced products. On the other hand, nitroaromatics are susceptible to attack by NaOH. A 4 h treatment of TNT at 80 °C and pH 14 causes a release of at least one nitro group (36). For this reason, the incubation at 95 °C might have caused a denitration of nonbound TNT and transformation products and of bound partially reduced TNT products. This may also explain the lack of nitro peaks in the HA spectrum shown in the first part of this paper (31).

^{15}N Distribution in Different Soil Fractions. Figure 6A shows the ^{15}N distribution of the anaerobic/aerobic treated soil. Thirty-three percent of the ^{15}N labeled compounds could be extracted with acetonitrile. This value was determined by measuring the ^{15}N content of extracted and nonextracted soil and calculating the difference of the two values. The extractable amount of TNT and metabolites calculated from the HPLC analyses (Table 1) is approximately 24%. This

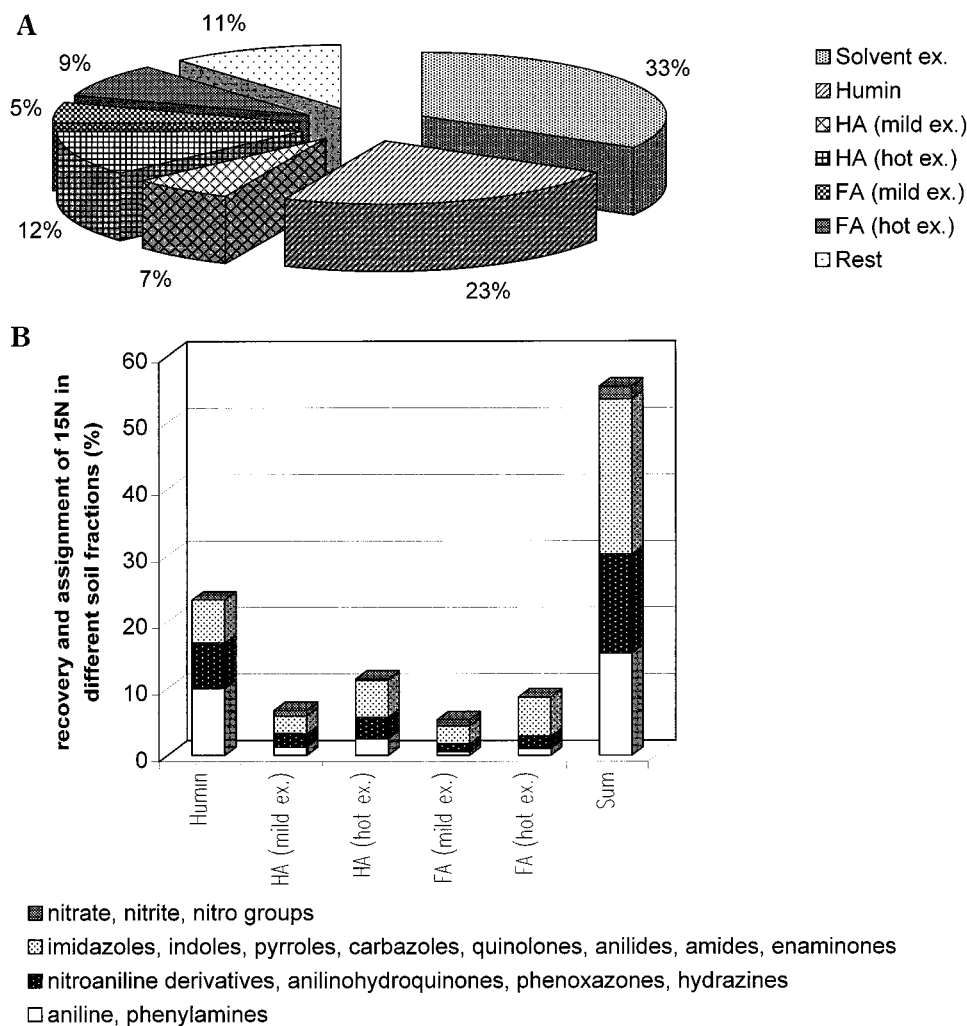


FIGURE 6. (a) ^{15}N distribution in the acetonitrile extract and humic fraction of a ^{15}N -TNT spiked soil after anaerobic/aerobic composting and (b) the relative ^{15}N intensity distribution of the corresponding solid-state ^{15}N NMR spectra.

indicates that during the composting process further acetonitrile extractable TNT transformation products, in addition to those listed in Table 2, were generated.

Thirty-three percent of the nonextractable ^{15}N compounds were present in the different HA and FA fractions. Seven percent was present in the HA extracted under mild conditions, whereas 12% could be detected in the fraction extracted with hot NaOH. Five percent of the ^{15}N was present in the FA extracted mild. Nine percent could be enriched in the fraction extracted with hot NaOH. Twenty-three percent of the labeled nitrogen remained in the humin fraction after the extraction process.

