Risø-PhD-14(EN)

Production of N_2O in grass-clover pastures

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Denmark
September 2005

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This thesis is submitted in partial fulfilment of the requirements for the Ph.D. degree at the Royal Veterinary and Agricultural University, Denmark.

Abstract:
Agricultural soils are known to be a considerable source of the strong greenhouse gas nitrous oxide (N_2O), and in soil N_2O is mainly produced by nitrifying and denitrifying bacteria. In Denmark, grass-clover pastures are an important component of the cropping system in organic as well as conventional dairy farming, and on a European scale grass-clover mixtures represent a large part of the grazed grasslands. Biological dinitrogen (N_2) fixation in clover provides a major N input to these systems, but knowledge is sparse regarding the amount of fixed N_2 lost from the grasslands as N_2O. Furthermore, urine patches deposited by grazing cattle are known to be hot-spots of N_2O emission, but the mechanisms involved in the N_2O production in urine-affected soil are very complex and not well understood.
The aim of this Ph.D. project was to increase the knowledge of the biological and physical-chemical mechanisms, which control the production of N_2O in grazed grass-clover pastures. Three experimental studies were conducted with the objectives of:

I assessing the contribution of recently fixed N_2 as a source of N_2O
II examining the link between N_2O emission and carbon mineralization in urine patches
III investigating the effect of urine on the rates and N_2O loss ratios of nitrification and denitrification, and evaluating the impact of the chemical conditions that arise in urine-affected soil

The results revealed that only 3.2 ± 0.5 ppm of the recently fixed N_2 was emitted as N_2O on a daily basis. Thus, recently fixed N released via easily degradable clover residues appears to be a minor source of N_2O. Furthermore, increased N_2O emission following urine application at rates up to 5.5 g N m^{-2} was not caused by enhanced denitrification stimulated by labile compounds released from scorched plant roots. Finally, the increase of soil pH and ammonium following urine application led to raised nitrification rate, which appeared to be the most important factor explaining the high initial N_2O emission from simulated urine patches.
The results are discussed in relation to the national N_2O inventory guidelines issued by the Intergovernmental Panel on Climate Change, and the environmental impact of organic farming practises are also considered. Suggestions for future research are outlined.

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ISBN 87-550-3463-2

Sponsorship:
The Danish Agricultural and Veterinary Research Council, and the Danish Research Centre for Organic Farming (DARCOF).

Cover :
Organic steers grazing a mixture of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) at Østeragergård, Osted. Photo: Henning Høgh Jensen

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Preface

This thesis constitutes a part of the requirements for obtaining the Doctor of Philosophy (Ph.D.) degree at the Royal Veterinary and Agricultural University (KVL). The Ph.D. project was carried out at the Ecosystems Programme, Biosystems Department, Risø National Laboratory. In addition, experimental work was conducted at Institut National de la Recherche Agronomique (INRA), Clermont-Ferrand, France, and at Højbakkegård, KVL Taastrup. The work was funded by the Danish Agricultural and Veterinary Research Council, and the Danish Research Centre for Organic Farming (DARCOF).

The Ph.D. project was part of the DARCOF project 'Dinitrogen Fixation and Nitrous Oxide Losses in Organic Grass-clover Pastures' (DINOG), which aimed at estimating the amount of nitrous oxide (N_2O) emitted from grazed grass-clover pastures. Data obtained by each partner in the project was made available for incorporation into the whole-farm nitrogen flow model, FASSET. During the Ph.D. study I have been enrolled in the Research School for Organic Agriculture and Food Systems (SOAR).

The thesis consists of two parts. Part A is an introduction to the thesis work, which describes the background of the project and gives an overview of the experimental work conducted. Outcomes of the work and the perspectives are discussed. Part B includes three paper manuscripts based on the three experimental studies conducted.

I would like to thank Per Ambus for his very competent supervision in all aspects of experimental research – ranging from silicone sealing to data analysis. I also wish to thank my supervisor at KVL, Henning Høgh Jensen, for sharing his knowledge when I had specific questions related to grass-clover and farming practises. The warm welcome to Clermont-Ferrand by Dr. Jean-François Soussana and his group is greatly acknowledged – a special thanks to Florence Teyssonneyre and Katja Klumpp for great collaboration. Also thanks to Dr. Xavier Le Roux for a very fruitful cooperation on one of the paper manuscripts from which I learned a lot. I also wish to acknowledge Liselotte Meltofte and Anja Nielsen for their assistance in the laboratory. Furthermore, Daniel Carter and Jane Hansen are acknowledged for their great help regarding language editing and proofreading of the thesis. Finally, I owe a dept of gratitude to Daniel for his full support and his big effort at home, especially during the last six months.

Sammendrag på dansk

Agerjorder er kendt for at bidrage betydeligt til udledningen af den kraftige drivhusgas lattergas (N_2O), og i jorder produceres N_2O primært af nitrifiserende og denitrifiserende bakterier. I Danmark er kløvergræs en vigtig bestanddel af sædsskiftet på både økologiske og konventionelle mælkebrug, og på europæisk plan udgør kløvergræs en stor del af de græssede arealer. Kvælstof (N) tilføres primært til disse systemer via biologisk N_2 fiksering i kløverplanten, men der mangler viden om hvor meget af det fikserede N_2 der udledes fra marken som N_2O. Derudover ved man at der sker stor N_2O udledning fra urinpletter afsat af græssende kvæg, men de mekanismer der ligger til grund for N_2O dannelsen i urinpletter er meget komplekse og ikke kendt i detaljer.

Det overordnede formål med dette Ph.D. projekt var at uddybe forståelsen af de biologiske og fysisk-kemiske mekanismer der er styrende for N_2O dannelsen i en græsset kløvergræs mark. Der blev udført tre eksperimentelle forsøg, som havde til formål at:

I kvantificere andelen af nyligt fikseret N_2 der frigives som N_2O

II undersøge sammenhængen mellem N_2O dannelse og kulstofmineralisering i urinpletter

III studere effekten af urin på hastigheden og N_2O tabsratioen for nitrifikationen og denitrifikationen, samt evaluere betydningen af de kemiske forhold som optræder i urinpletter

Resultaterne viste at kun 3.2 ± 0.5 ppm af det nyligt fikserede N_2 blev udledt som N_2O på daglig basis. Nyligt fikseret N frigjort via letomsætteligt kløvermateriale lader derfor til at være en ubetydelig kilde for N_2O produktionen. Derudover viste resultaterne at øget N_2O udledning efter tilførsel af urin i doser på op til 5.5 g N m^{-2} ikke skyldtes øget denitrifikation stimuleret af labile forbindelser frigivet fra afsvedne planterødder. Endelig fremgik det at øget pH og ammonium indhold i jorden efter urintilførsel forøgede hastighed af nitrifikationen, hvilket lod til at være den vigtigste årsag til den høje N_2O udledning umiddelbart efter urintilførsel.

Resultaterne diskuteres i relation til retningslinierne for de nationale N_2O opgørelser som er udgivet af 'Intergovernmental Panel on Climate Change'. Derudover bliver økologisk jordbrugs påvirkning af miljøet berørt, og forslag til fremtidige forskningsaktiviteter bliver fremlagt.

1 Background

Agricultural soils are known to be a considerable source of nitrous oxide (N_2O) (Kroeze et al., 1999) and at present this N_2O accounts for 5 % of the European release of anthropogenic derived greenhouse gases (EEA, 2002). Furthermore, N_2O is involved in ozone depletion in the stratosphere (Crutzen, 1981). In soils, N_2O is mainly produced in the bacterial processes of nitrification and denitrification (Granli and Bøckmann, 1994) (see Section 1.2). The level of soil inorganic nitrogen (N) influences the rate of emission. Therefore organic farming has been suggested as a means to reduce agricultural N_2O emissions, since no mineral fertilizer is used in this farming system and because the N input is lower than in conventional farming (Dalgaard et al., 2000; Kotschi and Müller-Sämann, 2004).

In organic as well as conventional dairy farming, grass-clover pastures are an important component of the cropping system. This is because grass-clover is an excellent cattle fodder, and because clover has the ability of fixing atmospheric dinitrogen (N_2) (see Section 1.1). According to the guidelines issued by the Intergovernmental Panel on Climate Change (IPCC), inventories for N_2O emissions from agricultural soils should be based on the assumption that 1.25 % of the total N supply is emitted as N_2O (IPCC, 1997). This emission factor is used as a standard for mineral fertilizer, manure and biologically fixed N_2, although the factor relies on experiments with fertilizer and manure only (Bouwman, 1996). The emission factor for biologically fixed N_2 may be lower than 1.25 %, because N is released only slowly into the soil. However knowledge is very sparse (see Section 1.5).

In Denmark, organic cattle must have access to grazing fields at least 150 days per year, and grazing cattle are known to promote substantial N_2O emission (Oenema et al., 1998; Anger et al., 2003). Nitrogen returned to the soil via urine and dung is likely to locally exceed the needs of the plants and is therefore at risk of being lost as N_2O. In addition, soil compaction due to treading may enhance denitrification, and thereby lead to increased N_2O losses. At present, however, the mechanisms responsible for the grazing-derived N_2O emissions are only partly understood.

The aim of this Ph.D. project was to increase the knowledge of the biological and physical-chemical mechanisms, which control the production of N_2O in grazed grass-clover pastures. Two specific topics were selected as areas of focus. Firstly, biologically fixed N_2 as a source of N_2O was selected, since very little is known about this topic. Secondly, the effect of urine deposition on the microbial nitrification and denitrification processes was selected. The reason for the latter choice is that urine deposition is possibly the most important factor influencing the N_2O loss from grazed pastures, partly because 60-65 % of N excreted by cattle is found in urine (Oenema et al., 1997) (see Section 1.4). The theoretical background of the Ph.D. project is described in more detail below.

1.1 Grass-clover swards in organic and conventional farming

About 3 % of the agricultural area in the European Union is managed by organic farming, but substantial differences in the proportion exist between the individual countries (Willer and Yussefi, 2005). In Denmark, approximately 6 % of the agricultural

area is under organic management (Danish Plant Directorate, 2004). In organic farming, the plant-soil system receives N via animal manure and via N_2 fixing grain or forage legumes. Grass-clover mixtures are almost an obligatory element of the organic crop rotation in Denmark, and in this way the Danish practise is unique compared to other countries. The grass-clover sward is used for forage and green manure, and typically persists for 2-3 years in the crop rotation. As grass-clover is an excellent cattle fodder, it is an important component of the cropping system in organic as well as conventional dairy farming, and here the swards may persist for a longer period.

In the farmers' yearly report on area utilization to the Danish Food Industry Directorate, the areas covered by grass-clover and pure grass are reported all together. Grass and grass-clover swards accounted for 32 % the area managed by organic farming in 2003, whereas its proportion of the conventionally managed area was just 13 % (Table 1). Thus, grass-clover swards are more abundant in organic farming than it is in conventional farming. Organic farming deviates from conventional farming by showing particular respect for the environment, nature and animal welfare (DARCOF, 2000). In Denmark, organic livestock must have access to grazing fields at least 150 days per year in order to meet with the aim regarding animal welfare (Danish Plant Directorate, 2005). About 88 % of the organic grass and grass-clover swards was grazed by livestock in 2003 and this proportion was similar for the conventional swards (Table 1).

Table 1. The agricultural area in Denmark, which was covered by organic and conventional grass and grass-clover swards in rotation as well as permanent swards in 2003 [a]

	Organic [b]		Conventional [c]	
	In rotation	Permanent	In rotation	Permanent
Grazed swards (1000 ha)	24	18	151	132
Ungrazed swards (1000 ha)	4	1	33	9
Swards (% of area)	19	13	7	6
Agricultural area (1000 ha)	149		2509	

[a] Permanent grass and grass-clover swards does not include fallow fields

[b] Area under conversion not included; Danish Plant Directorate (2004)

[c] Overall data for Danish agriculture was obtained from Statistics Denmark (2004) and Danish Food Industry Directorate, pers. comm.

On a European scale about 40 % of the agricultural area is covered by permanent grassland used for livestock farming (FAO, 2004). The grasslands range from intensively fertilized pure grass swards to extensively managed grass-legume mixtures and semi-natural grasslands, which are often found in mountainous areas or on moist lowland soils. The starting point for the present thesis is N_2O production in organic mixtures of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) grazed by dairy cattle. However, as focus is on the process level the results are also valid for conventional grass-clover swards, and part of the results are relevant for extensively managed grasslands in general.

1.2 Processes producing N_2O and the controlling factors

The bacterial processes of nitrification and denitrification are the most important sources of N_2O in soil (Granli and Bøckmann, 1994), and they will be described in more detail below. Other processes contribute substantially to the N_2O production under some conditions. In an Irish grassland for example, most of the N_2O was found to derive from fungal codenitrification (*i.e.* denitrification where nitrite-N (NO_2^-) combines with N from other sources) (Laughlin and Stevens, 2002). At low pH, N_2O may be formed via chemodenitrification, which is the chemical decomposition of nitrous acid (HNO_2) following reaction with organic (e.g. amines) or inorganic (e.g. Fe^{2+}) compounds (Van Cleemput, 1998).

N_2O from nitrification

Nitrification refers to the two-step process, in which ammonia (NH_3) is oxidised to nitrite, and nitrite is further oxidised to nitrate (NO_3^-) (Fig. 1). Most nitrification is conducted by chemoautotrophic bacteria (Simek, 2000), and two different groups of bacteria are involved at the two steps, namely NH_3-oxidizers and NO_2^--oxidizers (Bock et al., 1986). Although the true substrate for the NH_3-oxidizers is NH_3 (Wood, 1986), ammonium (NH_4^+) is often referred to as the substrate for nitrification, which is probably because NH_4^+ is in equilibrium with NH_3. The nitrifying bacteria gain energy to fix carbon dioxide (CO_2) to organic carbon (C) by oxidizing the reduced N compounds. In addition to autotrophic nitrification, heterotrophic nitrification is also known, which appears to be more common among fungi than bacteria (Wrage et al., 2001). The heterotrophic microorganisms do not gain energy from the nitrification process, but use organic carbon as energy and C source. However, heterotrophic nitrification is generally regarded as a minor source of N_2O (Wrage et al., 2001). Below, nitrification will therefore refer to autotrophic nitrification only.

