Incorporation studies of NH_4^+ during incubation of organic residues by ^{15}N-CPMAS-NMR-spectroscopy

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This study focuses on the processes occurring during incorporation of inorganic nitrogen into humic substances. Therefore rye grass, wheat straw, beech saw dust, sulphonated lignin and organosolve lignin were incubated together with highly ^{15}N-enriched ammonium sulphate in the laboratory for 600 days. Samples from the incubates were periodically analysed for weight loss, and carbon and nitrogen contents. The samples were also analysed by solid-state ^{13}C- and ^{15}N-CPMAS-NMR-spectroscopy to follow the turnover of the materials during incubation. Most of the detectable ^{15}N-signals was assigned to amide – peptide structures. The remaining intensities could be ascribed to free and alkylated amino groups, and those on the low field side of the broad amide–peptide signal to indole, pyrrole and nucleotide derivatives. Abiotic reactions of ammonia with suitable precursors and the formation of pyridine, pyrazine or phenyloxazone derivatives were not observed. Signals from ammonia and nitrate occurred only at the end of the incubation.

Introduction

Ammonia-N and its ionized form are the most important sources of nitrogen in soils for plants and microorganisms. Ammonia undergoes several biotic and abiotic reactions by which it is immobilized and remobilized from inorganic to organic forms and *vice versa* during longer or shorter periods (Jansson & Persson, 1982). The continuous exchange between mineral and organic bound N-forms is well established. This is also the reason why only 60% or less of fertilizer-N applied to soil is directly taken up by plants, whereas 40–60% or even more originates from continuously mineralized soil N. Application of ^{15}N-labelled ammonia fertilizer showed that after harvest 50–60% became immobilized in the soil organic matter (Broadbent & Carlton, 1978). Only during the first or second year did this immobilized N become preferentially available to plants, and thereafter this residual fertilizer-N behaved like the bulk of soil nitrogen (Stevenson & Kelley, 1985).

Immobilization and mobilization of ammonia depend mainly on the C/N ratio of plant residue during humification. Bacteria and fungi utilize ammonia during the degradation of carbon-rich plant material mainly as a source of N for the synthesis of their biomass. During prolonged incubation when the carbon source becomes exhausted and the C/N ratio decreases to less than 10, NH_3 is released and is present in soil as NH_4^+, or after nitrification as NO_3^-.

Microorganisms are the catalysts of most transformations in soils. In arable soils of western Europe they contain about 80–120 kg N ha^{-1}; but 60–70 times more of organic N (5000–8000 kg N ha^{-1}) is contained in humic components. During a growing season less than 1% of this immobilized N is available to plants.

Upon hydrolysis with hot 6 N HCl about 40–60% can be identified as amino acids, amino sugars and ammonia, whereas the rest remains unknown (Anderson *et al.*, 1989). This unknown nitrogen consists of both hydrolysable- and non-hydrolysable-N, but identified and unknown N participate in the N-cycle as well (Ivarson & Schnitzer, 1979).

Several authors assume that N-containing compounds are stabilized by covalent linkages to soil humic compounds as integral constituents (Flaig *et al.*, 1975). Stabilization could occur by nucleophilic addition of amino acid derivatives to quinones or by formation of heterocycles. Furthermore, reactions between carbohydrates and amino acids or peptides have been suggested (Maillard reaction: Cheshire *et al.*, 1990; Stevenson, 1994). Several reports indicate that ammonia gets abiotically fixed to soil organic matter, peat, coal or lignin (Söchtig & Flaig, 1974; Nömmik & Vathras, 1982; Preston *et al.*, 1982; Thorn & Mikita, 1992; Lapierre *et al.*, 1994; Bosatta & Ågren, 1995). Thorn & Mikita (1992) provided

Table 1 Elemental composition of the samples used. The C/N ratio was calculated in w/w from the data obtained from elemental analysis of the non-degraded plant and lignin material (before addition of $(^{15}NH_4)_2SO_4$) and after addition of $(^{15}NH_4)_2SO_4$ to the plant material – quartz sand mixture at the beginning of the incubation

Sample	C /g kg^{-1}	N /g kg^{-1}	C/N before addition of $(^{15}NH_4)_2SO_4$	C/N after addition of $(^{15}NH_4)_2SO_4$
Rye grass	380	52	7	7
Wheat straw	430	4	108	70
Beech sawdust	460	< 1	460	138
Sulphonated lignin	420	1.5	280	105
Organosolve lignin	600	1.3	462	160

Table 2 Possible assignments for the ^{13}C NMR spectra (referenced to tetramethylsilane) (Malcolm, 1989)

Chemical shift range /p.p.m.	Assignment
220–160	Carboxyl/carbonyl/amide carbons
160–140	Aromatic COR or CNR groups
140–110	Aromatic C-H carbons, alkene carbons
110–90	Anomeric carbon of carbohydrates, C-2, C-6 of syringyl units
90–60	Carbohydrate-derived structures (C-2 to C-5) in hexoses, C_α of some amino acids, higher alcohols
60–45	Methoxyl groups and C-6 of carbohydrates and sugars, C_α of most amino acids
45–0	Methylene groups in aliphatic rings and chains, terminal methyl groups

^{15}N NMR-spectroscopic evidence that most of the nitrogen incorporated into humic acids by abiotic reaction of NH_3 in aqueous solution appears to be in the form of indole and pyrrole, in addition to pyridine, pyrazine, amide and aminohydroquinone nitrogen.

From hydrolysates produced by hot 6 N HCl-treatment of whole soil or humic materials, hydroxy- and oxoindols, pyrrole-, quinoline-, piperidine-derivatives and other heterocyclic components were identified (Schnitzer & Spiteller, 1986). However, it is, questionable whether such compounds are formed during the harsh hydrolysis treatment (Anderson *et al.* 1989).

The non-hydrolysable N of the soil humus is considered to consist significantly of heterocyclic N. Pyrolysis of whole soil samples detected several heterocyclic N-products, in addition to aliphatic and aromatic amines, cyanides and ammonia (Granada *et al.*, 1991; Schulten & Schnitzer, 1992). Such components were also involved in a structural scheme of humic acids presented by Schulten (1994). Questions, however, arise if such compounds are present in humic compounds or are artefacts formed during the high temperatures of pyrolysis (Saiz-Jimenez, 1994).

