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ORIGINAL ARTICLE

Characterization of fecal nitrogen forms produced by a sheep fed with ^{15}N labeled ryegrass

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Abstract Little is known about nitrogen (N) forms in ruminant feces, although this information is important to understand N dynamics in agro-ecosystems. We fed ^{15}N labeled ryegrass hay to a sheep and collected ^{15}N labeled feces. Nitrogen forms in the feces were characterized by chemical extractions, solid-state cross polarization ^{15}N nuclear magnetic resonance spectroscopy (SS CP/MAS ^{15}N NMR) and Curie-point pyrolysis–gas chromatography/mass spectrometry (Cp Py-GC/MS). A 4 months incubation experiment was conducted to assess N release from the feces. Half of the fecal N could be ascribed to bacterial and endogenous debris and a third to undigested dietary N. About a tenth of the fecal N was mineralized during the incubation experiment. The ^{15}N abundance of nitrate released during the incubation remained constant and close to the ^{15}N abundance of the total feces N. The NMR analysis of the feces showed that most of the N was present in proteins, while some was present as heterocyclic N, amino acids and ammonium. The Cp Py-GC/MS analysis confirmed the presence of proteins, amino acids and heterocyclic N in the feces. Comparing these results to those obtained from the ^{15}N labeled hay suggests that some N compounds present in the plant were not digested by the animal, and that the animal excreted de novo synthesized N compounds. The low content in ammonium and amino acids, the low rate of N release from these feces during the incubation and the relatively high fecal protein content, particularly the hard to mineralize undigested and microbially bound forms, can explain the low transfer of N from these feces to crops observed in a previous work.

Keywords Ryegrass hay · Sheep feces · ^{15}N labeling · Curie-point pyrolysis–gas chromatography/mass spectrometry · Solid state cross polarization ^{15}N nuclear magnetic resonance

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Introduction

In agricultural systems where crop and animal production are integrated nitrogen (N) and phosphorus

excreted in animal manure can be recycled to crops (Schröder 2005). However, the application of animal manure in excess of plant needs, or at a time when plants cannot take up nutrients, will result in nutrient losses to the atmosphere and to water bodies (Robertson and Vitousek 2009). Nitrogen release from manure to plants must be therefore correctly quantified so as to optimally recycle animal manure to crops. This release is, however, difficult to assess, as besides manure, soils also deliver N to crops. Labeling animal manure with ^{15}N and applying the labeled manure to the field allows assessing the N transfer from the manure to the soil and then to the plants without ambiguity (Powell et al. 2004; Sørensen and Thomsen 2005; Cusick et al. 2006b; Berntsen et al. 2007).

Manure is composed of variable proportions of urine, feces and animal bedding materials. Urine contains most of its N in the form of urea and ammonium (NH_4^+). Urea is rapidly transformed into NH_4^+ through hydrolysis by urease. Ammonium can then be sorbed on soil particles, taken up by microbes or plants, transformed into nitrate (NO_3^-) or lost to the environment. Urine derived N is taken up by plants as well as water soluble mineral fertilizer N (Sørensen and Jensen 1996; Jensen et al. 1999; Langmeier et al. 2002; Bosshard et al. 2009) and is rapidly incorporated into soil N compounds (Bosshard et al. 2008). The nitrogen forms contained in ruminant feces are complex. The release of N from feces to the soil solution and to plants is very variable. Overall, N forms present in feces have a limited effect on crop N nutrition on the short term (Jensen et al. 1999; Langmeier et al. 2002; Bosshard et al. 2009). Apart from data obtained from chemical fractionation (Langmeier et al. 2002; Powell et al. 2006; Morvan and Nicolardot 2009) there is little information on the forms of N present in ruminant feces. Powerful methods (as solid state cross polarization/magic angle spinning ^{15}N nuclear magnetic resonance [SS CP/MAS ^{15}N NMR], or Curie-point pyrolysis–gas chromatography/mass spectrometry [Cp Py-GC/MS]) are available to resolve the chemical structures in which N is found in feces. Whereas Cp Py-GC/MS has been used to characterize carbon (C) and N forms in forage, soil organic matter, composts, municipal solid wastes (Ralph and Hatfield 1991; Reeves and Francis 1997; Leinweber and Schulten 2000; Gonzalez-Vila et al. 2001; Franke et al. 2006, 2007) and more recently in manure (Calderon et al. 2006; Schnitzer et al. 2007), fewer works used NMR to characterize N forms in these organic compounds because of the low concentration of ^{15}N at natural abundance in these samples (Morier et al. 2008). To our knowledge animal feces have not yet been characterized by SS CP/MAS ^{15}N NMR.

This paper is the third of a series in which we used the same ^{15}N labeled sheep feces. Bosshard et al. (2009) observed in a field experiment that the uptake of N derived from a single application of ^{15}N labeled feces by 3 subsequent crops (wheat, soybean, maize) was much lower than the uptake of N derived from ^{15}N labeled urine or mineral fertilizer. Bosshard et al. (2008) showed that, compared to urine N and mineral fertilizer N, less feces N was recovered in the organo-mineral fraction and in the light fraction of the soil sampled in the same field experiment after harvest of wheat, suggesting that feces N had been used to a lower degree by soil microbes and plant than urine or mineral fertilizer N. Besides, the results obtained by Bosshard et al. (2009) also suggest that significant amounts of feces N had been removed from the upper soil horizon of the microplots where ^{15}N labeled feces had been added by leaching and/or by earthworms. The aim of the present paper is to evaluate whether information on the forms of N in these feces would contribute to explain their low N availability observed in the field.

To reach this aim the feces of a sheep fed with ^{15}N labeled ryegrass hay were analyzed by chemical extractions, Cp Py-GC/MS and SS CP/MAS ^{15}N NMR and the rate of N release from the feces was quantified in a four-month incubation experiment. The ryegrass hay fed to the sheep was also characterized by chemical extractions and Cp Py-GC/MS. The results obtained in this work allow discussing (1) the pathway of ^{15}N enrichment of feces, (2) the limits of this labeling technique for the production of feces homogeneously labeled with ^{15}N, and (3) the relation between feces N forms and their availability to crops as observed in the field.