Nitrifying bacteria are usually considered as weaker competitors for NH_4^+ than heterotrophic microorganisms and plant roots (e.g. Verhagen et al., 1995). Compared to plant roots, though, the spatial distribution of microorganisms matches better with the distribution of available NH_4^+ in the soil, as both may be present on soil particle surfaces

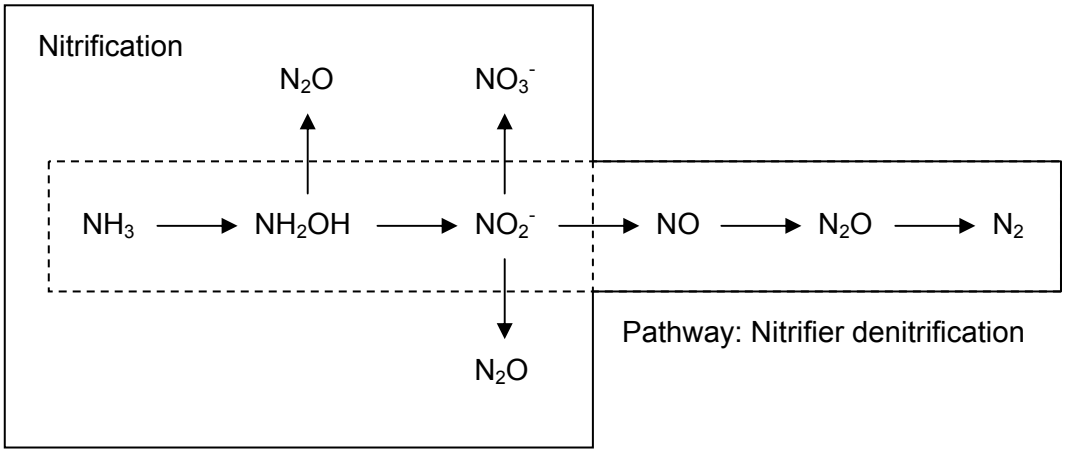

Figure 1. Chemoautotrophic nitrification, including the nitrifier denitrifcation pathway. Modified after Wrage et al. (2001).

(Jackson et al., 1989). This enables significant nitrification rates in grassland soils (Firestone and Davidson, 1989).

Nitrifiers produce N_2O in two ways, by nitrification and by nitrifier dentrification (Wrage et al., 2001) (Fig. 1). In nitrification, N_2O is formed as a by-product of NH_3 oxidation due to chemical decomposition of intermediates such as NH_2OH or the product NO_2^- itself. This is usually considered a special form of chemodenitrification. In addition, incomplete oxidation of NH_2OH may lead to production of N_2O. Nitrifier denitrification is a pathway of nitrification, which is performed by only one group of microorganisms, namely autotrophic NH_3-oxidizers. In nitrifier denitrification, the oxidation of NH_3 to NO_2^- is followed by the reduction of NO_2^- to N_2O and N_2 (Fig. 1). Thus, N_2O is produced as an intermediate in the pathway. Most often authors do not distinguish between N_2O production via nitrification and nitrifier denitrification, partly because a simple method to differentiate between the two sources of N_2O is still lacking. Nitrifier denitrification takes place when oxygen is limiting due to the fact that NH_4^+ oxidizing bacteria use NO_2^- as an electron acceptor instead of O_2 (Poth and Focht, 1985). This mechanism not only allows the organisms to conserve limited O_2 for the oxidation of NH_4^+, but also avoids the concentration of NO_2^- to reach toxic levels and maintains optimal redox levels (Hutchinson and Davidson, 1993).

Factors affecting production of N_2O via nitrification

The rate of N_2O production via nitrification is determined by two parameters, being 1) the rate of nitrification and 2) the ratio of N_2O-N lost per NO_3^- produced. The two parameters are illustrated in Figure 2 as the flow through the pipe and the hole in the pipe, respectively (Firestone and Davidson, 1989). They are affected by environmental factors independently of each other.

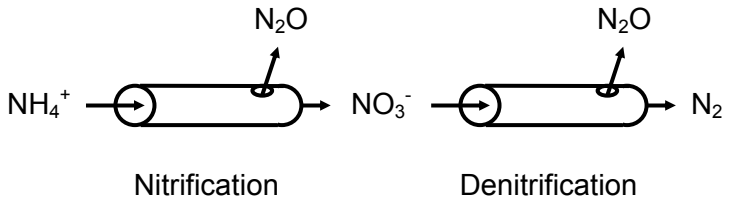

Figure 2. An illustration of the two parameters that influence the loss of N_2O from nitrification and denitrification. Modified after Firestone and Davidson (1989).

The key factors influencing the rate of nitrification are the concentration of NH_4^+ and of free oxygen (O_2), by being substrates for the process (Firestone and Davidson, 1989). At low water content, bacterial activity and transport of NH_4^+ is restrained. However, at high water content, diffusion of O_2 is slow. Grundmann et al. (1995) found that in a sandy loam soil maximum nitrification occurred at 50 % water filled pore space (WFPS). Optimum temperature for nitrification in this study was 25 °C, however the optimum temperature may vary among soils and compared to the other factors temperature is less important for the rate of the process (Schmidt, 1982; Firestone and Davidson, 1989). The pH optimum for the process appears to be about 8 (Kyveryga et al., 2004, and references within). The rate of nitrification decreases below pH 6 and the process typically stops at

pH 4 (Schmidt, 1982). In temperate areas nitrification is usually highest in spring and autumn, and lowest in summer and winter (Paul and Clark, 1996).

The ratio of N_2O/NO_3^- produced (hole in the pipe) rises when the oxygen level decreases (Goreau et al., 1980) and when the pH decreases (Martikainen, 1985). However, at both of these conditions the rate of nitrification is low (Firestone and Davidson, 1989). Usually, the N_2O/NO_3^- ratio of nitrification is less than 1 % (Firestone and Davidson, 1989; Hutchinson and Davidson, 1993).

N_2O from denitrification

Nitrate produced in nitrification can be subject to 1) assimilation by plants or microorganisms, 2) leaching, 3) dissimilatory reduction to NH_4^+, or 4) reduction to NO, N_2O or N_2 via denitrification (Tiedje, 1988). Several processes are involved in the reduction of NO_2^- or NO_3^- to NO, N_2O or N_2, and could thus fit within a broad definition of denitrification. Among these processes are chemodenitrification, respiratory NO_3^- reduction by microbes (typically referred to as denitrification) and nonrespiratory NO_3^- reduction (Tiedje, 1988). The nonrespiratory processes that produce N_2O include bacterial NO_3^- assimilation and bacterial dissimilation of NO_3^- to NH_4^+. In the following, denitrification will refer only to anaerobic respiration in bacteria during which ATP formation is coupled to electron transport from an inorganic or organic source to nitrogen oxides via the cytochrome system (Tiedje, 1988; Paul and Clark, 1996). The process is shown in Figure 3.

Figure 3. Pathway of denitrification (Granli and Bøckman, 1994).

Denitrifying bacteria prefer O_2 for respiration, however when O_2 is lacking they are capable of using nitrogen oxides instead (Tiedje, 1988). Denitrification is mostly done by heterothrophic bacteria, which use organic carbon compounds as their energy source, cell C source and electron donor (Paul and Clark, 1996). All denitrifying bacteria are able to complete the whole process, however sometimes they stop when reaching the NO- or N_2O-step (Firestone and Davidson, 1989).

Factors affecting production of N_2O via denitrification

The rate of N_2O production via denitrification depends on two parameters, just as in the case of nitrification. These are the rate of denitrification and the ratio of N_2O-N lost per N_2+N_2O produced (Fig. 2). Requirements for denitrification to occur are: 1) the presence of bacteria having the metabolic pathway, 2) the availability of suitable reductants such as carbon, 3) a low level of oxygen and 4) the supply of NO_3^- or other nitrogen oxides (Firestone and Davidson, 1989). It can be assumed that if the requirements of carbon, N-oxides, and limited O_2 are fulfilled, then bacteria having the metabolic pathway will occupy the denitrification niche. Presence of O_2 will most often be the limiting factor for denitrification, followed by NO_3^- availability in unfertilized soils and carbon availability in well-aerated soils (Tiedje, 1988; Robertson, 1989).

The supply of O_2 in the soil is determined by soil moisture and soil structure (Robertson, 1989; Hutchinson and Davidson, 1993). Denitrification generally occurs when the soil water content is high enough to restrict the supply of O_2 via diffusion (Hutchinson and Davidson, 1993). Thus, denitrification is usually associated with soil water content above 60 % WFPS (Davidson, 1991). Supply of NO_3^- is a requirement for denitrification. As formation of NO_3^- via nitrification needs aerobic conditions, while denitrification needs anaerobic conditions, high denitrification rates especially occur when the conditions chance from aerobic to anaerobic. This chance may take place in time, for example during wetting-up of dry soil by rainfall (Nieder et al., 1989). The chance may also take place in space; for instance the conditions may be aerobic outside a soil aggregate, while it is anaerobic inside the aggregate (Tiedje, 1988).

The denitrification process is dependent on carbon availability (Beauchamp et al., 1989; Benckiser, 1994). A study including 17 soils showed that denitrification capacity was correlated with total soil organic carbon content, but much better correlations existed with water-soluble or easily decomposable organic carbon (Burford and Bremner, 1975). One reason for the latter may be that O_2 was consumed during decomposition of the labile compounds and thereby increased the demand for nitrogen oxides as electron acceptor (Tiedje, 1988; Azam et al., 2002). Another reason may be that the denitrifying bacteria prefer the easily decomposable organic matter as their energy source, cell C source and electron donor. In line with this, a study conducted at 60-90 % WFPS demonstrated that the denitrification rate was limited by the supply of easily decomposable carbon at high NO_3^- availability (Weier et al., 1993).

Denitrification occurs when the temperature is between 5 and 75 °C, and the rate increases with temperature (Paul and Clark, 1996). Furthermore, the rate of denitrification is influenced by soil pH, being less in acidic soils than in neutral or slightly alkaline soils (Simek and Cooper, 2002). The effect of pH may be indirect, for example the availability of organic carbon and N mineralization are reduced under acidic conditions, resulting in a smaller microbial community and, thus, a smaller denitrifying component. There exists no universal optimum for denitrification, since the denitrifying microflora can adapt to soil pH (Simek and Cooper, 2002). Annual denitrification rates in grasslands have been reported in the range 1-28 kg N ha^{-1} (Table 2).

The ratio of N_2O/N_2 produced depends on the availability of oxidant (e.g. nitrate) and reductant (e.g. organic carbon), on the O_2 availability, and on the overall rate of denitrification. When the availability of oxidant exceeds the supply of reductant to a great extent, then the nitrogen oxide may be incompletely reduced, thus resulting in a high N_2O/N_2 ratio of end products (Firestone and Davidson, 1989; Hutchinson and Davidson, 1993; Weier et al., 1993). In contrast, when the rate of denitrification is limited by the supply of oxidant, nearly all of the N oxide is reduced to N_2.

Oxygen seems to inhibit the synthesis and activity of all enzymes involved in denitrification (*viz.* NO_2^- reductase, NO reductase and N_2O reductase), but the expression of the enzymes show different O_2 sensitivity, which furthermore deviate among different species of denitrifiers (Tiedje, 1988; Conrad, 1996). When O_2 availability in the soil changes the N_2O/N_2 product ratio of denitrification depends on the effect of O_2 on the expression of these enzymes. In general, the N_2O/N_2 ratio increases with increasing O_2 concentrations (Tiedje, 1988).

An analysis based on the Michaelis-Menten model of enzyme kinetics predicts that the fraction of N_2O should increase whenever an environmental factor slows the overall rate

of reduction below the maximum that can be supported by the existing enzyme (Betlach and Tiedje, 1981). This is supported by numerous studies showing that when the pH of a soil is decreased, denitrification liberates more N_2O and the N_2O/N_2 ratio is increased (Simek and Cooper, 2002).

Table 2. Annual denitrification rates in grasslands. References form Nieder et al. (1989) and Barton et al. (1999)

Location	Vegetation	Fertilizer kg N $ha^{-1}y^{-1}$	Denitrification kg N $ha^{-1}y^{-1}$	
New Zealand	Pasture	0	5	[a]
New Zealand	Pasture	0	4.5	[b]
New Zealand	Grass-clover	0	3.4	[c]
The Netherlands	Ryegrass	250	24.6	[d]
United Kingdom	Grass	100	10	[e]
United Kingdom	Permanent grass	250	11	[f]
United Kingdom	Grass	200	1-28	[g]

[a] Ledgard et al. (1997), [b] Luo et al. (1994), [c] Ruz-Jerez et al. (1994), [d] De Klein and Van Logtestijn (1994), [e] Barraclough et al. (1992), [f] Ryden (1983), [g] Egginton and Smith (1986)

Total N_2O loss from nitrification and denitrification

Inspired by "hole-in-the-pipe" model of Firestone and Davidson (1989; Fig. 2) an equation was constructed, which describes the total loss of N_2O from nitrification and denitrification (E):

$$E = N \times L_N + D \times L_D \ ,$$

where N and D are the rates of nitrification and denitrification, respectively, L_N is the N_2O loss ratio of nitrification (*i.e.* N_2O-N lost per NO_3^- produced) and L_D is the N_2O loss ratio of denitrification (*i.e.* N_2O-N lost per N_2+N_2O produced).

The formed N_2O will diffuse towards the soil surface, but before it escapes into the atmosphere some N_2O may be reduced to N_2 by bacteria situated other places in the soil (Davidson, 1991). Organisms reported to be capable of reducing N_2O include 1) denitrifying bacteria, 2) some of the bacteria that dissimilate NO_3^- to NH_4^+ and 3) autotrophic NH_3-oxidizing bacteria (*i.e.* via nitrifier denitrification) (Poth, 1986; Conrad, 1996).

High rates of N_2O emission are most often related to denitrification activity rather than to nitrification, but denitrification rates are spatially and temporally very variable (Firestone and Davidson, 1989). In contrast, nitrification is a rather constant process and may therefore give rise to considerable N_2O losses (Firestone and Davidson, 1989).

1.3 N_2O emission from grassland

Estimates of annual N_2O emissions from ungrazed grasslands fertilized with less than 200 kg N ha^{-1} y^{-1} have been reported in the range 0.2-1.6 kg N ha^{-1} (Table 3). Velthof et al. (1996) observed large N_2O losses from grassland during spring, summer and autumn but relatively small losses during winter. Ambus and Christensen (1995) found that N_2O emissions from grassland peaked in autumn. Soil water content is often found to be a key factor controlling N_2O emissions from grasslands (e.g. Ruz-Jerez et al., 1994; Carran et al., 1995; Merino et al., 2001a). Under certain conditions grassland soils may act as net sinks of atmospheric N_2O due to high N_2O reduction activity (Glatzel and Stahr, 2001; Wrage et al., 2004a).

In a nitrogen-15 (^{15}N) labelling experiment from May to September, both nitrification and denitrification was found to contribute to the N_2O production in a grass-clover pasture (Ambus, 2005). However, nitrification seemed to be the major source. Kerster et al. (1997) measured N_2O emission from fertilized (325 kg N ha^{-1}) perennial ryegrass sward. Nitrification dominated the N_2O production in spring, whereas denitrification was the main source of N_2O in autumn. The N_2O loss ratio of denitrification ($N_2O/(N_2+N_2O)$) varied between 8 and 21 %, the median being 15 %. Merino et al. (2001b) found that denitrification contributed with about 80 % of the N_2O emission from an unfertilized Spanish grass-clover sward in autumn. From the results it appears that the N_2O loss ratio of denitrification in this study was about 40 %, whereas less than 0.2 % of the NH_4-N entering nitrification was lost as N_2O.

