



Nature of organic nitrogen in fine particle size separates of sandy soils of highly industrialized areas as revealed by NMR spectroscopy

Heike Knicker*, Michael W.I. Schmidt¹, Ingrid Kögel-Knabner

Department of Soil Science, Technische Universität München, 85350 Freising-Weihenstephan, Germany

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Abstract

The structure of nitrogen-containing compounds of the fine particle size fractions ($< 20 \mu\text{m}$) of two Podzols obtained from the highly industrialized Ruhr area in Germany, were examined by means of solid-state ^{15}N nuclear magnetic resonance (NMR) spectroscopy. In order to improve the signal-to-noise ratio of the spectra, the samples were treated with hydrofluoric acid (HF), prior to NMR analysis. Comparing the solid-state ^{15}N NMR spectra of plant incubates obtained before and after HF treatment revealed no major alteration of the nitrogen fraction induced by HF. From 60 to 90% of the nitrogen detectable in the solid-state ^{15}N NMR spectra of the soil particle size fractions were assigned to amides. A smaller signal derives from free amino groups, leading to the conclusion that most of the nitrogen was derived from peptide-like structures. The calculated high contribution of peptides to the total organic carbon and nitrogen of the samples confirms earlier studies demonstrating that peptide-like material plays a more important role in refractory soil organic matter formation than commonly thought. Major contributions of N-containing heterocyclic aromatic compounds, formed by recondensation reactions or deriving from the input of coal and soot particles from coal processing industries, were not identified. Obviously, in these fractions, contamination did not significantly alter the chemical composition of the organic nitrogen. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The importance of refractory organic nitrogen in the overall nitrogen cycle is well recognized, but little is known about its chemical composition or the mechanisms responsible for its resistance. Several models explaining the formation of refractory soil organic matter have been proposed (Schnitzer, 1985; Kelly and Stevenson, 1996). Some of these postulate that oligomers or monomers derived from degraded biomacromolecules recombine as heterocyclic compounds,

eventually forming the brown colored macromolecular network of refractory soil organic matter (Flaig et al., 1975; Kelly and Stevenson, 1996). Some of the proposed heterocyclic products were identified by means of chromatographic and pyrolytic techniques (Schnitzer and Spiteller, 1986; Schulten et al., 1995; 1997). On the other hand, recent ^{15}N NMR spectroscopic investigations indicated that in soils, the concentrations of such compounds may not be high enough to account for most of the humified organic nitrogen (Knicker et al., 1993; 1995b; 1997a, b). According to these studies, soil organic nitrogen occurs mainly in amide functional groups, most probably of biogenic origin. This result is in strong contrast to the above mentioned recondensation pathways and indicate that commonly thought labile biomolecules can survive microbial degradation.

* Corresponding author. Tel.: +49-8161-71-4423; fax: +49-8161-71-4466.

E-mail address: knicker@weihenstephan.de (H. Knicker).

¹ Present address: Max-Planck-Institut für Biogeochemie, P.O. 100164, 07745 Jena, Germany.

Table 1
Mass distribution and distribution of C and N in bulk soils and their fine particle size fractions

Horizon	Particle size	Mass% of bulk	C_{org}		N		C-to-N
			g kg^{-1}	% of bulk	g kg^{-1}	% of bulk	
<i>Podzol Flaesheim</i>							
Aeh (0–12 cm)	bulk	100	14.5	100	0.4	100	36
	medium silt	2.2	102.5	16.1	2.7	15.3	38
	fine silt	2.0	263.8	37.6	5.7	29.4	46
	clay	1.5	393.7	42.0	10.9	42.2	36
	total ^a	5.7	760.0	95.7	19.3	86.9	39 ^b
Bh (31 cm)	bulk	100	34.5	100	1.0	100	35
	medium silt	3.7	196.3	22.4	5.5	21.6	36
	fine silt	3.7	285.1	32.5	8.4	33.0	34
	clay	3.8	272.7	31.9	14.7	42.0	26
	total ^a	11.2	754.1	86.8	28.6	96.6	26 ^b
<i>Podzol Grafenwald</i>							
Aeh (0–8 cm)	bulk	100	102.0	100	3.8	100	27
	medium silt	5.5	294.9	16.6	11.2	16.9	26
	fine silt	2.1	288.4	6.2	12.8	7.4	23
	clay	1.6	311.3	5.1	18.2	8.0	17
	total ^a	9.2	894.6	27.9	42.2	32.3	21 ^b

^a Sum of the medium silt, fine silt and clay fraction.

^b C-to-N ratios of the fine particle size fractions were calculated from the sum of their C_{org} (g kg^{-1}) content divided by the sum of their N contents (g kg^{-1}).

Recently, protection of biomolecules during humification by their interaction with the mineral phase has been discussed (Christensen, 1996). Several reports have shown that clay minerals can protect organic molecules against microbial degradation (Ensminger and Gieseck, 1942) and that minerals are an important site of amino acid binding (Rosenfeld, 1979). It was shown that refractory organic matter in soils is intimately associated with the silt and clay fractions in soils (Christensen, 1992), although some young organic material is present in these fractions. An understanding of the chemical structure and composition of the organic nitrogen in these fractions may give some insights into the mechanisms involved in the stabilization of soil organic matter. Therefore the emphasis of our examination was to reveal some insights into the role of the finer soil particle size fractions of soils in nitrogen stabilization by subjecting the fine particle size fractions (medium silt, fine silt and clay) of two Podzols deriving from the Ruhr area, Germany to solid-state ^{15}N NMR spectroscopy. As the location of the study sites has been highly industrialized since the beginning of the century, accumulation of disperse atmospheric organic particles (coke, coal dust) from coal-processing industries is likely (Schmidt et al., 1996; Schmidt, 1997). Such particles may alter the composition of stabilized organic nitrogen as well as the processes involved in the humification of biogenic compounds. Their influence on the chemical composition and stabilization of soil organic nitrogen in the

fine particle size fractions of the examined soils was evaluated.

