Partitioning of N in growing plants, microbial biomass and soil organic matter after amendment of N-ammonoxidized lignins

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Abstract

Nitrogen (N) availability is a crucial factor for maintaining soil productivity, but application of mineral N-fertilizer raises environmental concerns. Based on earlier humification models, ammonoxidized technical lignins were suggested as potential slow N-release fertilizers. In order to obtain first insights on their efficiency as plant fertilizer, their impact on soil organic matter composition and stability, and its role within the N cycle in soils, pot experiments were performed in which perennial ryegrass (*Lolium perenne* L.) was grown on a typical Andalusian soil (*Luvisol, chromic*) after amendment of N-lignins highly enriched in ¹⁵N (Sarkanda and Indulin ammonoxidized lignins) for 75 days. For comparison, the incubation study was also carried out with soils with and without ¹⁵NO₃ fertilization. Among these experiments, the addition of K¹⁵NO₃ resulted in the greatest aboveground plant production.

However, most of the growth occurred during the first 28 days. Thereafter, a fast decrease of the bioavailable N pool occurred. The application of ammonoxidized lignins altered the pH and electrical conductivity of the soil. At higher concentrations a retardation of seed germination was evidenced. After 75 days, the plant shoots from the pots amended with ^{15}N -Indulin and ^{15}N -Sarkanda accumulated 8% and 20% of the initial ^{15}N present in the amended soils at the beginning of the experiment ($^{15}\text{N}_0$). In the ^{15}N -Indulin pots the N was efficiently sequestered from fast release or leaching and most of $^{15}\text{N}_0$ remained in the soil (64%). In contrast, the ^{15}N -Sarkanda pots showed a lower efficiency in N retention. After 28 days of incubation only 42% of $^{15}\text{N}_0$ was retained in the ^{15}N -Sarkanda amended soil, but more than 17% was recovered within the soil microbial biomass. Until the end of the incubation time, the $^{15}\text{N}_0$ detected in the soil microbial biomass decreased to less than 3%, whereas the amount associated with the soil matrix maintained around 37%. The notable increase of ^{15}N in the above-ground plant production (20%) evidences an efficient use of ^{15}N released from the ^{15}N -Sarkanda for plant production. Solid-state nuclear magnetic resonance (NMR) spectroscopy revealed that the ^{15}N of the added ^{15}N -lignins was quickly transformed into peptide-type N, most tentatively of microbial origin, without major alteration of the lignin backbone. This indicates that in soils the competition for nutrients favors N immobilization into biomass with its subsequent sequestration within recalcitrant biopolymers rather than its stabilization via covalent binding to lignins.

Keywords: N fertilization; plant-available N; recalcitrance of technical lignins; soil amendment, soil organic N stabilization

1. Introduction

Nitrogen (N) represents an important limiting nutrient in agricultural ecosystems. Most of the soil N is bound in organic forms, of which at least half is relatively inert and only gradually available to higher plants through microbial fixation and mineralization. Consequently, the use of N fertilizer (15 Tg yr^{-1} in Western and Central Europe; (Heffer and Prud'homme, 2011)) is necessary to maintain constant levels of crop production. However, the intensive use of synthetic N fertilizers for agricultural crops has an unintended cost to the environment and human health due to surplus and inefficient application. Nitrogen runoff from farms threatens to contaminate surface and groundwater. Ammonia from fertilized cropland has become a major source of air pollution, whereas emissions of nitrous oxide form a potent greenhouse gas. Therefore, it is very important to find a rational fertilization strategy that can ensure a competitive production with minor impact on the environment.

As an alternative to costly conventional chemical N fertilizers the use of N-lignins produced by ammonoxidation of technical lignins was suggested (Neff et al., 2003). The latter are by-products of the pulp and paper industry. Although generated in kilotons p.a. only about 2% of them (1 Mt yr^{-1} lignosulphonates, 0.1 Mt yr^{-1} Kraft lignin) are used commercially (Gosselink et al., 2004). However, since lignin represents an important precursor of soil organic material (SOM), its amendment to soils is expected to be helpful in the battle against desertification and to improve the global C balance towards C sequestration.

The ammonoxidation (also known as ammoxidation) was developed in the 1930s by Franz and Palm (Franz and Palm, 1930) as an industrial process for the production of nitriles using ammonia and oxygen. Based on the assumption that condensation of lignin with ammonia represents an important process during

humification, the technology was later advanced for converting technical lignin into N-rich organic fertilizers (Flaig and Söchtig, 1967). The process was further improved in the 80s by introducing an ambient pressure technology that afforded products that contained lower amounts of N-heteroaromatic compounds in favor of higher portions of N-binding forms more easily mineralizable in soil and lower degree of structural lignin alteration (Fischer and Schiene 2002). Here, the incorporation of N is achieved by the application of gaseous oxygen and aqueous ammonia at slightly elevated temperature which partly breaks down aromatic lignin moieties and introduces N in the form of urea, amides and amines. Although the respective products of this reaction were not yet detected in notable concentrations in natural soils (Schnitzer, 1985; Knicker, 2011), their assumed high resistance against microbial degradation may justify the application of N-lignins in agriculture. However, before they can be used, it has to be assured that their production and application is economically viable and environmentally sustainable. The latter requires that the N is bioavailable and can be used efficiently for plant growth. Further, it has to be ensured that neither the amendment nor the degradation products are harmful to the environment.

First tests of ammonoxidized lignins as slow N-release fertilizers in pot experiments with *Sorghum* confirmed that in comparison to fertilization with ammonium sulphate the application of N-lignins can lower N-leaching rates (Ramírez et al., 1997). Toxic effects on the plants were not reported. Thus, in theory, amendment of N-lignins may represent a feasible solution to reduce water pollution by nitrates from conventional fertilizers or liquid cattle manure with a concomitant increase of the recalcitrant soil organic C pool. However, further studies with *Sorghum* (Cano et al., 2001) indicated lower yields after application of N-lignins than after fertilization with urea during the first growing period. Only, during

the second growing cycle, the N-lignin amended plants performed better than those treated with urea, supposedly because then the nitrogen from the N-lignin became readily available. Still, the mechanisms, involved in this process are not well understood. Comparably scarce is the knowledge about the impact of their degradation products on SOM composition and stability.