Table 3. Annual N_2O emissions from grasslands

Location	Vegetation	Fertilizer kg N $ha^{-1}y^{-1}$	N_2O emission kg N_2O-N $ha^{-1}y^{-1}$	
The Netherlands	Ryegrass	0	0.5-1.0	[a]
Germany	Diverse	0-80	0.2-1.2	[b]
Germany	Grass-clover	175	1.3-1.6	[c]
Germany	Diverse	0-120	0.2-0.4	[d]

[a] Velthof et al. 1996, [b] Kammann et al. 1998, [c] Kaiser et al. 1998, [d] Anger et al. 2003

In a ryegrass pot experiment, Beck and Christensen (1987) showed that N_2O emission increased when all above-ground grass was removed. In a similar way, N_2O emission increased as grass leaves turned yellowish. The results indicated that easily available organic matter from the roots stimulated the denitrification when the plants were damaged and during senescence. Thus, the authors expected that grass cutting might result in a marked N loss through denitrification. Kammann et al. (1998) found that increasing the number of cuts per year (2, 3 and 6) reduced N_2O emissions from a semi-natural non-grazed grassland. The authors concluded that the ability of plant roots to take up NO_3^- increased with increasing cutting frequencies, therefore reducing the amount of NO_3^- available for soil denitrifying microorganisms. In contrast, Kaiser et al. (1998) reported small increases in the N_2O emission after cutting of grass-clover swards.

Yamulki et al. (1997) observed that N_2O emission from grassland decreased with increasing acidity (pH 7.6, 5.9 and 3.9). This fits with the reported decline in the rate of nitrification and denitrification in acidic soils.

1.4 N_2O formation in urine patches

Excretion of N via urine

The major part of the Danish grass-clover swards are grazed by dairy cattle (Table 1) and the same is the case for a large part of the European grasslands. Dairy cows grazing on grass-clover pastures excrete 60-65 % of the excess N intake via urine (Oenema et al., 1997). The concentration of N in urine may vary from 1 to 20 g N l^{-1}, as a result of large variations in the N content of the diet and in the intake of water. Urea is the major component of urine and typically accounts for over 70 % of the urine-N content. Oenema et al. (1997) estimated the N deposition rate to vary between about 20 and 80 g N m^{-2} in cattle urine patches. In comparison, Vertès et al. (1997) measured the amount of urine-N deposited by grazing heifers and found that 90 % of the urine patches contained between 3 and 50 g N m^{-2}. The variability was due to variation in volume and N concentration of urine.

Chemical conditions in urine-affected soil

Urine patches deposited on the pasture are known to be hot-spots for N_2O formation (e.g. Allen et al., 1996;Yamulki et al., 1998). Williams et al. (1999) estimated that N_2O losses within the first 24 hours after urine deposition accounted for approximately 8 % of the annual N_2O emission from a grassland. Sherlock and Goh (1983) found that more N_2O was emitted after application of urine-N than after equivalent amounts (20 g N m^{-2}) of urea-N or ammonium-N dissolved in similar volume of liquid, and the difference was mainly related to the initial effect on the N_2O emission. Thus, elevated soil water content and availability of inorganic N only explain part of the urine-induced N_2O emission.

The mechanisms responsible for the raised N_2O production in urine-affected soil are very complex and not well understood. They may vary according to abiotic factors, e.g. soil type, moisture and pH (Clough et al., 1998; Clough et al., 2004), and the amount of urine-N deposited (Petersen et al., 2004). It is well known that soil pH increases temporarily following urine deposition. This is because of enzymatic hydrolysis of urea, which is typically completed within 24 hours after deposition (Haynes and Williams, 1992; Bol et al., 2004). Urea hydrolysis is expressed as

$$(NH_2)_2CO + 3\ H_2O \rightarrow 2\ NH_4^+ + OH^- + HCO_3^-$$

The alkaline products cause an increase in soil pH. Hydrogen carbonate ion (HCO_3^-) is the conjugate base of carbonic acid (H_2CO_3), which is relatively unstable and decomposes into CO_2 and water. Ammonium is in equilibrium with dissolved ammonia ($NH_{3(aq)}$) in the soil solution, and the equilibrium is dependent on soil pH. At pH 6, 7, 8 and 9 $NH_{3(aq)}$ accounts for 0.1, 1, 10 and 50 % of the ammoniacal pool, respectively (Schmidt, 1982). Thus, the pH increase following urea hydrolysis shifts the NH_4^+ – $NH_{3(aq)}$ equilibrium towards $NH_{3(aq)}$. Dissolved ammonia is furthermore in equilibrium with gaseous ammonia ($NH_{3(g)}$) in the air-filled pore space of the soil, and $NH_{3(g)}$

volatilization from urine patches may vary between 3 and 52 % of urine-N (Petersen et al., 1998).

As a result of urea hydrolysis, the level of soil NH_4^+ increases immediately following urine application, whereas a lag phase of 2-14 days often passes before NO_3^- accumulates (Thomas et al., 1988; Bol et al., 2004). The reason for the delay of nitrification is initial microbial stress caused by $NH_{3(aq)}$ toxicity and/or low osmotic potential. In soils at near-neutral pH, nitrification is probably only inhibited by these stress conditions when the urine-N concentration exceeds 16 g N l^{-1} (Monaghan and Barraclough, 1992). Soil pH in urine patches decline gradually as a result of NH_3 volatilization, nitrification and plant uptake of NH_4^+. In a soil core study, pH returned to near original level within 40 days after urine application (Clough et al., 2004).

Mechanisms suggested to be involved in urine-induced N_2O formation

In general, the N_2O emission rises immediately after urine application (e.g. Allen et al., 1996; Flessa et al., 1996; Koops et al., 1997, Yamulki et al., 1998). Nitrite oxidation is more readily inhibited by $NH_{3(aq)}$ and/or low osmotic potential than the NH_4^+ oxidation (Harada and Kai, 1968), which in some cases leads to NO_2^- accumulation in urine-affected soil (e.g. Petersen et al., 2004). Thus, the initial N_2O could be produced following oxidation of urea-derived NH_4^+, either due to chemical decomposition of NO_2^- or due to reduction of NO_2^- via nitrifier denitrification (Fig. 1). This is consistent with Wrage et al. (2004b), who observed increased N_2O production via nitrifier denitrification in urine-affected soil. Sherlock and Goh (1983) suggested that the initial N_2O might be produced via chemodenitrification, which they defined as a chemical reaction between minor urine components (e.g. amino acids) and soil constituents. Koops et al. (1997) found that initial N_2O from urine applied on very dry top-soil was mainly produced via nitrification, however, in less dry soil denitrification was reported to be the dominant source of the initial N_2O (De Klein and Van Logtestijn, 1994).

Williams et al. (1999) proposed that the initial N_2O was not derived from urine-N, but rather a result of urine-induced increase of dissolved organic carbon (DOC), which promoted enhanced denitrification of soil N. However, concentrations of soluble C in cow urine are too low to account for large increases in metabolic activity in soil (Lovell and Jarvis, 1996). The content of DOC in the soil increases following urine application because of solubilization of organic C caused by increased pH and $NH_{3(aq)}$ concentration (Monaghan and Barraclough, 1993), but these compounds can be rather recalcitrant (Petersen et al., 2004). Finally, the content of labile DOC may increase following urine application because of release of carbon compounds from plant roots scorched by $NH_{3(aq)}$ (Shand et al., 2002). Monaghan and Barraclough (1993) suggested that these labile compounds stimulate denitrification activity and thereby are part of the reason for the initial N_2O emission from urine patches. However, studies that focus on this mechanism are rare, whereas plant scorching per se is a well-known phenomenon. Several studies have reported that plants were scorched by NH_3 following applications of high rates of urine-N (> 20-30 g N m^{-2}) (e.g. Petersen et al., 1998; Ritchey et al., 2003). Scorching was typically detected as shoot death; either caused by root death after exposure to dissolved ammonia (Richards and Wolton, 1975) or because the shoot material had been in direct contact with NH_3 (Petersen et al., 1998).

The hypothesis that initial N_2O-N is mainly derived from soil N was supported by the study of Bol et al. (2004), which showed that urea-derived N only accounted for 3-4 %

of the N_2O emission on the day of urine application. This fraction increased to 13-23 % when measured 14 days after application. They observed dilution of the pool of NH_4^+ derived from urea-N, which they ascribed to root damage caused by scorching or to exchange with the microbial pool in connection with an osmoregulation response. Also, a laboratory study revealed that other sources than urea-N contributed to the N_2O emission (Clough et al., 2004). As possible sources the authors suggested glycine added in the urine, and soil inorganic N released via mineralization or deamination of soil organic matter under the high pH condition following urea hydrolysis. Furthermore, the results indicated that the N_2O production probably occurred at sites of original soil pH following diffusion of inorganic N from the urine-affected zone. In another laboratory study, analysis of phospholipid fatty acid composition indicated that initial inhibitory effects of urine on the microbial population after a few days were replaced by increased microbial activity and net growth (Petersen et al., 2004).

The N_2O emission usually peaks 10-20 days after urine application (Monaghan and Barraclough, 1993; Allen et al., 1996; Flessa et al., 1996; Lovell and Jarvis, 1996; Yamulki et al., 1998; Clough et al., 1996; Van Groenigen et al., 2005b), and in some cases this is followed by a second N_2O pulse. The delayed N_2O peak might arise from the mineralization and subsequent nitrification of urine-N (Williams et al., 1999). In conclusion, the high N_2O emission from urine patches is partly caused by nitrification and denitrification of N derived from other sources than the urine itself. Thus, urine seems to increase the availability of inorganic N in general. Several mechanisms have been suggested, e.g. soil inorganic N released via mineralization or deamination of soil organic matter under the high pH condition (Clough et al., 2004) and release of nitrogenous compounds from plant roots scorched by $NH_{3(aq)}$ (Bol et al., 2004). Furthermore, urine application may stimulate the denitrifying microorganisms by increasing the availability of labile carbon compounds; either because increased pH and $NH_{3(aq)}$ concentration cause solubilization of organic C, or because C compounds are released from scorched plant roots (Monaghan and Barraclough, 1993).

Indication of linked N_2O production and C mineralization

An increase in soil respiration occurring simultaneously with the initial N_2O emission from urine patches would support the view that initial N_2O formation is caused by enhanced denitrification stimulated by raised availability of labile C compounds. In a study by Bol et al. (2004) the urine-induced N_2O emission was not linked to an increase of soil respiration, whereas Lovell and Jarvis (1996) found that soil respiration increased significantly following application of urine, especially real cow urine. They partly ascribed this to microbial respiration of soil organic C, which was solubilized following urine application. Emissions of N_2O and CO_2 may therefore both increase as a result of urine deposition, but the gasses may be produced in two different microbial processes. For instance, Petersen et al. (2004) reported that nitrifier denitrification was responsible for increased N_2O emission in urea-amended soil, whereas the increased CO_2 emission was caused by enhanced microbial turnover stimulated by urea-N. In a six-week study, the emission of N_2O and CO_2 was determined following urine application to a grass-clover sward, where the vegetation had been ^{13}C-labelled prior to the study (Ambus, unpublished). Soil respiration increased following urine application, and within 6 weeks the amount of urine-induced CO_2-C corresponded to the C content of urine. However, 50 % of the CO_2-C was derived from the plants, possibly due to scorching, and the remaining 50 % was derived from soil C and urine-C. Furthermore, a temporal delay in

the N_2O emission compared to the urine-induced CO_2 emission signified that N_2O production was not linked with C mineralization. In conclusion, of the mentioned studies, only that reported by Lovell and Jarvis (1996) indicated that N_2O production in urine-affected soil could be linked with C mineralization via denitrification.

1.5 Greenhouse gas inventories

The greenhouse effect

The rate and duration of the global warming of the 20th century is probably larger than any other time during the last 1000 years. Since 1979, the global average surface temperature has increased by 0.15 ± 0.05 °C per decade (IPCC, 2001). The observed warming may be a result of raised concentration of greenhouse gasses in the atmosphere. Briefly, the greenhouse effect is based on the fact that the Earth absorbs radiation from the Sun and this energy is re-emitted to space as heat. An increase in the concentration of greenhouse gases in the atmosphere will reduce the efficiency with which the Earth's surface returns heat to space. This tends to warm the lower atmosphere and the surface of Earth. The anthropogenic greenhouse gases include CO_2, N_2O, methane and fluorocarbons.

The contribution of N_2O

Nitrous oxide has a rather high global warming potential. The global warming potential takes into account the relative radiative effect of the gas compared to CO_2 and the lifetime of the gas in the atmosphere. The global warming potential of one ton of N_2O-N is 296 times higher than of one ton of CO_2-C, when calculated over a period of 100 years (IPCC, 2001). In greenhouse gas inventories all emissions are converted to CO_2 equivalents. On a global scale N_2O has been estimated to account for 6 % of the present greenhouse forcing rate ascribed to anthropogenic derived gases (IPCC, 2001). Anthropogenic sources of N_2O include agricultural soils, livestock management, biomass burning and industrial activities, and agricultural soils accounts for about 50 % of the anthropogenic N_2O emission (IPCC, 2001). Some variation exists in the relative contribution of N_2O between different parts of the world. Thus, N_2O emitted from agricultural soils alone has been estimated to account for 5 % of the European release of anthropogenic derived greenhouse gases (EEA, 2002), whereas this source accounts for approximately 14 % of the Danish input of anthropogenic greenhouse gasses (Olesen et al., 2001). The major sink for N_2O is the stratospheric reaction with atomic oxygen, and the resulting nitric oxide (NO) initiates the destruction of the stratospheric ozone layer (Crutzen, 1981). At present, the concentration of N_2O in the atmosphere is 319 ppb, which is increasing with 0.8 ppb per year (IPCC, 2001).

The Kyoto Protocol

The Kyoto Protocol was finalised in Kyoto, Japan, in 1997 and aims at reducing the anthropogenic release of greenhouse gasses in order to prevent drastic changes of the climate on Earth in future. Before the protocol could be turned into action it should be ratified by 55 or more countries, which represent at least 55 % of the greenhouse gas emission from the industrialized countries (Danish Ministry of Environment and Energy,

2000). The requirements were met in February 2005 as Russia finally decided to ratify the protocol. More than 30 industrialized countries have signed the protocol, representing 61.6 % of the greenhouse gas release from the industrialized part of the world (Madslund, 2005). These countries are now committed to reduce their emissions in 2008-2012 with at least 5 % of the 1990 level. The European Union should collectively reduce the emission with 8 %, and Denmark has agreed to contribute with a 21 % reduction of the greenhouse gas emission.

National N_2O inventories

Worldwide, 192 countries have signed the United Nations Framework Convention on Climate Change, committing them to make national inventories for emissions of greenhouse gases. The Intergovernmental Panel on Climate Change has made guidelines to be used for calculating the emissions (IPCC, 1997). According to the IPCC guidelines, inventories for N_2O emissions from agricultural soils should be based on the assumption that 1.25 % of added N is emitted as N_2O. This emission factor is used as a standard for mineral fertilizer, manure and biologically fixed N_2, although the factor relies on experiments with mineral fertilizer and manure only (Bouwman, 1996).

Legumes may give rise to N_2O by supplying the microbial community in the soil with N compounds. In addition, many strains of the symbiotic N_2 fixing bacteria *Rhizobium* are able to denitrify nitrate that moves into the root nodules from the soil (O'Hara and Daniel, 1985). However, this ability was not found among the strains that form symbiosis with white clover (De Klein et al., 2001). Input to the systems via biological N_2 fixation in grass-legume swards is currently not considered as a source of N_2O in the IPCC guidelines (IPCC, 1997), partly due to uncertainties in quantifying the N_2 fixation in grasslands (Mosier et al., 1998). Hence, the agricultural greenhouse gas release may presently be underestimated. As organic farming to a very large extent utilises grass-legume mixtures as a source of N, the contribution from organic farming systems in particular may be underestimated.