Materials and methods

Throughout this paper we present absolute ^{15}N abundance in atom% ^{15}N which denotes the proportion of all N atoms of mass 15 on total N multiplied by 100.

Production of ^{15}N-labeled ryegrass hay and of ^{15}N-labeled sheep feces

Italian ryegrass (*Lolium multiflorum*, cultivar Axis) was grown in a greenhouse under controlled conditions (photoperiod 16 h, temperature 25°C during the day and 18°C during the night) in PVC-boxes (560 mm × 360 mm × 129 mm) that were perforated at the bottom and filled with 30 kg of quartz sand. Each box was fertilized with a nutrient solution containing N and other macro- and micronutrients. Nitrogen was applied at an amount of 7 g $K^{15}NO_3$ (15.90 atom% ^{15}N) per box and per cut. Each nutrient solution application was divided into two equal doses. For the first cut, the first dose was given after germination and the second dose 5–6 days later. For the following cuts, the first dose was added directly after cutting and the second dose 5–6 days later. All micronutrients were applied in the first dose after each cut. Nutrition solution that was leached was collected in containers installed below each box. These containers were filled with 10 l of water to avoid the crystallization of the leached solution and covered with a black foil to avoid growth of algae. The leached solution was pumped back daily to the ryegrass. The ryegrass was cut six times with the first cut being done 34 days after germination. All following cuts were performed in intervals of 21 days. After each cut the harvested ryegrass was dried for 40 h at 45°C and then the six cuts were thoroughly mixed. Unlabeled ryegrass hay was simultaneously produced under identical conditions. Unlabeled and labeled hay were analyzed for major nutrients. Labeled hay was additionally analyzed by Cp Py-GC/MS.

We fed an adult male sheep (101 kg live weight) first with unlabeled ryegrass hay for 7 days to adapt the animal to the forage, then during 9 days with the ^{15}N-labeled ryegrass hay (14.60 atom% ^{15}N) and finally, in order to evaluate the evolution of ^{15}N-enrichment in the excreta after having stopped ^{15}N-feeding, we fed it again with unlabeled ryegrass hay for another 3 days as suggested by Sørensen et al. (1994). For the collection of feces and urine the sheep was put into a crate (170 cm × 70 cm × 185 cm) which was fitted with an automatic drinking bowl and a feeder for the ryegrass hay. The urine could flow through the grid where the animal was standing on into a tray attached below the cage. The urine could drain off from the tray through a narrow channel into a can that was cooled from the outside with ice to minimize gaseous N losses. Feces were either collected directly from the grid or from another grid that was affixed above the urine sampling channel. Because of animal welfare regulations it was not possible to keep the sheep in the crate over the entire experimental period of 19 days. Therefore a quantitative collection of feces and urine was done only on day 16, 17 and 18 when the highest ^{15}N-enrichment in the excreta was expected. From day 6 through 19 representative samples of both types of excreta were taken to determine the evolution of atom% ^{15}N. The excreta were collected daily and frozen at −20°C until use. Feces collected at day 16 were analyzed by chemical extractions, Cp Py-GC/MS and SS CP/MAS ^{15}N NMR and used in an incubation experiment. This animal experiment was approved by the Cantonal Veterinary Office of Zug, Switzerland.

Nutrient content of ryegrass hay

The ryegrass hay was finely ground using a ball mill Retsch® (Retsch GmbH, Germany) prior to element analyses. Concentration of phosphorus (P), magnesium (Mg), calcium (Ca) and sodium (Na) was determined on 0.5 g of dried ryegrass that had been ashed at 550°C for 8–10 h. The ashes were dissolved in 2 ml 20% HCl, the solution was heated shortly, filtered (Whatmann no. 40), distilled H_2O was then added to a volume of 50 ml, and the element concentrations were measured on an ICP-OES Liberty 220 (Varian, USA).

Total N and ^{15}N abundance analyses were carried out on a continuous flow Roboprep CN Biological Sample Converter coupled to a Tracermass Mass Spectrometer (Europa Scientific, Crewe, England). Nitrogen bound to neutral detergent fiber (NDF-N), i.e. associated to plant cell structural components (i.e. lignin, hemicellulose and cellulose) was obtained by boiling 5 g of milled ryegrass hay with a neutral detergent solution (Van Soest et al. 1991) for 1 h in a Fibretec System M (Tecator Ltd., Hønegäs, Sweden). The detergent-insoluble fibrous material was washed with boiling distilled water, dried over night at 103°C and subsequently milled using a ball mill Retsch® (Retsch GmbH, Germany). Total N and ^{15}N abundance of NDF were measured as described above. Selected characteristics of the labeled and unlabeled hay are given in Table 1.

Table 1 Composition of unlabeled and ^{15}N-labeled ryegrass hay (*NDF* neutral detergent fiber, *OM* organic matter, *nm* not measured)

Ryegrass	OM (g kg^{-1} DM)	NDF (g kg^{-1} DM)	P (g kg^{-1} DM)	Mg (g kg^{-1} DM)	Ca (g kg^{-1} DM)	Na (g kg^{-1} DM)	Total N (g kg^{-1} DM)	NDF-N (g kg^{-1} DM)	Total N (atom $\%^{15}N$)	NDF-N (atom $\%^{15}N$)
Unlabeled	894	492	5.9	4.4	3.4	0.5	24.9	nm	0.37	nm
Labeled	888	550	7.0	4.6	3.6	0.5	24.3	13.3	14.6 a	13.9 b

Means of atom% ^{15}N of ryegrass hay fraction followed by *different letters* are significantly different ($P < 0.05$)

Total and mineral N content of feces and ^{15}N abundance of total N in feces and urine

Thawed feces and urine samples collected from day six onwards were acidified with concentrated H_2SO_4 prior to freeze-drying to minimize gaseous N losses. After freeze-drying the feces samples were finely ground using a ball mill Retsch® (Retsch GmbH, Germany). Total N and C content in the feces and urine were measured with a Nitrogen Analyzer 1500 (Carlo Erba, Italy). Comparison of the total N amount in freeze-dried feces and urine, respectively, with the total N amount in thawed samples revealed an average loss of 5.8% of N from urine despite the addition of H_2SO_4 during freeze-drying while no N was lost from feces. Total N and ^{15}N abundance in feces and urine were measured on the previously mentioned Tracermass Mass Spectrometer. Mineral N (NH_4^+-N and NO_3^--N) in feces collected at day 16 was extracted with 2 *M* KCl (5 g of fresh feces in 100 ml) as described in Langmeier et al. (2002) and measured colorimetrically on a SAN^{plus} Analyzer (Skalar, Netherlands).

