Characterization of ^{15}N-TNT Residues After an Anaerobic/Aerobic Treatment of Soil/Molasses Mixtures by Solid-State ^{15}N NMR Spectroscopy. 1. Determination and Optimization of Relevant NMR Spectroscopic Parameters

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Solid-state ^{15}N NMR was applied to a humic acid, extracted from ^{15}N-2,4,6-trinitrotoluene (TNT) enriched soil treated in an anaerobic/aerobic composting system to characterize the nitrogen functionality of the transformation products bound to the soil organic material. Signals assignable to aniline derivatives and condensation products were identified, indicating that the anaerobic/aerobic treatment caused a reduction of nitro groups followed by condensation reactions with the soil organic material. Relevant parameters for routine application of the cross polarization magic angle spinning technique were determined and optimized. The proton spin-lattice relaxation times of all peaks in the ^{15}N NMR spectrum of the humic acid did not exceed 30 ms. Due to the fast relaxation, the application of ^{15}N NMR spectroscopy to soils with lower enrichment of ^{15}N-TNT is feasible. The influence of spinning sidebands on the intensity distribution was shown to be minimal at spinning speeds between 5.5 and 6.5 kHz. Contact times between 0.7 and 1 ms resulted in spectra with representative intensity distribution of all visible ^{15}N-TNT transformation products. However an underestimation of unreacted TNT must be considered. The results imply that CPMAS ^{15}N NMR is a valuable tool for the examination of bound residues of TNT in soils. (Figure 1).

Introduction

In the environment of military institutions and ammunition plants, 2,4,6-trinitrotoluene (TNT) represents a major soil contaminant. During manufacturing and handling, large amounts of the explosive and related nitroaromatics and aminonitroaromatics are introduced into the environment. Considering the toxicity of these compounds (*1*−*3*), a remediation of contaminated sites is urgently needed. Composting has been investigated intensively as bioremediation technique for soils contaminated with explosives (*4*−*7*). Besides classical composting procedures, anaerobic/aerobic treatments have lately gained major interest (*8*−*10*). As previous investigations showed that TNT is extremely recalcitrant against microbial mineralization (*7*), the aim of a composting procedure is thus the formation of bound residues of TNT transformation products. Under composting conditions the explosive is transformed first successively to aminodinitrotoluenes (ADNT) and then to diaminonitrotoluenes (DANT). If redox potentials below −200 mV are reached, 2,4,6-triaminotoluene (TAT) is also generated. These reduction products can form nonextractable residues in soil and compost (*4*, *5*, *11*). The application of composting as a remediation strategy involves that the bound residues are ecologically acceptable in the environment and harmless to human health. For the evaluation of these aspects, however, the chemistry and the binding stability of the immobilized residues have to be well understood.

As the immobilization of TNT and its degradation products in soils seems to be closely related to their nitrogen containing groups (*12*, *13*). The technique of choice for the examination of processes involved in the formation of bound residues and the chemistry of the nitrogen functionality of TNT transformation products is solid-state ^{15}N NMR spectroscopy. For the study of pollutants in soils and soil-related systems, ^{15}N NMR spectroscopy has severe sensitivity problems due to the low natural abundance of the ^{15}N isotope. For an NMR experiment, this isotope is approximately 50 times less sensitive than the ^{13}C nucleus. In general, the concentrations of N-containing pollutants in soils are not high enough to be ^{15}N NMR spectroscopically distinguishable from the bulk composition, but this disadvantage can be overcome by using ^{15}N-tracer. Under the premise that the concentration of the label is high enough, distinguishable peaks will occur in the solid-state ^{15}N NMR spectrum. Chemical transformation of the labeled compounds can then be followed by alteration of their signal intensities or by a change in their chemical shifts in the spectrum. This requires that the relative intensity distribution of the signals observable in the NMR spectrum correlates with the relative concentration of their different functional groups in the sample. In a solid-state NMR spectrum, however, the observed signal intensities do not depend on the concentration of the causing nuclei alone. Intra- and intermolecular interactions can obscure the relative intensity distribution. Only if the influence of these interactions are considered and the relevant acquisition parameters correctly adjusted, can quantitative results be obtained (*14*, *15*).