In order to fill this gap and to obtain a better understanding of the partitioning of N derived from N-lignins among growing plants, microorganisms and the SOM pool, pot experiments were performed using a typical soil of the Andalusian region (Southern Spain) on which *Lolium perenne* was grown for 75 days under the controlled conditions of a greenhouse. We applied ammonoxidized lignins with high ^{15}N -enrichments (^{15}N -Sarkanda and ^{15}N -Indulin) and followed the partitioning of the released ^{15}N among the biological pools (plants and microorganisms) and SOM as a function of incubation time. In addition, soil without amendment and soil with K^{15}NO_3 as fertilizer were tested for comparative purposes.

Compared to many other tracer studies, considerably high ^{15}N enrichments of up to 2.9 atm% for the ^{15}N -lignins and 98 atm% for the K^{15}NO_3 were used. With this we ensured that the insensitive solid-state ^{15}N nuclear magnetic resonance (NMR) spectroscopy can be applied as a tool for characterizing the chemistry involved in N immobilization and mobilization pathways. Analysis of the organic C fraction by solid-state ^{13}C NMR spectroscopy gave information about the impact of the lignin addition and N-availability on SOM quality.

2. Materials and Methods

2.1 Soil sampling and characteristics

The soil matrix for incubation was obtained from the A horizon of a Terra rossa (*Luvisol, chromic*) (IUSS Working Group WRB, 2007) located below an olive tree plantation, close to Aljarafe in the province of Seville (SW Spain; 37° 21.32' N, 6° 4.07' W). Such soils are typical arable soils of Mediterranean regions and are described in detail by Mudarra Gómez (1988). Prior to the incubation experiments, the soil was dried and sieved (<2 mm) and small branches, fresh mosses and plant remains, as well as roots were removed manually.

2.2 Production of ¹⁵N enriched ammonoxidized lignins

The ammonoxidation of the two technical lignins (Pine kraft lignin Indulin AT™, MeadWestvaco, Charleston, USA; Sarkanda grass soda lignin, Granite, Switzerland) was carried out in a stirred reactor using a 10 % (v/v) aqueous solution of ammonia enriched with the ¹⁵N isotope by a factor of ten in relation to its natural abundance. Reaction conditions were as follows: 0.5 MPa O₂, 100°C, 180 min.

During the ammonoxidation process the C content of ¹⁵N-Indulin decreased from 59.9 to 53.5%, whereas nitrogen contents increased by a factor of four (from 1.0 to 4.2%). Enrichment to 2.9 atm% was achieved for ¹⁵N. Similar values were obtained for ¹⁵N-Sarkanda lignin changing the carbon and nitrogen content from 59.6 to 52.7% and from 1.5 to 5.9% respectively. Here, the ¹⁵N enrichment comprised 2.6 atm%. The final C/N ratios (12.7 and 8.9 respectively) are appropriate for being used as a fertilizer (<20) (Meier et al., 1994).

2.3 Incubation experiments

A pre-experiment was carried out to determine i) the appropriate amount of lignin amendment to avoid an overload of nitrogen, and ii) the maximum running time

of the incubation experiment. Therefore pots (250 cm³) were filled with 150 g of soil and the impact of adding 150, 300, 500, 1000, 1500 and 2000 mg of N-Indulin (not enriched with ¹⁵N) on the growth of 0.5 g grass seeds (*Lolium perenne* L.) was tested in triplicate. After adjusting the soil humidity to 55% of the maximal water holding capacity, the pots were placed into a greenhouse under similar conditions to those reported by de la Rosa and Knicker (2011), briefly, 22 °C for 75 days (14 h light day⁻¹) and water supply equivalent to 700 mm yr⁻¹. The bottom of the jars was perforated to allow leaching of surplus water. The leachate was collected but its amount after freeze drying was not sufficient for further analysis. The aboveground plant biomass production (PBP_{above}) was measured after 14, 28, 50, 60 and 75 days of incubation. Therefore, the shoots were cut, dried (24 h at 40 °C) and weighed at each of those intervals.

Based on the results of this pre-experiment and taking into account the C, N and ¹⁵N contents of the soil and ¹⁵N-lignins, the volume of the pots (250 cm³), as well as the detection limit of solid-state ¹⁵N NMR spectroscopy, an addition of 2.0 grams of ¹⁵N-lignin per pot was chosen for the final incubation study. For each experiment, pots for four sampling dates were prepared in triplicate according to the conditions described above. For comparison, an additional series of pots with soil amended with K¹⁵NO₃ (99 atm%) was set up. Here, an initial ¹⁵N concentration of 10 mg 100 g⁻¹ soil was used to ensure that even after the expected fast removal of N by leaching enough ¹⁵N was present to allow solid-state ¹⁵N NMR spectroscopy. Pots without any amendment were prepared and used as controls.

The PBP_{above} was determined after 18, 28, 42, 58 and 75 days of incubation and is given as the sum of each harvest. At each sampling, PBP_{above} collection was performed in the same way than described above. After 28, 58 and 75 days of

incubation pots were removed from the greenhouse and the soil was manually separated from the root residues before being dried (48 h at 40 °C) and homogenized for further analysis.

2.4 Elemental analysis

Total C, total N (TN) and ^{15}N contents of the starting ^{15}N -lignins, soil mixture and the harvested shoots were measured in duplicate using an Elementar Vario EL microanalyzer coupled to a quadrupole mass spectrometer (QMS) (GAM-200, In Process Instruments GmbH, Bremen, Germany). Here the ^{15}N concentration was determined as its contribution to the sum of all N isotopes of the sample (atm%). Using acetanilide at natural ^{15}N abundance ($n = 10$), a standard deviation of 0.007 atm% was calculated. The combustion temperature was 975 °C. The total organic C (TOC) of the soil matrix was calculated from the difference between TC and the C remaining after heating the samples in a muffle oven at 550 °C for 5 h. In order to obtain the portion of the added ^{15}N which was incorporated into $\text{PBP}_{\text{above}}$ at each sampling date ($^{15}\text{N}_{\text{PBP}}$), the amounts of ^{15}N recovered with each grass harvest were determined separately and subsequently summed up.

