Incorporation of ^{15}N -TNT transformation products into humifying plant organic matter as revealed by one- and two-dimensional solid state NMR spectroscopy

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Abstract

Solid-state double cross polarization magic angle spinning (DCPMAS) ^{15}N ^{13}C nuclear magnetic resonance (NMR) spectroscopy was applied to study the incorporation of TNT transformation products into humifying plant organic matter. For this approach, ^{13}C -enriched plant material (*Lolium perenne*) was mixed with quartz sand and aerobically incubated for 11 months after addition of $^{15}\text{N}_3$ -2,4,6-trinitrotoluene (TNT). After successive extraction of the incubate with water, methanol and ethyl acetate, approximately 60% of the ^{15}N added as $^{15}\text{N}_3$ -TNT ($^{15}\text{N}_{\text{add}}$) remained in the solid organic residue (SOR-fraction). The acid insoluble fraction (AI) obtained after NaOH and HCl extractions contained approximately 20% of $^{15}\text{N}_{\text{add}}$. For both fractions, ^{15}N NMR spectroscopy revealed an almost complete reduction of the TNT after 11 months of aerobic incubation. Most of the reduced nitrogen groups underwent further condensation. The corresponding DCPMAS NMR spectra allowed the identification of amides that are further substituted by alkyl groups that resist even acid hydrolysis. This assigns them to relatively stable compounds rather than to newly synthesized microbial peptides. The results of this study suggest further that the covalent binding of TNT transformation products to plant derived organic matter is mediated by alkylation and acetylation reactions, rather than by 1,4 addition of TNT-derived nitrogenous groups to quinones of the humic material.

Keywords: TNT-immobilization; Formation of amides; Polyamide-type macromolecule; Remediation; Double cross polarization magic angle spinning ^{15}N ^{13}C NMR; Isotopic labeling

1. Introduction

During World Wars I and II, considerable amounts of the explosive 2,4,6-trinitrotoluene (TNT) and its by-products were released into soils, either by accident or due to improper handling

during production, application and disposal. In order to diminish the potential hazardous impact on the environment and the risk for human health of those contaminated sites, much effort has been directed towards a better understanding of the fate of TNT in soils.

Commonly, the examination of the chemical transformation of TNT in soils is performed by analyzing the supernatants obtained after extraction

of the soil with various organic solvents. However, a considerable part of the TNT and its transformation products are found to become immobilized into the solid soil phase (Hundal et al., 1997; Drzyzga et al., 1998; Achtnich et al., 1999b). Thus, persisting even after exhaustive extraction, those TNT compounds are often neglected with the use of common wet chemical approaches. Consequently, little is known about their chemical composition and the processes responsible for their immobilization in soils.

Besides sorption phenomena (Hundal et al., 1997; Li et al., 1997; Sheremata et al., 1999), covalent binding between TNT and humic material was proposed to be responsible for TNT immobilization. It was suggested that in a first step, the nitro groups of the TNT are reduced and subsequently anilinoquinones are formed by Michael addition of the aminonitrotoluene to quinones of the soil organic matter (Thorn, 1997). Alternatively, covalent binding of 2,4-diamino-6-nitrotoluene through laccase was proposed (Dawel et al., 1997). Evidence for the occurrence of condensation reactions of TNT in the presence of humic material was recently obtained by means of solution- and solid-state ^{15}N nuclear magnetic resonance (NMR) spectroscopy (Thorn, 1997; Achtnich et al., 1999a; Knicker et al., 1999, 2001; Bruns-Nagel et al., 2000). However, the broadness of the resonance lines, in particular in the solid-state ^{15}N NMR spectra, aggravates a more specific assignment of the signal intensity to certain N-functional groups.