However, countries are allowed to develop their own inventory methodology based on local measurement data. Some countries, e.g. Denmark (Mikkelsen et al., 2005) and Switzerland (Schmid et al., 2001), include quantitative estimates of biological N_2 fixation in grasslands in their N_2O inventory, using the default IPCC emission factor of 1.25 % as for other N inputs. This factor nonetheless seems to be unrepresentative for biologically fixed N_2, as substituting fertilizer N with biological N_2 fixation is often found to reduce N_2O emissions from grasslands (e.g. Garrett et al., 1992; Ruz-Jerez et al., 1994; Kaiser et al., 1998). Reasons for this may be that only a part of the fixed N is mineralized during the lifetime of the crop (Petersen and Olesen, 2002), and because the mineralization occurs slowly (Velthof et al., 1998).

According to the IPCC methodology, the N_2O loss from legume pastures includes N_2O emission from grazing animals' excreta. Here it is assumed that 20 % of the N contents in urine and dung is lost as NH_3, and that 2 % of the remaining N is emitted as N_2O. Furthermore, the IPCC methodology operates with a background N_2O emission from cultivated organic soils, the so-called Histosols that include soils developed on old boggy land. Thus, for legume swards located on Histosol a default background emission of 5 kg N_2O-N ha^{-1} y^{-1} is added in the N_2O inventory. The default background emission is 5 kg N_2O-N ha^{-1} $year^{-1}$. The N_2O loss from ploughing in legume swards is calculated as 1.25

% of the N left in plant residues (IPCC, 1997). Presently, only N input via above-ground residues is considered in the Danish N_2O inventory (Mikkelsen et al., 2005).

Current knowledge on N_2O emission factors

The N_2O emission factor for livestock urine deposited on grassland has been studied intensively and the results were reviewed by Oenema et al. (1997), De Klein et al. (2001) and Van Groeninnigen et al. (2005a). Generally, between 0.1 and 4 % of the N in urine is emitted to the atmosphere as N_2O. The emission factors were generally higher for pastures grazed by dairy cows than for pastures grazed by sheep or beef cattle. Furthermore, there seems to be contrasting views as to whether artificial urine under- or overestimates the N_2O emission factor for real cow urine (De Klein et al., 2003; Van Groenigen et al., 2005a). In a review of 10 field studies, Van Groenigen et al. (2005a) found that median N_2O emission factor for real urine was 0.9 %. However, based on a laboratory study the authors concluded that in camping areas the combined effect of compaction and dung addition on urine-affected soil may increase the N_2O emission factor for urine. This conclusion was verified by a related field study (Van Groenigen et al., 2005b). Realistic emission factors should therefore consider the frequency of camping areas. Within the range of urine-N rates typically found in urine patches, the N_2O emission factor appears to be rather unaffected by the amount of urine-N applied (Bol et al., 2004; Ambus, 2004; Van Groenigen et al., 2005a, 2005b). On two out of eight occasions, though, Van Groenigen et al. (2005b) observed considerably higher N_2O emission factors for larger amounts of urine-N.

So far, the N_2O emission factor for biologically fixed N_2 in grass-legume swards has only been estimated via modelling (e.g. Schmid et al., 2001) or determined indirectly by relating total N_2O emission to measured N_2 fixation (e.g. Ruz-Jerez et al., 1994). Via modelling, Schmid et al. (2001) estimated the N_2O emission factor for biologically fixed N_2 in permanent grasslands to be 0.22 %. However, after steady state in soil carbon and nitrogen was reached, the emission factor increased to 0.56 %. Obviously, these emission factors are associated with extremely large uncertainties. Ruz-Jerez et al. (1994) found that the annual N_2O emission represented about 1 % of the N input by legume fixation in grazed grass-clover swards. The N_2O loss often increased following a grazing period, mainly because of N return in animal excreta. Thus, the estimated emission factor includes the contribution from N_2 fixation as well as the effect of grazing. In contrast, the effect of grazing animals is accounted for separately in the IPCC methodology.

Experimental data on the N_2O emission factor for biologically fixed N_2 in grass-clover swards is currently missing and the controlling factors are rather unknown. Therefore, a brief literature review was conducted in order to theorize which biotic and abiotic factors could potentially control the emission factor (see Section 1.7). The future aim could be to include N_2O emissions from legume pastures in the national inventories. However, will 1.25 % be a suitable emission factor for biological N_2 fixation in grass-clover pastures? To enlighten this question, an estimate of the emission factor was computed based on data from the literature. Before this work is presented a description is given of the processes and controlling factors on the way from biologically N_2 fixation until the fixed N is available for nitrifying bacteria in the soil. Whenever possible, data for white clover are presented, however on occasions it has been necessary to supplement with data for other species.

1.6 Biological N_2 fixation and release of fixed N into the soil

Biological N_2 fixation

Dinitrogen constitutes about 80 % of the atmosphere, but due to its triple bond it is inaccessible to most organisms except for certain prokaryotic species (Buchanan et al., 2000). White clover live in symbiosis with rhizobial populations of the strains *Rhizobium leguminosarum* bv. *trifolii*, which are capable of fixing atmospheric N_2 (Frame et al., 1998). The bacteria are localized in root nodules in the clover plant, where they have differentiated into bacteroids. Dinitrogen fixation in the bacteroid follows this equation:

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

The reaction is catalysed by two enzymes in one complex, dinitrogenase reductase and dinitrogenase (Buchanan et al., 2000). During the process ATP is used, which is supplied from respiration of carbon compounds provided by the plant. Plant metabolism in the nodule generates organic acids that both feed the bacteria and provide carbon skeletons for the N-transport compounds used to transport fixed N to the rest of the plant. Thus, for the plant there is a cost of living in symbiosis with N_2 fixing bacteria. The plant has to form nodules and deliver carbon compounds for N-transport and as fuel for the bacterial ATP synthesis. An amount of 6-12 g carbohydrates is consumed per g fixed N (Marschner, 1995). Thus, this process is considerably more energy-demanding than uptake of a similar quantity of inorganic N.

The amount of N_2 fixed via biological N fixation in agricultural systems is uncertain (Mosier et al., 1998). In a review of 29 studies carried out in New Zealand on atmospheric derived N in white clover biomass (including herbage, stubble and roots), about 50 % of the estimated annual N_2 fixation rates were within the range of 100-400 kg N ha^{-1} (Crush, 1987). For Danish pastures of white clover and perennial ryegrass, estimates of the amount of N derived from biological N_2 fixation in harvested clover biomass (above 6 cm) has been reported in the ranges 47-235 kg N ha^{-1} y^{-1} (Table 4). In the mentioned studies, biological N fixation supplied 74-99 % of the N in white clover, with large variation occurring within each study.

Table 4. Nitrogen derived from biological N_2 fixation in harvested clover biomass (above 6 cm) determined in Danish swards of white clover and perennial ryegrass

N derived from fixation kg N $ha^{-1}y^{-1}$	Reference
49-79	Eriksen et al. (2004)
47-114	Høgh-Jensen and Schjoerring (1997)
59-125	Hansen and Vinther (2001)
209-235	Vinther and Jensen (2000)

Factors affecting biological N_2 fixation

Legumes have a mechanism to suppress nodule formation and function if NO_3^- or NH_4^+ is available in the soil (Marschner, 1995). Furthermore, in grass-clover pastures high concentrations of inorganic N influences the competitive balance between clover and grass, thus reducing the clover content of the mixture. In line with this, Høgh-Jensen and Schjoerring (1997) estimated N_2 fixation in a grass-clover pasture, and found that the amount and the proportion of atmospheric derived N in clover decreased with increasing supply of fertilizer N. However, the proportion of atmospheric derived N never dropped below 80 % of total clover N.

The proportion of clover N derived from N_2 fixation tends to decrease with pasture age (1, 2 and 8 years) (Eriksen et al., 2004). Furthermore, the proportion changes with season, being highest in the middle of the growing season (Vinther and Jensen, 2000). This is because the level of soil inorganic N is low in the middle of the growing season, due to high plant growth rate and low mineralization. Other factors affecting N_2 fixation include soil acidity, which has an adverse effect on the *rhizobium*-white clover relationship. Low soil pH potentially affects 1) rhizobial survival and multiplication prior to infection, 2) infection of the root, 3) nodule development and 4) white clover growth in general (Frame et al., 1998). Høgh-Jensen and Schjoerring (1994) found that cutting frequency (3 and 6 cuts per year) had no effect on N_2 fixation in a mixture of red clover, white clover and ryegrass.

Transfer of nitrogen from plant to soil

Nitrogen in plants can be divided into three main groups, being inorganic N (mainly NO_3^-, but also NH_4^+), low molecular organic compounds (free amino acids, amides, amines etc.) and high molecular organic compounds (proteins, nucleic acids etc.) (Buchanan et al., 2000). Nitrogen can be transferred from plant to soil either via litter, leaching of nitrogenous substances out of the leaves or via rhizodeposition (Rovira et al., 1979; Wetselaar and Farquhar, 1980).

Data on transfer of N via litter and leaching of N compounds from leaves is very sparse for common grassland species (Ledgard, 2001; Høgh-Jensen and Schjoerring , 2001). Lee and Pankhurst (1992) reported that root exudates and dead roots may contribute 30-40 % of total organic matter input to the soil, which indicates that litter and leaching compounds from leaves may make up 60-70 %. However, in grass-clover pastures a large part of the above-ground production is harvested during the growing season or removed by grazing cattle, leaving less biomass for litter formation. Factors affecting litter formation are for example drought stress and attack by herbivores or fungi.

Rhizodeposition

Rhizodeposition includes 1) root and nodule turnover, 2) exudates, *i.e.* compounds of low molecular weight which leak from root cells, 3) secretion, *i.e.* actively released compounds, and lysates, *i.e.* compounds released from autolysis of epidermal cells (Rovira et al., 1979; Dubach and Russelle, 1994).

In a 2-year field study based on ^{15}N leaf labelling technique, rhizodeposition in white clover-ryegrass mixture during the two years represented 70 % of total N in shoot, stubble, macro roots and rhizodeposition at the final harvest (Høgh-Jensen and

Schjoerring, 2001). The measured rhizodeposition included fine roots, and it only covered the stabilised fraction of the rhizodeposition during the two years. This means that re-uptake and loss was not accounted for. Finally, it should be noted that although the measured pool of N was referred to as rhizodeposition it cannot be excluded that some of the ^{15}N labelled nitrogen recovered in the soil after 2 years was derived directly from above-ground plant parts, either via leaching or via death and decay of above-ground tissues.

Root and nodule turnover is the major source of transferable N between white clover and grass (Laidlaw et al., 1996), thus it is also the major way of transfer between clover and soil. Laidlaw et al. (1996) estimated that in the period from July to October, white clover lost 8 mg N m^{-2} d^{-1} from roots in the top 10 cm of the soil. Nitrogen uptake was 12 mg N m^{-2} d^{-1}. Assuming that the loss was solely a result of turnover of roots and nodules, nitrogen in the root system was turned over once within the three months. The fast turnover rate of the root system may be due to continuous grazing of the sward, and thereby frequent defoliation resulting in death of roots and nodules. Since the estimate of N loss was made in autumn, the turnover rate for N in roots is likely to be higher than the mean for the whole grazing season.

The main region of exudation and secretion is near the root tip, with lysates occurring further away from the tip where the outer cells are dying (Bowen and Rovira, 1991). Often exudates, secretes and lysates are collectively referred to as exudates, since it is usually impossible to distinguish exudates experimentally from the other sources (Grayston et al., 1996). In the literature, exudates represent between 1.7 and 10 % of the carbon in rhizodeposition. The variation is primarily due to the use of two different methods for measuring root exudation, of which one underestimates and the other overestimates the exudation (Grayston et al., 1996). The minor contribution in mass from exudates is supported by a review stating that soluble exudation is most often in the range 10-100 mg g $root^{-1}$, whereas insoluble root derived material is often in the range 100-250 mg g $root^{-1}$ (Newman, 1985). Although the smaller contribution, exudates seems to be important in the flow from N_2 fixation to N_2O emission, because they are easy degradable and thus rapidly decomposed by the microbes.

Root exudates include sugars, amino acids, organic acids, fatty acids and enzymes. The dominant exudate components have nearly always been shown as sugars, amino acids and organic acids (Grayston et al., 1996). Of these, the only nitrogenous compounds are the amino acids. Paynel et al. (2001) studied exudation of N containing compounds from white clover roots. Ammonium and amino acids constituted the major part of the exudates, whereas the content of peptide and proteins was very low. Shepherd and Davies (1994) suggested that exudation of amino acids happens due to passive diffusion along a concentration gradient. However, the roots recapture most of the exuded amino acids (Jones and Darrah, 1994). Paynel et al. (2001) found that the amino acid composition of root extract and exudate deviated, which points towards a selective net-exudation of amino acids by the root system. Their results indicated that release of NH_4^+ and amino acids was accompanied by decay of epidermal cells and release of NO_3^-.

Factors affecting rhizodeposition

Studies on the effect of various factors on rhizodeposition are sparse for white clover. However, caution must be taken in making conclusions from studies of other species, since root exudates vary both in quality and quantity between species (Grayston et al.,

1996). The type and amount of rhizodeposition released from white clover depend on age and developmental stage of the plant. Generally, turnover of roots and nodules represents the major part of rhizodeposition. However, Paynel et al. (2001) reported that in two month old white clover plants, exudation of N compounds represented the major part of N release into the soil, whereas nodule and root turnover only played a minor role. At flowering, seed production is a strong sink for N, hence subterranean clover has been shown to allocate less N to the root system at flowering compared to at the vegetative stage (McNeill et al., 1997). This was accompanied by decreased recovery of clover N in soil total soluble N and microbial N, which indicated a decrease in rhizodeposition.

Factors leading to a reduction of the living above-ground biomass will inevitably lead to an adjustment of the size of the root system, and thereby to an increase in rhizodeposition due to death and decay of roots and nodules. This for example happens in autumn and as a result of grazing or cutting (Butler et al., 1959; Laidlaw et al., 1996). Presence of microorganisms, for example symbiotic organisms, may lead to increased root exudation. Paynel et al. (2001) shows that white clover released more amino acids in non-sterile soil than in axenic conditions. The increase in exudation is most likely due to an increase in sink strength caused by microbial degradation of exudates in the rhizosphere (Grayston et al., 1996). Other reasons may be increased root cell permeability caused by microbial production of plant hormones or physical damage of the root by the microorganisms (Grayston et al., 1996).

In most cases dry stress leads to enhanced root exudation (Grayston et al., 1996). Rising temperature increased the amount of root exudates and the relative proportion of amino acids exuded by subterranean clover (Rovira, 1959). Lack of oxygen enhances root exudation. Decreasing O_2 concentrations reduce the active transport over the cell membrane, thus also re-uptake of root exudates released via passive diffusion, leading to increased net-exudation (Grayston et al., 1996).