The nature of the soil and root material did not allow for a clear separation and a nonbiased recovery of the latter. This impeded a correct determination of the proportion of ^{15}N incorporated into the roots. Therefore, we limited our analysis to the uptake of the isotope into aboveground shoots. For the estimation of ^{15}N which may be lost by volatilization or leaching, we assumed a root-to-shoot ratio between 0.1 and 0.2 (Herndl et al., 2011) and a comparable C-to-N-to- ^{15}N ratio for the shoot and the root material.

2.5 Solid state ^{13}C and ^{15}N NMR spectroscopy

The mineral phase and paramagnetic ions in the sampled soil mixtures were removed by a treatment with 10% (w/w) hydrofluoric acid (HF) (Gonçalves et al., 2003) prior to NMR spectroscopy. Briefly, 10 g of air-dried soil was weighed in a polyethylene beaker and shaken with 50 mL of HF solution for 3 h. The supernatant was removed and discarded. This procedure was repeated five times. Subsequently the solid residue, containing the concentrated SOM was washed three times with distilled water to remove residual HF, and finally oven dried at 40°C. The solid-state ^{13}C NMR spectra of the HF-treated soils and the used ^{15}N -lignins were acquired with a Bruker DSX 200 NMR spectrometer, operating at a resonance frequency of 50.32 MHz. The cross polarization magic-angle spinning (CPMAS) was applied using a double bearing probe and zirconium oxide rotors of a diameter of 7 mm which were closed with KELF-caps. The spinning speed was 6.8 kHz. During the contact time of 1 ms a ramped ^1H -pulse was applied to circumvent Hartmann-Hahn mismatches. The pulse delay was 300 ms. Depending on the ^{13}C content of the samples, between 2,000 and 150,000 scans were accumulated. Line broadenings between 20 and 100 Hz were applied. The ^{13}C chemical shifts were calibrated relative to tetramethylsilane (0 ppm) with glycine (COOH at 176.08 ppm). The spectra were subdivided into different chemical shift regions according to Knicker and Lüdemann (1995): alkyl C (0–45 ppm), N-alkyl/methoxyl C (45–60 ppm), O-alkyl C (60–110 ppm), aromatic C (110–140 ppm), phenol C (140–160 ppm), carboxyl/amide C (160–185 ppm) and carbonyl C (185–245 ppm). The relative ^{13}C intensity distribution was determined by integrating the signal intensity in the different chemical shift regions with a modified integration routine supplied with the instrument software. Corrections concerning the intensity distribution of spinning side bands were made as described by Knicker et al.

(2005). The solid-state ^{15}N NMR spectra of the ^{15}N -lignins were obtained with a Bruker DMX 400 and a Varian Inova 600 operating at ^{15}N resonance frequencies of 40.54 MHz and of 60.81 MHz, respectively. The samples were placed into zirconium oxide rotors of 4 mm and 6 mm diameter and rotated with 5 and 8 kHz at the magic angle respectively, applying the cross polarization technique with a contact time of 1 ms and a pulse delay of 150 and 500 ms. Between 50,000 and 200,000 scans were accumulated.

2.6 Soil microbial biomass (SMB)

Quantification of the soil microbial biomass (SMB) was carried out after 28 and 58 days of the incubation applying the chloroform-fumigation-extraction method developed by Jenkinson and Powlson (1976). Briefly, soil samples with a moisture adjusted to 50-60% of their water holding capacity were fumigated with chloroform, then kept in the dark for 24 h at 25°C and subsequently extracted with 0.5 M K_2SO_4 . A control was prepared without chloroform fumigation. Three replicates were measured. Finally, after freeze-drying the samples were analyzed for their C, N and ^{15}N contents. Biomass C and N (BC and BN) were then determined using the following equations described by Brookes et al. (1985).

$$\text{BC} = [\text{C}_\text{F} - \text{C}_\text{C} / k_\text{C}]$$

$$\text{BN} = [\text{N}_\text{F} - \text{N}_\text{C} / k_\text{N}]$$

The amount of ^{15}N incorporated into the N-biomass (B^{15}N) was calculated on the base of the atm% of ^{15}N in the BN with the equation:

$$\text{B}^{15}\text{N} = \text{BN} \times (^{15}\text{N} \text{ (atm\%)}).$$

BC , BN and B^{15}N were obtained from the difference of C, N and ^{15}N in the extracts of the fumigated soil (C_F and N_F) and un-fumigated soil (C_C and N_C). The

required factors (k_C and k_N) are the proportion of biomass C and N (0.45 and 0.54 respectively according to Brookes *et al.*, 1985).

2.7 Statistical analysis

The significance of elemental analysis (TOC, TN and ^{15}N , *cf.* Tables 2 and 3) was tested using a Student's *t* test (Microsoft Excel 2010[®]). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Impact of ^{15}N -Indulin addition on the above-ground plant biomass production

The addition of N-lignin to the soil in the pre-experiment caused a decrease in the pH values (from 8.6 to 8.0) and a rise of the electrical conductivity (EC) from 14.5 to 30.1 mS m^{-1} (Table 1). The extent of those changes increased with N-lignin addition. Additions of 500 mg of N-ammonoxidized lignin resulted in the highest $\text{PBP}_{\text{above}}$ at the early stage of the experiment (132.1 ± 28.3 mg within the first 14 days of the incubation). Increasing the amount to 1,500 mg and higher, reduced the harvest mostly due to retardation of germination. However, a considerable part of the harvest losses caused by the low germination rate in those pots was regained until day 50. In fact, at day 14 and 50, $\text{PBP}_{\text{above}}$ of the pots amended with 1,500 and 2,000 mg N-lignin were twice of those treated with 500 mg. After 75 days of incubation, $\text{PBP}_{\text{above}}$ in all experiments decreased considerably. Possibly, nutrient availability or the size of the pots were insufficient to permit further development of the plants. Based on this observation, for the experiments with the ^{15}N -lignins a maximum duration of 75 days was chosen. Due to the low sensitivity of solid state ^{15}N NMR

spectroscopy, the retarded germination was accepted and an addition of 2,000 mg of ^{15}N -lignins per pot was selected for studying the partitioning of N derived from ^{15}N -lignins in soil and plant compartments after their amendment.