One possibility to obtain more insights into the nature of the functional groups involved in the condensation reaction of TNT transformation products is via solid-state double cross polarization (DCP) magic angle spinning (MAS) ^{15}N ^{13}C NMR spectroscopy. It is based on a magnetization transfer from ^1H to ^{15}N and subsequently to ^{13}C . The latter is finally detected. The key of this approach is that the resulting spectrum shows only signals of ^{13}C that couple with ^{15}N . Expanding this experiment to a two-dimensional (2-D) version, interactions, indicating covalent bonds, are revealed by cross peaks correlating the signal intensity of the ^{13}C NMR spectrum with that of the ^{15}N NMR spectrum.

The application of this technique, however, encounters severe sensitivity problems and is only feasible if substances are used that are highly enriched in ^{13}C and ^{15}N (Knicker, 2002). This, on the other hand, turns into an advantage if one intends to reveal some insights on possible covalent binding between two specific components during humification. Labeling one of the reactants with ^{13}C and the other with ^{15}N , the respective DCPMAS ^{15}N ^{13}C NMR spectrum will show only signals of the corresponding interacting functional groups.

As known to the author, in the present preliminary study, this technique is applied for the first time to reveal possible covalent binding between humifying material and N-containing xenobiotics. For this purpose, ^{13}C -enriched plant residues (*Lolium perenne*) were mixed with $^{15}\text{N}_3$ -TNT and subjected to aerobic degradation. Because our effort was directed towards the insoluble organic fraction, the soluble components were removed and only the insoluble organic residue and its humic acid fraction were subjected to 1-D and 2-D DCPMAS ^{15}N ^{13}C NMR analysis. With this approach, it was hoped to detect the functional groups that are involved in the immobilization of TNT and its transformation products into humifying plant organic matter.

2. Methods

2.1. Sample material

^{13}C -enriched plant material was obtained from pot experiments during which *Lolium perenne* was grown on commercially available sterilized gardening substrate mixed with quartz sand (1:3) in a closed fiber glass chamber located in a phytotron (Knicker, 2002). ^{13}C -enriched CO_2 (99 atm%) was introduced into the chamber via tubing, after exchanging the air present with CO_2 -free air. After reaching a height of approximately 4 cm, the stems were cut and harvested. The pots with the cut plants were reintroduced into the growing chamber for further growth. After yielding enough stem material, the pots were removed from the chamber and the roots were separated from the growing

Table 1

Total carbon (C_t) and nitrogen (N_t) concentrations and concentration of the added ^{15}N from TNT ($^{15}\text{N}_{\text{add}}$) in the fresh plant material/ $^{15}\text{N}_3$ -TNT mixture and the extracts of a plant material (*Lolium perenne*) incubated for 11 months under aerobic condition

Sample	C_t (mg g ⁻¹)	N_t (mg g ⁻¹)	C_t/N_t (w/w)	$^{15}\text{N}_{\text{add}}$ (mg g ⁻¹)	$^{15}\text{N}_{\text{add}}$ recovered (%)
Plant material/ $^{15}\text{N}_3$ -TNT	405.9	29.1		19.6	
Methanol extract	170.6 ± 9.6	84.2 ± 4.1	2	10.1 ± 0.4	3.9 ± 0.1
Ethyl acetate extract	681.1 ± 1.1	17.5 ± 0.7	39	6.2 ± 0.2	1.3 ± 0.1
H ₂ O-fraction	97.4 ± 0.2	68.0 ± 0.3	1.4	8.7 ± 0.9	12.3 ± 1.3
SOR fraction ¹	199.7 ± 2.0	15.7 ± 0.1	13	4.0 ± 0.1	59.6 ± 1.0
AI fraction ²	416.5 ± 1.8	47.9 ± 0.5	9	13.5 ± 0.4	19.9 ± 0.5
Recovery (%) ³	31	49			77

¹ Organic residue after extraction with methanol, ethyl acetate and water.

² Acid-insoluble fraction of SOR that is soluble in NaOH but precipitates after addition of HCl.

³ Recovery corresponds to the sum recovered in the SOR, methanol-, ethyl acetate- and H₂O-fractions with respect to the initial non-incubated sample.

substrate. Both stems and roots were air-dried, milled to a fine powder and mixed in a 1:2 ratio.