Mineralization

Mineralization of organic N refers to degradation of proteins, amino sugars, and nucleic acid into NH_4^+. Litter and dead roots differ from root exudates by being decomposed more slowly, on a scale of weeks or months. This is due to the fact that polysaccharides and polypeptides require enzymatic degradation before they can be assimilated by the microorganisms (Grayston et al., 1996). In contrast, exudates, secretes and lysates are more labile and therefore likely to be quickly incorporated into microbial biomass (McNeill et al., 1997). Whether NH_4^+ is released into the soil or immobilized during decomposition of organic matter depends on whether the microorganisms are in lack of N for growth. By having a low C/N ratio exudates may act as primers for the degradation of exciting soil organic matter and thus lead to increased nutrient availability (Grayston et al., 1996, and references within; Laidlaw et al., 1996). Mineralization rates are not solely dependent on the C/N ratio of the material, but also on the chemical composition. For instance the polyphenol content was found to control the N mineralization of legume residues (Paul and Clark, 1996). In permanent pastures, the annual immobilization of N into soil organic N has been estimated to be 50-150 kg N ha^{-1} (Jackman, 1964; Ledgard, 2001).

A very simplified overview of the fates of organic N in soil could be as follows: When plant nitrogen enters the soil in high or low molecular organic compounds, it may be

subject to decomposition and incorporation into microbial biomass, followed by a number of cycles between the NH_4^+ pool and the microbial biomass, perhaps 8-10 times per year (Coleman et al., 1983). This pool of nutrients may represent only a few grams per square meter (Coleman et al., 1983). Later on the nitrogen may be found 1) as NH_4^+ adsorbed to clay or humus or fixed in clay interlayers, 2) taken up by clover or grass, or 3) in a large pool of slowly decomposable and more stable compounds, constituting many kilograms per square meter. Nitrogen may enter this pool for example via incorporation into microbial cell wall, by NH_4^+ forming quinone-NH_2 complex with soil organic matter (Paul and Clark, 1996) or by being part of more persistent plant compounds. This pool of N may turn over very slowly, maybe less than once every century (Coleman et al., 1983). Furthermore, 4) NH_4^+ may have been subjected to nitrification into NO_3^-.

Factors affecting mineralization

Rates of decomposition and nutrient cycling are determined by the rate of input to the soil of plant material, the nature of this material, environmental conditions (e.g. temperature, soil moisture and nutrient availability) and the available decomposer community (Grayston et al., 1996). The rate of mineralization increases with increasing N availability. De Neergaard and Gorissen (2004) found that, in a grass-clover mixture, C released into the soil via rhizodeposition passed through the soil microbial biomass faster than it did in a pure ryegrass stand. The authors suggested that this difference was caused by a high N availability in the rhizosphere of white clover, which made the conditions more favourable for microbial utilisation of the grass-clover rhizodeposits.

1.7 N_2O emission factor for biologically fixed N_2

Parameters possibly affecting the emission factor

Season of the year and developmental stage of clover could potentially be important factors affecting the emission of N_2O-N derived from biological N_2 fixation. For example, McNeill et al. (1997) found that rhizodeposition of nitrogenous compounds from subterranean clover decreased at flowering compared to vegetative stage, since seed production is a strong sink for N. Furthermore, in late summer microbial activity, and thus mineralization, nitrification and denitrification, may be reduced due to low soil water content. In addition, plants strongly compete for the mineralized N, leaving less for nitrifying and denitrifying bacteria. This may be some of the reasons for the low N_2O emissions often observed from grasslands during summer time (e.g. Ambus and Christensen, 1995).

In autumn, a part of the clover leaves, stolons, roots and nodules senesce and die (e.g. Goodman, 1991). At this time of the year, the temperature is still high enough to allow microbial activity, thus decomposition of the plant material will be initiated and possibly mineralization of N also. However, plant growth is reduced, which means reduced competition for mineralized NH_4^+ between plants and microorganisms. Furthermore, increasing soil moisture in autumn may favour nitrification, since it facilitates transport of NH_4^+ and enables bacterial activity. This may imply a high rate of nitrification, and thus N_2O production. In addition, anaerobic conditions in microsites (e.g. inside soil aggregates) allows for high rates of denitrification, and may thereby give rise to high

N_2O losses from the process. Several studies on N_2O emissions from pastures have reported denitrification to be the major source of N_2O in autumn (e.g. Kerster et al., 1997; Merino et al., 2000b). Collectively, this may result in considerable emission of N_2O-N originating from biological N_2 fixation. Emission factors are calculated on yearly basis, and an emission factor for biologically fixed N_2 in grass-clover pastures should therefore take this seasonal variation into account.

Similar to the situation in autumn, cutting and grazing could lead to increased emission of N_2O-N derived from biological N_2 fixation. Cutting frequency has been shown not to influence biological N_2 fixation in grass-clover pastures (Høgh-Jensen and Schjoerring, 1994). However, removal of above-ground plant parts inevitably leads to adjustment of the size of the root system, and thus death and decay of roots and nodules (Butler et al., 1959). This is followed by decomposition, mineralization of N, nitrification and maybe denitrification – the rate of each process depending on environmental factors. Plant defoliation could therefore result in increased N_2O production. This hypothesis is supported by Bech and Christensen (1987), who found increased N_2O emission in relation to removal of above-ground plant parts and senescence of grass. However, they explained the result by release of labile compounds from the roots, being used by denitrifying bacteria. According to this, the emitted N_2O did not necessarily contain N, which had recently been released from the plants. In contrast, Kammann et al. (1998) found that increasing the numbers of cuts reduced N_2O emissions. The authors explained the results by increased uptake of NO_3^- by the plant roots, and thereby reduced amounts of NO_3^- available for denitrifying bacteria. However, the frequency of measurements (about once per week) may have been insufficient to catch increased N_2O emissions following cutting. In line with this, a study based on daily flux measurements reported increased N_2O emission after cutting (Kaiser et al., 1998).

The N_2O loss ratios of nitrification and denitrification are higher in acidic soils than in neutral soils, but the rates of the two processes are lower. Consistent with this, Yamulki et al. (1997) found that the N_2O emission from grassland decreased with increasing acidity. Thus, the N_2O emission factor for biologically fixed N may be lower for grass-clover pastures on acidic soils. However, soil acidity adversely affects biological N_2 fixation in white clover and therefore the production in such a pasture may be poor.

High soil inorganic N due to fertilization will reduce biological N_2 fixation directly through a reduction of the proportion of N, which is derived from the atmosphere, and indirectly through a reduced clover content due to more competition from the grass in the mixture. This will tend to reduce N_2O originating from biological N_2 fixation, but the emission factor may be unchanged. Furthermore, the total N_2O emission from the pasture may increase with increasing amounts of fertilizer, since fertilizer-N is a source of N_2O emission. However, in itself, a decrease in the clover content of the mixture may increase the efficiency in which clover N released into the soil is utilised by the companion grass, and thereby reduce the N_2O emission factor for biologically fixed N.

An estimate of the emission factor

Release of N derived from biological N_2 fixation may in itself give rise to a N_2O emission, meaning that the fixed N is recovered in the emitted N_2O-N. However, as stated above, release of root exudates may act as a primer for decomposition of more resistant soil organic matter, leading to mineralization and potentially to N_2O emission. Furthermore, labile carbon compounds released by clover may act as reductants for

denitrifying bacteria and in that way influence the production of N_2O. These are indirect N_2O emissions caused by the presence of the clover plant, and could also be included in the emission factor for biologically fixed N_2.

A rough estimate of the emission factor was made based on the data presented above. A Danish white clover-perennial ryegrass pasture was chosen as point of reference, and focus was on the background N_2O emission of N originating from biological N_2 fixation. Thus, grazing-derived N_2O emission was not taken into account. Data for N in white clover herbage, stubble, macro roots and stabilised rhizodeposition was obtained from Høgh-Jensen and Schjoerring (2001) (Table 5). The authors noted that clover contributed 30 % of shoot biomass and 36 % of stubble biomass, and clover was therefore assumed to account for 33 % of the macro root biomass. As only the stabilised fraction of the rhizodeposition could be measured in the present study, a non-stabilised fraction was added. This was done by assuming that the non-stabilised fraction represented 20 % of the total N transfer from clover to soil during two years. This gives 16 kg N ha^{-1} y^{-1} available for nitrification. Assuming a N_2O loss ratio for nitrification of 1 % (Firestone and Davidson, 1989; Hutchinson and Davidson, 1993) results in a N_2O production of 0.16 kg N ha^{-1} y^{-1}.

Table 5. Estimation of N_2O emission factor for biologically fixed N in grass-clover pastures

	Clover-derived N kg N ha^{-1}	
Herbage per year	57	a b
Stubble per 2 years	29	a
Macro roots per 2 years	16	a
Stabilised rhizodeposition per 2 years	131	a
Non-stabilised rhizodeposition available for nitrification per year	16	c
N_2O emission from nitrification per year	0.16	d
Annual denitrification rate	4	e
N_2O emission from denitrification per year	0.60	f
Total N_2O emission per year (0.16+0.60)	0.76	
N_2 fixation per year (0.9×(57+(29+16+131)/2+16))	145	
N_2O emission factor for biologically fixed N (0.9×0.76×100/145)	0.47 %	

[a] Høgh-Jensen and Schjoerring (2001)

[b] Calculated from dry matter production (700 g m^{-2} y^{-1}), clover content (0.30) and % N (2.7 %)

[c] Assumption

[d] N_2O loss ratio from Firestone and Davidson (1989); Hutchinson and Davidson (1993)

[e] Based on data presented in Table 2

[f] N_2O loss ratio from Kester et al. (1997)

On the basis of rates of denitrification measured in grasslands (Table 2), the denitrification of clover-derived N was expected to be 4 kg N ha^{-1} y^{-1}. The N_2O loss ratio of denitrification was assumed to be 15 %, which is the median of reported losses from a fertilized grassland in The Netherlands (Kerster et al., 1997). Thus, N_2O emission from denitrification was estimated to be 0.60 kg N ha^{-1} y^{-1}, giving a total N_2O emission derived from clover-N of 0.76 kg N ha^{-1} y^{-1}. This is within the range of reported N_2O emissions from grasslands (Table 3). Biologically fixed N was assumed to account for 90 % of the clover-derived N, and thereby amounted to 145 kg N ha^{-1} y^{-1}. Finally, the emission factor for biologically fixed N was estimated to be 0.47 %. However, the calculation is extremely uncertain in all the steps. For instance the N_2O loss ratio of denitrification is very variable and the ratio used may be unrepresentative as a yearly average for grass-clover pastures. Nonetheless, the estimated emission factor is fairly consistent with results obtained via modelling by Schmid et al. (2001). Through simulation they estimated the emission factor for biologically fixed N_2 in a subalpine site to be 0.56 %. This suggests that the emission factor is possibly lower than the 1.25 % recommended by IPCC for crops other than legume pastures.

2 The conducted studies

Three experimental studies were conducted as part of the Ph.D. project. The studies are numbered from I to III, and a detailed description of each study appears in the corresponding paper found in Part B of the thesis.

2.1 Brief introduction to each study

Study I – From N_2 fixation to N_2O production

The objective of this study was to develop a method to measure N_2 fixation and N_2O production. Furthermore, the objective was to assess the contribution of recently fixed N_2 as a source of N_2O and the transfer of fixed N from clover to companion grass.

A mixture of white clover (*Trifolium repens* L. cv. Klondike) and perennial ryegrass (*Lolium perenne* L. cv. Fanda) was sown in pots using topsoil from an organic crop rotation. The ^{15}N-labelling approach consisted of enriching the atmosphere in a growth cabinet with $^{15}N_2$ (0.4 atom% excess) to trace the biological N_2 fixation. A minimum-volume closed-system growth cabinet was developed, which could host 12 pots of 15 cm × 15 cm (Fig. 4). In this cabinet, three 14-day incubations were conducted with grass-clover mixtures at 16, 26 and 36 weeks of age. Immediately after labelling, half of the grass-clover pots were sampled and the N_2 fixation during the incubation was established by relating the excess ^{15}N content of the plant and soil fractions to the ^{15}N enrichment of the atmospheric N_2.

The remaining half of the grass-clover pots were examined for emission of ^{15}N labelled N_2O for another eight days using a static chamber method (Fig. 5). Generally, the static chamber method refers to measurement of gas emissions (e.g. N_2O) by enclosing the soil surface with a cover and thereby forming a headspace of known volume. Samples for determination of N_2O concentration are collected from the headspace at regular intervals.

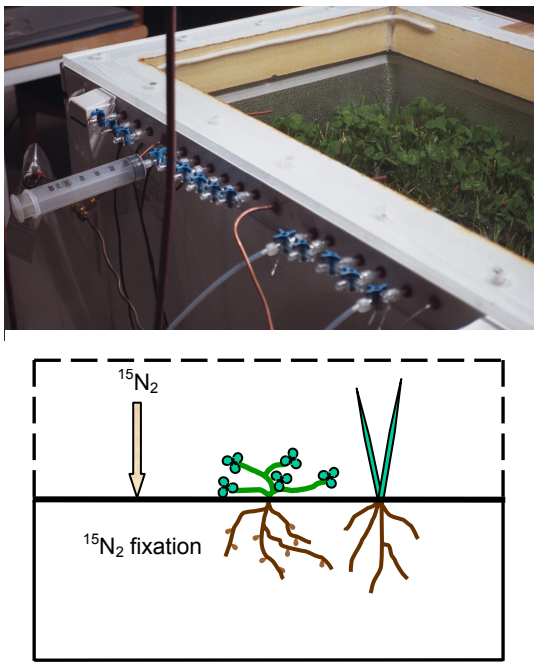

Figure 4. The closed-system growth cabinet and the labelling approach.

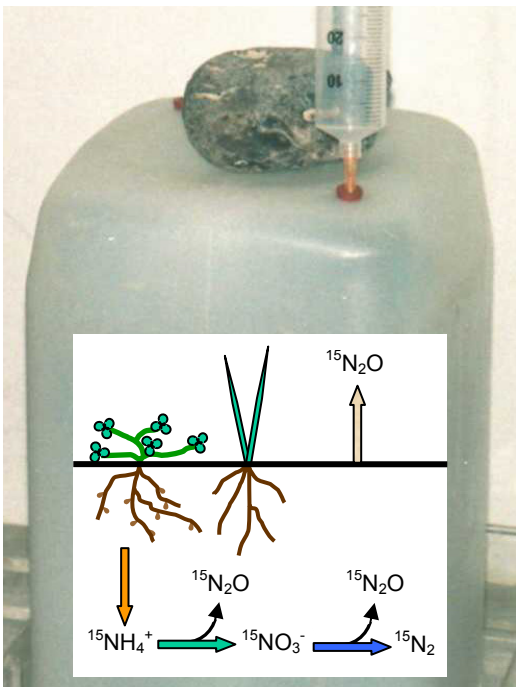

Figure 5. Static chamber method for measurement of $^{15}N_2O$ emission.
Mineralization, *Nitrification*
Denitrification.

Finally, the flux of N_2O is calculated from the linear increase of N_2O concentration in the headspace during the cover period.

Study II – Carbon mineralization and urine

The aim of this study was to examine the link between N_2O emission and carbon mineralization in urine patches. More specifically, the objective was to test the hypothesis that increased N_2O emission after urine deposition is in part due to enhanced denitrification stimulated by labile carbon compounds leaching from NH_3-scorched plant roots.