2. Materials and methods

2.1. Description of the soils

Soil material was obtained from the Aeh horizon of the Haplic Podzol Grafenwald located near Bottrop on the northern border of the highly industrialized Ruhr area in Germany. The magnetic susceptibility of $81 \times 10^{-8} \text{ m}^3 \text{ kg}^{-1}$ and microscopic studies showed that this horizon contains contamination from nearby coal-processing industries (Schmidt, 1997). A second sample set was obtained from the Aeh and Bh horizons of the Haplic Podzol from Flaesheim (Germany), situated approximately 30 km north-west of the first site. Their magnetic susceptibility was lower ($1 \times 10^{-8} \text{ m}^3 \text{ kg}^{-1}$) compared to Podzol Grafenwald from Bottrop, indicating that industrial emissions did not accumulate to a greater extent in this soil. However, due to its location and magnetic susceptibility measurements of the O horizons, contamination from industrial emissions cannot be completely excluded. Both soils have been under forest since the beginning of this century. The vegetation of the Podzol Grafenwald is mixed deciduous and spruce forest, whereas the Podzol Flaesheim is under a spruce stand. Soil description, sampling and classification were per-

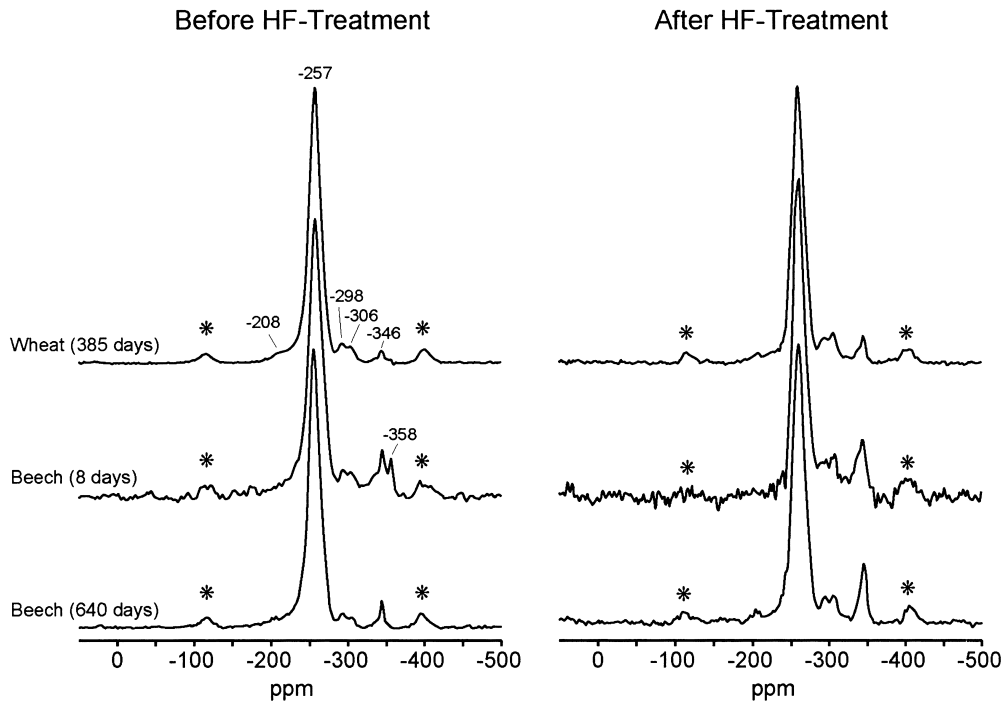


Fig. 1. Solid-state ^{15}N NMR spectra of ^{15}N -enriched plant incubates before and after treatment with 10% hydrofluoric acid (HF). Asterisks indicate spinning sidebands.

formed according to established procedures (FAO, 1990). A more detailed description of the soils is given by Schmidt (1997).

2.2. Sample preparation

Soil samples were sieved (<2 mm) and subjected to particle size fractionation. Fractionation was performed by a combination of wet sieving and sedimentation after dispersion with ultrasonic treatment according to Schmidt et al. (1997). For further analysis, separates of medium silt (6.3 to 20 μm), fine silt (2 to 6.3 μm) and clay fractions (<2 μm) were isolated and their amounts of total organic carbon and total nitrogen were determined in duplicate with a Leco CNS 2000 and an Elementar Vario EL (Table 1). The minimum detection quantities were $0.1 \pm 0.3 \text{ g kg}^{-1}$ for C and N.

2.3. HCl hydrolysis

For determination the amount of hydrolyzable amino acids and the content of the nonhydrolyzable nitrogen fraction, the silt and clay separates of the Flaesheim Podzol were hydrolyzed with 6 M HCl as described in detail by Kögel-Knabner (1995). The amino acid content was determined colorimetrically using the ninhydrin procedure.

2.4. Extraction with HF

To improve the signal-to-noise ratio of the solid-state ^{15}N NMR spectra of soils at natural ^{15}N abundance all samples were treated with 10% HF solution, prior to ^{15}N NMR spectroscopic analysis. Then 5 g of sample material was mixed with 40 ml of 10% (v/v) HF and shaken in a closed polyethylene beaker. After allowing the suspension to settle for 12 h, the supernatant was removed and the HF treatment was repeated five times. The residues were washed with distilled water and freeze-dried. Possible alterations of the nitrogen fraction that may have been induced by the HF treatment were elucidated by comparison the solid-state ^{15}N NMR spectra of microbiologically degraded plant material before and after HF extraction. This material comprises ^{15}N -enriched degraded wheat material incubated under water saturation conditions for 385 d (Knicker and Lüdemann, 1995) and beech sawdust samples incubated after addition of ^{15}N enriched ammonium sulfate, at a sample moisture of 60% of the maximal water holding capacity for 8 and 639 d, respectively. A more detailed description of the samples and the incubation procedure were given by Knicker (1993); Knicker and Lüdemann (1995); Knicker et al. (1997b).