Approximately 5 g of the plant residues and 84.3 g of quartz sand (0.3–0.5 mm) were added under continuous stirring to 100 mg of fine grained $^{15}\text{N}_3$ -TNT (99 atm%). The sample was put into a glass beaker and inoculated with an aqueous extract from fresh compost. A sample moisture of 60% of the maximal water-holding capacity was adjusted and the mixture was aerobically incubated in the dark at 30 °C under a slow stream of moist air for 11 months. After this time, 31% of the total carbon and 49% of the total nitrogen were recovered in the organic fractions of the incubated sample (Table 1). The humified plant residue/quartz sand mixture was distributed into two centrifuge beakers to each of which 80 ml H₂O was added. After sonification, the samples were centrifuged for 20 min (4500 r.p.m.). The supernatants were combined and freeze dried (H₂O-fraction). The residues were further extracted with 80 ml methanol and 80 ml ethyl acetate, respectively. The supernatants were recovered and dried under a stream of N₂. In order to increase the concentration of ^{15}N in the extraction residue, and thus its sensitivity for the NMR spectroscopy, the quartz sand was mechanically separated. Therefore, the residues were combined and transferred to a glass beaker filled with distilled water. While the quartz sand settled quickly to the bottom of the beaker, the organic material was floating in and above the

water phase, which allowed its removal by decanting. The remaining quartz sand was carefully washed with distilled water to recover organic remains that stuck to the quartz sand. The washing water was added to the organic matter/water mixture. Then, the water was removed by rotor evaporation and freeze-drying, resulting in the solid organic residue (SOR)-fraction.

Approximately 1 g of the SOR-fraction was extracted with 25 ml aqueous 0.5 M NaOH solution and the supernatant was acidified with HCl (32%) until the solution reached a pH = 1. During this process the acid-insoluble fraction (AI) precipitated. Then, the mixture was centrifuged and the supernatant was removed. Prior to freeze-drying, the AI-fraction was desalted by dialysis against deionized water using dialysis tubing with a pore width of 2.4 nm (cut-off = 12 000–14 000 Da).

Subsamples of the SOR- and AI-fractions comprising 252 mg and 147 mg, respectively, were subjected to acid hydrolysis with 6 M HCl for 12 h at 105°C. The non-hydrolyzable residues were filtered and washed with deionized water. The filtrates were freeze-dried for further analysis.

2.2. Determination of the elemental composition and the ^{15}N and ^{13}C concentrations

Total organic carbon (C_t) and total nitrogen (N_t) were measured in doublets by dry combustion (975 °C) using an Elementar Vario EL detecting

N as N_2 and C as CO_2 . The detection limits for C and N are 0.4 and 1 μg , respectively. The ^{15}N contents of the samples were measured by leading an aliquot of the helium/combustion gas mixture (flow rate = 200 ml/min helium) into a quadrupole mass spectrometer (QMS) (GAM-200, InProcess Instruments GmbH, Bremen, Germany) connected to the Elementar Vario EL. During the release of N_2 , the masses 28 ($^{14}\text{N}_2$), 29 ($^{14}\text{N}^{15}\text{N}$) and 30 ($^{15}\text{N}_2$) were scanned with a dwell time of 50 ms. The ^{15}N concentrations were calculated from the $^{14}\text{N}/^{15}\text{N}$ and N_t content of the sample after manual integration of the signal intensities of the three masses. The contribution of ^{15}N from $^{15}\text{N}_3\text{-TNT}$ ($^{15}\text{N}_{\text{add}}$) was determined by subtracting the natural abundant ^{15}N ($0.365 \pm 0.01 \text{ atm}\%$) detected for the plant material before incubation from the measured ^{15}N content. The atomic ratio $^{13}\text{C}/\text{C}_t$ of the starting plant material was calculated to be 13 and was determined from the integrals of the signal intensities of the masses 28 (^{12}CO) and 29 (^{13}CO) scanned after release of CO_2 .