The study was conducted in grassland monoliths placed in temperature controlled transparent enclosures kept under natural daylight, which were part of a ^{13}C-labelling facility situated at Institut National de la Recherche Agronomique (INRA), Clermont-Ferrand, France. Starting 22 April 2003, ^{13}C-labelling of plants was initiated by providing the enclosures with CO_2 depleted in ^{13}C. To simulate grazing, the vegetation of six monoliths was cut on 22 April and 9 June, and within two weeks artificial urine was applied to three of the monoliths at a rate of 3.1 and 5.5 g N m^{-2}, respectively.

Prior to urine application, small PVC pipes were installed in each monolith between the plants. Every pipe was connected to a sampling valve on the outside of the enclosure via a silicon tube (Fig. 6). Four days before urine application (Day -4), the pipes were sealed by rubber stoppers to determine below-ground production of N_2O and CO_2 as well as $\delta^{13}C$ signal of evolved CO_2 via the static chamber method. Determination of gas emissions from urine-treated and control monoliths was done on day -4, -1, 0, 1, 2, 4, 5, 6 and 7 after urine application. If increased N_2O emission following urine application

was a result of higher denitrifying activity due to supply of labile compounds released from scorched plant roots (more depleted than other soil C pools in the monoliths studied), then $\delta^{13}C$ of soil respiration was expected to decline.

The $\delta^{13}C$ signal was also determined for soil organic matter, dissolved organic C and CO_2 evolved by microbial respiration. In addition, denitrifying enzyme activity and nitrifying enzyme activity were measured at the first urine application event.

Figure 6. Equipment for measurement of gas emission from grassland monolith placed in a ^{13}C-labelling facility.

Study III – Nitrification, denitrification and urine

The objective of this study was to investigate how deposition of urine affects the N_2O losses from nitrification and denitrification. More specifically, the objective was to examine the effect of urine on the rates and N_2O loss ratios of the two processes and evaluate the importance of the chemical conditions arising in urine-affected soil.

Figure 7. Measurement of N_2O emission from microplots.

The study was conducted in an organic sward consisting of white clover, red clover (*Trifolium praténse* L.) and perennial ryegrass, where microplots were established in December 2002 by pushing 30-cm diameter PVC cylinders into the soil. The effects of artificial urine (52.9 g N m^{-2}) on the nitrification and denitrification processes relative to a water control were examined in an experiment that took place in September 2003. To evaluate the impact of the chemical conditions occurring in urine-affected soil, these results were compared with the effects of an ammonium solution, which were assessed in a similar experiment conducted 14 days prior.

In each experiment, ^{15}N technique was used in the field to determine the rate

Figure 8. Injection of acetylene into a soil core prior to incubation in a glass jar.

of nitrification and the amount of N_2O produced via nitrification or denitrification. Thus, in sets of microplots either soil NH_4^+ or soil NO_3^- was labelled with ^{15}N. Subsequently, the emission of ^{15}N labelled N_2O was determined by a static chamber method (Fig. 7). Nitrification rate was established in the microplots via $^{15}NO_3^-$ pool dilution during a period of 24 hours. The rate of denitrification was determined on soil cores in the laboratory using acetylene (C_2H_2) inhibition of the bacterial reduction of N_2O to N_2 (Fig. 8).

2.2 Stable isotope technique

Stable isotope technique was a central methodology in the Ph.D. project, as it was used in all three studies. The term covers a broad range of methods (Knowles and Blackburn, 1993; Lajtha and Michener, 1994). The technique relies on the fact that some elements consist of more than one isotope that vary in atomic mass due to differences in the number of neutrons in the nucleus. The methods used in the three studies were all based on tracer technique, which involves labelling a specific element found in the atmosphere or soil compartment by changing the abundance of one isotope. The flow of this element between the labelled pool and unlabelled pools within the biosphere-atmosphere system is subsequently monitored by measuring the change over time of the isotopic abundance in these pools. Tracer technique is a very useful tool when aiming to obtain detailed information on the processes involved in the transformation of nitrogen and carbon within the biosphere-atmosphere system.

Isotopic abundance is determined by isotope ratio mass spectrometry, and the value is referenced to a primary standard. The standard for carbon is a marine limestone fossil that has a $^{13}C/^{12}C$ ratio of 0.011 (Lajtha and Michener, 1994). For nitrogen the standard is atmospheric N_2, which has a ^{15}N abundance of 0.3663 atom% (Shearer and Kohl, 1993). The natural abundance of ^{15}N in various components of the biosphere is generally within 1 or 2 % of that of atmospheric N_2. Isotopic enrichment may be reported as atom% excess, which is the atom% of a labelled sample subtracted the atom% of an unlabelled sample. Small variation from the isotopic abundance of the standard is typically reported in delta (δ) unit, which is based on the ratio between the heavy and the light isotope and the deviation of this ratio between the sample and the standard in per mille of the standard, *viz.*:

$$\delta\ (‰) = 1000 \times (R_{sample} - R_{standard}) / R_{standard}\ ,$$

where R = abundance of heavy isotope / abundance of light isotope. Carbon dioxide in atmospheric air has a $\delta^{13}C$ of about -8 ‰ (Wahlen, 1994). Several physical and chemical processes lead to enrichment or depletion of the heavy isotope via isotopic fractionation (Lajtha and Michener, 1994). For instance, plants contain less ^{13}C than atmospheric CO_2

because discrimination against the heavier $^{13}CO_2$ takes place both during the diffusion of CO_2 in the leaf and during the enzymatic fixation of CO_2.

In the Ph.D. project, tracer technique was used to quantify N_2 fixation (I), assess the fate of fixed N via translocation and N_2O emission (I), detect mineralization of plant-derived carbon (II), determine and quantify the sources of N_2O (III), and measure the rate of nitrification (III). Equations used for the calculations and the assumptions on which they are based are found in the 'Materials and Methods' section of the respective paper. However, two of the equations are discussed in more detail below.

Determination of N_2 fixation by a legume exposed to ^{15}N-enriched atmosphere during a period in the middle of the growth

The argumentation presented below validates the use of the equation for calculating N_2 fixation in Paper I. When calculating the N_2 fixation by a legume exposed to a ^{15}N-enriched atmosphere, the proportion of total N in the plants derived from the atmosphere (P) is determined as

$$P = N_L^*/N_P^*, \quad (1)$$

where N_L^* is the ^{15}N atom% excess enrichment of the legume after exposure to an atmosphere with a ^{15}N atom% excess enrichment of N_P^* (Warembourg, 1993). Wood and McNeill (1993) showed that this P value is independent of the plant N pool at the start of the labelling period. However, the following argument establishes that the P value is also valid in cases where the plants accumulate N both before and after the labelling period. Legumes, which have been exposed to a $^{15}N_2$-enriched atmosphere during a period in the middle of their growth, have an N content (N_L) that can be divided into the following components:

$$N_L = N_L S + N_L F + N_L P_1 + N_L P_2 + N_L P_3, \quad (2)$$

where S, F, P_1, P_2 and P_3 is the proportion of N_L derived from soil, fertilizer, non-enriched N_2 before labelling, enriched N_2 during labelling and non-enriched N_2 after labelling, respectively. The amount of ^{15}N accumulated by the plants is

$$N_L(0.366 + N_L^*) = 0.366(N_L S + N_L F + N_L P_1 + N_L P_2 + N_L P_3) + N_L P_2 N_P^* \quad (3)$$

under the assumption that N supplied by soil, fertilizer and non-labelled atmosphere have the same ^{15}N abundance and that no $^{15}N_2$ remains during the last period. From equation (2) and (3):

$$P_2 = N_L^*/N_P^* \quad (4)$$

Thus, the amount of N originating from fixation during the labelling period ($N_L P_2$) is

$$N_L P_2 = N_L N_L^*/N_P^*, \quad (5)$$

where N_L is the N accumulated by the plants during their full growth, and P_2 is the proportion of that N originating from the enriched N_2 during the labelling period, which in this case is a period in the middle of the growth.

Equation for quantifying the sources of N_2O

The aim of the following is to clarify the choice of equation used to quantify the sources of N_2O in Paper III. There exists some deviation in the literature on how to calculate the

contribution of nitrification and denitrification to the N_2O production in ^{15}N tracer studies. Stevens et al. (1997) suggested the following equation:

$$d = (a_m - a_n)/(a_d - a_n), \quad (6)$$

where d is the fraction of the N_2O flux derived from denitrification, a_n and a_d are the ^{15}N atom% excess enrichment of the NH_4^+ and NO_3^- pool, respectively, and a_m is the ^{15}N atom% excess enrichment of the emitted N_2O, assuming that the initial N_2O concentration in headspace is negligible. The fraction of the N_2O flux derived from nitrification is calculated as 1-d. The amount of N_2O-N derived from denitrification is

$$Cd = (Ca_m - Ca_n)/(a_d - a_n), \quad (7)$$

where C is the increase in the headspace N_2O concentration during the cover period.

In Study III, the initial N_2O concentration in headspace accounted for a large part of the N_2O present at the end of the cover period. Thus, it could not be neglected, and the emission of ^{15}N labelled N_2O (CC^*) was therefore established as

$$CC^* = C_tC_t^* - C_0C_0^*, \quad (8)$$

where C_0 and C_t are the N_2O concentration at the start and end of the cover period, respectively, and C_0^* and C_t^* are the atom% excess enrichment of N_2O at the start and end of the cover period, respectively. The term CC^* corresponds to Ca_m in equation 7, and thus equation 7 could potentially be used to calculate Cd. For some treatments, though, emission of labelled N_2O was detected without net N_2O emission, and the intention was to include these results in the data set instead of regarding them as missing values. In these cases, the emission of ^{15}N labelled N_2O was calculated as

$$CC^* = (C_t^* - C_0^*)C_0 \quad (9)$$

However, if using equation 7 in these cases the results would not be corrected for the ^{15}N enrichment of the NH_4^+ pool (a_n), because the term Ca_n would be omitted, as the net emission (C) was nil. Consequently, the data set would be biased. Furthermore, in Study III gas measurements were initiated within 2.5 hours of solution application and therefore it could be assumed that the unlabelled N pool (*viz.* NH_4^+ or NO_3^-) had not yet been labelled via transformation of labelled N (Panek et al., 2000). Consequently, it was decided to 1) account for the initial N_2O present in headspace and 2) not account for the ^{15}N enrichment of the unlabelled N pool. Thus, the emission of N_2O-N derived from nitrification or denitrification (CP) was established as

$$CP = CC^*/N_i^*, \quad (10)$$

where N_i^* is the atom% excess enrichment of labelled N pool. Panek et al. (2000) made a similar calculation.

2.3 Development of a growth cabinet for $^{15}N_2$-labelling

The minimum-volume closed-system growth cabinet

In Study I, clover plants were provided with ^{15}N labelled N_2 to trace the fate of recently fixed N. The first suggested $^{15}N_2$-labelling approach consisted of sealing the root compartment from the above-ground compartment and then enriching the soil gas atmosphere with $^{15}N_2$. However, this approach was found to be inappropriate for clover

plants due to potential stolon formation in the root compartment, and therefore it was decided to label both the above- and below-ground atmosphere.

Because of the high cost of gaseous ^{15}N, a first task was to construct a minimum-volume closed-system growth cabinet suitable for this experiment since this facility was not in our possession. A detailed description of the constructed growth cabinet appears in the 'Materials and Methods' section of Paper I. Briefly, a chest freezer was found useful for this purpose, as it already contained a cooling system, required to remove excess heat from growth lamps. External growth lamps supplied light through a transparent window of plexiglas mounted in the lid of the freezer. A computer maintained a defined cabinet temperature within ± 0.4 ºC, which was an application developed in cooperation with University of Copenhagen. The computer also controlled light on/off as well as supply of CO_2 during specified periods to keep near ambient levels. The cabinet could host 12 pots of 15 cm × 15 cm and water was provided through a silicon tube to each pot connected to a valve on the outside.

Attempts to improve gas tightness

The constructed growth cabinet appeared to be gastight, at least for CO_2, as addition of 200 ml pure CO_2 resulted in a CO_2 concentration in the cabinet atmosphere of about 1500 ppm, which did not decline during the following 24 hours of monitoring by a Photoacoustic Multi-gas Monitor (1312, INNOVA AirTech Instruments, Ballerup, DK). However, the first $^{15}N_2$ incubation revealed that the cabinet was not completely tight for N_2, as the ^{15}N abundance of N_2 in the cabinet declined in an apparent exponential pattern during the incubation. Deviation in the cabinet's tightness for N_2 and CO_2 could result from the fact that N_2 is a smaller molecule than CO_2.

Before the second incubation, attempts were made to improve the seal of the growth cabinet and subsequently evaluate the effect on the tightness for N_2. Briefly, the cabinet was added between 20 and 80 litre pure CO_2 over about 12 hours and samples of cabinet atmosphere were then collected regularly for determination of N_2 concentration by gas chromatography (GC-14B, Shimadzu, Kyoto, JP). This was done before and after silicon sealing of the outside transition between casket and lid. The silicon seemed to improve the cabinet's tightness for N_2, as the N_2 concentration increased at a slower pace after the cabinet had been sealed (Fig. 9). However, the tightness for N_2 was not complete. The cabinet was therefore pressurized by adding about 60 litre CO_2 over 30 minutes and a couple of smaller leaks were then detected and blocked. Finally, at the onset of the following incubations silicon was used to seal the outside transition between casket and lid.

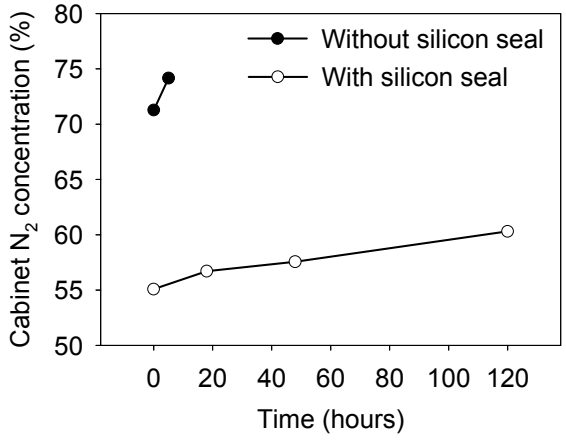

Figure 9. Test of the growth cabinet's tightness for N_2 without and with silicon sealing of the outside transition between casket and lid.

3 Summarizing discussion

3.1 Biologically fixed N_2 as a source of N_2O

Previous studies have demonstrated that growth of legumes may increase the annual N_2O emission by factor two to three compared to unfertilized grass swards (Duxbury et al., 1982; Veldkamp et al., 1998), thus biological N_2 fixation appears to contribute with significant amounts of N to the N_2O production in established leguminous crops. To my knowledge Study I is the first time biologically fixed N_2 as a source of N_2O has been measured directly. Thus, the study provides unique results. In addition, Paper I documents a novel method to assess the contribution of recently fixed N_2 to the N_2O production. Results from the grass-clover mixture revealed that only 3.2 ± 0.5 ppm of the recently fixed N_2 was emitted as N_2O on a daily basis, which accounted for 2.1 ± 0.5 % of the total N_2O-N emission. Thus, recently fixed N released via easily degradable clover residues appeared to be a minor source of N_2O. A large part of the remaining N_2O-N was most likely derived from previously fixed N_2, which indicated that long-term N release, through decay of more recalcitrant clover tissues, probably contributes considerably to the flow from N_2 fixation to N_2O emission. In addition, this is consistent with the process believed to be responsible for the transfer of fixed N to companion grass.