The use of ^{15}N NMR-spectroscopy in either liquid or solid form has proved valuable for analysing N-forms in humic compounds and melanins (Benzing-Purdie *et al.*, 1983). Because of the insensitivity of the ^{15}N-isotope in ^{15}N NMR (relative sensitivity: $^1H = 1$ and of $^{15}N = 3.85 \times 10^{-6}$) the signal intensity is greatly improved by using highly ^{15}N-enriched compounds such as plant incubates (Knicker & Lüdemann, 1995). This enhances the ability to assign signals.

The ^{15}N NMR spectroscopic analysis of ^{15}N-enriched plant residues during incubation amazingly showed that even up to 600 days, 80–90% of the total nitrogen signal intensity was found in the chemical shift region of amide–peptide structures (Knicker & Lüdemann, 1995). The remaining intensities occurred in other biological N-forms including amino-groups and ammonia, as well as nitrate. Signals for nitrogen containing heterocyclic compounds of the pyridine or phenazine type or nitriles were completely absent. The ^{15}N NMR-spectra of the non-hydrolysable residues indicated that the remaining N occurred mostly as amides, aliphatic amines and possibly indoles (Zhuo *et al.*, 1992; Knicker & Lüdemann, 1995).

The studies presented here focus on processes occurring during the incorporation of inorganic nitrogen, applied to decaying plant material, into humic substances by means of solid-state ^{15}N NMR spectroscopy. We used rye grass, wheat straw, beech wood sawdust, organosolve lignin, and sulphonated waste lignin from cellulose production for this purpose. We incubated them after amending them with 99% enriched ^{15}N-ammonium sulphate, under controlled laboratory conditions. In order to study the influence of oxygen content on incorporation of N-containing material into the humic matrix. We continued the incubation for approximately 600 days under aerobic or water-saturated conditions. Samples taken at time intervals were analysed for structural changes by solid-state ^{13}C and ^{15}N CPMAS NMR spectroscopy, weight losses and for different forms of N.

Material and methods

Sample preparation

Fourteen-day-old stems of rye grass *(Lolium perenne)* were collected from pot experiments, that had been treated with N-fertilizer at natural ^{15}N-abundance. Wheat straw was collected from a cultivated field after harvest. The beech wood sawdust was obtained from a saw mill. The organosolve and sulphonated lignin came from paper mills near Regensburg (Germany). The nitrogen and carbon contents of these samples are listed in Table 1. The samples were milled to fine powders. Approximately 200 g of quartz sand (0.3–0.4 mm) was mixed with plant or lignin residues in a ratio 100:6 and placed into glass beakers. Water was then added to reach a sample moisture of either 60% of the maximal water holding capacity (WHC) or water

Table 3 Possible assignments for the ^{15}N NMR spectra (referenced to nitromethane) (Martin *et al.*, 1981)

Chemical shift range /p.p.m.	Assignment to biological material	Other possible assignments
25 to –25	Nitrate, nitrite, nitro groups	
–25 to –90	Imine, phenazine, pyridine, Schiff-bases	
–90 to –145	Purine (N-7)	Nitrile groups
–145 to –220	Chlorophyll, purine/pyrimidine, indole, imidazole, pyrrole	Maillard products
–220 to –285	Amide/peptide, N-acetyl derivatives of aminosugars, tryptophane, proline	Lactame (pyrrole, indole)
–285 to –325	NH in guanidine	Aniline derivatives
–294	N_δ-arginine and N_α-citrulline	
–307	N_ε-arginine, N_ω-citrulline, urea, nucleic acids	
–325 to –375	NH_4^+, $-NH_3^+$, $-NH_2$, -NHR and $-NR_2$ groups, free amino groups in amino acids and amino sugars	Anilinium salts

Table 4 Relative signal intensities in different ^{15}N-shift regions of the incubated lignins

	25 to –25	–145 to –220	–220 to –285	–285 to –300	–300 to –325	–325 to –350	–350 to –375
Incubation time /days	/p.p.m.						
Organosolve lignin (60% WHC)							
13	–	3.9	81.4	3.3	4.7	5.7	1.0
31	–	3.6	79.1	3.9	5.8	8.5	3.0
81	–	3.1	77.9	3.9	6.2	6.5	2.4
322	–	3.5	86.4	3.9	3.4	2.5	0.3
573	1.3	4.8	80.8	3.9	4.5	3.6	1.1
Organosolve lignin (water saturation)							
13	–	1.4	83.3	3.8	5.0	5.4	1.1
31	–	2.1	84.3	3.7	4.1	4.9	0.9
81	–	1.8	84.0	3.9	5.1	4.5	0.7
322	–	3.4	86.0	3.4	3.6	3.2	0.4
573	–	5.8	84.4	3.5	2.9	2.8	0.6
Sulphonated lignin (60% WHC)							
13	–	4.5	83.2	2.8	2.6	5.9	1.0
81	–	2.4	85.5	2.6	3.2	4.2	2.1
212	–	3.8	83.6	2.3	2.8	3.4	4.1
471	–	4.5	79.9	2.4	2.9	4.1	6.2
573	–	3.0	82.3	3.0	3.3	3.8	4.6
Sulphonated lignin (water saturation)							
13	–	4.0	83.8	2.7	3.2	5.4	0.9
81	–	2.5	79.8	3.0	4.1	5.6	5.1
212	–	2.9	80.1	2.5	3.0	3.4	8.1
471	–	3.6	77.8	3.2	3.2	3.4	8.8
573	–	3.9	66.5	2.9	2.9	3.5	20.3

saturation. The ^{15}N-labelled ammonium sulphate (99.3% ^{15}N) and some other minerals were dissolved in the water so that N-addition corresponded approximately 1 mmol $(^{15}NH_4)_2SO_4$ per 12 g dry plant weight in each batch. The water, furthermore, contained an aqueous extract from 1 g of fresh compost for inoculation. The samples were placed in closed desiccators and aerated by a slow stream of moist, CO_2-free air. They were incubated in the dark at 25°C in an incubation chamber. Weight losses were determined at intervals from the lyophilized samples.