3.2 Impact of ^{15}N amendments and fertilization on typical soil parameters

As indicated in Table 2, at the beginning of the experiment, the addition of both ^{15}N -lignins to the soil resulted in an increase of TOC content from 8.3 ± 1.1 to 15.2 ± 1.9 and $16.0 \pm 1.7 \text{ mg g}^{-1}$ and of TN from 0.9 ± 0.1 to 1.5 ± 0.2 and $1.6 \pm 0.2 \text{ mg g}^{-1}$, for Indulin and Sarkanda respectively. Compared to the natural abundance of ^{15}N in soils (0.37 atm%), the addition of the soil amendment augmented also the ^{15}N contents available in the pots at the beginning of the experiments ($^{15}\text{N}_0$) ($1.288 \pm 0.021 \text{ atm\%}$ for Indulin and $1.376 \pm 0.021 \text{ atm\%}$ for Sarkanda). Comparably, the fertilization of pots with K^{15}NO_3 enhanced considerably the $^{15}\text{N}_0$ concentration to $10.195 \pm 0.174 \text{ atm\%}$ in the soil before starting the incubation.

The evolution of the ^{15}N contributions to the soil fraction varied in the course of the experiment and was depending on the used fertilizer. After 28 days, only 19% of the initial $^{15}\text{N}_0$ remained within the soil matrix in the pots fertilized with K^{15}NO_3 , which contrasted with the 42% and 74% obtained for the soils amended with ^{15}N -Sarkanda and ^{15}N -Indulin, respectively. After that, changes were less severe. In the pots with K^{15}NO_3 the ^{15}N recovered in the soil ($^{15}\text{N}_r$) slightly increased to 21% at day 58 and to 25% at day 75, possibly because some ^{15}N re-entered the SOM pool with the decaying root material. Comparably small changes of the $^{15}\text{N}_r$ were observed for the experiments with ^{15}N -Sarkanda (39% at day 58 and 37% at day 75) and ^{15}N -Indulin (70% after 58 days and 64% at day 75).

Comparable to the pre-experiment, the addition of ammonoxidized lignins caused some variation in the physical properties of the soils. The EC increased notable from 9.6 mS m⁻¹ (control soil) to 29.9 mS m⁻¹ (¹⁵N-Indulin) and 56.0 mS m⁻¹ (¹⁵N-Sarkanda) and the pH was modified from 8.6 to less alkaline values (~8.1). Although the extent of these differences decreased with time, both parameters did not reach the values of the control pots until the end of the experiment (Table 2).

3.3 Vegetation responses to soil amendments

Figures 1a and 1b show the partial and accumulated shoot growth of the grass in the pots with the different soil amendments. During the first month, the pots fertilized with K¹⁵NO₃ yielded the highest PBP_{above}, which was approximately two times higher than that of the control. In the series amended with ¹⁵N-lignins the germination occurred with retardation, as it was already observed in the pre-experiments. This explains why here, in spite of N fertilization, the PBP_{above} overran that of the controls only after 28 days. Grass shoot production (partial and total) for ¹⁵N-Indulin amended soils was greater than for those amended with ¹⁵N-Sarkanda lignin, but barely exceeded the total shoot production of the control samples at the end of the experiment.

3.3.1 ¹⁵N recovery in the grass material

Table 3 lists the TOC, TN and ¹⁵N contents of the recovered grass material. The C/N of the plant material of the control pots varied between 17.3 and 21.7, which is typical for grass plants grown under N-limiting conditions. The addition of N fertilizer in all experiments increased the N content of PBP_{above}, leading to a narrowing of the C/N ratio and indicating that N addition supported the production of protein-rich plant

shoots. Whereas those low values were maintained for the ^{15}N -lignins until the end of the experiment, the plants grown after $^{15}\text{NO}_3$ fertilization showed reduced production of N constituents, most tentatively because the stock of plant-available N was already used up.

After 28 days of incubation, only 2 to 3% of $^{15}\text{N}_0$ was used for shoot production in the ^{15}N -lignin amended pots. This can be explained by a low biomass production rate. Whereas this situation improved only slightly for ^{15}N -Indulin amended pots, a better availability of ^{15}N from the ^{15}N -Sarkanda lignin allowed that 20% of the $^{15}\text{N}_0$ was used for shoot production until the end of the experiment.

The highest ^{15}N incorporation into $\text{PBP}_{\text{above}}$ was achieved with K^{15}NO_3 ($37.648 \pm 1.191 \text{ atm\%}$ of ^{15}N at the end of the experiment), however most of this incorporation (around 80%) occurred during the first 28 days of the experiment, revealing the quick availability of ^{15}N from $^{15}\text{NO}_3$.

The amount of lost ^{15}N was estimated from the sum of $^{15}\text{N}_r$ recovered in the soil (Table 2), the $\text{PBP}_{\text{above}}$ ($^{15}\text{N}_{\text{PBP}}$, table 3), and the ^{15}N content of the roots ($^{15}\text{N}_{\text{root}}$). The latter was calculated considering an approximate root to shoot ratio of *Lolium perenne* of 0.1 to 0.2 (Herndl et al., 2011). This rough estimation indicates that during the experiment, approximately 30 to 50 % of $^{15}\text{N}_0$ were lost in the various pots with mineral fertilizer and about 40 to 56 % in those with ^{15}N -Sarkanda. The ^{15}N -Indulin amended soils showed a much better N retention. Here, only 23% of $^{15}\text{N}_0$ were lost after 28 days and approximately 27% after 75 days of incubation. The control soils without any fertilization, however, showed no detectable N-loss.