2.5. Solid-state ^{15}N NMR analysis

The solid-state ^{15}N NMR spectra were obtained on

Table 2
Relative intensity distribution of the solid-state ^{15}N NMR spectra of the incubated plant material before and after treatment with 10% HF

Sample	Incubation time (d)	25– –25 ppm	–25– –90 ppm	–145– –220 ppm	–220– –285 ppm ^a	–285– –325 ppm	–325– –350 ppm	–350– –375 ppm
Wheat	385	0	0	5	87	6	2	0
Wheat HF	385	1	0	3	83	9	4	0
Difference ^b		+1	0	–2	–4	+3	+2	0
Beech	8	1	1	7	73	9	6	4
Beech HF	8	1	1	2	73	12	9	1
Difference ^b		0	0	–5	0	+3	+3	–2
Beech	639	0	1	6	85	4	3	0
Beech HF	639	0	0	4	80	8	8	1
Difference ^b		0	–1	–2	–5	+4	+5	+1

^a Including the intensity of the spinning sides bands of the main peak at –257 ppm (–90 to –145 ppm and –375 to –430 ppm).

^b The difference was calculated by subtracting the intensity of the particular region of the spectra of the HF-treated samples from those of the untreated incubates.

a Bruker MSL 300 spectrometer at a frequency of 30.4 MHz, applying the cross polarization magic angle spinning technique (CPMAS) (Schaefer and Stejskal, 1976). A 90° ^1H pulse width of 5.5 μs and a contact time of 0.7 ms were used. During the NMR experiment the sample was spinning at the magic angle with 4.5 kHz. ^{15}N chemical shifts are reported relative to the nitromethane (=0 ppm) scale. Using this scale the chemical shift of liquid ammonia is reported at –381.9 ppm (Martin et al., 1981; Table 1). The spectra were obtained after accumulation of 500×10^{-3} to 770×10^{-3} scans. A more detailed description of the experimental conditions were given by Knicker and Lüdemann (1995).

3. Results

3.1. Chemical alterations induced by hydrofluoric acid

Recent ^{13}C NMR spectroscopic examination of composts and bulk soils (Skjemstad et al., 1994; Schmidt et al., 1997) revealed no major alterations of the organic carbon distribution due to HF treatment. From these studies, however, it cannot necessarily be assumed that this is also true for the behavior of the N-containing fraction. In order to estimate possible HF-related alterations in the chemical composition of the nitrogen fraction of the sample, the solid-state ^{15}N NMR spectra of degraded ^{15}N -enriched plant materials, obtained before and after treatment with 10% HF were compared (Fig. 1; Table 2).

After HF treatment, only slight changes in the features and the relative intensity distribution can be observed in the solid-state ^{15}N NMR spectra of the degraded plant material. The most obvious change is the disappearance of the ammonium signal at –358 ppm in the ^{15}N NMR spectrum of the beech after 8 d of incubation. Ammonium was clearly removed by the aqueous HF solution. Integration of the solid-state ^{15}N NMR spectra reveals a slight decrease of the relative intensity in the chemical shift regions between –145 to –220 ppm and –220 to –285 ppm in the spectra obtained after HF treatment (Table 2). The first chemical shift region is most tentatively assigned to the ring N in histidine or pyrrolic structures. The decrease in the chemical shift region between –220 to –285 ppm may result from hydrolysis of very labile amide structures. A relative increase in signal intensity can be observed in the chemical shift region assigned mainly to free NH_2 groups of basic amino acids. After HF treatment, an increase in relative signal intensity is also observed in the chemical shift region assigned to terminal amino groups of amino acids and sugars (–325 to –350 ppm). However, the alteration of the relative intensity in each chemical shift region ranges