Aliquots were used for the solid-state ^{13}C- and ^{15}N- CPMAS NMR measurements. In order to increase the concentration of carbon and nitrogen of the incubated materials the quartz sand was mechanically separated from the organic residues and washed with distilled water. Both the solid and aqueous residues

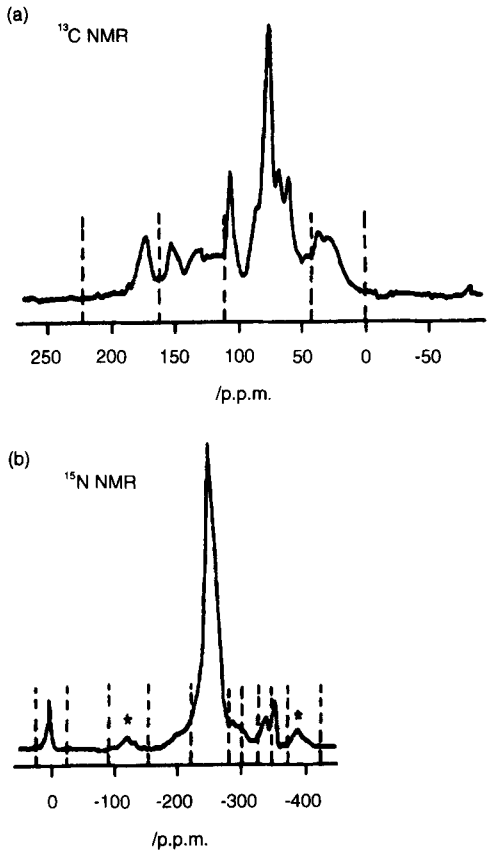

Figure 1 Typical solid-state ^{13}C and ^{15}N CPMAS NMR spectra of the incubated plant residues (^{15}N labelled wheat, incubated for 631 days at 60% WHC). Indication of the ^{13}C and ^{15}N signal ranges are shown in Tables 2 and 3. Asterisks indicate spinning side bands.

were combined and lyophilized. Carbon and nitrogen content were determined by elementary analysis of the lyophilized samples.

NMR methods

The solid-state ^{13}C-CPMAS NMR spectra were obtained with a Bruker MSL-100 spectrometer at a frequency of 25.2 MHz with magic angle spinning at 4 kHz. Approximately 20 000 scans were acquired for each spectrum. A commercial double bearing probe with 7 mm outside diameter and a phase-stabilized zirconium dioxide rotor and a single Hartmann–Hahn contact time of 1 ms was used. Previous experiments by Knicker & Lüdemann (1995) showed that all relevant relaxation times decreased strongly during incubation because free radicals were formed. Therefore, for samples obtained at the beginning of the incubation, a repetition time of 5 s was used, whereas for the incubated material a repetition time of 300 ms was sufficient (Knicker & Lüdemann, 1995).

The solid-state ^{15}N CPMAS NMR-spectra were obtained with a Bruker MSL-300 spectrometer at a frequency of

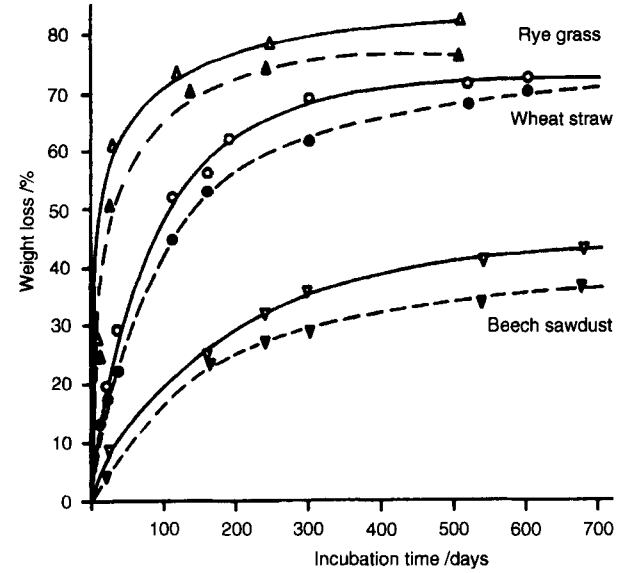

Figure 2 Weight losses of the composts from rye grass, wheat straw and beech sawdust. △, ○ and ▽ are at 60% WHC; ▲, ● and ▼ are at saturation.

30.4 MHz with a magic angle spinning of 4.5 kHz. A contact time of 0.7 ms and a repetition time between 5 s and 300 ms was used according to the degree of humification of the sample. Approximately 30 000–60 000 scans were accumulated.

The ^{13}C-chemical shifts are related to tetramethylsilane (= 0 p.p.m.) as external standard, whereas those from ^{15}N are related to nitromethane (= 0 p.p.m.) (Martin *et al.*, 1981). A more detailed description of the experimental procedure is given by Knicker & Lüdemann (1995). For quantification, the spectra were divided into different chemical shift regions assigned to specific carbon- or nitrogen-functional groups (Martin *et al.*, 1981; Malcolm, 1989; Knicker & Lüdemann, 1995). These assignments are shown in Tables 2 and 3, and in Figure 1.

Results

Chemical alterations during incubation

During incubation, the colour of the rye grass changed to a brownish green, whereas the wheat straw and the beech sawdust turned to more brownish under both aerobic and water saturated conditions. The lignin samples did not essentially change colour during incubation. After a few days, the surface layer of the water saturated samples was covered by a fungal mat, which remained throughout the whole incubation period. The pH of the sawdust- and organosolve-lignin incubates always remained nearly neutral (between pH 6.8–7.5), whilst the incubated wheat straw and rye grass became weakly alkaline (pH 9.5) in the first few weeks, then turned slightly acid. The pH of the incubated sulphonated lignin was already acidic (pH 3–3.5) at the beginning and remained so until the end.

Figure 2 shows weight losses during incubation of rye grass, wheat straw and sawdust at both aerobic and water saturated conditions of incubation. After 573 days of incubation weight losses from the sulphonated lignin amounted to 38% at 60% WHC and 32% at water saturation, those from the organosolve lignin amounted to 26% at 60% WHC and 9% at water saturation. Similar decomposition rates for lignocelluloses and lignin during soil incubation have been reported by Martin & Haider (1986).