2.3. NMR spectroscopy

All solid-state NMR spectra were obtained using a Bruker DMX 400 instrument with a triple resonance probe. The ^{15}N resonance frequency was 40.54 MHz; that of ^{13}C was 100.61 MHz. For the 1-D CPMAS ^{15}N NMR spectra 8240 scans and 23 950 scans were accumulated for the AI- and SOR-fractions, respectively, applying a pulse delay of 300 ms, a contact time of 0.7 ms and a spinning speed of 5.5 kHz (Knicker and Lüdemann, 1995; Knicker et al., 1999). A ramped ^1H -pulse shaped from 100% to 50% avoided Hartmann–Hahn mismatches (Peersen et al., 1993; Cook et al., 1996). A line broadening of 75 Hz was used before Fourier transformation.

The 1-D DCPMAS ^{13}C NMR spectra were accumulated after 32 768 and 516 732 single scans for the AI- and SOR-fractions, respectively. A pulse delay of 350 ms were used. A consecutive matched spin-lock transfer, first from ^1H to ^{15}N (contact time $t_1 = 0.7 \text{ ms}$) and then from ^{15}N to ^{13}C (contact time $t_2 = 3 \text{ ms}$), was performed. During the latter, protons were decoupled using a frequency-shifted Lee–Goldburg sequence (Bie-

lecki et al., 1990). During t_1 a ramped ^1H -pulse and during t_2 a ramped ^{15}N -pulse were used. Both pulses were shaped from 100% down to 50%. No line broadening was applied.

For the 2-D DCPMAS $^{15}\text{N}^{13}\text{C}$ NMR spectrum of the AI-fraction, a total of 16 spectra were obtained with mixing times increasing at an increment of 0.062 ms. For one spectrum, 32 768 scans were accumulated. A pulse delay of 350 ms was found to be sufficient to avoid saturation effects (Knicker et al., 1999; Knicker, 2002).

For all spectra, the ^{15}N chemical shifts and the ^{13}C chemical shifts are referenced to the nitromethane scale (=0 ppm) and to the tetramethylsilane (TMS) (=0 ppm), respectively, and were calibrated with glycine ($-\text{NH}_2 = -346.6 \text{ ppm}$; $-\text{COOH} = 176.04 \text{ ppm}$)

3. Results and discussion

3.1. 3.1 Elemental composition

Table 1 lists the concentration of C_t , N_t and the recovered ^{15}N that was added as $^{15}\text{N}_3\text{-TNT}$ prior to incubation ($^{15}\text{N}_{\text{add}}$) in the different fractions isolated from plant residues after 11 months of aerobic incubation. For comparison, the C_t , N_t and the $^{15}\text{N}_{\text{add}}$ concentrations of the fresh plant/ $^{15}\text{N}_3\text{-TNT}$ mixture are included in Table 1. After 11 months of incubation, only 4% and 1% of $^{15}\text{N}_{\text{add}}$ were extractable with methanol and ethyl acetate, respectively. Those low recoveries indicate an efficient transformation of the added $^{15}\text{N}_3\text{-TNT}$ into more polar products, some of which were extractable with the particulate organic matter of the H_2O -fraction (12% of $^{15}\text{N}_{\text{add}}$). More than half of $^{15}\text{N}_{\text{add}}$ (60%), however, could not be removed by water or the organic solvents and remained in the SOR-fraction, leaving approximately 23% of $^{15}\text{N}_{\text{add}}$ that was not recovered. The high $^{15}\text{N}_{\text{add}}$ -loss may be explained by binding of $^{15}\text{N}_3\text{-TNT}$ and its transformation products to organic matter residing in the quartz sand after mechanical separation. However, it may also be due to the loss of volatile N-compounds derived from the transformation and release of the $^{15}\text{NO}_3^-$ -groups of $^{15}\text{N}_3\text{-TNT}$.