Fractionation of N forms in feces

Feces collected at day 16 were subjected to the analytical protocol of Mason (1969) as modified by Kreuzer and Kirchgessner (1985) in order to produce the following N fractions: (1) undigested dietary N (UDN) mainly consisting of undigested, lignified cell wall components derived from the plant, (2) bacterial and endogenous debris N (BEDN) which contains mainly bacterial proteins from the rumen and the hindgut, some gut cell materials and few forage proteins and (3) water-soluble N (WSN) mainly originating from endogenous digestive secretions derived from pancreas, bile and the intestinal wall but also from soluble cell content of disrupted microbes (Kreuzer and Kirchgessner 1985). The N fractions were determined in separate subsamples of a homogenate made of 350 g fresh feces mixed with 3,500 ml distilled water. The UDN fraction was obtained by boiling 30 g of the homogenate with the neutral detergent solution for 1 h in a Fibretec System M (Tecator Ltd., Hønegäs, Sweden) after having shaken the homogenate subsample for 24 h at 4°C and 200 rpm in a water bath in order to remove microbial matter from truly undigested material. The detergent insoluble material was washed with boiling distilled water and dried overnight at 103°C. The water insoluble N associated with the particulate matter of the feces was determined by centrifuging another 30 g of the homogenate twice at 27,000 *g* for 30 min at 4°C. The supernatant was carefully discarded, the sediment containing the water insoluble N was recovered and lyophilized. All samples were finely ground using the Retsch mill prior to total N and ^{15}N analyses which were carried out as previously mentioned. For UDN, N concentration and ^{15}N abundance were directly determined. For WSN, these values were calculated as the difference between the values found for total feces N and water-insoluble N, while they were calculated for BEDN as the difference between water-insoluble N and UDN.

Incubation experiment with feces

To determine the ^{15}N-abundance of mineralized fecal N, fresh sheep feces collected at day 16 were incubated in quartz sand for 112 days. Fifty g of quartz sand that had been dried at 105°C for 3 days was mixed with 318 mg fresh feces containing 3.81 mg N and with the equivalent of 2.5 g dry matter of a soil containing 3.75 mg N added in a fresh form to stimulate microbial activity (Sørensen et al. 1994). The mixture was incubated in 250 ml bottles at 20°C in the presence of 0.2 ml distilled H_2O g^{-1} quartz sand so as to reach 55% of the water

holding capacity of the quartz sand. The bottles were covered by perforated lids of aluminum foil to reduce water losses. The moisture of the quartz sand was controlled weekly by weighing and if necessary readjusted. Three replicates of the sand-soil-feces-mixture (each equivalent to 25 g DM) were extracted with 80 ml 2 *M* KCl after 11, 47, 75, 97 and 112 days of incubation. Similarly, soil was also incubated in quartz sand without feces and treated as the sand-soil-feces mixture to evaluate the amount of N mineralized from the soil. The amount of N mineralized from the feces and its ^{15}N abundance were then corrected for the dilution by mineralized soil N obtained from the control treatment which accounted for 0.04–0.09 mg N g^{-1} soil DM.

NH_4^+-N and NO_3^--N in the KCl-extracts were analyzed colorimetrically as described above. ^{15}N abundance analyses of NH_4^+- and NO_3^--N in the KCl-extracts of the sand-soil-feces mixture and the sand-soil mixture were carried out on the mass spectrometer previously mentioned after diffusion of ^{15}N from the KCl-extracts to acidified glass filters as described by Goerges and Ditter (1998) and Sørensen and Jensen (1991).

Solid-state ^{15}N cross polarization magic angle spinning nuclear magnetic resonance of feces

Solid-state CP/MAS ^{15}N NMR was conducted on sheep feces collected at day 16. The ^{15}N solid-state spectrum was obtained on a Bruker DMX 400 at a frequency of 40.55 MHz with magic angle spinning at 5 kHz using freeze-dried, finely ground and not pretreated feces. A commercial double-bearing probe and a phase-stabilized zirconium rotor with an outside diameter of 7 mm were used. The Hartmann-Hahn contact time was 1 ms during which a ramped ^1H-pulse starting at 100% power and decreasing until 50% was applied to circumvent Hartmann-Hahn mismatches (Peersen et al. 1993). Approximately 2,000 scans were accumulated using a pulse delay of 0.3 s. Line broadening of 50 Hz were applied prior to Fourier transformation. The relative N distribution was determined by integration of the signal intensity in the different chemical shift regions using an adapted integration routine supplied with the instrument software. The spectrum was referenced to external nitromethane (=0 ppm).