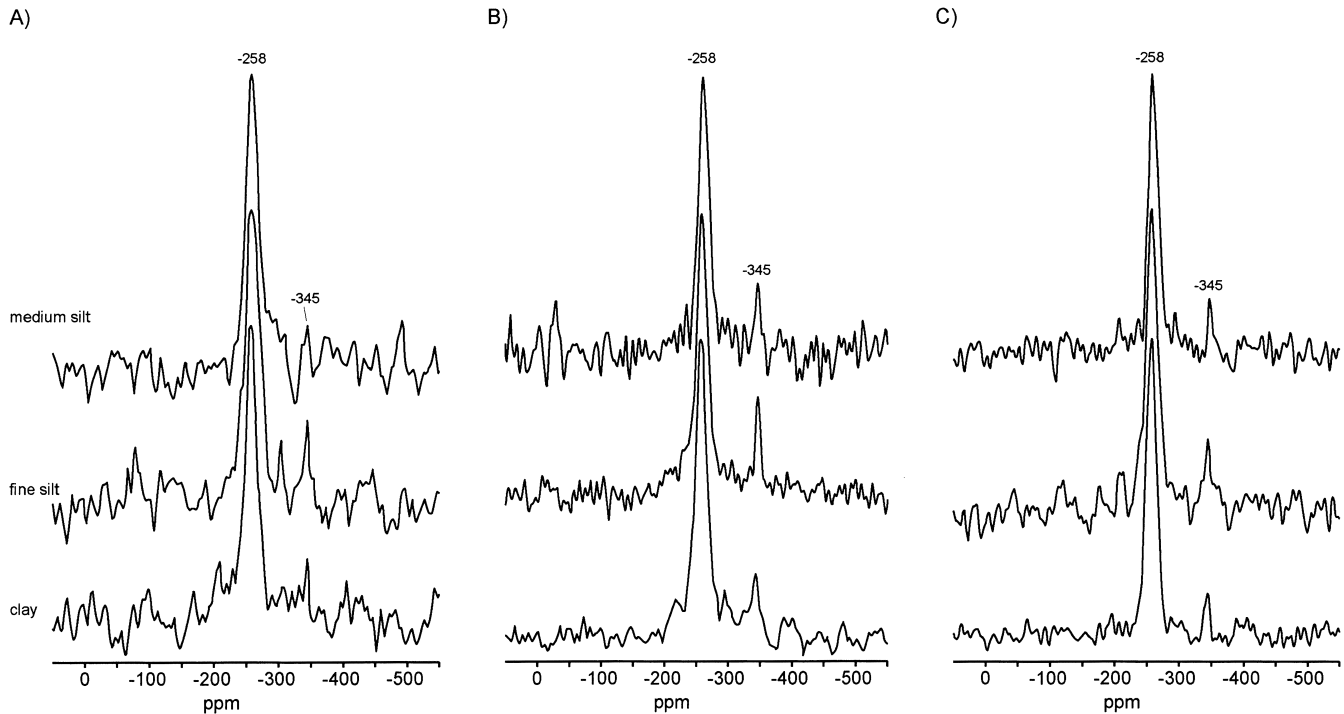


Fig. 2. Solid-state ^{15}N NMR spectra of medium silt, fine silt and clay fraction of Haplic Podzols from the Ruhr area, Germany. (A) Aeh horizon and (B) Bh horizon of Podzol located close to Flaesheim (Germany). (C) Aeh horizon of Podzol Grafenwald situated near Bottrop (Germany). Asterisks indicate spinning sidebands.

between 0 to 5% of the total signal intensity of the spectra. From these results it can be concluded that treatment with 10% HF does not lead to major changes of the chemical composition of organic nitrogen of soils. Thus, applying this treatment to soil samples, low in organic matter and therefore low in organic nitrogen allows their solid-state ^{15}N NMR spectroscopic analysis that would be impossible otherwise.

3.2. The Podzol Flaesheim

The Aeh horizon of the Podzol Flaesheim has a carbon content of 1.5% (w/w) and a nitrogen content of 0.04% (w/w), giving a C-to-N ratio of 36 that is characteristic for podzols (Table 1). Carbon and nitrogen contents increase to 3.4 and 0.1% (w/w), respectively, in the Bh horizon, while the C-to-N (w/w) ratio remains almost constant. This may be explained by accumulation of humified organic material by eluviation processes.

The fine particle size fractions (medium silt, fine silt and clay) account for only 5.7% of the total weight of the bulk sample of the Aeh horizon and 11.2% of that of the Bh horizon. In contrast 95.7 and 86.8% (w/w) of the total organic carbon of the Aeh and Bh horizon are associated with these fine fractions. Their nitrogen contents comprise 86.9 and 96.6% (w/w) of the total soil nitrogen, respectively. Comparable to previous stu-

dies, the relative enrichment in organic matter, however, is most pronounced in the clay fractions of these soil (Christensen, 1992 and references therein). These observations underline the prominent role of fine particle size fractions in controlling the dynamics of soil organic matter.

C-to-N ratios of the fine fractions show only slight alterations compared to those of the bulk soils, except for that of the fine silt fraction of the Aeh horizon and the clay fraction of the Bh horizon. Comparing the medium silt fractions and the clay fractions, a small decrease in the C-to-N ratio (w/w) from 38 to 36 for the Aeh horizon and from 36 to 27 for the Bh horizon can be observed. This follows the trend observed in other studies suggesting that organic matter decomposition increases with decreasing particle size (Baldock et al., 1992).

The solid-state ^{15}N NMR spectra of the fine silt, medium silt and clay fraction of the Aeh horizon of this Podzol, treated with HF, are dominated by peaks at -257 ppm indicating that most of the nitrogen is present in amide functional groups (Fig. 2). Comparable results were recently reported for the clay fractions of the Ah horizon of a Haplic Alisol and the Ap horizon of a Haplic Luvisol (Kögel-Knabner et al., 1996). Both soils were not influenced by deposition of atmospheric particles from coal processing industries. The dominance of the amide signal is also found in the solid-state ^{15}N NMR spectra of fine particle size

fractions of the Bh horizon. Additionally, signals between -325 and -350 ppm indicate the presence of primary aliphatic amines in amino acids and amino sugars. The latter signal is most pronounced in the silt fractions of both the Aeh and the Bh horizon. The resemblance of the spectra in Fig. 2 with those of the clay fraction of the Haplic Alisol and the Haplic Luvisol, mentioned above, points to the assumption that the dominance of amide structure is a common feature of fine particle size separates from soils.

An often discussed stabilization mechanisms in which clay minerals could have an effect on organic matter stabilization in soils represents the formation of melanoidins (Ikan et al., 1996). Here, organic nitrogen in soils is immobilized by condensation of carbonyl groups, i.e. carbohydrates, with ammonia or amino groups of amino acids or sugars via the Maillard reactions. These reactions comprise two major steps including the formation of Schiff bases and the Amadori rearrangement resulting in brown colored polymers, known as melanoidins or Maillard products (Ikan et al., 1996). As, Schiff bases show low reactivity at room temperature (Hedges, 1988), the formation of melanoidins may be in strong competition with microbial activity. On the other hand, it was suggested that the adsorption of the precursors onto clay minerals may create more favorable conditions for the Amadori rearrangement (Hedges, 1988). Consequently, if Maillard reactions are responsible for the stabilization of nitrogen in soils, their products are most likely enriched in the fine particle size fractions and should be detectable by means of solid-state ^{15}N NMR. Laboratory studies examining synthetic condensation products of sugars and amino acids revealed that some of their nitrogen is bound in pyridinic and pyrrolic compounds (Benzing-Purdie et al., 1983). In a ^{15}N NMR spectrum, signals of pyridinic-N are expected to occur in the chemical shift region between -25 to -145 ppm) where also nitrogen in imines or oximes can contribute to the signal intensity. In the spectra in Fig. 2, resonance lines, supporting the presence of higher amounts of such compounds, cannot be distinguished from the noise. Pyrrolic-N is expected to occur in the chemical shift region between -145 to -245 ppm. This region, however, is slightly overlapped by the broad amide signals (-220 to -285 ppm). A crude estimation reveals that such compounds do not exceed 15% of the total N intensity of the solid-state ^{15}N NMR spectra, shown here. The lack of considerable signal intensity of heterocyclic aromatic nitrogen or of aniline derivatives (-285 to -330 ppm) also indicates that condensation reactions between lignin degradation products or phenols and amino compounds (Flaig et al., 1975; Kelly and Stevenson, 1996) seems not to be a major pathway of nitrogen stabilization in the examined soils.