3.4 Effects on soil microbial biomass

Figure 2 shows the soil microbial biomass (SMB) determined with the chloroform fumigation extraction method after 28 and 58 days of incubation. After 28 days, the soil amended with ^{15}N -Sarkanda presented the highest content of SMB ($> 1,100 \mu\text{g C g}^{-1}$ dry soil). At this time, the series amended with ^{15}N -Indulin achieved similar SMB contributions than that fertilized with K^{15}NO_3 ($> 200 \mu\text{g C g}^{-1}$ dry soil). The ^{15}N recovered with the SMB varied for the different series. More than 17% were incorporated into SMB in the ^{15}N -Sarkanda amended soils, the respective ^{15}N recovery for ^{15}N -Indulin amended soils and the K^{15}NO_3 series was 6.6% and 2.4%, respectively. After 58 days, the SMB, and consequently the ^{15}N extracted with the SMB, decreased for all experiments. At this time, the ^{15}N recovered with SMB was 0.3%, 2.3% and 2.6% of $^{15}\text{N}_0$ in the soils amended with K^{15}NO_3 , ^{15}N -Indulin, and ^{15}N -Sarkanda.

3.5 Composition of soil organic matter

The fact that comparable solid-state ^{13}C NMR spectra were acquired from the original soil and the soils obtained after 28 days of incubation from the pots without any amendment and with $^{15}\text{NO}_3$ reveals that the latter caused no major alteration of the quality of the organic fraction (Fig 3; Table 4). All spectra are dominated by the signals in the O-alkyl C region (110 to 60 ppm) followed by alkyl C (45 to 0 ppm). The low alkyl C/carboxyl C (220 to 160 ppm) ratio < 2 indicates that most of this alkyl C derives from short chain residues, as they occur in peptides.

The ^{15}N -lignins result in solid-state ^{13}C NMR spectra with a typical lignin pattern giving signals at 147 ppm assignable to O-aryl C, i.e. in guaiacyl units and signals between 140 to 100 ppm derived mainly from unsubstituted aryl C positions. The pronounced signal at 56 ppm in both spectra reveals the presence of methoxyl C,

whereas the remaining signal intensity in the chemical shift region of O-alkyl C (100 to 45 ppm) and alkyl C (45 to 0 ppm) is attributed to lignin side chains. The resonance line at 173 ppm originates from carboxylic C which was formed either during the extraction of the lignin or during the ammonoxidation process.

The typical lignin pattern is still observable in the spectra of the SOM of the pots with the organic amendments (Fig. 3). This is in line with the fact that lignin addition almost doubled the TOC content of the sample (Table 2). Until the end of the experiment, a major decrease of the TOC contents or a relevant change neither in the signal pattern nor in the relative intensity distribution of the ^{13}C NMR spectra was observed. The remaining pattern of the spectra evidences that the lignin backbone was not subject of higher microbial degradation activity.

The ^{15}N NMR spectra of the used ^{15}N -lignins shown in Figure 4 reveal signals in the chemical shift region assignable to pyrrole-type N and nitrile N (–145 to –240 ppm), as well as considerable signal intensity in the chemical shift regions of aminobenzoquinones (–274 ppm), aminohydroquinones (–304 ppm) and aromatic amines (–330 ppm). The presence of similar structures in humic material treated with ^{15}N -labeled ammonium hydroxide has been proposed by Thorn and Mikita (1992) based on ^{15}N NMR spectroscopic studies. The formation of nitriles and N-heteroaromatic compounds from Indulin at elevated temperature ($T \geq 140^\circ\text{C}$) and oxygen pressure ($p\text{O}_2 \geq 1 \text{ MPa}$) has been described by Potthast et al. (1996). The respective spectrum of the untreated soil, on the other hand, shows its main signal at –260 ppm and a small signal around –346 ppm, typically assigned to peptide N and their terminal amino groups (Knicker et al., 1993). Note that here the ^{15}N occurs at natural abundance (0.37 atm%) which implies low sensitivity of those samples for ^{15}N NMR spectroscopy. Whereas the spectra of the ^{15}N -lignins were acquired with about

50,000 scans, the measurement time for the natural soil was about eight times longer.

In the ^{15}N NMR spectra of the soils from the pot with ^{15}N -lignin addition after 28 days of incubation the typical signals of the soil amendment disappear in favor to the dominating signal at -258 ppm and a small resonance line at -348 ppm. Considering that in this sample the ^{15}N derived from the original SOM contributes with less than 50% to the total ^{15}N of the sample at least half of the signal intensity must derive from compounds containing the ^{15}N , originally derived from the ^{15}N -lignins. The lack of signals assignable to the latter together with the appearance of the resonance lines typical for peptides evidences a quick transformation of the ^{15}N -groups of the soil amendments.

4. Discussion

Compared to the pot experiment with amendment of ^{15}N -lignins, the addition of K^{15}NO_3 resulted in a higher production of $\text{PBP}_{\text{above}}$. However, most of the growth occurred during the first 28 days. After that, the growth rate declined drastically, probably due to a quick decrease of the fast bioavailable N pool. This assumption is supported by several observations; i) after 28 days of incubation only 19% of the $^{15}\text{N}_0$ remained within the soil matrix (Table 2), whereas around 29% were already incorporated into $\text{PBP}_{\text{above}}$ (Table 3), ii) the $^{15}\text{N}_{\text{PBP}}$ in the grass collected in the subsequent harvests decreased (58.010, 48.749 and 37.648 atm% at 28, 58 at 75 days respectively; Table 3) and iii) after 28 days of incubation the development of the C and N contents of SMB in the pots with mineral fertilizer was much higher than in the control pots but they decreased severely after 58 days. Approximately 56% of $^{15}\text{N}_0$ was already lost after 28 days due to leaching and possibly also by volatilization.