Curie point pyrolysis–gas chromatography/mass spectrometry of hay and feces

We proceeded as described in Leinweber et al. (2009). The ^{15}N labeled ryegrass hay, the ^{15}N labeled feces and the unlabeled feces were pyrolyzed at 500°C for 9.9 s in a Curie-point-pyrolyzer Fischer 1040 PSC (Bonn, Germany). The pyrolysis products were separated on a gas chromatograph Varian 3800 (Walnut Creek, USA) equipped with a 25 m capillary column BPX 5 (SGE, Ringwood, Australia) that was coated with a 0.25 µm film thickness of a 5% phenylmethyl and 95% dimethyl polysiloxane stationary phase and had an inner diameter of 0.32 mm. Following split injection up to 45 s (splitless) at 300°C, the split ratio was 1:100 from 45 s up to 90 s and 1:5 from 90 s on. The flow rate of the helium carrier gas was adjusted to 2 ml min^{-1}. The starting temperature for the gas chromatographic program was 28°C (5 min), and heated at a rate of 5°C min^{-1}–280°C (30 min). The gas chromatograph was connected to a double focusing Finnigan MAT 212 mass spectrometer (Bremen, Germany). Conditions for mass spectrometric detection in the electron impact mode were 3 kV acceleration voltage, 70 eV electron energy, 2.2 kV multiplier, 1.1 s (mass decade)$^{-1}$ scan speed, and *m/z* (mass-to-charge ratio) 48–450 mass range. Mass spectra corresponding to peaks in the gas chromatograms were assigned by comparison with the Wiley mass spectral library, software edition 6.0. Their relative proportion was calculated by the peak area as a percentage of the total peak area of the chromatograms. The ^{15}N enriched compounds were identified by an increased isotope peak [M + 1] of the corresponding mole peak, when compared to the library spectra.

Statistical analysis

Except for Cp Py-GC/MS and SS CP/MAS ^{15}N NMR analyses, analyses were conducted with 3 analytical replicates. Analysis of variance was performed by using the statistical analyses package SYSTAT 11 (Systat Software Inc., USA) to compare the ^{15}N-enrichment of the neutral detergent fiber fraction with complete ryegrass hay as well as the ^{15}N-enrichment of the feces fractions and to test the effect of the incubation time on the ^{15}N-enrichment of mineralized fecal N. In case of significant effects, separation of means was tested using Tukey's HSD (honestly significant difference) test with a significance level of $P < 0.05$.

Results

Ryegrass hay

The ^{15}N-enriched ryegrass hay contained 24 g N kg^{-1} DM (weighted mean over six cuts) with an enrichment of 14.6 atom% ^{15}N (weighted mean over six cuts) (Table 1). Fifty-four percent of the originally applied ^{15}N was recovered in ryegrass shoots. The quality of the ryegrass hay fulfilled the requirements concerning energy and protein of adult non-performing small ruminants when fed as a sole feed (ALP 2006). Fiber-bound N (here assessed as NDF-N) made up almost 55% of the total N in the ryegrass and had a lower ^{15}N enrichment than total N.

Production of ^{15}N labeled urine and feces

Two days after the start of ^{15}N feeding, the ^{15}N enrichment in feces exceeded the one of urine and remained higher during the complete sampling period (Fig. 1) as shown in other studies with sheep (Sørensen and Thomsen 2005) or cows (Powell et al. 2004). The ^{15}N enrichment in urine reached its maximum at the end of the feeding experiment and decreased as soon as the sheep was fed unlabeled forage. The enrichment of the feces, in contrast, continued to increase slightly after terminating ^{15}N feeding. At the end of ^{15}N feeding total urinary N and total fecal N were enriched with ^{15}N at a level accounting for 60 and 77%, respectively, of that found in the ryegrass.

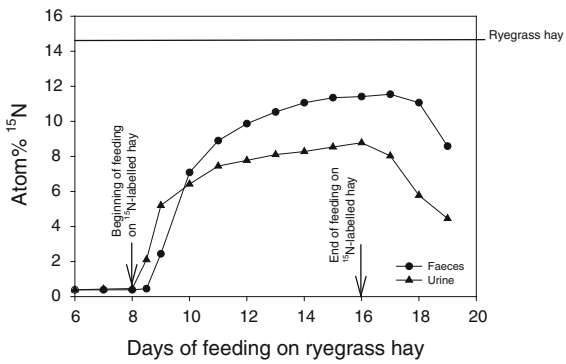

Fig. 1 Evolution of the ^{15}N enrichment in sheep feces and urine over 19 days

Table 2 Percentage of N and atom% ^{15}N in the different N fractions of the sheep feces collected at day 16 of the feeding experiment period

	Percentage of N in fraction (%)	Atom% ^{15}N (%)
Undigested dietary N (UDN)	33.0	10.7 (0.05) b
Bacterial and endogenous debris N (BEDN)	53.1	12.0 (0.16) a
Water-soluble N (WSN)	13.6	9.85 (0.50) c
Total N (TN)	100.0	11.3 (0.03) ab

Standard deviation is shown in brackets

Means of atom% ^{15}N of ryegrass hay fraction and feces fractions followed by *different letters* are significantly different ($P < 0.05$)

Total and mineral N and chemical N fractions in feces

The feces collected on day 16 had a dry matter content of 34.3% (with a standard deviation, SD of 9.5), a total carbon content of 459 g C kg^{-1} DM (SD of 0.5), a total N content of 35.0 g N kg^{-1} DM (SD of 0.5) and a content of KCl-extractable NH_4^+ of 1.3 g N kg^{-1} DM (SD of 0.15). Nitrate could not be detected in the feces.

The bacterial and endogenous debris N (BEDN) accounted for about half, the undigested dietary N (UDN) for a third and the water soluble N for 13.6% of the total fecal N (Table 2). These fractions had significantly different ^{15}N enrichments with the BEDN being the most enriched and the water soluble N the least enriched.

Incubation experiment

The amount of mineral N present as NO_3^- derived from the mineralization of fecal N increased from day 11 to day 97 when it reached 9.4% of the added fecal N and then decreased at day 112 (Table 3). No NH_4^+ could be detected during the incubation. Since there was no NO_3^- in the fresh feces, the NO_3^- observed in the incubation experiment was fully derived from the nitrification of fecal N. Between day 97 and 112 the amount of NO_3^- decreased from 3.3 to 1.8 mg g^{-1} of DM feces pointing out to an uptake of NO_3^- by microorganisms and/or to the occurrence of denitrification.