Table 3

α -Amino N content of the bulk samples of the Haplic Podzol Flaesheim (N_t) and their particle size fraction (N_{ps})

Horizon	Particle size	α -amino N (g kg ⁻¹ soil)	α -amino N/ N_{ps} (N_t) (%)
Aeh	bulk	0.4	52
	medium silt	2.7	31
	fine silt	5.7	34
	clay	10.9	43
Bh	bulk	1.0	21
	medium silt	5.5	23
	fine silt	8.4	20
	clay	10.4	21

The feature of the solid-state ^{15}N NMR spectra of the fine particle size separates show a similar pattern to those recently obtained from whole soils and from protein-rich biogenic material (Knicker et al., 1993; Knicker and Lüdemann, 1995). This observation strongly points to the assumption that organic nitrogen associated with the fine particle size fractions of soils has a comparable chemical composition than that in bulk soils and comprises predominantly proteinaceous structures. Such proteinaceous material may derive from nitrogen immobilized in labile, newly synthesized microbial metabolites, the residues of which were shown to also accumulate in the fine separates (Baladane, 1996). Such nitrogen, immobilized in microbial proteins is commonly thought to be easily hydrolyzed with 6 M HCl, a method generally believed to degrade most, if not all, proteinaceous compounds in soils and sediments (Anderson et al., 1989). After hydrolysis with 6 M HCl, less than 43% of the total soil organic nitrogen in the fine particle size fractions of the Aeh horizon and less than 23% of those obtained from the Bh horizon were hydrolyzed and identified as α -amino nitrogen (Table 3). Therefore, labile peptides can explain only a third to a half of the amide signal intensity in the solid-state ^{15}N NMR spectra of the fine particle size fractions. The remaining nitrogen seems to be related to refractory nitrogen, resistant to drastic chemical degradation. Such nitrogen was also detected in hydrolysis residues of plant residues, and sediments (Knicker and Lüdemann, 1995; Knicker and Hatcher, 1997) and were shown to occur mostly in amides. Thermochemolysis with tetramethylammonium hydroxide of the residues after HCl hydrolysis demonstrated that these chemically resistant amides are bound in peptide-like structures.

3.3. The Podzol Grafenwald

Particles from long-term disperse atmospheric input of coke and charred coal were shown to accumulate in

the Aeh horizon of the Podzol Grafenwald (Schmidt, 1997). These particles may explain its high carbon content of 10% (w/w) and high magnetic susceptibility of $81 \times 10^{-8} \text{ m}^3 \text{ kg}^{-1}$ (Table 1). The origin of the high amount of nitrogen of 0.4% (w/w) may also be associated with the atmospheric input of organic particles.

The fine particle size fractions of the Podzol Grafenwald comprise 14.1% of the total weight of the sample. Their relative contribution to the overall carbon and nitrogen content is 27.9 and 32.3% (w/w), respectively (Table 1). Compared to the Flaesheim Podzol these values are very low. On the other hand, the absolute amounts found for nitrogen and carbon in these samples are comparable to those found for the Flaesheim Podzol. These results suggested that most of the external input is located in the coarse fractions. However, although emissions seem to have accumulated mainly in the coarse fractions of the Aeh horizon, their presence may have affected humification processes, leading to a refractory material associated with the fine particle size fractions. The C-to-N ratio (w/w) of the medium silt fraction is 26 and within the range of the bulk soil. For the clay fraction the C-to-N ratio (w/w) decreases to 17, following the similar trend observed in the Podzol Flaesheim.

In a solid-state ^{15}N NMR spectrum, the presence of nitrogen derived from coal and ash particles, in the fine particle size separates, should be indicated by signals in the chemical shift region of pyridinic-N (–25 to –145 ppm) and of pyrrolic-N (–145 to –245 ppm) (Knicker et al., 1995a). Such signals are not visible in the spectra shown in Fig. 2. These are, similar to the ^{15}N NMR spectra of the Flaesheim Podzol, dominated by the resonance line peaking at –257 ppm and indicating peptides, followed by a pronounced peak in the chemical shift region assigned to terminal amino groups of amino acids and amino sugars (–325 to –350 ppm) and supporting the assumption that most of the signal intensity around –257 ppm originates from amide-like structures.

3.4. Contribution of peptides to the total soil organic matter

The low signal-to-noise ratios obtained for the shown spectra make a quantification via integration of different chemical shift regions of the solid-state ^{15}N NMR spectra difficult. However, considering a possible underestimation of pyridinic-N, pyrrolic-N and carbazolic-N, the relative contribution of amide-N to the total nitrogen of the sample was estimated to range from 65 to 90%.