Fig. 1. Solid-state CPMAS ^{15}N NMR spectra of ^{13}C -enriched plant residues that were incubated after addition of $^{15}\text{N}_3$ -TNT for 11 months. SOR is the fraction after successive extraction with methanol, ethyl acetate and water, while AI represents the fraction of SOR that is soluble in NaOH, but precipitates after addition of HCl.

Approximately 23% of the carbon of the SOR-fraction and 33% of its $^{15}\text{N}_{\text{add}}$ were isolated with the AI-extract. Thus, a considerable amount of the $^{15}\text{N}_3$ -TNT transformation products (20% of $^{15}\text{N}_{\text{add}}$) became associated with this fraction. Comparable values were found for corresponding extracts obtained from a compost of a soil-molasses mixture and soils that were incubated after addition of ^{14}C -labeled TNT (Hundal et al., 1997; Drzyzga et al., 1998).

3.2. CPMAS ^{15}N NMR spectra

Former CPMAS ^{15}N NMR studies showed that pure $^{15}\text{N}_3$ -TNT results in resonance lines at -14 ppm and -17 ppm (Witanowski et al., 1993; Knicker et al., 1999). The absence of those signals in the CPMAS ^{15}N NMR spectra in Fig. 1 demonstrates complete reduction of almost all $^{15}\text{N}_3$ -TNT transformation products that are associated with the SOR- and AI-fractions after 11 months

of aerobic incubation. The presence of the corresponding reduction products is evidenced by signals in the chemical shift region of aromatic amines between -270 and -340 ppm. In this region, a clear resonance line is detected at -320 ppm, a chemical shift typical for aniline (Witanowski et al., 1993). Substitution of the aromatic ring with an electron withdrawing group may explain the intensity between -270 and -320 ppm. Substitution of the amino-N, on the other hand, leads to a shift of the signal to higher fields. At the high-field site of the resonance at -320 ppm, however, considerable intensity indicating the presence of greater amounts of such compounds is not observed.

In the CPMAS ^{15}N NMR spectra of the SOR- and AI-fractions, the chemical shift region between -309 and -340 ppm accounts for 10% and 8% of the total ^{15}N -intensity, respectively. This corresponds to approximately 6% and 2% of $^{15}\text{N}_{\text{add}}$ that became immobilized as non-substituted aromatic amine in the SOR- and AI-fractions. Those relatively low amounts point to the conclusion that during humification and immobilization most of the reduced N-groups of the $^{15}\text{N}_3$ -TNT-metabolites experienced further transformation, probably by reacting with the degrading organic residues of the plant material.

Such condensation reactions between aromatic amines and humic material were previously examined by Thorn et al. (1996). Assuming quinones as the major reaction partner, they discussed the formation of aminodiphenylamines, hydrazine, anilinoquinone, phenoxazines and anilinoquinones. The chemical shifts of those compounds are expected to result in resonance lines at -293 ppm, -285 ppm, -307 ppm, -303 ppm and -277 ppm, respectively, and may explain the signals between -270 and -309 ppm. They account for 21% and 22% of the total ^{15}N -intensity of the spectra of the AI- and SOR-fraction, which correspond to 4% and 13% of $^{15}\text{N}_{\text{add}}$, respectively. Beside those compounds, hydroxylamines, suggested to be formed during an early stage of TNT transformation (Lewis et al., 1997; Hawari et al., 1999) may also contribute to this chemical shift region.