Parallel to the weight losses, the analytically determined C/N ratios of the incubated materials decreased. In the wheat straw these ratios decreased from approximately 72 after addition of the ammonium sulphate in the beginning to 12 and 17 at day 600 under aerobic and H_2O-saturated conditions, respectively. The corresponding values for the sawdust decreased from 140 at day zero to 73 and 91 at day 600. In the incubated sulphonated lignin these ratios decreased from 105 to 47 at 60% WHC and to 100 at water saturation, whilst those of the organosolve lignin incubates from 160 to 140 and 125, respectively. The rye grass incubates had a C/N ratio of 7 at beginning which remained constant in both of the variants of incubation.

The losses of N were insignificant during incubation of wheat straw and sawdust under both 60% WHC and water saturation, as they were nearly zero from both the lignin samples. The rye grass incubates with their small C/N-ratios, however, lost about 70% of their nitrogen by volatilization of ammonia or denitrification of nitrate, or both.

Analysis of the incubated material by quantitative solid-state ^{13}C- and ^{15}N-CPMAS NMR spectroscopy

Incubated rye grass, wheat straw and beech sawdust. Figure 3 shows several of the solid-state ^{13}C NMR spectra from rye grass, wheat straw and beech sawdust samples taken at different incubation times from batches kept at 60% WHC. From day 8 to about day 600 the spectra of rye grass, wheat and sawdust incubates still have their main ^{13}C-signal intensities in the range from 45 to 110 p.p.m. (mainly polysaccharides) and 110–160 p.p.m. (aromatics in lignins). In the spectra the signal in the chemical shift region of 160–220 p.p.m. (C = O-, COOH-groups or amides) becomes more pronounced after prolonged incubation.

Figure 4 shows a strong time-dependent relative decrease in the ^{13}C-NMR signal intensities in the carbohydrate chemical shift range and a relative increase in particular in the range of aromatic (110–160 p.p.m.), alkyl (0–45 p.p.m.) and slightly in the region of carbonyl or carboxylic or amide (160–220 p.p.m.) carbons for rye grass, wheat straw and saw dust incubates. This accords with observations from similar incubation studies (e.g. by Inbar *et al.* 1989). The N-rich rye grass incubate had the greatest increase of intensities in the alkyl region (0–45 p.p.m.).

Immediately after mixing with $(^{15}NH_4)_2SO_4$ the samples

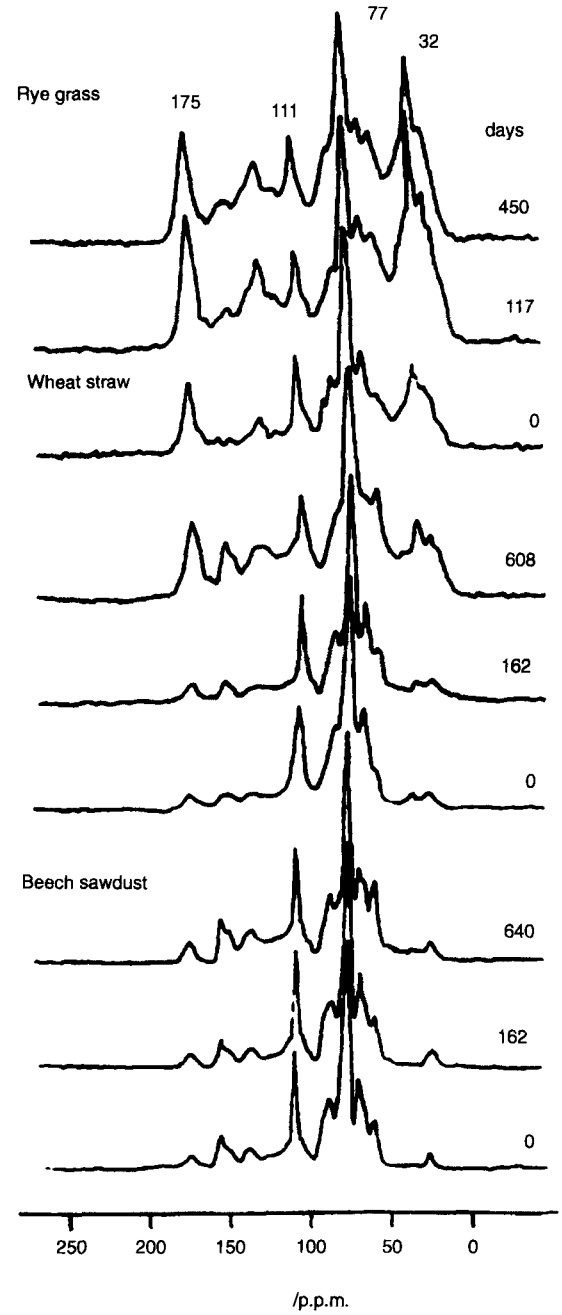

Figure 3 Solid-state ^{13}C CPMAS NMR spectra of the incubated rye grass, wheat straw and beech saw dust with addition of ^{15}N-ammonium at 60% WHC.

showed a strong signal at – 358 p.p.m. assigned to the NH_4^+ ion. Figure 5 shows that already after 8 days of incubation, ^{15}N-signals assigned to ammonium ions or ions of tertiary amines or both (–325 to – 375 p.p.m.) are weak, indicating that the added ammonium sulphate was readily assimilated by the microflora into the biomass. This incorporation is demonstrated by a prominent signal ranging from – 220 to – 285 p.p.m. and is most likely assigned to nitrogen bound in