In the present study, solid-state ^{15}N NMR spectroscopy was used as a possible means of examining the nature of nitrogen-containing products formed and immobilized during ^{15}N-TNT degradation during an anaerobic/aerobic composting procedure of ^{15}N TNT spiked soil. The main goal of part I of this study is to provide a parameter setup, which may allow routine application of solid-state ^{15}N NMR spectroscopy for the examination of TNT transformation products bound to organic material in soil and soil-related systems. Part II of this investigation is a systematic application of the spectroscopic technique described here, to whole compost and different humic fractions. For adjusting an appropriate NMR parameter setup, a humic acid of a soil

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TABLE 1. Tentative Assignment of Chemical Shift Regions to ^{15}N Functional Groups (*22*, *23*)

chemical shift region (ppm)	assignment
148 to 50	azo compounds
50 to −25	nitro groups
−25 to −120	imines, phenoxazinones, pyridines, quinolines
−120 to −165	nitriles, oxazoles
−165 to −270	imidazoles, indoles, pyrroles, carbazoles, quinolone, anilides, amides, enaminones
−270 to −310	aniline derivatives, phenoxazones, hydrazines
−310 to −350	aniline, phenylamines
−359	ammonium

incubated with high concentrations of ^{15}N-TNT was isolated and subjected to solid-state ^{15}N NMR spectroscopy. The high enrichment in ^{15}N in this sample allowed the application of advanced NMR techniques for the determination of relevant NMR parameters. The quantitative limits of solid-state ^{15}N NMR spectroscopy for the investigation of bound residues of TNT transformation products are discussed critically.

Material and Methods

Sample Material. TNT-contaminated soil from a former ammunition plant "Tanne" near Clausthal-Zellerfeld, Lower Saxony, Germany, was enriched with ^{15}N-TNT to a final concentration of about 20 g/kg dry soil. This high concentration was used to increase the sensitivity of the sample for solid-state ^{15}N NMR spectroscopy to a level that allows the determination and optimization of relevant acquisition parameters. The soil was characterized in more detail previously (*16*). Uniformly labeled ^{15}N-TNT was synthesized by the Department of Chemistry (research group of Dr. K. Steinbach) of the Philipps University, Marburg, Germany. The ^{15}N-TNT-enriched soil was mixed with 20% (w/w) molasses slivers, and then the mixture was incubated for 6 weeks anaerobically and 10 weeks aerobically. During the aerobic phase, fresh compost material and dry sugar-beet powder (dried and milled sugar-beet) were added to stimulate biological activity. A detailed description of the experimental setup is given in part II of this investigation (*17*).

After the anaerobic/aerobic treatment, the compost mixture was extracted with methanol to remove nonbound TNT and transformation products. Analyses of these extracts are presented in part II of the study. The extracted material was incubated for 3 h at 95 °C with 30% NaOH (w/v) to extract humic and fulvic acid. Finally, the humic acid was precipitated by acidification with 32% (v/v) HCl. The humic acid was suspended in distilled water and then dialyzed against distilled water to remove NaOH and acid residues. The dialyzed humic acid, dried at 50 °C, yielded a dark brown substance was obtained by this procedure (The extraction procedure is described in detail in part II of the study (*17*).)

NMR Spectroscopy. The solid-state ^{15}N NMR spectra were obtained with a Bruker DMX 400 spectrometer at a frequency of 40.55 MHz. A commercial Bruker double air bearing probe with 7 mm o.d. rotors was used. The chemical shift scale was calibrated with neat glycine and is reported relative to nitromethane (= 0 ppm). Using this scale, the chemical shift of liquid ammonia is reported at −381.9 ppm (*18*). Tentative peak assignments are given in Table 1. The cross polarization magic angle spinning (CPMAS) NMR spectra (*19*) were obtained at a spinning speed of 3.3, 5.5, and 6.5 kHz after accumulation of 2000−5000 single scans with a pulse delay of 300 ms. To improve the signal-to-noise ratio, a line-broadening of 10 Hz was applied. A 90° ^{1}H-pulse width of 5.6 μs was used. By varying the contact time in a series of CPMAS spectra, the time relevant for the polarization transfer, T_{NH}, and the proton-spin−lattice-relaxation-time in the rotating frame, $T_{1\rho H}$, were determined. The proton-spin−lattice-relaxation-time, T_{1H}, was determined indirectly by detecting the ^{15}N magnetization via an inversion recovery experiment (*20*).

For the single pulse excitation NMR spectra, 1800 scans were accumulated on the same instrument using the same probe described above and applying an acoustic ring down pulse sequence with inverse gated proton decoupling. A 90° ^{15}N-pulse width of 7.5 μs and a spinning speed of 5.5 kHz were used. Applying normal inversion recovery techniques for the exact determination of the spin−lattice-relaxation-time, T_{1N}, would consume weeks of spectrometer time because of spectrometer drifts would probably not yield quantitative results. To obtain preliminary estimates of the spin−lattice-relaxation-times, T_{1N}, spectra were collected with delay times of 30 and 60 s.