In previous ^{15}N NMR spectroscopic studies, an additional weak but broad signal was detected in the chemical shift region assigned to pyridine-type N or imine N (-25 to -120 ppm). The latter were proposed to result from Schiff-Bases formed by ketones or aldoses occurring in natural soil organic matter and the amino groups of aromatic amines (Parris, 1980; Thorn et al., 1996). Further intramolecular condensation could lead to phenoxazinone (-42 ppm) and quinoline (-65 ppm). In the spectra shown here, the contribution of the region between -25 and -120 ppm to the total ^{15}N -intensity is $<3\%$. However, in this context, it should be noted that the ^{15}N CPMAS technique is often criticized to underestimate non-protonated N (Schulten et al., 1995; Thorn et al., 1996). Therefore, in a previous study (Knicker et al., 1999) the relative intensity distribution in a CPMAS ^{15}N NMR spectrum of $^{15}\text{N}_3$ -TNT transformation products immobilized into a humic acid of a composted soil and that of the corresponding single pulse excitation (SPE) NMR spectrum were compared. For the latter, the ^{15}N nucleus is directly excited and detected without taking advantage of intensity enhancement by cross polarization transfer from the ^1H to the ^{15}N spin system. In relation to the CPMAS spectrum, this comparison revealed higher intensities in the chemical shift regions from 148 to -120 ppm and in that between -350 ppm and -450 ppm in the SPE ^{15}N NMR spectrum. On the other hand, due to the low signal-to-noise ratio of the SPE ^{15}N NMR spectrum, this increase was mainly caused by intensity contribution from the noise. But even if the total additional intensity in the SPE ^{15}N NMR spectrum derived from N-compounds that were not detected by the CPMAS-technique, the underestimation of non-protonated N in the respective CPMAS ^{15}N NMR spectrum did not exceed 50% . However, if this amount of non-protonated N had been underestimated in the spectra presented in Fig. 1, pyridine-type compounds would contribute to less than 7% of the total $^{15}\text{N}_{\text{add}}$ recovered in the SOR- and AI-fraction. Corresponding to maximal 4% of the total added $^{15}\text{N}_{\text{add}}$, such condensation products are thus unlikely to play a major role for the immobilization of TNT-metabolites.

Other possible condensation products such as pyrroles (-130 to -250 ppm), carbazoles (-262 ppm) and anilidines (-240 to -250 ppm) may add to the signal between -162 and -270 ppm comprising between 60% and 70% of the total intensity of the spectra in Fig. 1. Amides that were found to represent the major N-fraction in naturally formed humic material of biogenic origin (Knicker, 2000) are also expected to contribute to this chemical shift region. For the spectra of the present study, the ^{15}N derived from the plant material was calculated to account for approximately 1% of the total ^{15}N signal intensity. After subtracting this value from the intensity between -120 and -270 ppm, the amount of $^{15}\text{N}_3$ -TNT-derived ^{15}N that adds to the intensity in this region was calculated to be 40% of $^{15}\text{N}_{\text{add}}$ for the SOR-extract and 14% for the AI-fraction.

The dominance of the resonance line between -162 and -270 ppm was also detected in CPMAS ^{15}N NMR spectra of anaerobically treated $^{15}\text{N}_3$ -TNT-spiked soils and soil-slurries (Bruns-Nagel et al., 1998; Knicker et al., 2001). Thus, the mechanisms involved in the formation of products adding to this signal are likely to represent an important immobilization pathway of TNT-metabolites, both under aerobic and anaerobic conditions. However, as mentioned above, the region between -120 and -270 ppm embraces the chemical shifts of various compounds. Each of those could be formed by a different pathway, which aggravates to name the specific reactions, responsible for the covalent binding of TNT-residues to the humic material.

3.3. DCPMAS NMR spectroscopy

In Fig. 2 the 1-D DCPMAS ^{13}C NMR spectra of the SOR- and AI-fractions are presented. They show two dominant signals at 174 ppm and 55 ppm. Due to the low sensitivity of this NMR technique, they can only originate from carbons derived from the ^{13}C -enriched degrading plant material which interact with the ^{15}N of the transformed $^{15}\text{N}_3$ -TNT. Consequently, those signals can be taken as the first direct evidence that covalent binding between TNT transformation products and humifying material occurred.

Fig. 2. Solid-state DCPMAS ^{13}C NMR spectra of ^{13}C -enriched plant residues that were incubated after addition of $^{15}\text{N}_3\text{-TNT}$ for 11 months. SOR is the fraction after successive extraction with methanol, ethyl acetate and water, while AI represents the fraction of SOR that is soluble in NaOH, but precipitates after addition of HCl.