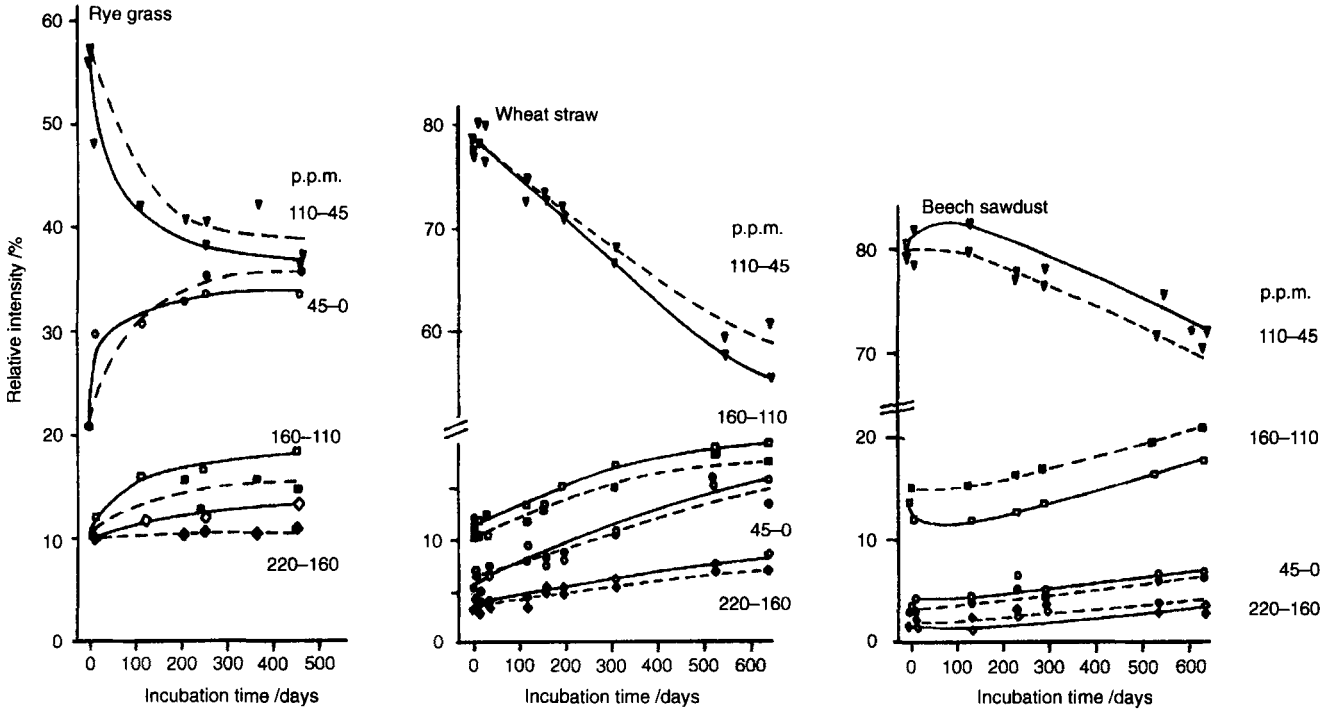

Figure 4 Relative intensities in different ranges of the solid-state ^{13}C CPMAS NMR spectra from wheat straw and beech sawdust incubates as a function of the incubation time.

peptides and amides, and possibly to lactams (Martin *et al.*, 1981). The signal of unsubstituted pyrrole is expected at – 235 p.p.m., which could be covered by the broad signal, peaking at – 257 p.p.m. The low field shoulder of this signal can also partly camouflage signals from substituted pyrroles (–145 to – 220 p.p.m.) originating from partly degraded chlorophyll. Signals from phenazines, phenoxazines, pyridines or Schiff-bases (–25 to – 90 p.p.m.) as well as signals from nitriles (–90 to – 145 p.p.m.) could not be detected at any time during incubation.

Changes in the relative signal intensity distribution of the solid-state ^{15}N CPMAS NMR spectra from rye grass, wheat straw and sawdust incubates at 60% WHC and water saturation, as a function of time are presented in Figure 6. With exception of the rye grass incubates, the relative intensity of the 'peptide-N-range' (–220 to – 285 p.p.m.) increased greatly until day 200 or 300 of incubation and then remained constant. Assimilation of the added ammonia is also indicated by the appearance of signals in the chemical shift ranges between – 145 and – 220 p.p.m. and – 285 and – 325 p.p.m. The resonance lines between – 145 to – 220 p.p.m. can originate from the NH-functions of purines, pyrroles or histidines. Specific signals from NH-groups in guanidine, such as N_ε and N_γ arginine, N_ω and N_α in citrulline, and NH_2-groups in uric acids or nucleotides are seen between – 285 to – 325 p.p.m. Signals between – 325 to –375 p.p.m. most likely originate from free or ionized NH_2-groups of amino acids and sugars, and N in ammonium ions (Martin *et al.* 1981).

Lignin samples. Both lignin samples were incubated without any additional easily degradable carbon sources. They, however, contained hemicelluloses that supported microbial growth. This became evident almost instantaneously by the appearance of a fungal mat on the surface of the incubates. The consumption of hemicelluloses in the sulphonated lignin sample was indicated by an initially strong solid-state ^{13}C CPMAS NMR resonance line at 75 p.p.m. (Figure 7), which continuously decreased during incubation in the sulphonated lignin samples at 60 or <100% WHC. From this figure, a relative increase in the methoxyl signal at 56 p.p.m. and that of the aromatic and phenolic signals at 160–90 p.p.m. is observed. Alterations in the shape and peak heights of the signals with increasing time of incubation were less in the organosolve than in the sulphonated lignin. Because of the small carbohydrate content and the high aromaticity of lignins, quantification of the aromatic ^{13}C-NMR-region was expanded from 90 to 160 p.p.m. to include the aromatic syringyl carbons at the low field side (160–140 p.p.m.) and the phenolic carbons at the high field side (120–90 p.p.m.).

The change in relative intensities in different regions of ^{13}C signal intensity with increasing incubation time for both the sulphonated and the organosolve lignins at 60% WHC and waterlogging is shown in Figure 8. Here, a strong increase in the relative intensity of the aromatic region of the sulphonated lignin samples during in the first 150 days can be seen. This region contributes approximately 55% to the total relative intensity. In the samples incubated at 60% WHC the region