Rochette and Janzen (2005) reviewed a number of papers on N_2O emission from legume fields, among others a short version of Paper I published on the Internet (Thyme and Ambus, 2004). They concluded that the legume-induced N_2O emission largely relates to the N release from root exudates and from decomposition of crop residues, rather than from biological N_2 fixation as such (*i.e. Rhizobium* dentrification). Therefore, the authors suggested that "the biological fixation process itself is removed from the IPCC N_2O inventory methodology". Furthermore, they proposed that "N_2O emission induced by the growth of legume crops should be estimated solely as a function of crop residue decomposition using an estimate of above- and below-ground residue inputs, modified as necessary to reflect recent findings on N allocation". These suggestions will be considered when formulating the 'Revised 2006 IPCC Guidelines for National Greenhouse Gas Inventories', which will replace the current IPCC guidelines (*viz.* IPCC, 1997).

I agree that the N_2 fixation process itself should be removed from the IPCC N_2O inventory methodology. The suggested changes will definitely improve the estimation of the legume-induced N_2O emission in annual grain legume crops. Up till now this source of N_2O has probably been overestimated because part of the fixed N_2 was assumed to contribute twice, *viz.* during the fixation process and during decomposition of crop residues (Olesen et al., 2001). However, the suggested changes leave the N_2O loss from permanent grass-legume swards underestimated, because N input from biological N_2 fixation via rhizodeposition is not recognised as a significant source of N_2O.

The rough estimate presented in Section 1.7 showed that the N_2O emission factor for biologically fixed N_2 in grass-clover swards could amount to 0.47 %, which was rather consistent with the emission factor of 0.56 % obtained via modelling (Schmid et al., 2001). More studies are needed in order to establish the emission factor for biologically fixed N in legume pastures. The studies should be based on long-term measurements and the $^{15}N_2$-labelling technique would therefore be inappropriate. In contrast, ^{15}N leaf labelling technique could be part of the experimental approach. In Section 1.7, parameters were identified that could possibly affect the emission of N_2O-N derived from biologically fixed N_2 in grass-clover pastures. The biotic and abiotic parameters

expected to be the most important based on a brief literature review were season of the year, cutting or grazing, pH, application of fertilizer and clover content of the pasture. These parameters should be carefully selected when setting up experiments to estimate the N_2O emission factor for biologically fixed N_2. Preferably, the effect of each parameter should be determined in order to obtain a balanced estimate for the N_2O inventories.

3.2 Mechanisms involved in N_2O formation in urine patches

Root scorching by NH_3

The high initial N_2O emission from urine patches could result from enhanced denitrification stimulated by labile compounds released from scorched plant roots. However, increased N_2O emission following urine application at rates up to 5.5 g N m^{-2} was not paralleled by increased mineralization of root-derived C (II). Thus, apparently root scorching and associated release of labile compounds was not involved in the urine-induced N_2O emission in this study. Even when a higher rate of urine-N (52.9 g N m^{-2}) was applied there was no evidence for release of labile compounds from scorched plant roots (III). However, previous studies reported increased soil content of DOC in urine-affected soil, possibly as a result of root scorching (Monaghan and Barraclough, 1993; Shand et al., 2002). The degree of scorching depends on the amount of NH_3 formed (Ritchey et al., 2003), which is influenced by the amount of urea-N applied, soil pH and the cation exchange capacity (CEC) of the soil (Bolan et al., 2004). Thus, root scorching is probably most pronounced when high rates of urine-N are deposited on calcareous soils with pH above 8 or on soils with low CEC. In these cases, urea hydrolysis leads to high pH and availability of NH_4^+, and the high pH shifts the $NH_4^+ - NH_{3(aq)}$ equilibrium greatly towards $NH_{3(aq)}$. Consequently, mineralization of root-derived C could potentially be involved in the initial N_2O production in high N-urine patches deposited on calcareous soils or on soils with low CEC.

Increased nitrification in urine-affected soil

Study II and III supported findings of Monaghan and Barraclough (1992) showing that in soils at near-neutral pH, the nitrifying bacteria are not stressed by $NH_{3(aq)}$ and low osmotic potential when the urine-N concentration is below 16 g N l^{-1}. In fact both studies pointed at increased nitrification rate as an important factor explaining the initial N_2O emission from urine patches. Accordingly, urine (conc. 0.7 g N l^{-1}, rate 3.1 g N m^{-2}) did not affect denitrifying enzyme activity, whereas nitrifying enzyme activity was enhanced two days after urine application (II). Furthermore, application of urine (conc. 15.6 g N l^{-1}, rate 52.9 g N m^{-2}) caused a greater N_2O loss from nitrification than addition of equivalent amounts of N as NH_4^+, which appeared to result from a higher nitrification rate in the urine-treated soil (III). Thus, in the urine treatment, nitrification seemed to be increased, not only by the elevated NH_4^+ content following urea hydrolysis, but also by the raised soil pH. Finally, the increased nitrification rate in the urine-treated soil caused raised N_2O losses from the process, and furthermore the produced NO_3^- gave rise to increased N_2O losses from denitrification. As a result, nitrification and denitrification contributed equally to the high initial N_2O emission from the simulated urine patches.

3.3 Methodological reflections

When having conducted an experiment, it will usually appear that minor details of the experimental protocol should have been changed slightly in order to conduct the ideal experiment. Important experience obtained during the three conducted studies appears below.

Study I: Dilution of $^{15}N_2$

Some diffusion of N_2 apparently occurred through the boundaries of the minimum-volume closed-system growth cabinet used for the $^{15}N_2$-labelling in Study I. Most likely the leak took place through the 12 mm plexiglas window. Dilution overtime of $^{15}N_2$ in the growth cabinet was expected, as this problem was also faced in other $^{15}N_2$ incubation studies conducted in a growth cabinet consisting of plexiglas (Wood and McNeill, 1993; McNeill et al., 1994). However, in the present study the rate of $^{15}N_2$ dilution was faster than expected. Thus, although the leak was compensated for by multiple additions of $^{15}N_2$, the mean $^{15}N_2$ enrichment in the growth cabinet over the 14-days incubation turned out to be less than planned for.

No emission of N_2O derived from fixed N was detected when measured 26 and 36 weeks after plant emergence. However, N_2 fixation was reduced at 26 and 36 weeks due to a sever aphid attack and in combination with the relatively low ^{15}N labelling of N_2 this may have caused inadequately labelling of the clover component, and thereby hindered the detection of recently fixed N in N_2O.

Study I: Aphid attack

The severity of the aphid infestation was in part caused by my own and the gardener staff's lack of experience in handling organic pot experiments. Here, plant health should mainly rely on the use of biological pest control as prevention against pest attack, e.g. via frequent distribution of predator mites in the glasshouse or growth chamber. Our experience from Study I shows that organic means of pest control, like smothering agents and biological pest control, may reduce the growth rate of an aphid population, but do not effectively stop an aphid attack in progress.

In the field, N_2 fixation by white clover is very variable, and occurrence of pests may explain part of this variation (Ledgard and Steele, 1992). Thus, the intensity of the aphid attack at 26 and 36 weeks after plant emergence and the resulting N_2 fixation was probably comparable to conditions that may arise in the field.

Study II: Urine-N rate

When planning the visit to INRA Clermont-Ferrand, the urine-N application rate was discussed intensively with the host Dr. Jean-François Soussana and his group. My wish was to study the mechanisms involved in N_2O formation under conditions similar to that found in moderate to high N-urine patches deposited by grazing cattle in the field. However, the urine application served as fertilization in the main experiment conducted in the ^{13}C-labelling facility. The host therefore insisted to apply the urine-N to the grassland monoliths at a uniform rate and not as urine patches. This decision resulted in the low urine-N application rate and hindered a study of the processes taking place in grassland affected by moderate to high urine-N rates. Having mentioned this, I would

like to emphasise the in all other ways terrific collaboration with Dr. Jean-François Soussana and his group, and acknowledge their great flexibility when adjustment of the main protocol was needed in order to enable my study in their experimental facility.

Study II: ^{13}C-labelling

In Study II, the labelling of the plant component was less than expected. Originally the $\delta^{13}C$ of the fossil fuel derived CO_2 used for labelling was measured to be -41 ‰. Based on this value a model was constructed that forecasted the change in $\delta^{13}C$ of the different carbon pools over time. The model was used to select the time of urine application based on the requirement that the root system should be adequately labelled in order to detect increased mineralization of root-derived C. However, the root compartment turned out to be less labelled than expected, and later it was discovered that the isotope ratio mass spectrometer used for determining the $\delta^{13}C$ of the fossil fuel derived CO_2 was not calibrated to measure delta values of ^{13}C. Thus, the $\delta^{13}C$ of the CO_2 used for labelling was not -41 but -35 ‰, resulting in a $\delta^{13}C$ signal of CO_2 in the enclosures of about -21.5 ‰ after the ^{13}C depleted air was mixed with atmospheric air (for details see Klumpp, 2004). The above mentioned inaccuracy had significant implications for the conduced study, as it resulted in a weaker labelling of the plant component and thus made the detection of mineralization of root-derived C more difficult.

Study III: ^{15}N-labelling of soil NH_4^+ or NO_3^-

Non-uniform labelling of the soil NH_4^+ or NO_3^- pool is a common problem in ^{15}N tracer studies, even in laboratory studies where the ^{15}N label is applied to homogenised soil samples (e.g. Stevens et al, 1997). The wish in Study III was to use ^{15}N tracer technique to examine the N_2O formation via nitrification and denitrification *in situ*, *i.e.* in undisturbed soil in the field. Therefore, ^{15}N labelled urine, ammonium solution and water were applied on the soil surface in microplots established in the field. Concerning the subsequent soil sampling, it was decided only to sample in one soil layer to avoid a laborious set up, as no assistance was available in the laboratory and soil samples had to be extracted on the day of sampling. The 0-10 cm soil layer was chosen to allow the ^{15}N labelled NO_3^- to move slightly in the soil profile during the 24 hours between the two soil samplings for determination of nitrification rate via $^{15}NO_3^-$ pool dilution.

However, in many cases the ^{15}N enrichment of the emitted N_2O exceeded the enrichment of soil NH_4^+ or NO_3^- measured about 5 hours after solution application in the 0-10 cm soil layer. In some cases it also exceeded the initial enrichment calculated for the 0-10 cm soil layer. Thus, the ^{15}N enrichment of the labelled pool being nitrified or denitrified appeared to be higher than the mean enrichment in the 0-10 cm soil layer. The added solutions were therefore assumed to affect the 0-2 cm soil layer, as this was the penetration depth. Thus, the calculated enrichment in the 0-2 cm soil layer was used to quantify the sources of N_2O.

More stratified soil sampling may have solved a part of this problem, *i.e.* 0-2.5 cm, 2.5-5 cm and 5-10 cm instead of pooling the 0-10 cm soil layer. However, the associated workload would have exceeded the resources available for this study. In a similar ^{15}N-tracer study, the effect of urine on the sources of N_2O was examined in grassland monoliths of 58 cm × 30 cm × 10 cm deep (Monaghan and Barraclough, 1993). Soil was sampled in the 0-5 cm and 5-7 cm soil layers. However, also in this study the ^{15}N

enrichment of N_2O exceeded the measured enrichment of the soil NO_3^- in the 0-5 cm layer. The authors explained the observation by non-uniform labelling and called for improvement of the ^{15}N technique for measuring denitrification losses with the aim of adapting the method for routine field use. Based on my experience from Study III I support this suggestion.

The problem of non-uniform labelling might be overcome by ensuring that the addition of labelled N by far exceeds the pre-existing soil N, as it will result in the presence of only one significant pool, which is practically uniform (Bergsma et al., 1999). This was the case for the ammonium/$^{15}NH_4^+$ treatment in Study III.

4 Conclusions and perspectives

Biologically fixed N_2 as a source of N_2O – implications for organic farming

Recently fixed N_2 is a minor source for the N_2O production in grass-clover swards. Thus, the known rise in the N_2O emission induced by the presence of legumes seems to result from long-term mineralization of fixed N.

Organic farming differs from conventional agriculture by aiming to avoid pollution of the environment caused by the farming practice (DARCOF, 2000). The organic plant-soil system receives N via animal manure and via N_2 fixing legumes, and in Denmark grass-clover mixtures used for forage and green manure is an important element of the organic crop rotation.

Substituting fertilizer N with biological N_2 fixation is often found to reduce N_2O emissions from grasslands, hence the N_2O emission factor for biologically fixed N_2 is probably lower than for mineral fertilizers. This was supported by estimated emission factors of 0.47 and 0.56 % (Section 1.7; Schmid et al., 2001). Consequently, the use of grass-clover pastures in organic dairy farming appears to be less polluting in terms of N_2O emissions as compared to the fertilized grass swards, which account for part of the pastures in conventional dairy farming.

However, when grass-clover swards are used for green manure in organic farming systems the associated N_2O loss could possibly exceed the N_2O emission factor for mineral fertilizers in conventional agriculture. This is because a comparison of the two N sources should include the N_2O loss caused by ploughing in of the grass-clover sward, which may be twice the amount of N_2O released during the growth of the sward (Kaiser et al., 1998). In unfertilized organic crop rotations, Vinther et al. (2004) estimated the N_2O emission factor for mineralization of organic matter to vary between 1.8 and 2.9 % in bare soil plots. Thus, it is of crucial importance that the N release from grass-clover residues are synchronized with the N demand of the following crop, if the N_2O loss related to grass-clover as green manure are to be similar or even less than the N_2O emission factor for mineral fertilizers. Synchronization may for example be improved by postponing ploughing in of the sward until right before sowing of the succeeding crop (Baggs et al., 2000). Finally, the entire effect of biologically fixed N versus fertilizer N on the N_2O losses from farming systems ought to be evaluated on a long-term scale, because part of the N input remain in the soil after the growing season and may affect the N_2O emission for several years (e.g. Vinther et al., 2004).

N_2O formation in urine patches – ideas for further research

Urine patches deposited by grazing cattle are known to be hot-spots of N_2O emission. Restrictions on grazing have been suggested as a means to reduce N_2O emissions from dairy farming systems, because the N_2O emission factor is much higher for urine and dung patches than for slurry which has been applied to the soil properly (Velthof and Oenema, 1997; Oenema et al., 1997; 1998). The current knowledge on the mechanisms involved in the N_2O formation in urine patches is unsatisfactory and needs to be improved. In the long term, this will hopefully result in development of new dairy farming practises that reduce the N_2O losses from urine patches deposited by grazing cattle, and thereby make it easier for organic dairy farming to simultaneously meet with its aims regarding high animal welfare (*i.e.* grazing) and low environmental impact.