Table 3 ^{15}N-enrichment of mineralized fecal N, mineralized fecal N and recovery of fecal N after increasing incubation time in quartz sand amended with feces collected at day 16 of the feeding experiment period and inoculated with soil

Incubation time (days)	Atom % ^{15}N of mineralized fecal N (%)	Mineralized fecal N (NO_3^-) (mg N g^{-1} feces DM)	Recovery (% of added fecal N)
11	10.76 (0.1)	1.4 (0.2) b	4.0
47	11.34 (0.1)	2.1 (0.2) ab	6.0
75	11.30 (0.1)	2.6 (1.0) ab	7.4
97	11.80 (0.2)	3.3 (1.2) a	9.4
112	10.58 (0.3)	1.8 (0.9) b	5.1

Standard deviations are shown in brackets

Results are only presented for NO_3^- as NH_4^+ was below detection limit during the incubation

Within a parameter means followed by *different letters* are significantly different ($P < 0.05$) by Tukey's multiple range test

The ^{15}N enrichment of the mineralized NO_3^- did not vary significantly during the incubation. It reached an average of 11.1% which was very close to the abundance of total fecal N (11.3%). If we assume that UDN was not mineralized during this incubation due to its suggested stable nature (Kreuzer and Kirchgessner 1985), we can estimate that WSN and BEDN contributed in average 40% and 60%, respectively, to the N mineralized from the feces.

Solid-state ^{15}N cross polarization magic angle spinning nuclear magnetic resonance of feces

The NMR spectrum observed for the feces was very similar to the spectrum obtained for *Lolium perenne* enriched in ^{15}N by Knicker and Lüdemann (1995). The NMR spectrum of the sheep feces was dominated by a signal between −220 and −285 ppm, peaking at −262 ppm (Fig. 2) that was assigned to amide N most likely in proteinaceous material (Knicker et al. 1997). It comprised more than 70% of total ^{15}N signal intensity. The low-field shoulder of the main peak covered signals from heterocyclic-N in histidine, nucleic acid derivates and substituted pyrroles in the chemical shift range of −145 to −220 ppm (Knicker and Lüdemann 1995). Within this range, a weak signal was detected at a chemical shift of −204 ppm which could be assigned to substituted pyrroles (Knicker et al. 1997) and accounted for 5.3% of the total peak area. The resonance lines between −300 and -325 ppm and between −325 and −370 ppm were tentatively assigned, respectively, to NH_2-groups of basic amino acids which accounted for 2.2% of total peak area and to NH_4^+-ions which accounted for 3.2% of total peak area (Knicker and Lüdemann 1995). The identified species accounted then for 80% of the total peak area, while the remaining 20% could not be attributed to specific N species.

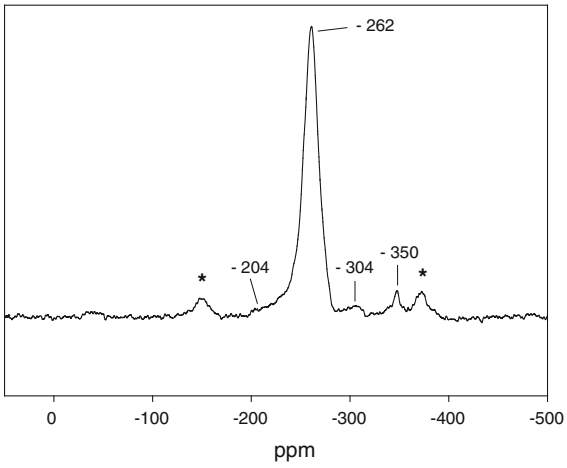

Fig. 2 Solid-state CP/MAS ^{15}N NMR spectrum of ^{15}N-enriched sheep feces collected at day 16 of the feeding experiment period. The spectrum is referenced to external nitromethane (=0 ppm). Asterisks (*) indicate spinning side bands

Curie point pyrolysis–gas chromatography/mass spectrometry of hay and feces

Data shown in the Table 4 give a summary of groups of organic compounds detected in the ^{15}N labeled ryegrass, in the unlabeled feces, and in the ^{15}N labeled feces. Similar results were obtained for the ^{15}N labeled feces and for the unlabeled feces confirming that ^{15}N labeling of the hay had no great influence on the metabolism of the animal. The results obtained with these feces were similar to those

Table 4 Groups of compounds obtained from the pyrolysis of ryegrass hay and feces collected at day 16 of the feeding experiment period

	^{15}N labeled Ryegrass	Unlabeled feces	^{15}N labeled feces
Nitrogen heterocyclic compounds	11.8	10.0	11.2
Substituted furans	2.1	0.9	0.8
Benzene and substituted benzenes	13.4	14.1	14.9
Phenols and substituted phenols	10.9	5.0	4.9
Aliphatic compounds	6.9	27.9	29.4
Carbocyclic compounds	6.5	25.4	27.2
Polycyclic aromatic hydrocarbon	nd	0.5	0.5
Unidentified compounds	48.4	16.2	11.0

Results are expressed in percent of the peak area

presented by Schnitzer et al. (2007) on chicken manure. Whereas only 11–16% of the compounds remained unidentified in feces, this fraction reached 48% for the ryegrass hay. The higher proportion of unidentified compounds in the ryegrass might be related to its higher concentration in carbohydrates, such as cellulose, which are pyrolyzed in compounds that are difficult to identify (Ralph and Hatfield 1991). Aliphatic and carbocyclic compounds were present in much higher proportion in the feces compared to the ryegrass whereas higher proportions of substituted furans and of phenol compounds were found in the ryegrass hay. The results on the N compounds are presented afterwards in detail. We first present the results of the other organic compounds to show in which chemical environments these N compounds were found.