The application of ammonoxidized lignins to the soil caused an increase in the total N content from 0.9 ± 0.1 (control soil) to 1.5 ± 0.2 and 1.6 ± 0.2 mg g^{-1} in the ^{15}N Indulin and ^{15}N Sarkanda amended soils, respectively (Table 2). This change did not result in higher yields of $\text{PBP}_{\text{above}}$ than in the pots with mineral fertilizer (1.0 ± 0.2 mg N g^{-1} at time 0), and merely after 42 days for ^{15}N -Indulin and 58 days for ^{15}N -Sarkanda the accumulated harvest grass exceeded that of the control pots (Fig. 1). This was mainly due to the retarded seed germination observed for these series. The chosen design of our experiments did not allow determining the reason of this retardation, but it is most tentatively caused by some constituents of the lignin. For instance *p*-hydroxybenzoic acid, cinnamic acid, syringic acid, catechol or vanillic acid are common plant allelochemicals (Capasso, 1997). Phenolic allelochemicals can inhibit plant root elongation, cell division, change cell ultra-structure, and then interfere with the normal growth and development of the whole plant (Cruz-Ortega et al., 1998; Inderjit, 2001). They are also discussed to delay seed-germination (Inderjit et al., 1999). In addition, the change in the physical properties of the soils (decrease of pH and increase of EC) caused by the addition of N-ammonoxidized material may have contributed to this negative effect. Possibly, applying the fertilization sufficiently earlier than seeding may allow a first transformation of the allelochemicals towards lower toxicity, but certainly more research is needed to ascertain this possibility and to obtain a better understanding of the content of such products in N-ammonoxidized lignins and their role in plant-soil interaction.

After 75 days, the plant shoots on the soils treated with ^{15}N -Indulin and ^{15}N -Sarkanda accumulated only 8% and 20% of the $^{15}\text{N}_0$ (Table 3). In the ^{15}N -Indulin pots most of the unused ^{15}N (64%) remained in the soil (Table 2), indicating that in this case ^{15}N was efficiently sequestered from fast release or leaching. The ^{15}N -Sarkanda

pots showed a different behavior. After 28 days of incubation only 42% of the $^{15}\text{N}_0$ were retained in the soil, but over 17% were recovered with the SMB. The latter comprised only 7% for ^{15}N -Indulin. After 58 days of incubation, the ^{15}N in the SMB decreased to 3% for ^{15}N -Sarkanda (Fig. 2b) and the amount remaining in the soil matrix was 39% (Table 2). At the end of the incubation time the ^{15}N in the above-ground plant yields increased ($> 20\%$) suggesting that in this case the added ^{15}N was not efficiently sequestered in the soil matrix and $\text{PBP}_{\text{above}}$ were considerably richer in proteins.

Concerning the nature of the N forms in the ^{15}N -lignin amended soils, the ^{15}N NMR spectra demonstrated some major chemical changes. ^{15}N -signals attributed to heterocyclic N and in particular to aminoquinones and aminohydroquinones abundant in the original ^{15}N -lignins disappeared in the ^{15}N NMR spectra of the HF-treated incubated soils in favor to resonance lines assignable to amide N, typically attributed to peptide-type N. Note that the pattern of the ^{15}N NMR spectra of the incubated soils are comparable to those of ^{15}N NMR spectra of biomass (Knicker, 2011) and also to the spectrum obtained from the soils amended solely with K^{15}NO_3 . This change is best explained by the use of the N, released from the ^{15}N -lignin as N-source for microbial growth. The high ^{15}N -recovery in the microbial biomass of the pot incubated for 28 days after amended with ^{15}N -Sarkanda supports this finding.

The fact that only minor changes of the solid-state ^{13}C NMR spectra were identified points to the conclusion that the release of ^{15}N groups from the ^{15}N -lignin occurred without major alteration of its aromatic backbone. This observation is questioning the high biochemical recalcitrance assumed for heterocyclic N compounds (Knicker and Skjemstad, 2000) or aromatic amines. However, they are in line with a recent study demonstrating the high availability of N incorporated into the

heteroaromatic network of grass biochar (de la Rosa and Knicker, 2011). The quick transformation of the ^{15}N groups associated with ^{15}N -lignins and other heteroaromatic structures indicates that comparable compounds suggested to be formed during humification via recondensation and auto polymerization (Schnitzer, 1985; Dail et al., 2001) may not be stable enough to be responsible for long term N sequestration in soils.

On the other hand, the quick incorporation of ^{15}N derived from the ^{15}N -lignins into newly formed biomass most likely prevented bigger losses by leaching or volatilization. The efficient immobilization of this ^{15}N into microbial biomass implies that some of it is incorporated into biochemically recalcitrant biopolymers which resist further degradation even after death of the microorganisms. Thus, although the added N does not persist within the amended N-lignin its subsequent immobilization within the microbial biomass and later within the SOM represents an efficient N-sequestration pathway.

5. Conclusions

Examining the partitioning of N derived from ammonoxidized lignins added to soils on which grass was grown, we observed that only a small part of its N was used for plant production. Most of it remained within the soil matrix. However, this N sequestration seems to be mainly caused by a transfer of N from N-lignin to microbial biomass. The efficiency of this transfer was different for ^{15}N -Sarkanda and ^{15}N -Indulin, demonstrating that it clearly depends upon the used material. The application of higher amounts of ammonoxidized lignins resulted in retardation of seed germination, possibly due to allelopathy or high salt concentrations. This observation

certainly needs further considerations and asks for more research before N-enriched technical lignins can be used in agriculture.