Calculating the relative contribution of nitrogen of such compounds to the total nitrogen of the bulk soil reveals that nitrogen in peptides associated with the fine particle size fractions comprise 56 to 78% and 63

Table 4

Relative contribution of peptide-N of each particle size fraction to the total N (N_t) of the bulk horizon

	Particle size	Peptide-N of N_t of bulk soil (%) ^a
Podzol Flaesheim: Aeh	medium silt	10–14
	fine silt	19–26
	clay	27–38
	total ^b	56–78
Podzol Flaesheim: Bh	medium silt	14–19
	fine silt	21–30
	clay	27–38
	total ^b	63–87
Podzol Grafenwald: Aeh	medium silt	11–15
	fine silt	5–7
	clay	5–7
	total ^b	21–29

^a Calculated from the relative contribution of peptide-N (N_{amide}) to the total N (N_{ps}) of the sample estimated from the ^{15}N NMR spectra multiplied by the contribution of nitrogen of the single particle size fraction (N_{ps}) to the total nitrogen of the bulk sample (N_t) (Table 1). The lower values were calculated for $N_{\text{amide}} = 65\%$ and the higher for $N_{\text{amide}} = 90\%$.

^b Here the values are calculated for the sum of the medium silt, fine silt and clay fraction. The lower values were calculated for $N_{\text{amide}} = 65\%$ and the higher for $N_{\text{amide}} = 90\%$.

to 87% of the total nitrogen of the bulk Aeh and Bh horizons of the Flaesheim Podzol, respectively. In both horizons, the relative contribution of peptide-N to the total nitrogen increases with decreasing particle size of the samples (Table 4).

In the medium silt and clay fractions of the Podzol Grafenwald, peptide-N contributes 11 to 15% and 5 to 7% to the total nitrogen of the bulk sample, respectively. These values are in accordance with the low relative contribution of organic matter associated with the fine particle size fractions to the bulk composition of this horizon. The influence of peptide-C (C_{peptide}) on the composition of organic matter becomes more evident calculating the relative contribution of peptide carbon to the total carbon content of each fraction. For this calculation the following Eq. (1) was used.

$$(N_{\text{ps}} \times 1.16 \times 4 \times N_{\text{amide}}) / C_{\text{ps}} = C_{\text{peptide}} \quad (1)$$

N_{ps} and C_{ps} represent the nitrogen and carbon content of each particle size sample in g kg^{-1} soil, respectively. N_{amide} is the nitrogen associated with amides in % of N_{ps} (65 to 90%) and is estimated from the ^{15}N NMR spectra. The factor 1.16 accounts for the atomic nitrogen-to-carbon ratio. The factor 4 indicates that in peptide structures every nitrogen is associated with approximately four carbon nuclei. Using this equation (Table 5) a relative contribution of peptide-C of 7 to 12% to the total carbon, present in the fine particle size separates of the Aeh horizon of the Flaesheim

Table 5
Relative contribution of peptide-C (C_{peptide}) to the total carbon of each particle size fraction (C_{ps})

Horizon	Particle size	Peptide-C (C_{peptide}) of C_{ps} of particular particle size fraction (%) ^a
Podzol Flaesheim: Aeh	medium silt	8–11
	fine silt	7–9
	clay	8–12
	total ^b	8–10
Podzol Flaesheim: Bh	medium silt	8–12
	fine silt	9–12
	clay	16–23
	total ^b	10–13
Podzol Grafenwald: Aeh	medium silt	11–16
	fine silt	13–18
	clay	18–24
	total ^b	14–20

^a Calculated according to Eq. (1). The lower values were calculated for $N_{\text{amide}}=65\%$ and the higher for $N_{\text{amide}}=90\%$.

^b Here the values are calculated for the sum of the medium silt, fine silt and clay fraction. The lower values were calculated for $N_{\text{amide}}=65\%$ and the higher for $N_{\text{amide}}=90\%$.

Podzol is determined. No major differences in these values is observed between the individual fractions.

In the samples of the Bh horizon, the relative contribution of peptide-C to the total carbon of the samples increases from 8 to 12% in the medium and fine silt separates to 16 to 23% in the clay separate. While contributions of peptide-C are similar for medium and fine silt fractions in both horizons, contributions for the clay fraction of the Bh horizon are twice as high as the clay fraction of the Aeh horizon. This suggests that in contrast to the Aeh horizon the Bh horizon exhibits a relative accumulation of peptide structures in the clay fractions.

The relative contributions of peptide-C to the total carbon of the medium silt and fine silt fraction of Aeh horizon from the Bottrop Podzol are slightly higher than those from the Flaesheim soil. They account for 11 to 16% and 13 to 18%, respectively. The peptide-C contribution of the clay fraction of the Podzol Grafenwald (18 to 24%) is in the range found for the clay fraction of the Bh horizon from the Flaesheim Podzol.

These estimations indicate relatively high contributions of peptides to the total organic matter in fine particle size fractions, although peptides are commonly thought to be mineralized rapidly. Consequently, their role in formation of refractory soil organic matter may have been more important than commonly thought.

3.5. Effects of paraffinic structures on the chemical composition of fine particle size separates

Several studies have indicated that during humification paraffinic compounds are enriched relative to carbohydrates (Hatcher et al., 1983; Kögel-Knabner and Hatcher, 1989). Solid-state ^{13}C NMR spectroscopic studies demonstrated that such paraffinic com-

pound are a major constituent of the organic material in the clay fraction of soils (Baldock et al., 1990). Previously, it was reported that paraffinic structures may be involved in the stabilization of labile biomolecules by encapsulating those into a hydrophobic network (Knicker and Hatcher, 1997). During particle size fractionation such structures would presumably be recovered in the fine separates. Alternative to protection via adsorption onto the mineral surface, their presence in the fine particle size separates may be an explanation for the high contribution of peptide-like material in those fractions. In order to elucidate this possibility, the contribution of methylenic chain carbon to the bulk organic matter of the particle size separates was estimated from the relative intensity distribution of their published corresponding solid-state ^{13}C NMR spectra (Schmidt, 1997). However, performing such an elucidation, one has to bear in mind that in a solid-state ^{13}C NMR spectrum signals of the aliphatic chain of peptides overlap with those of other methylenic structures in the alkyl-C region from 45 to 0 ppm. The C_2 carbon of almost all amino acids contributes to the signal intensity in the chemical shift region between 60 and 45 ppm (Kalinowski et al., 1984) a region that is generally included in the O-alkyl-C region (110 to 45 ppm). Amides give signals in the chemical shift region between 180 to 160 ppm, that is commonly referred to as carboxyl-C region. Carbons associated with aromatic amino acids result in resonance lines in the chemical shift region between 160 to 110 ppm.