The spectra were integrated by an integration routine supplied with the instrument software using the chemical shift regions compiled in Table 1.

Results and Discussion

Chemical Shift Assignment. Figure 1a shows the solid-state CPMAS ^{15}N NMR spectrum of the humic acid extracted from a soil that was enriched with ^{15}N-TNT and treated in an anaerobic/aerobic composting system. The main peak of the spectrum is observed in the chemical shift region between −100 and −350 ppm followed by a broad signal between −40 and −100 ppm. Smaller resonance lines occur between 200 and 80 ppm and between −370 ppm and −400 ppm. This spectrum shows a completely different pattern from solid-state ^{15}N NMR spectra of natural soils and their humic fractions (*21*, *22*). The latter are dominated by a signal peaking around −260 ppm followed by a signal around −345 ppm, assigned to amide functional groups and terminal amino groups of amino sugars and amino acids, respectively. Such signals cannot be distinguished in the spectrum shown here but may be overlapped by the broad signals peaking at −240 and −322 ppm. However, due to the high ^{15}N-enrichment of the added TNT, such signals are not expected, since the concentration of ^{15}N isotopes in natural soil organic material is far too low to be detectable in a solid-state ^{15}N NMR spectrum obtained after accumulation of 5000 single scans.

As indicated in the solid-state ^{15}N NMR spectrum in Figure 2, obtained from pure ^{15}N-TNT, signals of nitrogen groups of unreacted TNT are expected in the region between −10 and −20 ppm (*23*). The lack of considerable signal intensity in this region of the solid-state ^{15}N NMR spectrum of the humic acid indicates that most of the nitro groups of added ^{15}N-TNT were reduced during the incubation. That remaining unreacted nitrogen functionalities of TNT were destroyed by the drastic conditions of the alkaline hydrolysis cannot be excluded. Since the nitrogen from the natural soil cannot be detected and the humic acid fraction was obtained after extracting the soil with methanol, it can be assumed that the signals of the spectrum originate from transformation products of TNT that are bound to the natural humic material of the soil.

The peak at −322 ppm lies within the chemical shift region of free amino groups of anilines (*22*, *23*) and derives from reduction products of TNT. The microbial transformation of TNT is suggested to occur via monoamines (4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene) to diamines (2,4,-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene) and possibly 2,4,6-triaminonitrotoluene. The broad shoulder of this resonance line between −270 and −322 ppm occurs in the chemical shift region, assigned to secondary amines of aniline derivatives. This indicates that the reduction products of TNT were involved in further polymerization

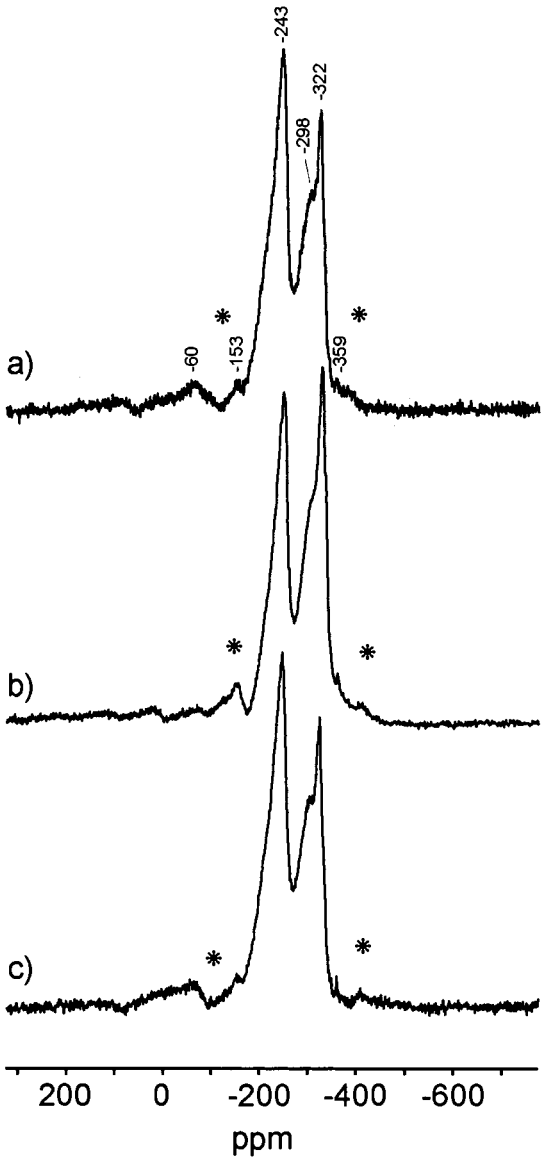

FIGURE 1. Solid-state ^{15}N NMR spectra of a ^{15}N-TNT-enriched humic acid obtained with the cross polarization magic angle spinning (CPMAS) technique at a spinning speed of 5.5 kHz (a), 3.3 kHz (b), and 6.5 kHz (c). A contact time of 1 ms and a pulse delay of 300 ms was used.