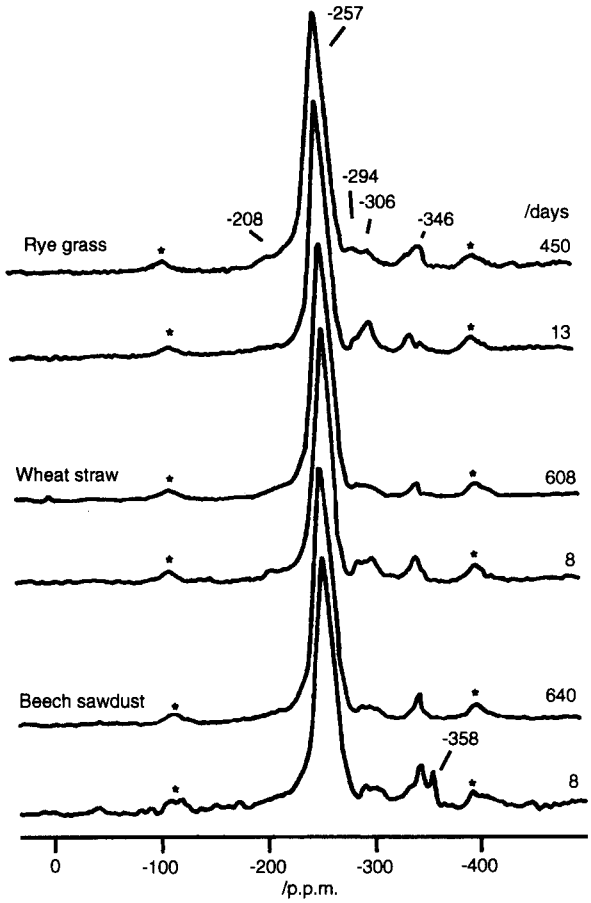

Figure 5 Solid-state ^{15}N CPMAS NMR spectra of incubated rye grass, wheat straw and beech saw dust with addition of ^{15}N-ammonium at 60% WHC.

that contains the residual hemicellulose NMR shifts and the resonances from the lignin side chains (60–90 p.p.m.) decreased dramatically at first and then more slowly. In the waterlogged samples the intensity in this region remained fairly constant after an initial decrease. This initial decrease can be explained by the metabolization of the residual carbohydrates (strong signal at 75 p.p.m. at day 13 which finally disappeared) and also by the microbial attack on the lignin side chains.

The relative intensities of all other regions of the spectra of the sulphonated lignin initially show a slight change and then remained nearly constant throughout incubation. The small relative increase in the alkyl region may be explained by the accumulation of microbial bioproducts. As shown in Figure 8 the organosolve lignin shows no major changes in the relative intensity distribution of their solid-state ^{13}C NMR spectra as function of incubation time, indicating that only little degradation occurred.

In spite of the little degradation of the lignin itself, already after 13 days of incubation, more than 80% of the added ammonia was incorporated into peptides and other biological nitrogen forms (Figure 9). This is also indicated by Table 4 which shows the time dependence of the relative intensities of ^{15}N NMR signals in different regions of the lignin incubates. The contributions from terminal aliphatic NH_2 groups (e. g. lysine and N-terminal amino acids in peptides from –325 to –350 p.p.m.), which amounted to about 5–8% during the first few weeks of incubation, decreased with time, whereas the signal at –358 p.p.m. from ammonium (–350 to –375 p.p.m.), greatly increased after day 573, and contributed more than 20% to the relative intensities of the waterlogged sample of suphonated lignin. Generally, no signals from nitrate ions were detected in the spectra, and only a small signal at 0 p.p.m. was visible in the organosolve lignin incubated for 573 days at 60% WHC (Figure 9). This result is also presented in Table 4, which shows that the large contribution of the relative intensities from the 'peptide region' (–220 to –285 p.p.m.) is decreasing with prolonged incubation and indicates an ongoing autolysis of the microbial biomass. Throughout incubation of the lignin samples no signals in the range from –25 to –90 p.p.m. appeared, indicating the resonances from pyridines, pyrimidines, or between –90 and –145 p.p.m., indicating nitriles. The change with time with the organosolve lignin incubates was similar, but not as pronounced as the sulphonated lignin samples.

Discussion

This study and incubation studies of ^{15}N-labelled plant residue materials by Knicker & Lüdemann (1995) show that the ^{15}N-NMR-spectra consist of signals from products of biological origin. These spectra are dominated by a signal between –220 and –285 p.p.m. with maximum intensity at –257 p.p.m., indicating nitrogen in peptide linkages. In the region between –285 and –325 p.p.m. two weak, but clearly resolved peaks, are most probably attributable to amino groups and alkylated amino groups. We assign the resonances between –325 to –375 p.p.m. to protonated free or alkylated amino groups and ammonium ions.

Although ammonium was added initially, its signal disappeared fairly soon after starting incubation. In the wheat straw and beech wood samples, of which polysaccharides are still the main constituents, no significant ammonium signals appeared during prolonged incubation. Only in the incubated lignin, which is less rich in easily available carbon, are there signals from ammonia and nitrate. Such signals were also present in the degraded organic matter obtained from ^{15}N-labelled green wheat (Knicker & Lüdemann, 1995). One should, however, bear in mind that only nitrogen compounds containing much ^{15}N can be recognized by their NMR signals. Plant residues such as rye grass with an initial large natural N content may dilute the added ^{15}N-labelled nitrogen. Therefore, conversion products such as ammonia or nitrate which develop during incubation of the unlabelled plant nitrogen may be underestimated by ^{15}N NMR.