Aside from these practical aspects, our study provides some insights with respect to understanding of processes involved in N sequestration in soils. The idea of using ammonoxidized lignins as slow N-release fertilizer is mainly based on the assumption that comparable compounds are essential intermediates in the formation of humic substances and the stabilization of soil organic N. However, according to our results, in natural soils organic N forms immobilized by condensation of ammonia with lignin compounds have lower biochemical stability than commonly thought. Possibly due to the high competition for essential nutrients, their N seems to be quickly liberated from the lignin backbone by microbial activity allowing its subsequent use for the build-up of new plant and microbial biomass. Thus, even if N-lignins are formed during humification, our findings indicate that their resistance towards biochemical transformation will be too low to be responsible for long-term organic N sequestration in soils. On the other hand, with its immobilization into microbial biomass, some N will be incorporated into biochemically more recalcitrant structures, which after the death of the microbes and entering the SOM pool can contribute to an efficient sequestration of soil organic N.

Acknowledgements:

The authors would like to thank the DFG (Germany) (KN 463/9-1), the FWF (Austria) (I 154-N19), and the MICINN (Spain) (CGL2009-10557) for financial support. J.M. De la Rosa thanks the MINECO (Spain) for funding his JAE-DOC contract. The Lehrstuhl für Bodenkunde of the TU München is acknowledged for

allowing us to use their elemental analyzer and their NMR spectrometers, Dr. Anne Berns from Forschungszentrum Jülich for facilitating some of the solid-state ^{15}N NMR measurements.

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FIGURE CAPTIONS:

Figure 1. Above-ground plant biomass production (PBP_{above}) of pots with soils (Luvisol, *chromic*) incubated with and without addition of $K^{15}NO_3$, ^{15}N -Indulin, ^{15}N -Sarkanda. The yields are given per pot (150 g of soil) and as function of incubation time (days). The error bars show the mean $\pm$ standard deviation ($n=3$). a) Partial grass biomass production within one sampling period. b) Cumulative biomass production.

Figure 2. Soil microbial biomass C (BC), N (BN), and ^{15}N ($B^{15}N$) extracted from soils (Luvisol, *chromic*) incubated with and without addition of $K^{15}NO_3$, ^{15}N -Indulin, ^{15}N -Sarkanda for 28 and 58 days. The values were calculated according to Brookes et al. (1985). Total amounts of $B^{15}N$ in SMB are plotted per pot (150 g of soil). Percentages of $B^{15}N$ are related to the initial amounts of ^{15}N in each experiment ($^{15}N_0$). The error bars represent the standard deviation ($n=3$).

Figure 3. Solid state ^{13}C NMR spectra of ^{15}N -lignins, the HF-treated original soil (Luvisol, *chromic*) and the HF-treated soils incubated with and without addition of $K^{15}NO_3$, ^{15}N -Indulin, ^{15}N -Sarkanda for 28, 58 and 75 days.

Figure 4. Solid state ^{15}N NMR spectra of ^{15}N -Indulin, ^{15}N -Sarkanda lignins and the HF-treated soils incubated with and without addition of $K^{15}NO_3$, ^{15}N -Indulin, ^{15}N -Sarkanda for 28 and 58 days.

Table 1. Partial above ground plant biomass production (PBP_{above}) (*Lolium perenne*), in mg per growing time unit and pot (150 g of soil from a Luvisol, *chromic*) with standard deviation ($n=3$), pH (1:5 in H_2O) and electrical conductivity (EC; 1:5 in H_2O ($mS\ m^{-1}$)) after amendment with increasing amounts of N-Indulin (mg).

150 mg			300 mg			500 mg			1 000 mg			1 500 mg			2 000 mg			
Time	PBP _{above}	pH	EC	PBP _{above}	pH	EC	PBP _{above}	pH	EC	PBP _{above}	pH	EC	PBP _{above}	pH	EC	PBP _{above}	pH	EC
0 d	-	8.5	14.5	-	8.5	16.4	-	8.4	18.5	-	8.3	22.5	-	8.2	26.1	-	8.1	30.1
14 d	90 ± 20			84 ± 20			132 ± 28			97 ± 22			25 ± 9			23 ± 7		
28 d	53 ± 17	8.6	13.3	49 ± 16	8.6	14.0	40 ± 16	8.5	15.0	78 ± 18	8.5	18.0	71 ± 18	8.3	21.5	81 ± 21	8.3	27.4
50 d	64 ± 17			55 ± 17			42 ± 12			71 ± 19			73 ± 19			83 ± 20		
60 d	15 ± 6	8.6	12.3	11 ± 6	8.6	12.5	10 ± 3	8.6	14.2	9 ± 3	8.6	14.9	13 ± 4	8.4	20.7	22 ± 6	8.4	25.5
75 d	14 ± 6	8.7	12.5	10 ± 4	8.7	13.0	10 ± 4	8.6	14.6	9 ± 3	8.6	14.1	14 ± 4	8.5	18.1	10 ± 4	8.5	23.7

Table 2: Electrical conductivity (EC) and pH, both measured in H₂O, total organic carbon (TOC), total nitrogen (TN) and relative abundance of ¹⁵N (given as percentage of ¹⁵N of TN; atm%) of soil material (Luvisol, *chromic*) on which *Lolium perenne* was grown after addition of ¹⁵N-lignins or K¹⁵NO₃. The N and ¹⁵N recovery in the soil (N_r and ¹⁵N_r) is given as % of N and ¹⁵N present in the soil at the beginning of the experiment (N₀ and ¹⁵N₀).