reactions. Examining the reactions of aniline with humic acids by means of solution ^{15}N NMR spectroscopy, Thorn et al. (*24*) suggested phenoxazines, anilinohydroquinones, hydrazines, and aminodiphenylamine as possible contributors to the signal intensity in this chemical shift region. These products may be formed by covalent binding of the aromatic amines with the naturally occurring soil organic matter either through nucleophilic addition reactions with the carbonyl functional groups of the latter or through oxidative coupling (e.g. free radical coupling) reactions.

The signal between −200 and −270 ppm may be indicative for condensation reactions leading to pyrroles (−205), indoles (−247 ppm), or quinolone (−246 ppm). As substitution of the latter will shift the signal toward lower fields (*22*), the signal peak at −240 ppm most likely represents substituted indole derivatives. Anilides (−248 ppm) may contribute to the upfield shoulder of this peak. The shoulder on the downfield site of the main peak between −160 to −200 ppm may originate from substituted pyrroles (−145 to −220 ppm), imidazoles, or oxazoles. Nitrile groups can also occur in this specific chemical shift region. The chemical shift region

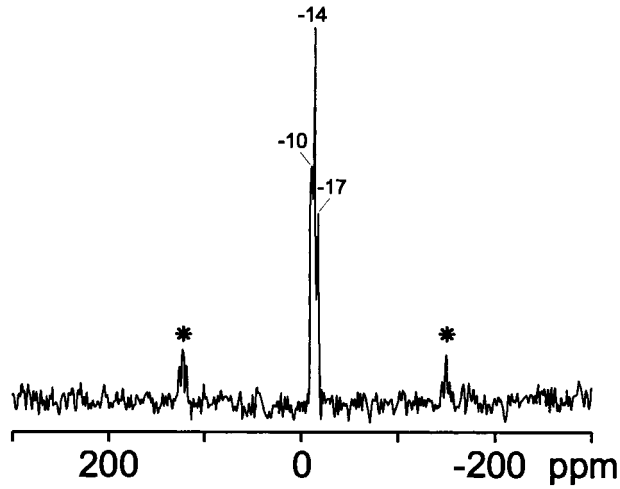

FIGURE 2. Solid-state CPMAS ^{15}N NMR spectra of unreacted ^{15}N-TNT obtained at a spinning speed of 5.5 kHz. Asterisks indicate spinning sidebands.

between −20 and −90 ppm is assigned to pyridinic N and imine structures. The latter may result from Schiff-bases between the ketones or aldoses occurring in the natural soil organic matter and the amino groups of the reduced TNT. Further intramolecular condensation could lead to phenoxazinone (−42 ppm) and quinoline (−65 ppm).

From these preliminary results, it can be concluded that during anaerobic/aerobic composting of ^{15}N-TNT spiked soil the explosive is reduced to aromatic amine, and some of the TNT transformation products are covalently bound to the natural soil organic matter.

Quantification of Solid-State CPMAS ^{15}N NMR Spectra. The emphasis of the NMR spectroscopy is to determine, via chemical shift assignments, the gross chemical structure of this material and to attempt a quantitative correlation between the different signal intensities and the chemical composition. Quantification of solid-state NMR spectra, obtained after application of the cross polarization magic angle spinning technique, requires that certain effects obscuring the relative signal intensity distribution can be neglected. In the following discussion, the influence of spinning sidebands, spin−lattice relaxation, and cross polarization dynamics on the observed signal intensities will be discussed.

Spinning Sidebands. One advantage of solid-state NMR spectroscopy for examining of soil-related systems lies in its independence from sample solubility. Applying common techniques used in liquid NMR spectroscopy, this advantage, however, results in broad overlapping resonance lines. In many cases, the resulting spectra do not allow a determination of the gross chemical structure via chemical shift assignment.