Both ryegrass and feces yielded methyl substituted furans which can be derived from carbohydrates such as cellulose (Bracewell et al. 1989; Pouwels et al. 1989). In the ryegrass hay the most important benzene compound were methyl-benzene with 6.1% of the total peak area and styrene (3.3%) which can be a pyrolysate product of lignin (Ralph and Hatfield 1991). The main benzene compounds in the feces were also methyl substituted benzenes (methyl-benzene with about 6.2% and 1,4-dimethylbenzene with 3.3–3.6%). These benzene compounds might be derived from aromatic compounds (Schnitzer et al. 2006). The most important phenol compounds were phenol (2.9%), 3-methyl-phenol (3.0%), 2-methoxy-phenol (4.8%) in the ryegrass and 4-methoxy-phenol (2.4–2.7%) in the feces. Phenol pyrolyzates might be derived from lignin, from other phenolic compounds or from the pyrolysis of tyrosin (Ralph and Hatfield 1991). According to Pouwels et al. (1989) the pyrolysis of cellulose can also produce phenols. The aliphatic compounds observed in the ryegrass hay were 1,3,5-hexatriene, a diene, two alkanes, two aldehydes and one aliphatic carboxylic acid. Only the aliphatic carboxylic acid (9,12-Hexadecadienoic acid, methyl ester) was present in relatively high concentration (2.8% of the total peak) while all other compounds were present in low level (less than 2%). In the feces we obtained ten dienes with 1,4-pentadiene representing 5.2–7.9%, 1,3-pentadiene, 2.0–2.8% and 1,2-pentadiene 2.3–5.5%. Besides, six alkenes, ten alkanes, three aldehydes and three alcohols were also observed in the feces. All these compounds were present in low concentrations (less than 2%). According to Schnitzer et al. (2007) Curie-point pyrolysis of fatty acids can produce alkanes and alkenes while other aliphatic compounds might be derived from other lipids. Finally the carbocyclic compounds were dominated by 2-cyclopenten-1-one (2.7%) in the ryegrass and by cyclohexene, 1-methyl-4-(methyl-ethenyl)-R (20.0–20.4%) in the feces. These carbocyclic compounds are probably derived from polycarboxylic acids (Bracewell et al. 1989). Pouwels et al. (1989) suggest that cyclopenten derivatives could be produced from cellulose.

The nitrogen containing compounds represented 11.8% of the total peak area for the ryegrass hay and 10.0–11.2% for the feces (Table 4). The detailed results on N containing compounds are given in the Table 5. Some of the N-containing pyrolysis products pyrroles, pyridines and pyrazoles were observed both in the ryegrass hay and in feces. There are two possible origins of these molecules: (1) they can be genuine constituents of the organic materials as all organisms contain heterocyclic N, and (2) they can be formed due to the flash pyrolysis and electron impact ionization in Cp Py-GC/MS (Schnitzer et al. 2007). On the basis of the (flash) Cp Py-GC/MS alone it is impossible to distinguish between these two possible origins (Kögel-Knabner 2000; Schulten and Leinweber 2000). However, the signal assignments in the SS CP/MAS ^{15}N NMR spectrum (Fig. 2) call for the

Table 5 N-containing and ^{15}N-enriched pyrolysis products and their abundance (expressed in % of peak area) in ryegrass hay and feces collected at day 16 of the feeding experiment period tentatively assigned by pyrolysis–gas chromatography/mass spectrometry (Py-GC/MS), mass weight (MW), elemental composition and possible origin

Pyrolysis products	MW	Elemental composition	Abundance in RH or F	^{15}N-enriched in	Possible origin	References
1H-Pyrrole	67	C_4H_5N	RH (0.9%), F (2.0%)	RH	Pr; AA (hypro/pro)	Kögel-Knabner (2000), Sorge et al. (1993)
1H-Pyrrole, 1-methyl-	81	C_5H_7N	RH (1.0%), F (0.4%)	RH, F	Pr; AA (hypro/pro)	Nguyen et al. (2003)
1H-Pyrrole, 2-methyl-	81	C_5H_7N	F (0.8%)		Pr; AA (hypro/pro)	Nguyen et al. (2003)
1H-Pyrrole, 3-methyl-	81	C_5H_7N	F (1.6%)		Pr; AA (hypro/pro)	Nguyen et al. (2003)
1H-Pyrrole, 2,5-dimethyl-	95	C_6H_9N	RH (0.3%), F (0.3%)	RH, F	Pr; AA (hypro)	Chiavari and Galletti (1992)
Pyrrole, 2,4-dimethyl-	95	C_6H_9N	RH (0.3%)	RH	Pr; AA (hypro)	Chiavari and Galletti (1992)
1H-Pyrrole, 1-ethyl-	95	C_6H_9N	RH (0.3%), F (0.6%)	RH	Pr; AA (hypro)	Chiavari and Galletti (1992)
Pyrrole, 3,4-diethyl-2-methyl-	137	$C_9H_{15}N$	F (0.8%)		Lig	Niemann et al. (1995), Van Arendonk et al. (1997)
1H-Pyrrole, 1-pentyl-	137	$C_9H_{15}N$	F (0.4%)		Lig	Niemann et al. (1995), Van Arendonk et al. (1997)
Pyridine	79	C_5H_5N	RH (4.4%), F (0.8%)	RH	Pr; AA (ala/tyr)	Chiavari and Galletti (1992), Nguyen et al. (2003)
Pyridine, 4-methyl-	93	C_6H_7N	F (0.04%)	F	Pr; AA (ala/cys)	Chiavari and Galletti (1992), Kögel-Knabner (2000)
Pyridine, 4-methyl-1-oxide-	108	C_6H_7ON	F (0.9%)		Pr; AA (tyr); Lig	Schulten (1996), Sorge et al. (1993)
Pyridine, 2,3-dimethyl-	107	C_7H_9N	RH (0.2%)		Pr; AA (tyr)	Sorge et al. (1993)
Pyridine, 2,5-dimethyl-	107	C_7H_9N	RH (0.2%)	RH	Pr; AA (tyr)	Sorge et al. (1993)
Pyridine, 5-ethenyl-2-methyl-	119	C_8H_9N	RH (0.2%)		Pr; AA (thr)	Sorge et al. (1993)
Pyrimidine, 4-methyl-	94	$C_5H_6N_2$	RH (0.2%)	RH	Pr; Tannin	Niemann et al. (1995)
1H-Pyrazole, 1-methyl-	82	$C_4H_6N_2$	F (2.5%)		Pr; AA (his)	Sorge et al. (1993)
1H-Pyrazole, 3-methyl-	82	$C_4H_6N_2$	RH (0.8%), F (0.6%)		Pr; AA (his)	Sorge et al. (1993)
Piperidine, 1-methyl-	99	$C_6H_{13}N$	RH (1.5%)		Pr; peptide	Schulten (1996), Van Arendonk et al. (1997)
1H-Imidazole, 2-ethyl-	96	$C_5H_8N_2$	RH (0.8%)		Pr; AA (lys)	Sorge et al. (1993)
4-Imidazolidinone, 5-(phenylmethyl)-2-thioxo-	206	$C_{10}H_{10}OS_2$	RH (0.3%)	RH	Unknown	
Ethanone, 1-(4-methyl-1H-imidazol-2-yl)-	124	$C_6H_8ON_2$	RH (0.2%)		Lig	Poirier et al. (2005), Reeves and Francis (1997)