Incubation time	Sample	pH	EC (mS m ⁻¹)	TOC (mg g ⁻¹)	TN (mg g ⁻¹)	¹⁵ N (atm %)	N _r (% of N ₀)	¹⁵ N _r (% of ¹⁵ N ₀)
0 days	¹⁵ N-Indulin	8.1	29.9	16.0±1.7**	1.5±0.2**	1.288±0.021**		
	¹⁵ N-Sarkanda	8.0	56.0	15.2±1.9**	1.6±0.2**	1.376±0.034**		
	K ¹⁵ NO ₃	8.5	12.1	8.4±1.4 ^{ns}	1.0±0.2*	10.195±0.174**		
	Control	8.6	9.6	8.3±1.1	0.9±0.1			
28 days	¹⁵ N-Indulin	8.3	27.4	15.8±1.8**	1.3±0.3**	1.106±0.078**	91	74
	¹⁵ N-Sarkanda	8.1	48.5	14.5±2.1**	1.2±0.3**	0.802±0.131**	73	42
	K ¹⁵ NO ₃	8.5	13.1	11.0±1.7**	1.0±0.2 ^{ns}	1.904±0.157**	88	19
	Control	8.4	15.3	8.9±1.3 ^{ns}	0.9±0.1 ^{ns}		97	
58 days	¹⁵ N-Indulin	8.4	25.1	15.6±1.4**	1.3±0.2**	1.031±0.103**	88	70
	¹⁵ N-Sarkanda	8.2	38.2	13.1±1.5**	1.2±0.2**	0.714±0.134**	70	39
	K ¹⁵ NO ₃	8.6	14.5	12.2±1.2**	0.9±0.2 ^{ns}	2.389±0.057**	90	21
	Control	8.5	14.0	9.9±1.3*	0.9±0.2 ^{ns}		98	
75 days	¹⁵ N-Indulin	8.5	23.7	15.0±1.5**	1.2±0.3**	0.947±0.201**	82	64
	¹⁵ N-Sarkanda	8.3	24.3	13.0±1.6**	1.1±0.2*	0.783±0.023**	65	37
	K ¹⁵ NO ₃	8.6	16.9	12.3±1.3**	1.0±0.2 ^{ns}	2.741±0.592**	95	25
	Control	8.6	12.8	10.6±1.2**	1.0±0.1 ^{ns}		99	

ns: not significant; * and **: significant relative to the control at 0 days at *p* < 0.05 and 0.01 respectively.

Table 3. Total organic carbon (TOC), total nitrogen (TN) and relative abundance of ^{15}N (given as percentage of ^{15}N of TN; atm%) in the shoots of *Lolium perenne* grown on soil material from a Luvisol *chromic*, after addition of ^{15}N -lignins or K^{15}NO_3 and harvested after different incubation time periods. The ^{15}N recovery in the total aboveground biomass production ($^{15}\text{N}_{\text{PBP}}$) is given as % of ^{15}N which was present in the soil at the beginning of the experiment ($^{15}\text{N}_0$). From those numbers the ^{15}N recovery in the roots ($^{15}\text{N}_{\text{root}}$) was estimated according to Herndl et al. (2011) reporting a root-to-shoot-ratio of *Lolium perenne* of 0.1 to 0.2.

Incubation time		TOC (mg/g)	TN (mg/g)	C/N	^{15}N (atm%)	$^{15}\text{N}_{\text{PBP}}$ (% of $^{15}\text{N}_0$)	$^{15}\text{N}_{\text{root}}$ (% of $^{15}\text{N}_0$)
28 days	^{15}N -Indulin	396.5±80.3 ^{ns}	38±8**	10.4	1.910±0.013	3	0.3–0.6
	^{15}N -Sarkanda	336.0±41.2*	41±10**	8.2	2.014±0.010	2	0.2–0.4
	K^{15}NO_3	324.8±56.3*	33±7**	9.8	58.010±1.444	29	2.9–5.8
	Control	391.0±82.3	18±4	21.7			
42 days	^{15}N -Indulin	390.0±75.4 ^{ns}	45±10**	8.7	2.080±0.089	6	0.6–1.2
	^{15}N -Sarkanda	376.2±42.4*	47±11**	8.0	2.229±0.061	4	0.4–0.8
	K^{15}NO_3	358.4±56.1**	27±6**	13.3	48.749±0.845	33	3.3–6.6
	Control	383.1±47.3 ^{ns}	22±5*	17.3			
75 days	^{15}N -Indulin	334.1±52.7**	30±8**	11.1	1.884±0.080	8	0.8–1.6
	^{15}N -Sarkanda	298.2±39.2**	35±9**	8.5	20.122±2.385	20	2.0–4.0
	K^{15}NO_3	375.8±44.3*	20±6**	18.8	37.648±1.191	37	3.7–7.4
	Control	372.4±46.7*	18±4 ^{ns}	20.7			

ns: not significant; * and **: significant relative to the control at 28 days at $p < 0.05$ and 0.01 respectively

Table 4. Intensity distribution in the ^{13}C NMR spectra of soils (Luvisol, *chromic*) amended with ^{15}N -lignins and K^{15}NO_3 before growing grass (*Lolium perenne*) as a function of incubation time. Prior to NMR analysis the soils were demineralised with 10% (v/v) HF.

Incubation time	Sample	Ketone, aldehyde	Carboxyl, amide	Aromatic	O-Alkyl	Methoxyl N-Alkyl	Alkyl
		245-185 ppm	185-160 ppm	160-110 ppm	110-60 ppm	60-45 ppm	45-0 ppm
0 days	Original soil	1.9	13.1	20.1	31.2	10.2	23.5
28 days	^{15}N -Indulin	1.7	8.4	34.3	26.4	11.7	17.4
	^{15}N Sarkanda	1.8	9.7	31.9	24.6	12.7	19.3
	K^{15}NO_3	2.9	11.9	16.2	33.1	10.4	25.4
	Control	2.7	11.6	18.7	32.8	10.3	24.1
58 days	^{15}N -Indulin	3.0	9.9	30.1	24.4	12.0	21.5
	^{15}N -Sarkanda	3.7	10.9	32.8	20.5	12.3	19.9
	K^{15}NO_3	1.2	12.7	15.8	30.8	11.5	28.0
	Control	1.0	12.9	17.0	27.7	11.6	29.8
75 days	^{15}N -Indulin	3.5	9.2	35.3	21.0	12.4	18.6
	^{15}N -Sarkanda	2.8	10.4	30.3	24.5	11.5	20.6
	K^{15}NO_3	1.7	10.8	16.9	35.8	10.4	24.4
	Control	1.0	10.7	15.9	42.3	9.1	21.0

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Figure 3

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