The line broadening results from dipolar, quadrupolar, and chemical shift interactions, which are orientation dependent. In liquids, these interactions are averaged by the fast molecular motions. In solids, these anisotropic interactions can be reduced by spinning the sample with sufficient speed at the magic angle (54°44′). For the complete removal of any of these anisotropic interactions, the magnitude of the spinning frequency must be at least as high as the line broadening caused by the interaction itself. In the case of the chemical shift anisotropy, this line broadening is dependent upon the applied static field and can have values of up to 500 ppm. NMR spectroscopic investigation of ^{15}N at magnetic fields higher than 9 T requires spinning speeds greater than 7 kHz for the complete removal of the chemical shift anisotropy. Such speeds can hardly be reached with

TABLE 2. Relative Intensity Observed in the Solid-State CPMAS ^{15}N NMR Spectra of a ^{15}N-TNT-Enriched Humic Acid Obtained at Various Spinning Speeds

	(ppm)							
speed (kHz)	148/50	50/−25	−25/−120	−120/−165	−165/−270	−270/−310	−310/−350	−350/−450
3.3	2	2	3	4	40	19	25	5
5.5	1	2	4	2	49	19	20	3
6.5	1	3	4	2	50	19	18	3

TABLE 3. Relaxation and Cross Polarization Parameters as Determined from the Solid-State ^{15}N NMR Spectra of a ^{15}N-TNT-Enriched Humic Acid

	ppm				
	−60	−150	−240	−300	−322
T_{1H}	28 ms	23 ms	22 ms	22 ms	23 ms
T_{NH}	206 μs	46 μs	63 μs	52 μs	109 μs
$T_{1\rho H}$	26 ms	4 ms	6 ms	4 ms	4 ms
$t_{optimal}$	>0.5 ms	0.2 ms	0.2 ms	0.2 ms	0.2 ms
$I(0)$	0.08	0.06	1.06	0.45	0.45
$I(t=1$ ms)	0.08	0.05	0.9	0.36	0.36
Calcdd intensity loss at $t=1$ ms	0%	27%	25%	20%	20%
$I(t=0.7$ ms)	0.08	0.05	0.98	0.38	0.39
Calcd intensity loss at $t=1$ ms	0%	27%	8%	15%	13%

common probes. Spinning sidebands appear in the spectrum. They are observed on each side of the isotropic signal at a frequency distance equal to the spinning frequency and can overlap other resonance signals occurring in their chemical shift region. An assignment of peaks to chemical structures then becomes difficult.

To evaluate the influence of spinning sidebands, spectra were obtained at various spinning speeds (Figure 1a−c). Table 2 lists the relative intensity of different chemical shift regions determined by integration. Compared to the spectrum obtained at a spinning speed of 5.5 kHz (Figure 1a), that obtained at 3.3 kHz (Figure 1b) shows higher peak intensity in the chemical shift region between −120 and −165 ppm (tertiary nitrogen in heterocyclic compounds) and in the chemical shift region between −310 and −350 ppm (primary amino groups of anilines). This can be explained by the contribution of signal intensity of the spinning sidebands of the main peak at −240 ppm in the spectra obtained at 3.3 kHz. The main intensity of the shoulder between −350 and −450 ppm moved upfield. While in the spectra obtained at 3.3 kHz this shoulder can be assigned to a spinning sideband of the peak at −322 ppm, in the spectra obtained at 5.5 kHz spinning sidebands of the main peak at −240 ppm are expected in that region. A small narrow peak indicating the presence of ammonium occurs at −359 ppm. This signal may be overlapped by the spinning sideband in the spectra obtained at 5.5 kHz. This signal can also be observed in the spectra obtained at a spinning speed of 6.5 kHz (Figure 1c). Here, the upfield spinning sideband occurs at −408 ppm. Its relative intensity contribution is <2%. The upfield spinning sideband is hidden by the signal near −60 ppm, but here it can be assumed that its relative intensity contribution has no major influence on the intensity determination for this region. Comparing the intensity distribution of the spectra obtained at 6.5 and at 5.5 kHz, differences exceeding the range of experimental error are not observed. These results indicate that at a spinning speed of 5.5 kHz the incomplete removal of anisotropic interactions does not have a major effect on the relative intensity distribution.