In none of the incubates, however, are signals from nitrogen

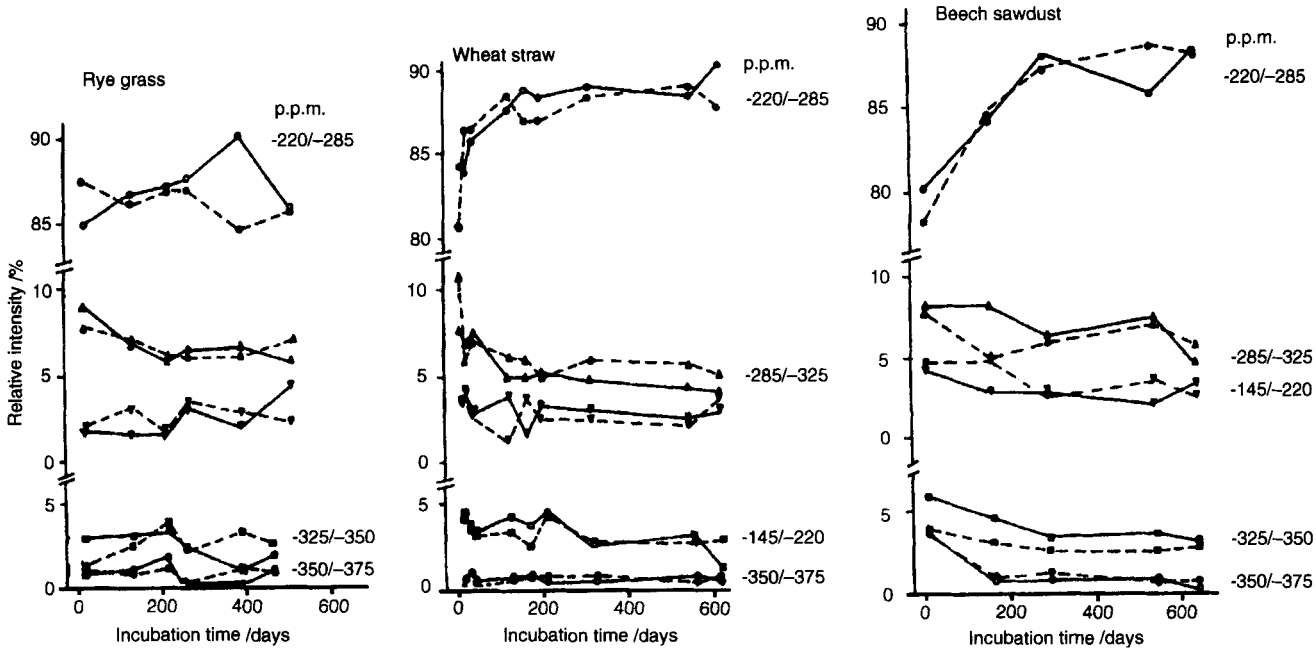

Figure 6 Relative intensities in different ranges of the solid-state ^{15}N CPMAS NMR spectra from rye grass, wheat straw and beech sawdust composts as a function of the composting time.

in aromatic heterocyclic compounds, Schiff-bases, imines, or nitriles with resonance lines in the range from –25 to –145 p.p.m. detectable. However, often it is argued that pyridinic-N is not visible in CPMAS NMR, because there are no protons in the immediate neighbourhood. Recently, however, signals of pyridinic-N were detected in solid-state ^{15}N CPMAS NMR spectra of artificial melanoidins (Knicker *et al.*, 1996; Benzing-Purdie *et al.*, 1983). The lack of such signals in the solid-state ^{15}N CPMAS NMR spectra presented here strongly indicate that such compounds are not formed in considerable amounts during incubation. Weak signals from indoles, nucleotides, substituted pyrroles and imidazoles probably occur in the broad unresolved foot on the low field side of the main amide – peptide peak between –145 and –250 p.p.m. (N_1 and N_3 of several purine and pyrimidine bases). Integration of the region between –145 to –220 p.p.m., however, suggests (Figure 5 and Table 4) that these structures contribute at most 5–8% to the total N in the incubates.

Certainly, there should be sufficient opportunities during transformation and ripening of the samples for abiotic incorporation of ammonia with suitable precursors to form heterocyclic and N-compounds with chemical shifts in the regions from –25 to –145 p.p.m. Theoretically, such abiotic reactions could occur during later stages of incubation of the N-rich rye grass or the lignins. Reactions of ammonia with aromatic ring degradation products from lignin were reported by Musso (1963) which form, by consequent reactions, pyridine and phenoxazone derivatives.

The formation of heterocyclic N, however, does not seem to be a main reaction for stabilization of N in incubates or soils (Knicker *et al.*, 1993). Not yet excluded are nucleophilic-addition of ammonia or peptides to quinone structures, since N in such structures should also have signals in the region from –300 to –325 p.p.m. and below. However, during incubation such a reaction would be indicated by an accumulation of the end products of such an 1,4-addition and therefore, appear as an increase in signal intensity in the region of their chemical shift. We found no substantial increase of signal intensity in this region during incubation for 2 years.

The main N-portion in soil seems to consist of stabilized polypeptides or other N-containing biological materials. Their stabilization may occur through hydrogen linkages or sorptive interactions with humic compounds, as suggested by Haworth (1971). Also, stabilization in soil, occurs by physicochemical reactions of humic compounds with the inorganic soil matrix, mainly with clays. The observation that up to 75% of the nitrogen from humic and fulvic acids can be removed by hot water extraction by passage over cation-exchange resins (Khan & Sowden, 1972) or become substantially mineralized by repeated drying and rewetting of soil (Cabrera, 1993) also suggests that a major portion of the nitrogen compounds in humic substances are not attached by covalent linkages.

Obviously, there always is a significant portion of non-cellular proteinaceous material present in soil which is apparent by the great variety of active cell free enzymes. It is of a remarkable stability, a fact generally believed to be result from their association with clay and humus in soil. Boyd & Mortland (1990) suggested that this occurs through non-polar interactions between the hydrophobic portions of the enzyme and the hydrophobic surfaces of the clay-organic complexes. Enzymes

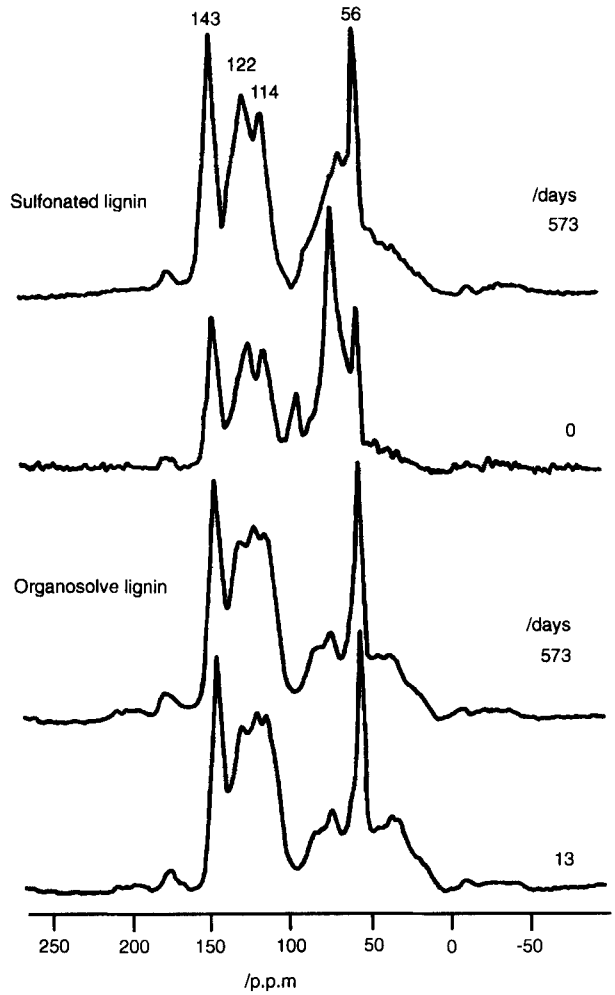

Figure 7 Solid-state ^{13}C CPMAS NMR-spectra from sulphonated lignin and organosolve lignin incubates at different incubation times and 60% WHC.

absorbed in such complexes even resist hydrolysis by microbial proteases and mineral acids (Skujins, 1976).