The signal around 55 ppm is assigned to N-alkyl C, demonstrating that amino groups of the reduced $^{15}\text{N}_3\text{-TNT}$ were substituted by alkyl compounds. The signal between 190 and 160 ppm occurs in the chemical shift region of carboxyl C, unveiling covalent binding via amide-bonds. However, nitrile C (around 160 ppm) and imine C (around 190 ppm) may also contribute to the intensity in this region. On the other hand, no considerable signal intensities of nitrile N (around -145 ppm) and imine N (between -25 and -60 ppm) were detected in the corresponding 1-D CPMAS ^{15}N NMR spectra. If present, their concentrations are below the detection limit.

A more precise assignment of the signals in the 1-D DCPMAS ^{13}C NMR spectra allows the 2-D DCPMAS ^{15}N ^{13}C NMR spectrum of the AI-fraction in Fig. 3. Unfortunately, it was not possible to acquire a corresponding spectrum from the SOR-fraction, due to its low ^{15}N concentration of 4 mg g^{-1} . In the 2-D NMR spectrum of the AI-extract, the intense cross peak correlates the car-

boxyl region of the ^{13}C dimension (190–160 ppm) with the region between -200 and -270 ppm of the ^{15}N dimension, which supports the presence of amides. They may result from condensation between the amino groups of the reduced $^{15}\text{N}_3\text{-TNT}$ transformation products and aromatic or aliphatic acids of the degrading plant material. Acetylation can explain the signal at 174 ppm in the ^{13}C -dimension. The reaction with aromatic acids, on the other hand, would lead to benzamides that may contribute to the weak ^{13}C -resonance at 164 ppm. Their corresponding ^{15}N -signals are expected around -280 ppm. In Fig. 3, however, no cross peak is identified at that chemical shift, emphasizing their low importance for the incorporation of TNT transformation products into soil organic matter. Another possible explanation for the ^{13}C -signal between 160 and 170 ppm could be the formation of amide bonds via formylation, a reaction that was reported to occur during biotransformation of TNT by *Phanerochaete chrysosporium* (Hawari et al., 1999). If such products are present, the ^{13}C signal at 164 ppm should correlate with a ^{15}N signal at -247 ppm in the 2-D DCPMAS ^{15}N ^{13}C NMR spectrum, which is the case in that of the AI-fraction.

Fig. 3. Two-dimensional solid-state DCPMAS ^{15}N ^{13}C NMR spectra of the acid-insoluble fraction obtained from ^{13}C -enriched plant residues that were incubated after addition of $^{15}\text{N}_3\text{-TNT}$ for 11 months.

A further interesting observation is the absence of a cross peak correlating the aromatic amine region between -309 and -340 ppm of the ^{15}N dimension to any C functional groups. This supports the assignment of the signal at -320 ppm in the CPMAS ^{15}N NMR spectrum to unsubstituted aromatic amines. The N-alkyl C region of the ^{13}C -dimension (60 – 45 ppm), on the other hand, is correlated to the amide N region of the ^{15}N -dimension, resulting in a cross peak with a comparable intensity to that at 174 ppm of the ^{13}C -dimension. This indicates that most of the amide N are also alkylated.

Beside those discussed cross signals, only one more small signal, assigning the ^{15}N -region around -250 ppm to the aromatic C region in the ^{13}C -dimension (160 – 90 ppm) is detected. This signal could derive from pyrrole- or indole-type compounds, but may also be identified as a spinning side band of the amide signal. Such spinning side bands occur due to incomplete removal of the chemical shift anisotropy by insufficiently high rotation of the sample (here, 5.5 kHz) around the magic angle.