Proton-Spin−Lattice-Relaxation-Time T_{1H}. Quantification of NMR spectra requires sufficient signal-to-noise ratios. To obtain such spectra, numerous single scans are accumulated, leading to an averaging of the noise and an enhancement of signal intensity. In single pulse excitation NMR spectra, the relative intensity of a signal is considered to be directly proportional to the relative concentration of the chemical species, if saturation effects are avoided. Such saturation effects occur if, between the single scans, the spin system was not allowed to relax to its thermal equilibrium. The time needed for this process is determined by the spin−lattice-relaxation-time, T_1, and is dependent upon the chemical and physical environment of the specific nucleus. In a heterogeneous mixture, nuclei of different functional groups may therefore show different relaxation behavior. To obtain spectra which can be quantified, the time span between two single pulse experiments has to be approximately five times larger than the longest spin−lattice-relaxation-time T_{1max} observed for the nucleus under study. For the ^{15}N nucleus in solids only limited spin relaxation pathways are available, resulting in long relaxation times. If the ^{15}N is directly observed in a solid-state NMR experiment it becomes difficult to obtain spectra with acceptable signal-to-noise ratios. This problem can be overcome by the application of the cross polarization (CP) technique. Here the magnetization of the ^1H-spin system is transferred to that of the ^{15}N isotope via dipole−dipole interactions. The delay between the single pulses then becomes dependent upon the proton-spin−lattice-relaxation-time, T_{1H}, which is, due to efficient spin diffusion, that is much faster than that for ^{15}N.

T_{1H} was determined by a series of spectra accumulated with the application of a 180° ^1H-pulse and a variable pulse delay, d, before the conventional cross polarization sequence. The signal intensity $I(d)$, of the various peaks were determined via integration as function of the pulse delay, d. T_{1H} was then calculated using eq 1 and a program supplied with the software of the NMR instrument.

$$I(d) = I(0) * \exp(-d/T_{1H}) \quad (1)$$

Table 3 summarizes the T_{1H}-values. When evaluating these data, it has to be noted that in a solid-state NMR spectrum of a heterogeneous mixture, one peak can cover the signals of different groups which vary in their chemical and physical environment. T_{1H}-values determined for such peaks thus represent the weight average value for all nuclei contributing to the observed signal.

For the sample under study, short T_{1H}s between 20 and 30 ms were determined, revealing efficient proton relaxation pathways. This may be due to the fairly high concentration of free radicals observed for soils and humic acids. Comparable numbers were obtained for humic acids of incubated plant material (*24*). From the small variation between the T_{1H} of the single peaks, it can be assumed that efficient spin diffusion and, therefore, efficient exchange of spin information between adjacent protons occurs.

The short T_{1H}s observed for the sample under study have promising implications for further studies of TNT humification in soils by means of solid-state ^{15}N NMR spectroscopy. Since saturation effects can be avoided by applying pulse delays as short as 150 ms, samples with less enrichment can be examined. A ^{15}N NMR spectroscopic investigation of ^{15}N-enriched TNT transformation products bound to soil organic matter in bulk soils becomes feasible (see part 2 (*17*)). However, by applying such short pulse delays, the amount of unreacted ^{15}N-TNT may be underestimated due to saturation. In contrast to the ^{15}N nuclei of the humic acid isolate, those of the pure ^{15}N-TNT show long T_{1H}s of 16 s. These result from the lack of paramagnetic material in this sample and with low spin diffusion expected for more ordered structures.

Cross Polarization. In a cross polarization experiment, the magnetization transfer occurs during the contact time, *t*, at which the ^1H and the ^{15}N spin systems are irradiated simultaneously. During *t*, the ^1H system is kept in an excited state. Some relaxation still occurs and is described by the spin−lattice-relaxation-time in the rotating frame, $T_{1\rho H}$. The magnetization is then transferred with the cross polarization time, T_{NH}, from the ^1H system to the ^{15}N system. The cross polarization dynamics can be described as follows:

$$I_t = I_0\,(T_{1H},\, T_{NH},\, T_{1\rho H},\, t) \qquad (2)$$

Here, I_t is the intensity observed after the contact time *t*. I_o is directly proportional to the number of nuclei in the sample. With increasing *t*, the ^{15}N-magnetization and therefore the signal intensity of the observed ^{15}N increases with the first-order rate constant, $1/T_{NH}$. After the optimal contact time, ($t_{optimal}$), the ^{15}N-magnetization and therefore the ^{15}N signal intensity decreases exponentially because of loss of magnetization due to $T_{1\rho H}$. The efficiency of the magnetization transfer is dependent upon a number of factors such as molecular motion and the distance of the ^{15}N nucleus from the protons. In general, T_{NH} is shorter for $^{15}NH_2$ and ^{15}NH than for $^{15}NH_3$, which in turn is shorter than for nonprotonated ^{15}N nuclei. Considering this fact, it becomes obvious that in a cross polarization experiment the maximal signal intensity can only be achieved if the polarization transfer is complete before $T_{1\rho H}$ has started. For this reason, intensity loss due to cross polarization dynamics must always be contemplated. However, the observed signal intensity becomes close to maximum at $t_{optimal}$ if the inequality $T_{1\rho H} \gg T_{NH}$ is applicable.