Drzyzga et al. (17) performed similar composting experiments as described here with ^{14}C -TNT. They, however, used soil contaminated with about 1180 mg of TNT/kg of dry soil which was spiked with 100 mg of ^{14}C -TNT/kg of dry soil. Under two different composting conditions they could extract 9.4 and 44.7% of the radioactivity with water, ethyl acetate, and methanol, respectively. HA and FA were extracted with 50% NaOH (g/mL) by boiling the soil material for 2 h in a reflux unit. This procedure yielded in a recovery of 4.9% and 9.5% of the radioactivity in the FA, 13.1% and 11.1% in the HA, and 28.8% and 62% in the humin fraction, respectively. Comparing these results to the data presented here, the ^{15}N balance indicates a higher portion of TNT residues incorporated into the FA and HA fraction than the ^{14}C balance. This might be because the two-step extraction (mild/drastic) was more effective than simply one hot extraction. At present,

comparing investigations with ^{14}C - and ^{15}N -TNT are under way to evaluate the comparability of the ^{14}C - and ^{15}N -balance.

^{15}N Assignment to Different Chemical Structures. With the data presented in Table 2 (peak areas) and Figure 6 it was possible to assign the ^{15}N residues of the ^{15}N -TNT present in the various soil fractions after the anaerobic/aerobic composting process to different chemical structures. Most interestingly, the major portion of ^{15}N (approximately 58%) was found in a bound manner (Figure 6B). The sum of all five fractions shows that 23% of ^{15}N was present in mostly heterocyclic structures, whereas 15% was bound in a covalent manner. Fifteen percent could be detected as amino functions and 2% as nitro functions. The low molecular weight FA contained mainly heterocyclic bound ^{15}N . In contrast, almost similar amounts of ^{15}N are assigned to heterocyclics and possibly covalent bonds in the humin and HA fractions.

In this paper, we have extensively characterized anaerobic/aerobic composted ^{15}N -TNT enriched soil via solid-state ^{15}N NMR spectroscopy. It is a well-known fact that composting of TNT-contaminated soil does not lead to a mineralization of this xenobiotic (1, 4, 17, 22). It has long been assumed that the disappearance of the explosive in this process is due to coupling reactions with humic constituents. However, experimental evidence for this notion has long been lacking. Dawel et al. (37) recently presented evidence for a nucleophilic addition of 2,4-diamino-6-nitrotoluene (2,4-DANT) to a guaiacol dimer mediated by laccase. The importance of this reaction for bioremediation

methods, however, remained unclear. Experiments performed by Thorn et al. (27) and Thorn (28) are closer to soil systems. However, again, no bioremediation method was simulated by these authors. In their experiments, FA and HA extracted from different soils were incubated with ^{15}N labeled aniline, ADNT, and DANT, respectively. The authors found analogous to our results that a major fraction of aromatic amines was bound in heterocyclic and/or condensed structures. Furthermore, they found only minor amounts of imines and concluded that in contrast to a recent suggestion (38), imines are not the predominant binding form for these aromatics. Our solid-state ^{15}N NMR spectra of the bulk soils and humic fractions of anaerobic/aerobic composted ^{15}N -TNT spiked soils demonstrate that similar reactions as in the experiments performed with model compounds do occur under composition conditions and underline the concept developed in these in vitro studies.

At present, it can only be speculated which reaction pathways lead to the different bound residues formed during the anaerobic/aerobic composting process. Known biochemical reaction mechanisms are not suitable to explain their generation. Thorn et al. (27) have extensively discussed different chemical reactions that may be involved. The actual relevance of these mechanisms for bioremediation processes, however, remains unknown. Experiments with different humin model chemicals, extracted humic compounds, and different TNT reduction products are required to achieve more precise information about the coupling pathways and products.

Recent research results about the biodegradation of herbicides might contribute to the explanation of the generation of heterocyclic TNT residues containing structures. Sandermann et al. (39) published a proposed reaction pathway of 3,4-dichloraniline to a succinimide catalyzed by the fungi *Phanaerochaete chrysosporium*. The identification of formylated TNT reduction products in cultures of *P. chrysosporium* (21) as well as in anaerobic/aerobic compost systems (16) demonstrates that certain catalytic properties of this fungi are not only restricted to this organism. For this reason, a formation of succinimides should also be discussed for composting treatments of TNT contaminated soils.

A major result of the experiments presented here is the fact that nonextractable residues of TNT, after an anaerobic/aerobic composting, seem to be bound in a very stable manner to different humic compounds. Even a hydrolysis of the bonds under drastic conditions was not possible. This supports the strategy to use humification of TNT transformation products as a remediation technique (Figure 1). However, comparing the nitrogen-containing products of the humification process of TNT with those formed under natural conditions, some caution is due. Several studies examining the humification of biogenic precursors in natural settings by solid-state ^{15}N NMR spectroscopy have shown that most of the nitrogen is immobilized in amide functional groups (40, 41). Considerable signals of heterocyclic structures were only detected in fossilized samples (42). The different chemistry of TNT humification products from those of naturally formed soil organic nitrogen may have considerable implication on the biochemistry of remediated soils. In future work, the understanding of this implication will be a major aspect, in addition to the further characterization of the TNT humification products.

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