Besides physicochemical stabilization the selective enrichment of amide functional groups can also be caused by refractory biological material. Evidence of refractory amide structures in biological materials has been reported for fungal melanins (Knicker *et al.*, 1995).

The most straightforward explanation of these results is that the major pathway of nitrogen metabolism in these incubates involves the uptake of ^{15}N-labelled ammonia and a synthesis of bacterial and fungal proteins and biological nitrogen-containing structures, some of which may resist degradation. Upon ageing of the incubates and particularly in soil this nitrogen containing materials may become more stabilized, possibly through reactions with soil organic matter and clays or through selective enrichment of sterically or physically protected refractory biological macromolecules, but they do not undergo essential new transformations, either abiotic or biotic.

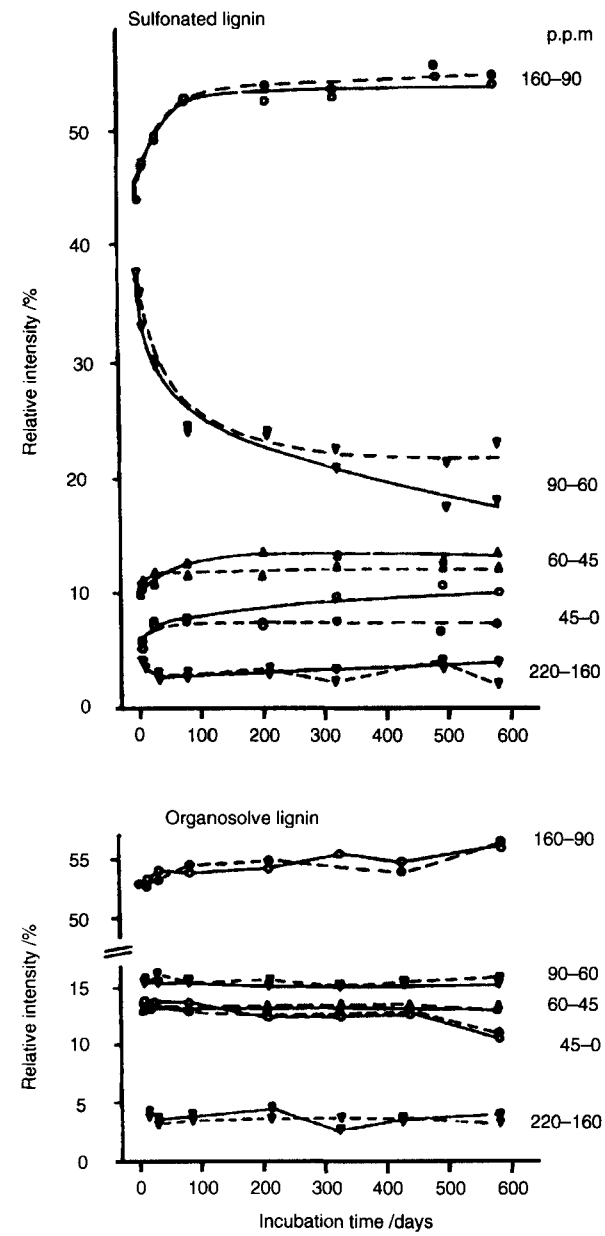

Figure 8 Relative intensities in different ranges of the solid-state ^{13}C CPMAS NMR spectra from sulphonated lignin and organosolve lignin incubates as a function of the incubation time.

Addendum

Whilst our paper was being reviewed, Clinton *et al.* (1995) reported the immobilization of ^{15}N ammonia and ^{15}N nitrate in a forest soil and found almost the entire ^{15}N signal intensity after 135 days of incubation was attributable to ^{15}N in peptides, nucleic acids, and aliphatic amino groups. The results of these ^{15}N NMR studies were consistent with the view that biological processes dominate over direct chemical processes for incorporation of N in litter, degrading organic material, and soil. On the other hand Schulten *et al.* (1995) found by Curie point

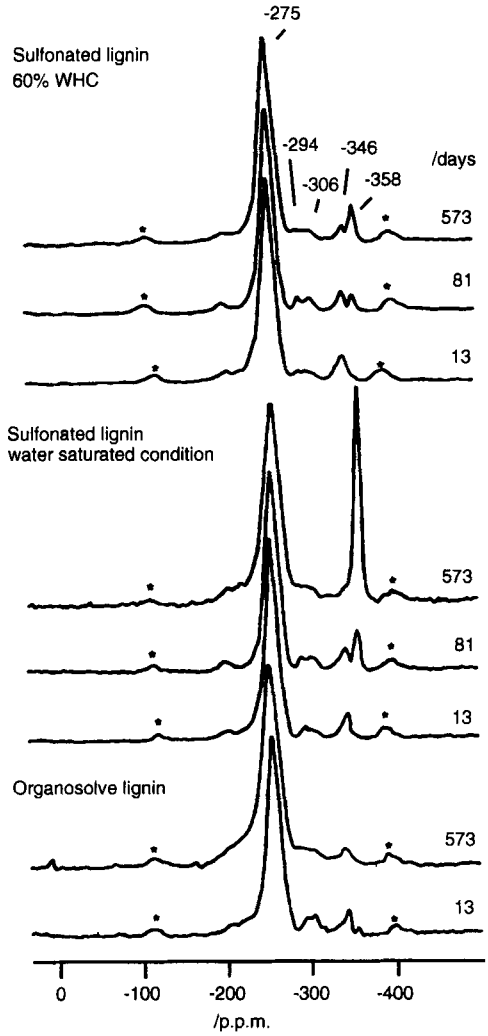

Figure 9 Solid-state ^{15}N CPMAS NMR spectra from incubates of sulphonated and organosolve lignins incubated with ^{15}N-ammonium sulphate at 60% WHC.

pyrolysis a wide variety of nitrogen-containing heterocycles including pyridines, pyrazines, imidazoles, pyrroles as well as alkylnitriles. These latter results are at variance with all ^{15}N NMR spectroscopic studies reported by us and previous authors.

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