T_{NH} and $T_{1\rho H}$ were estimated from a determination of the spectral intensities, *I*, as a function of contact time, *t*. Figure 3 compiles the results for the signals peaking at −60, −240, and −320 ppm. The lines drawn through the experimental points result from standard computer programs used for the calculation of T_{NH} and $T_{1\rho H}$. Within the precision of the intensities measured, each curve can be described by one T_{NH} and one $T_{1\rho H}$. However, as already stated above for the T_{1H}, these numbers should not be taken to mean that the magnetic relaxation of all fractions of these complex mixtures hidden under one signal can really be characterized by a single exponential function. The times determined here should rather be taken as practical hints for the optimization of the experimental parameter in the CPMAS experiments.

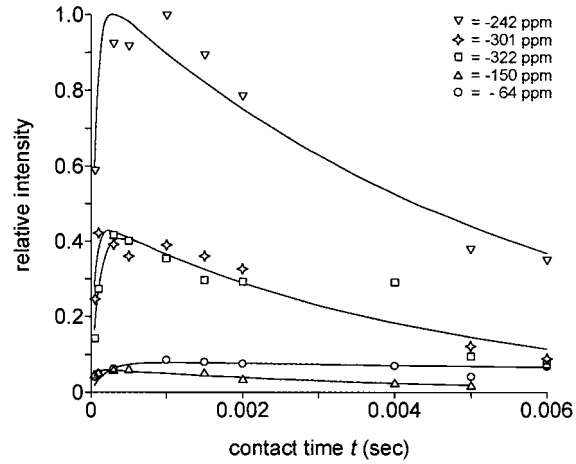

FIGURE 3. Relative intensity of various peaks in the solid-state ^{15}N NMR spectra of a ^{15}N-TNT-enriched humic acid as function of the contact time *t*.

The functions show a steep initial increase of *I* at the application of fairly short contact times and a slow decrease of *I* at longer *t*. For these signals, as listed in Table 3, the inequality $T_{NH} \ll T_{1\rho H}$ is fulfilled. Compared to those functions that of the peak at −60 ppm shows a slower initial increase. This can be explained by the low concentration of protons in the direct vicinity of nitrogen in pyridines and imines, decreasing the efficiency of the cross polarization rate. A T_{NH} of 0.3 ms was determined, indicating that magnetization transfer still occurs at an advanced stage of $T_{1\rho H}$ relaxation. The highest observable signal intensity of the peak at −60 ppm is reached after a contact time of 0.5 ms. This decreases only slightly thereafter, due to a long $T_{1\rho H}$ of 26 ms.

Except for the peak at −60 ppm, all signals show their maximal signal intensity at a contact time of approximately 0.2 ms. If this contact time is applied, maximal signal intensity will not be obtained for the peak at −60 ppm. According to theoretical considerations *t* should be at least three times larger than T_{NH}. For our sample, a contact time of 700 μs would thus be needed. The intensity losses expected at this contact time and those calculated for a contact time of 1 ms are listed in Table 3 and highest for the peak at −150 ppm. No intensity loss was calculated for the peak around −60 ppm. In this case, the low intensity of the peak may obscure the results. If the experimental error is considered, it can be assumed that spectra obtained with contact times between 0.7 and 1 ms represent a fairly quantitative intensity distribution of functional groups detectable with the CPMAS technique.

Single Pulse Excitation Experiment. In evaluating the possibility of quantification of a solid-state CPMAS ^{15}N NMR spectrum, one must to bear in mind that nitrogen groups in a vicinity with no or low concentrations of protons may not be observable. For example for nitrogen in unreacted TNT the maximum signal intensity was reached at a contact time of approximately 4 ms. Applying longer contact times up to 8 ms gave no significant decrease in observed signal intensity for this sample. Such nitrogen groups should be visible in an experiment in which the ^{15}N is directly observed. In general, direct observation of ^{15}N nuclei in heterogeneous mixtures with a simple single pulse excitation experiment, results in spectra in which the resonance lines are overlapped by a signal derived from the ringing of the probe during irradiation. Baseline and phase correction cannot eliminate the influence of this signal. To avoid such a signal, a special acoustic ring down sequence was applied.