AA amino acids, *ala* alanine, *cys* cysteine, *F* feces, *his* histidine, *hypro* hydroxyproline, *Lig* lignin, *lys* lysine, *Pr* protein, *pro* proline, *RH* ryegrass hay, *thr* threonine, *tyr* tyrosine

predominance of proteinaceous material although it also showed the presence of low concentrations of heterocyclic N such as substituted pyrroles. Pyrroles observed in Cp Py-GC/MS were shown to be thermal degradation products of hydroxyproline (Chiavari et al. 1998). Other N-containing pyrolysis products such as pyrimidines and piperidines which can also originate from protein-derived amino acids

(Niemann et al. 1995; Schulten 1996; Van Arendonk et al. 1997) were exclusively observed in the forage. The N-containing compounds imidazole and methylimodazol-ethanone known to derive from cell wall components (Niemann et al. 1995; Reeves and Francis 1997; Van Arendonk et al. 1997) were also exclusively found in the forage.

Of the N-containing compounds in feces obtained by Cp Py-GC/MS nine were ^{15}N-enriched in the ryegrass hay and three were ^{15}N-enriched in the feces. Some of these ^{15}N enriched compounds were present both in the hay and in the feces, while some were ^{15}N enriched only in the hay or only in the feces. The three ^{15}N enriched pyrolyzates observed in the feces have been derived from proteins or/and for pyridine-4-methyl, from cysteine (Munson and Fetterolf 1987).

Discussion

Pathways of ^{15}N-enrichment of feces

The lower abundance of pyrolyzates derived from cellulose and the higher abundance of pyrolyzates derived from fatty acids, other lipids and polycarboxylic acids found in the sheep feces in comparison to the ryegrass hay reflected well its digestion by the animal.

Dietary N enters the digestive tract of the sheep as non-protein-N (e.g. free amino acids, peptides, nucleic acids, urea, amines, amides, NO_3^-) and protein-N (Leng and Nolan 1984). Most non-protein-N and a large proportion of protein N is converted to NH_4^+ in the rumen whereof a part is used by rumen microbes for their own protein synthesis (Leng and Nolan 1984). Feed N compounds that are not degradable in the rumen are often also resistant to digestion in the small and the large intestine (van der Walt 1993). This indigestible feed N will pass the digestive tract and be recovered as the UDN fraction of the feces. Plant proteins are degraded in the rumen by a two-step process. First extracellular proteases produced by proteolytic microorganisms hydrolyze the amide bonds in peptides releasing low molecular weight peptides and amino acids which are then deaminated to NH_3 by microbial deaminases (Leng and Nolan 1984). The newly synthesized ruminal microbial protein is either digested in the small intestine or excreted with feces. The same holds for microbial proteins synthesized in the hindgut. Microbial proteins make up the major proportion of the BEDN fraction of feces. Additionally, water-insoluble peptides and degradation products may be recovered in the BEDN.

The ^{15}N-enriched compound 1H-pyrrole, 1-methyl- which was identified in ryegrass hay and feces (Table 5) was described to originate from proline by Nguyen et al. (2003). The ^{15}N-enriched 1H pyrrole, 2,5-dimethyl- identified in ryegrass hay and in feces may originate from hydroxyproline (Chiavari and Galletti 1992). Ruminal degradation of proline-containing peptides is known to be low (Wallace et al. 1993). A fraction of the ^{15}N enriched pyrroles found in both the ryegrass hay and in the feces could also be derived from real pyrroles originally present in the ryegrass hay. The presence of a low pyrrole concentration in the feces was suggested by the SS CP/MAS ^{15}N NMR analysis (Fig. 2). Thus ^{15}N-enriched proline-containing peptides and ^{15}N enriched heterocyclic N compounds might have passed the digestive tract unchanged and directly be transferred to the UDN fraction of feces.

In contrast the protein-derived pyridine, 4-methyl- was only present in feces (Table 5). Pyridine, 4-methyl in the pyrolyzates might be derived from cysteine (Chiavari and Galletti 1992). From an isotope study using ^{35}S, Bird and Thornton (1972) concluded that up to 24% of fecal ^{35}S resulted from the transfer of ^{35}S from the blood into the large intestine where it was incorporated into cysteine (and methionine) by bacteria present in the large intestine and excreted as BEDN. Thus pyridine, 4-methyl- may have become ^{15}N-enriched due to de novo synthesis of cysteine by microorganisms and then excreted in BEDN. This microbial de novo synthesis of proteins could explain the higher enrichment of BEDN observed in our study (Table 2).

Were the feces homogeneously labeled with ^{15}N?

The feces were not homogeneously labeled with ^{15}N (Table 2). First of all the grass fed to the sheep could not be homogeneously labeled. The fiber bound N (NDF) was indeed slightly less labeled than the rest of the N (Table 1). Enzymatic reactions controlling N assimilation in plants have been reported to fractionate between ^{14}N and ^{15}N in natural abundance studies

(Werner and Schmidt 2002). The results of the Cp Py-GC/MS analysis also shows this inhomogeneous labeling of the hay as only 9 of the 15 N-containing compounds were ^{15}N-enriched (Table 5). The differential labeling of the N fractions in the feces might also be explained by N isotopic fractionation caused by processes taking place in the digestive tract after the digesta has passed the rumen. This could involve isotopic discrimination taking place by digestive enzymatic reactions and the release of body protein (bile, enzymes, intestinal cells etc.) to the digestive tract which is differently labeled with ^{15}N as it had been synthesized before feeding the ^{15}N enriched ryegrass.