In order to obtain a better understanding of the effects of peptide-C on the features of ^{13}C NMR spectra, their relative contribution to the intensity of the relevant chemical shift region was calculated. For this calculation a relative contribution of amide-N to the total nitrogen of the samples between 65 and 90% was

Table 6

Relative contribution of peptide-C to the relative signal intensity of the single regions of the ^{13}C NMR spectra of Podzol Flaesheim based on previous results by Schmidt (1997)

			Carboxyl-C	Aromatic-C	O-Alkyl-C	Alkyl-C
Aeh	medium silt	^{13}C intensity distribution	6	24	36	34
		% C_{peptide} of total intensity	2–3	ND ^a	2–3	4–6
		% C_{peptide} of single region	33–50	ND	6–8	12–18
	fine silt	^{13}C intensity distribution	8	31	28	33
		% C_{peptide} of total intensity	2	ND	2	4
		% C_{peptide} of single region	33	ND	7	12
	clay	^{13}C intensity distribution	6	18	30	46
		% C_{peptide} of total intensity	2–3	ND	2–3	4–6
		% C_{peptide} of single region	33–50	ND	7–10	9–13
Bh	medium silt	^{13}C intensity distribution	10	29	25	25
		% C_{peptide} of total intensity	2–3	ND	2–3	4–6
		% C_{peptide} of single region	20–33	ND	7–10	16–24
	fine silt	^{13}C intensity distribution	12	40	27	21
		% C_{peptide} of total intensity	2–3	ND	2–3	4–6
		% C_{peptide} of single region	17–25	ND	7–11	19–29
	clay	^{13}C intensity distribution	10	37	31	22
		% C_{peptide} of total intensity	4–6	ND	4–6	8–12
		% C_{peptide} of single region	40–60	ND	13–19	36–55

^a Not determined.

assumed based on the results of their solid-state ^{15}N NMR spectra. It was further considered that each nitrogen nucleus in peptides is associated with one carbon detectable in the chemical shift region of carboxylic groups (220 to 160 ppm), one carbon in the chemical shift region of O-alkyl or N-alkyl groups (110 and 45 ppm) and two carbons observed in that of aliphatic carbons (45 and 0 ppm) of the corresponding ^{13}C NMR spectrum. The relative contribution of peptide-C ($C_{\text{peptide/region}}$) to the intensity of each chemical shift region of the ^{13}C NMR spectra can be calculated applying the following Eq. (2). The aromatic content of some amino acids will not be considered, because it was not possible to estimate their relative contribution to the total nitrogen from ^{15}N NMR spectroscopy.

$$N_{\text{amide}} \times 1.16 \times F \times N/C \text{ (w/w)} = C_{\text{peptide/region}} \quad (2)$$

N_{amide} represents the contribution of amide-N to the total sample nitrogen. The term $1.16 \times N/C$ (w/w) accounts for the atomic nitrogen to carbon ratio of the sample. The factor F accounts for the number of carbons expected in the single chemical shift region derived from peptides. For the chemical shift region assigned to carboxylic-C and O-alkyl-C, F will be taken as 1. For the region assigned to aliphatic carbon F will be 2. In Tables 6 and 7, the results of this calculation are given.

In the fractions of the Aeh horizon of the Flaesheim Podzol (Table 6), peptide-C was estimated to contribute maximal 10% to the signal intensity observed in the O-alkyl region and maximal 13% to that in the alkyl region. Consequently, as expected, most of this signal intensity is attributed to O-alkyl carbon and methylene carbon of paraffinic structures or fatty

Table 7

Relative contribution of peptide-C to the relative signal intensity of the single chemical shift regions of the ^{13}C NMR spectra of the Aeh horizon of Podzol Grafenwald based on previous results by Schmidt (1997)

		Carboxyl-C	Aromatic-C	O-Alkyl-C	Alkyl-C
Medium silt	^{13}C intensity distribution	7	29	38	26
	% C_{peptide} of total intensity	3–4	ND ^a	3–4	6–8
	% C_{peptide} of single region	42–57	ND	8–11	23–31
Fine silt	^{13}C intensity distribution	5	22	41	32
	% C_{peptide} of total intensity	3–5	ND	3–5	6–10
	% C_{peptide} of single region	60–100	ND	7–12	19–32
Clay	^{13}C intensity distribution	7	25	38	30
	% C_{peptide} of total intensity	5–6	ND	5–6	10–12
	% C_{peptide} of single region	71–86	ND	13–16	32–38

^a Not determined.

Table 8

Ratio of ^{13}C NMR signal intensity of the chemical shift region of alkyl-C to carboxyl-C with and without considering contribution of peptide-C

Horizon	Particle size	Alkyl-C/carboxyl-C	Alkyl-C- $C_{\text{pra}}^{\text{a}}$ /carboxyl-C- $C_{\text{prc}}^{\text{b}}$
<i>Podzol Flaesheim</i>			
Aeh	medium silt	5.7	7.5–9.3
	fine silt	4.1	4.8
	clay	7.7	10.5–13.3
Bh	medium silt	2.5	2.4–2.6
	fine silt	1.8	1.7
	clay	2.2	2.3–2.5
<i>Podzol Grafenwald</i>			
Aeh	medium silt	3.7	5–6
	fine silt	6.4	13–22
	clay	4.3	10–8

^a C_{pra} is the contribution of peptide-C to the chemical shift region of aliphatic-C (45 to 0 ppm). The values are given using the contribution of amide-N to the total nitrogen of the ^{15}N NMR spectra of 65 and 90%.