If saturation effects are avoided, the signal intensity distribution in single pulse excitation NMR spectra is

TABLE 4. Relative Intensity Distribution Observed in the Single Pulse Excitation ^{15}N NMR Spectra of a ^{15}N-TNT-Enriched Humic Acid Obtained after a Pulse Delay of 30 and 60 s Compared with that Obtained from the Corresponding CPMAS ^{15}N NMR Spectrum

NMR−technique	pulse delay (s)	(ppm) 148/50	50/−25	−25/−120	−120/−165	−165/−270	−270/−310	−310/−350	−350/−450
CPMAS	0.3	1	2	4	2	49	19	20	3
Single pulse	30	4	4	9	4	42	11	19	7
Single pulse	60	4	5	8	5	42	11	19	7

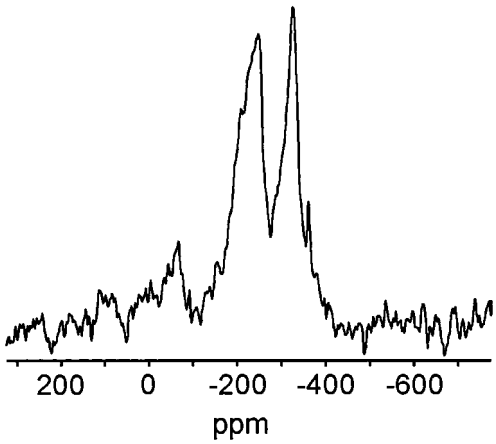

FIGURE 4. Solid-state ^{15}N NMR spectra of a ^{15}N-TNT-enriched humic acid obtained with the single pulse technique at a pulse delay of 60 s.

considered to widely represent the real chemical composition of the sample. A comparison of the spectrum accumulated with the single pulse sequence with that obtained after application of the CPMAS technique will therefore provide a good evaluation of the limitations of CPMAS NMR spectroscopy for quantification humic fractions, spiked with ^{15}N-TNT.

At the present stage of development, no values for T_{1N} of immobilized TNT in humic material are available in the literature. Unfortunately, due to the low sensitivity and the expected long relaxation times of such compounds in the solid-state, it is considered technically unfeasible to attempt the T_{1N} via inversion recovery experiments. A raw estimation can be made by comparing two spectra with variable pulse delay. If saturation was avoided in both experiments, the relative intensity distribution should not change, and it can then be assumed that the shorter pulse delay should be long enough to allow quantification.

Table 4 shows the relative intensity distribution of the ^{15}N NMR spectra, which were obtained with the single pulse excitation pulse sequence after pulse delays of 30 and 60 s. To increase the signal-to-noise ratio, a line broadening function of 200 Hz was applied. No major alterations in signal intensity distribution are observed with increasing pulse delay, leading to the conclusion that in both spectra saturation effects were avoided. The spectrum presented in Figure 4 shows the same pattern already observed for those obtained with the CPMAS technique (Figure 1a). No additional peaks are detectable, indicating that in the CPMAS ^{15}N NMR spectrum, peaks of most if not all ^{15}N-labeled functional groups of the sample are visible. A comparison of the relative intensity distribution of the spectra accumulated with the single pulse technique with those obtained with the CPMAS technique at a spinning speed of 5.5 kHz and a contact time of 1 ms is given in Table 4.

In relation to the CPMAS spectrum, the single pulse excitation NMR spectrum reveals higher intensities in the chemical shift regions from 148 to −120 ppm and in that between −350 and −450 ppm. This may cause underestimation of nonprotonated nitrogen in the CPMAS NMR spectrum. Considering the low signal-to-noise ratio of the single pulse excitation NMR spectrum, it seems likely that this increase is caused by an intensity contribution from the noise.

The present study shows, for the first time, that bound residues of ^{15}N-TNT-metabolites, produced by anaerobic/aerobic treatment, can be characterized chemically. Our investigations indicate that solid-state ^{15}N NMR spectroscopy is a promising tool for the identification of ^{15}N-labeled transformation products of xenobiotics such as TNT immobilized into soil organic material. Aromatic amines and their condensation products were found to be closely associated with the humic fraction and probably by covalent bonding. Although some intensity loss in the chemical shift region of nonprotonated nitrogen and underestimation of the amount of unreacted TNT has to be considered, CPMAS ^{15}N NMR spectroscopy represents an additional valuable technique for revealing the nature of the insoluble fraction of TNT transformation products in soils and the mechanisms involved in their immobilization into the soil organic matter matrix.

Acknowledgments

This work was supported by the Federal Ministry of Education, Science, Research and Technology (BMBF), by the state of Lower Saxony and the Industrieverwaltungsgesellschaft AG (IVG), Bonn, Germany. Furthermore, we thank Dr. Förster (Bruker, Karlsruhe) for his invaluable advice and support in applying the specific NMR sequences. Mrs. E. Schuhbauer is gratefully acknowledged for carefully preparing the figures.

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Received for review April 27, 1998. Revised manuscript received October 19, 1998. Accepted October 20, 1998.

ES980422X