Whereas in absolute terms these feces cannot be considered as homogeneously labeled, we consider as Cusick et al. (2006a), Sørensen and Thomsen (2005) and Berntsen et al. (2007) that these can nevertheless be used in N cycling studies as the ^{15}N enrichment of N released during an incubation period relevant for plant growth remained very close to that of the total N feces. The discussion presented in the next section shows that the results obtained with these labeled feces were actually relevant to study the fate of N in soil/plant systems.

The availability of fecal N to plants

The results presented in this paper explain largely those obtained by Bosshard et al. (2008 and 2009) in a field experiment conducted with the same ^{15}N labeled sheep feces. Figure 3, which is adapted from Bosshard et al. (2009), shows that only 10% of the ^{15}N derived from these feces was taken by wheat in the year of application, while the following soybean took up 3.3% and the following maize 1.5% of this fecal N. The recovery rate of ^{15}N by wheat was 3–4 times higher when N was added as mineral fertilizer or urine. A physical fractionation of soil organic matter on soil sampled after wheat harvest in the same field experiment showed that more ^{15}N had been incorporated in the organo-mineral fraction and in the light fraction when added as urine than when added as sheep feces (Bosshard et al. 2008). These results suggest that more urine-N had been taken up by plants and microorganisms and then recycled as plant and microbial residues into the light fraction and organo-mineral fraction than feces-N, and/or that more NH_4^+ derived from urine had been sorbed onto

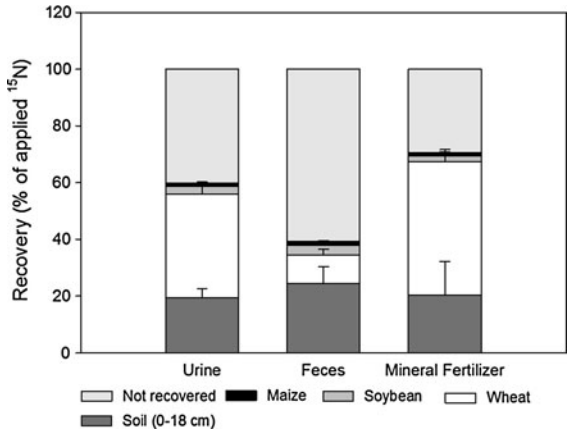

Fig. 3 Recovery of a single N application added as ^{15}N labelled sheep urine, sheep feces or mineral fertilizer (NH_4NO_3) in a field experiment in three subsequent crops (wheat in 2003, soybean in 2004 and maize in 2005) and in the 0–18 cm horizon of soil sampled at maize harvest in 2005. Data adapted from Bosshard et al. (2009)

clay minerals. Altogether these results suggest that these feces were a rather poor source of N for plants and microorganisms. Two hypotheses can be made to explain the low N availability from feces (Bosshard et al. 2009): either the forms of N present in the feces were very stable and slowly mineralized or significant amounts of feces N had been removed from the upper soil horizon of the microplots where ^{15}N labeled feces had been added. The results obtained in the present paper rather support the first hypothesis.

The ten percent of N derived from the feces and taken by wheat are very similar to the 9.4% of N which were released from feces during the first 3 months of incubation under laboratory conditions. Considering the undigested dietary N in the feces as stable, we come to the conclusion that 40% of the N mineralized during the incubation was derived from water soluble N forms (making up 1.3 mg mineralized N g^{-1} DM) while 60% was derived from the bacterial and endogenous debris N (making up 2.0 mg mineralized N g^{-1} DM). The 1.3 mg N g^{-1} DM mineralized from water soluble N forms corresponds to the concentration of NH_4^+ found in the feces by KCl extraction (1.3 mg N g^{-1} DM) and by SS CP/MAS ^{15}N NMR (which would be 1.1 mg N g^{-1} DM if the feces would be homogeneously labeled) suggesting, logically, that NH_4^+-feces is an important source of N for crops. The rest of the mineralized N probably derived from bacterial

proteins present in the BEDN fraction of the feces (Kreuzer and Kirchgessner 1985). Another source of N for crops might have been the amino acids observed in SS CP/MAS ^{15}N NMR analysis as these are known to be rapidly transformed into NH_4^+ and NO_3^- (Jan et al. 2009).

The preceding paragraph discussed the forms of feces N that can be rapidly mineralized, but under which forms is the unavailable feces N? The chemical fractionation shows that a third of feces N are in the undigested dietary fraction. The presence of undigested N compounds deriving from proline or pyrroles in the feces was confirmed by the Cp Py-GC/MS analysis. These forms will probably also be very stable in soils. Proteins can also enter the soil and be stabilized over long periods by various processes as discussed by Rillig et al. (2007). The results presented in this work and in Bosshard et al. (2008, 2009) suggest that a large fraction of the proteins present in feces are stable in the soil.

The low availability of N from ruminant feces for plants has also been observed by Jensen et al. (1999) and Langmeier et al. (2002). Jensen et al. (1999) showed that 6% of N derived from ^{15}N labeled sheep feces was taken up by barley in the year of manure application about 4% of this N was taken up by the following grass and 1–2% by grass in its 2nd year. Langmeier et al. (2002) showed that the recovery of N by *Lolium multiflorum* from ^{15}N labeled cow feces was much lower than that from ^{15}N labeled urine or mineral fertilizer (20% for feces vs. 64% for urine vs. 76% for mineral fertilizer). The coefficient of recovery measured by Langmeier et al. (2002) was higher than what has been observed by Bosshard et al. (2009). However, the fraction of undigested dietary N was lower in the cow feces than in the sheep feces and Langmeier et al. (2002) included six harvests of ryegrass grown in small pots in a glasshouse thereby maximizing soil exploration by roots.

Altogether we can conclude that low content in ammonium and amino acids, the low rate of N release from these feces during the incubation and the relatively high fecal protein content, particularly the hard to mineralize undigested and microbially bound forms, can explain the low transfer of N from sheep feces to crops.

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