^b C_{prc} is the contribution of peptide-C to the chemical shift region of carboxylic-C (220 to 160 ppm). The values are given using the contribution of peptide-N to the total nitrogen of the ^{15}N NMR spectra of 65 and 90%.

acids, respectively. However, a considerable amount (approximately 50%) of the signal intensity in the carboxyl region was estimated to be assignable to peptide structures.

The calculation for the samples of the Bh horizon reveals a contribution of peptides of approximately a quarter to a third to the relative intensities of the carboxylic regions of the spectra of the medium and fine silt fractions, respectively. For the clay fraction, an increase of contribution to approximately two thirds was calculated. A steady increase of peptide-C contribution with decreasing particle size can also be observed for the O-alkyl and alkyl region. The values are in the range estimated for that of the Aeh horizon. For the aliphatic region of the Bh horizon, calculated contributions of peptide-C (16 to 55%) are twice as high as those calculated for the Aeh horizon.

In the ^{13}C NMR spectra of the Podzol Grafenwald, the peptide contribution to the intensity in the carboxylic region becomes even more prominent (between 42 and 100%). Similar to the O-alkyl region of the particle size fractions of both horizons of the Flaesheim Podzol, the contributions of peptide-C to the O-alkyl region is <20%. For the samples of the Aeh horizon the contribution of peptide-C to the aliphatic region increases with decreasing particle size and accounts for 23 to 31% in the medium silt fraction and 32 to 38% in the clay fraction, respectively. The values for the medium and fine silt fraction of this Aeh horizon are in the range of those obtained for the corresponding fractions of the Bh horizon of the Flaesheim Podzol

The average chain length of the methylenic chains may be estimated by the ratio of signal intensity of the alkyl-C region to that of the carboxyl-C region (Table 8). For the Aeh horizon of the Flaesheim Podzol,

ratios between 4 and 8 were found. Each carboxyl-C not associated with nitrogen accounts for 5 to 14 methylenic carbons not derived from peptides. Smaller ratios of 2 to 3 for the Bh horizon suggest the accumulation of short chain acids, e.g. amino acids. However, here it has to be kept in mind that the higher carboxyl-C content may be caused by the oxidation of lignin compounds or sugars. Alkyl-C-to-carboxyl-C ratios of 4 to 6 were obtained for the fractions of the Podzol Grafenwald. Considering the presence of peptides in the medium silt fraction, each carboxyl-C not associated with peptide structures accounts for 5 to 6 alkyl-C not derived from peptides. Values between 10 to 18 were calculated for the clay fraction of this soil. The highest ratios of 13 to 22 is found for the corresponding fine silt separates. These values strongly support the assumption that paraffinic structures are major contributors to the alkyl-C of these samples. Low ratios of alkyl-C not associated with peptides to carboxyl-C not derived from amides of 2 and 3 for the fraction of the Bh horizon lead to the conclusion that here, short chain acids — possibly from oxidation products of microbial degradation — dominate over longer methylenic carbon chains.

4. Conclusions

The solid-state ^{15}N NMR spectra of all fine particle size fractions of two Podzols deriving from the highly industrialized Ruhr area in Germany revealed that the major part of the organic nitrogen is bound in amides, most probably as part of peptide-like material. A considerable fraction of this material resists acid hydrolysis, and thus, is unlikely to derive from living microbial biomass. A major contribution of N-contain-

ing heterocyclic aromatic compounds was not observed, indicating that here recondensation reactions played a minor — if any — role in soil organic nitrogen stabilization. The lack of heterocyclic aromatic nitrogen in the studied samples, further indicates that contamination from atmospheric coal and ash particles did not significantly alter the chemical composition of the organic nitrogen fraction. From the results obtained it can be assumed that some of the identified amides in the fine particle size separates of soils is present in a form resistant to microbial and chemical degradation. They may be stabilized by chemical interactions of the substrate with the mineral matrix (Marshmann and Marshall, 1981; Samuelsson and Kirchman, 1990; Mayer, 1994a, b), or organic structures complexed by polyvalent cations (e.g. Fe^{3+} or Al^{3+}) (Sollins et al., 1996).

Another explanation for the presence of peptide-like compounds in these samples may be the physical protection within micro- or macroaggregates (Tisdall and Oades, 1982; Muneer and Oades, 1989; Skjemstad et al., 1993; Tisdall et al., 1997) or within the network of refractory biopolymers, proposed to explain the presence of amide-N in the insoluble residues of a 4000 year old algal sapropel (Knicker and Hatcher, 1997). Trapped into such aggregates or encapsulated within hydrophobic recalcitrant biopolymers, peptides could be unavailable to hydrolysis by HCl or soil enzymes.

In the fine particle size fractions of the Podzol Grafenwald, with its high content of paraffinic constituents, encapsulation of peptides into a paraffinic network may be in addition to organo–mineral interactions responsible for their preservation. However, the low contribution of long chain methylenic structures to the organic matter in the fine particle size fractions of the Bh horizon of the Podzol Faesheim makes the encapsulation mechanism unlikely to be responsible for peptide sequestration in these samples. Here protection by interactions of the organic material with the mineral matter may be the predominant form of peptide sequestration. From our results one may conclude that in soils several pathways for the protection of labile organic matter may be possible. Depending on the chemical composition of the organic material input and the physical and chemical properties of the soils, one or the other pathway may be favored.

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