According to Thorn et al. (1996), anilinoquinones and anilinohydroquinones result in ^{15}N signals around -275 ppm and -307 ppm, respectively. The respective ^{13}C -signal of the N-substituted aromatic carbons are expected between 160 and 140 ppm in the ^{13}C dimension. A cross peak connecting those two chemical shift regions cannot be detected in Fig. 3. Thus, during 11 months of the incubation, 1,4 addition of the aromatic amine to quinones (Parris, 1980) seem not to be a major pathway for the immobilization of TNT into organic matter derived from degrading plant litter.

Due to the low signal-to-noise ratios of the DCPMAS ^{13}C ^{15}N NMR spectra, smaller signals indicating the formation of heteroaromatic N may be hidden by the noise. Thus, their formation cannot be excluded. On the other hand, the clearly observable cross peaks demonstrate that the formation of amide linkages and alkylation of their N represent an important reaction during incorporation of N derived from TNT into humifying organic material. However, the 2-D DCPMAS ^{15}N ^{13}C NMR spectrum in Fig. 3 shows a comparable

pattern to that recently obtained from the AI-fraction of ^{13}C -enriched plant residues, incubated after addition of K^{15}NO_3 (Knicker, 2002). This feature of the latter was interpreted to be caused by peptide compounds, which were formed by the immobilization of inorganic ^{15}N during microbial degradation of plant residues. Considering a possible release of nitro groups during TNT transformation, it is also likely that the amides and N-alkyl groups observed in the present study, originate from proteinaceous microbial components that were synthesized using the TNT-derived nitrogen source. Evidence for the incorporation of TNT transformation products into microbial biomass were indeed found by Boopathy et al. (1994), who added ^{14}C -TNT to an aerobic consortium of *Pseudomonas* and, after 15 days of incubation, recovered 8% of the ^{14}C as biomass.

In order to obtain a first idea about the origin of the amides observed in our study, the two samples were subjected to hot acid hydrolysis. This treatment is commonly thought to hydrolyze most of the peptides that are associated with biomass. After hydrolysis, 80% of the $^{15}\text{N}_{\text{add}}$ of the AI-fraction and 90% of the $^{15}\text{N}_{\text{add}}$ of the SOR-extract were recovered in the non-hydrolyzable residues, indicating that most of their amides are resistant against harsh hydrolysis. This supports their assignment to covalently bound $^{15}\text{N}_3$ -TNT transformation products, rather than to labile proteinaceous compounds of the microbial biomass. This finding is in accordance with former studies by Carpenter et al. (1978), who examined the microbial degradation of ^{14}C -labeled TNT in activated sludge systems. No essential radioactivity was detected in triglycerides, phosphatides or amino acids. Most of the radioactivity was found in the precipitates that separated from the fractions after storage at 10°C in the dark. Based on the solubility properties and their infrared spectra, it was suggested that those precipitates contain recalcitrant cross-linked macromolecular structures of the polyamide-type, formed by condensation of cellular compounds with biotransformed TNT.

4. Conclusion

In the present work, DCPMAS ^{15}N ^{13}C NMR was applied for the first time to investigate the

immobilization of TNT during humification of plant organic matter in quartz sand. Although the low sensitivity of this technique restricted this approach to compounds highly enriched in ^{13}C and ^{15}N and yielded spectra with low signal-to-noise ratios, the obtained spectra clearly demonstrate covalent binding of TNT transformation products to humifying organic matter. Such direct evidence is hardly achievable with common wet chemical approaches that restrict the analysis to soluble, thus mobile TNT metabolites. In future, improvements of spectral resolution and the signal-to-noise ratio may be obtained by the use of higher magnetic fields. However, even the spectra accumulated with the equipment available to us have already allowed the identification of two major C groups, namely amides and N-alkyl C, that are involved in the incorporation of TNT transformation products into humic material. Summarizing the discussed mechanisms for the formation of amide bonds during the transformation of TNT in soils, the observed pattern of the DCPMAS NMR spectra is in accordance, and indeed best explained, with the presence and accumulation of macromolecular polyamide-type structures. Future research on TNT immobilization is still needed and has to concentrate on a more detailed understanding of the nature of those polyamides as well as their behavior during humification.

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