The main conclusions of the urine studies are that increased N_2O emission following urine application at rates up to 5.5 g N m^{-2} was not caused by enhanced denitrification stimulated by labile compounds released from scorched plant roots (II). Furthermore, the rise of soil pH and NH_4^+ following urine application (52.9 g N m^{-2}) led to increased nitrification rate, which appeared to be the most important factor explaining the high initial N_2O emission from simulated urine patches (III). To my knowledge, Paper III is the first time that raised soil pH has been suggested to increase the rate of nitrification in urine patches, and thereby be part of the reason for the urine-induced N_2O emission.

Finally, $NH_{3(aq)}$ concentration is a crucial factor controlling which mechanisms are involved in the N_2O formation in urine patches, as high concentrations may lead to microbial stress and root scorching. The soil $NH_{3(aq)}$ concentration following urea hydrolysis increases with the amount of urea-N applied and with soil pH, but decreases with rising soil CEC. The obtained results point at new potential research activities based on similar experimental approaches, but focusing on the mechanisms responsible for the N_2O formation in high N-urine patches deposited on soils representing a range in pH and CEC.

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Paper I

Biologically fixed N_2 as a source for N_2O production in a grass-clover mixture, measured by $^{15}N_2$

(Accepted for publication in Nutrient Cycling in Agroecosystems)

Biologically fixed N_2 as a source for N_2O production in a grass-clover mixture, measured by $^{15}N_2$

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Abstract

The contribution of biologically fixed dinitrogen (N_2) to the nitrous oxide (N_2O) production in grasslands is unknown. To assess the contribution of recently fixed N_2 as a source of N_2O and the transfer of fixed N from clover to companion grass, mixtures of white clover and perennial ryegrass were incubated for 14 days in a growth cabinet with a $^{15}N_2$-enriched atmosphere (0.4 atom% excess). Immediately after labelling, half of the grass-clover pots were sampled for N_2 fixation determination, whereas the remaining half were examined for emission of ^{15}N labelled N_2O for another eight days using a static chamber method. Biological N_2 fixation measured in grass-clover shoots and roots as well as in soil constituted 342, 38 and 67 mg N m^{-2} d^{-1} at 16, 26 and 36 weeks after emergence, respectively. The drop in N_2 fixation was most likely due to a severe aphid attack on the clover component. Transfer of recently fixed N from clover to companion grass was detected at 26 and 36 weeks after emergence and amounted to 0.7 ± 0.1 mg N m^{-2} d^{-1}, which represented 1.7 ± 0.3 % of the N accumulated in grass shoots during the labelling period. Total N_2O emission was 91, 416 and 259 µg N_2O-N m^{-2} d^{-1} at 16, 26 and 36 weeks after emergence, respectively. Only 3.2 ± 0.5 ppm of the recently fixed N_2 was emitted as N_2O on a daily basis, which accounted for 2.1 ± 0.5 % of the total N_2O-N emission. Thus, recently fixed N released via easily degradable clover residues appears to be a minor source of N_2O.

Key words

emission factor, nitrogen fixation, nitrogen transfer, nitrous oxide, $^{15}N_2$, white clover

Abbreviations

CONT – control

EMI – determination of N_2O EMIssion

FIX – determination of N_2 FIXation

START – sampled at the start of labelling

Introduction

In temperate organic farming, biological N_2 fixation in grass-legume swards provides a major N input to the system, but knowledge is sparse regarding the amount of fixed N_2 lost from the grasslands as N_2O. Agricultural soils are known to be a considerable source of N_2O (Kroeze et al. 1999) and at present this source accounts for 5 % of the European release of anthropogenic derived greenhouse gases (EEA 2002). Furthermore, N_2O is involved in ozone depletion of the stratosphere (Crutzen 1981). In soils, N_2O is mainly produced in the bacterial processes of nitrification and denitrification (Firestone and Davidson 1989). Thus, legumes may give rise to N_2O by supplying the microbial community in the soil with N compounds. In addition, many strains of the symbiotic N_2 fixing bacteria *Rhizobium* are able to denitrify nitrate that moves into the root nodules from the soil (O'Hara and Daniel 1985). However, this ability was not found among the strains that form symbiosis with white clover (De Klein et al. 2001).

According to the guidelines issued by the Intergovernmental Panel on Climate Change, inventories for N_2O emissions from agricultural soils should be based on the assumption that 1.25 % of the total N supply is emitted as N_2O (IPCC 1997). This emission factor is used as a standard for all N inputs, although the factor relies on experiments with fertilizer and manure only (Bouwman 1996). Input to the systems via biological N_2 fixation in grass-legume swards is currently not considered as a source of N_2O in the IPCC guidelines (IPCC 1997), partly due to uncertainties in quantifying the N_2 fixation in grasslands (Mosier et al. 1998). Hence, the agricultural greenhouse gas release may presently be underestimated. As organic farming to a very large extent utilises grass-legume mixtures as N source, the contribution from organic farming systems in particular may be underestimated. However, countries are allowed to develop their own inventory methodology based on local measurement data. Some countries, e.g. Denmark and Switzerland, include the contribution from biological N_2 fixation in grasslands in the national N_2O inventory, using the standard emission factor of 1.25 % (Schmid et al. 2001; Mikkelsen et al. 2005). This factor nonetheless seems to overestimate the contribution from biologically fixed N_2, as substituting fertilizer N with biological N_2 fixation is often found to reduce N_2O emissions from grasslands (e.g. Garrett et al. 1992; Ruz-Jerez et al. 1994).

So far, the N_2O emission factor for biologically fixed N_2 in grass-legume swards has only been estimated via modelling (e.g. Schmid et al. 2001) or determined indirectly by relating total N_2O emission to measured N_2 fixation (e.g. Ruz-Jerez et al. 1994). Therefore a $^{15}N_2$-tracer-experiment was initiated on grass-clover to assess the contribution of recently fixed N_2 as a source of N_2O and the transfer of fixed N from clover to companion grass. The $^{15}N_2$-labelling technique is the sole direct measure of N_2 fixation, and in many cases it is the only method to assess the fate of biologically fixed N_2 (Warembourg 1993). To our knowledge the present study is the first where the $^{15}N_2$-labelling technique is used to determine the contribution of N_2 fixation to the N_2O production.

Materials and methods

Establishment of grass-clover

Air-dried and sieved (1 cm) topsoil from an organic crop rotation was packed in 15 × 15 cm pots to a bulk density of 1.46 g cm^{-3}. The soil was a loamy sand with total N content of 0.12 %, total C content of 1.4 %, pH in water of 7.6 and water-holding capacity of 0.23 g water g^{-1} dry soil. Each pot was either sown with a mixture of white clover (*Trifolium repens* L. cv. Klondike) and perennial ryegrass (*Lolium perenne* L. cv. Fanda) or with perennial ryegrass only. All pots were placed in a glasshouse with a day/night regime of 16/8 h, minimum temperature 21/16 ºC and minimum light intensity of 120 µmol m^{-2} s^{-1} (PAR) provided by fluorescent tubes. Seedlings emerged around 21 March 2002, and after three weeks the plant density was reduced to 14 seedlings per pot (grass:clover, 1:1). Grazing was simulated by cutting to a height of 6 cm every second week. Six weeks after emergence, the pots were transferred outdoors. Ammonium sulphate corresponding to 25 kg N ha^{-1} was added at 8, 14, 21 and 26 weeks after emergence. At 28 weeks, pots were transferred to a growth chamber with a day/night regime of 16/8 h, temperature 20/15 ºC and light intensity of 300 µmol m^{-2} s^{-1} (PAR). From 26 weeks after emergence, attempts were made to control aphids on clover via smothering agents and biological pest control by the Asian lady beetle (*Harmonia axyridis*), an aphid midge (*Aphidoletes aphidimyza*) and a parasitic wasp (*Aphidius colemani*).

Growth cabinet for $^{15}N_2$-labelling

The ^{15}N-labelling approach consisted of introducing $^{15}N_2$ into the atmosphere in a minimum-volume closed-system growth cabinet in order to trace the symbiotic N_2 fixation. The labelling cabinet (Figure 1) was a modified chest freezer (model TMW300, Frigor, Viborg, DK) in which the volume was reduced to 48 (width) × 86 (length) × 42 (depth) cm by installing a raised floor (4 mm aluminium sheeting). The cabinet could host twelve 15 × 15 cm pots, which were placed in plastic bags and elevated slightly above the floor to hinder water exchange between pots. External growth lamps supplied light through a transparent window of 12 mm plexiglas mounted above a 44 × 82 cm hole cut into the lid of the freezer. To improve the seal between lid and casket, an EPDM rubber gasket was fitted to the sealing edge of the freezer. Circulation of air within the cabinet was achieved using a fan (60 × 60 mm) to blow air from the bottom to the top of the cabinet through a 7.5 cm diameter PVC Flex Pipe.

Temperature was maintained by a computer, which controlled the compressor of the freezer. The computer also controlled light on/off as well as the supply of CO_2 during defined periods in order to keep CO_2 near ambient levels. The concentration of CO_2 in the cabinet was monitored by an infra-red gas analyser (IRGA; EGM-2, PP Systems, Hitchin, UK). The atmosphere of the system was circulated externally around a closed loop made from copper tubing ($^1/_8$" OD) by a timer-controlled diaphragm pump. A CO_2 scrub could be integrated in the closed loop in order to remove excess CO_2 produced during night. The scrub consisted of 1 *M* potassium hydroxide (KOH) in a 0.5 litre screw capped serum bottle, mounted with a rubber stopper pierced by two tubes. This scrub was later replaced by a 0.6 litre solid-state soda lime scrub (75 % $CaOH_2$, 3.5 % NaOH), as KOH foam had started to corrode the rubber stopper. A 12 litres tedlar bag attached to

the closed loop prevented over-pressure in the system. The closed gas loop was equipped with a sampling port for collecting gas samples and introducing $^{15}N_2$ to the system. Water was provided through a silicon tube to each pot connected to a valve on the outside. The irrigation was adjusted to obtain a soil water content slightly below the water-holding capacity based on initial transpiration measurements, experience from the former labelling event and water status of control pots. Condensate that accumulated at the floor of the cabinet was sucked out daily via a silicone tube connected to a valve on the outside.

$^{15}N_2$-labelling

Three 14-day incubations were conducted with grass-clover mixtures at 16, 26 and 36 weeks of age. At each labelling event, 16 grass-clover and 12 grass pots were cut to a height of 6 cm. Eight grass-clover and eight grass pots were placed in an ordinary growth chamber with a day/night regime of 16/8 h, temperature at 20/15 ºC and light intensity of 300 μmol m^{-2} s^{-1} (PAR). The remaining eight grass-clover and four grass pots were placed in the labelling cabinet under similar conditions. The following day (Day 1), four grass-clover and four grass pots from the growth chamber were sampled to establish the amount of N in the plant material at the start of the labelling period (START pots - soil not analysed). The remaining four grass-clover and four grass pots in the growth chamber were controls and were sampled on day 14 (CONT pots). On day 1, two litres 98 atom% $^{15}N_2$ were added to the labelling cabinet and on day 8, a volume of 0.7 litres was added, resulting in a mean enrichment of the atmosphere over the 14-day incubation period of 0.4 atom% excess. To compensate for a leaky diaphragm pump during the incubation at 36 weeks after emergence, the $^{15}N_2$ addition on day 8 was substituted by addition of about 0.5 litres on day 4, 7 and 11. A sample of the cabinet atmosphere was taken daily and stored in an evacuated 120 ml serum bottle fitted with rubber stopper before analysis for ^{15}N abundance of N_2, concentration of N_2O, and sometimes (5/14 days) ^{15}N abundance of N_2O. On day 14, four grass-clover and four grass pots from the labelling cabinet were sampled to establish the N_2 fixation during the labelling period (FIX pots).

Measurement of $^{15}N_2O$ emission

The remaining four grass-clover pots from the labelling cabinet (EMI pots) were transferred to the ordinary growth chamber. During the following eight days, emission of ^{15}N labelled N_2O was measured daily from these pots using a static chamber method. Beforehand, water-holding capacity was determined on a set of pots by removing plant shoots and saturating the soil with water. The pots were covered by plastic to hinder evaporation and were then allowed to drain for two days before weighing and determination of gravimetric water content (oven drying at 105 °C for 24 h). At least one hour before onset of gas measurements, the EMI pots were irrigated to reach 60-65 % of the water-holding capacity. For analysis of initial N_2O concentration and ^{15}N abundance, two evacuated 3.5 ml Venoject vials and two evacuated 120 ml serum bottles were filled with samples of growth chamber atmosphere using 5 and 60 ml Plastipak syringes, respectively. The same procedures were used when sampling headspace gas during the following cover period. Each pot was placed on an 11.5 × 11.5 × 1.3 cm platform above a shallow (1 cm) tray of water. The pot was then enclosed within an 18 × 18 × 29 cm plastic cover fitted with a rubber stopper to allow sampling, and weighted down to

ensure a complete water-seal. After 45, 90, 135 and 180 minutes of cover period, a 3.5 ml sample of the headspace gas was removed through the rubber stopper for analysis of N_2O concentration. At the end of the cover period (180 minutes), a 120 ml sample was taken for analysis of ^{15}N abundance of N_2O. The EMI pots were harvested after eight days of gas measurement. At 36 weeks after emergence, emission of N_2O was also measured for unlabelled grass pots. Once during each experiment, ^{15}N abundance of emitted N_2O was determined on unlabelled grass-clover pots. The result was at natural abundance or slightly below, thus 0.3663 atom% was used as the background value in the calculations.

The 3.5 ml gas samples were added 2 ml N_2 before they were analysed for N_2O in a gas chromatograph (GC-14B, Shimadzu, Kyoto, JP) fitted with a HaySep Q column and an electron capture detector (column and detector temperature were 30 °C and 300 °C, respectively). Concentration of N_2O in gas samples from the labelling cabinet was determined in the same way. Gas samples of 100 µl from the labelling cabinet and from the 120 ml samples taken during the cover periods at 16 weeks were analysed manually for ^{15}N abundance of N_2 using an elemental analyser (EA 1110, Carlo Erba, Milano, IT) fitted with an injection port and coupled in continuous flow mode to an isotope-ratio mass spectrometer (IRMS; Finnigan MAT Delta E or Finnigan MAT Delta Plus, Bremen, DE). All 120 ml samples from the cover periods as well as selected samples from the labelling cabinet were analysed for ^{15}N abundance of N_2O following removal of CO_2 and cryogenic focusing of N_2O on a trace gas concentration unit (PreCon Finnigan MAT, Bremen, DE) in continuous flow mode to an IRMS (Finnigan MAT Delta Plus, Bremen, DE).

Sampling of pots

Shoot material was harvested and sorted into clover and grass. At 26 and 36 weeks after emergence, a dead shoot fraction was also determined for the grass-clover pots. Fresh weight of shoot material from FIX pots was established in order to calculate needed irrigation of EMI pots to reach 60-65 % of water-holding capacity. A root and a soil fraction were obtained by sieving (6 mm), and the root fraction was subsequently cleaned of soil by repeatedly being immersed in water then washed into a fine sieve.

Analyses of plants and soil

Dry matter of plant samples was determined (oven dried at 80 °C for 24 h). Plant samples and samples of air-dried soil were finely ground and analysed for total N and ^{15}N on the elemental analyser and IRMS (Finnigan MAT Delta Plus, Bremen, DE). In addition, total carbon was measured on soil from grass-clover CONT pots sampled at 16 weeks after emergence. This treatment was also used to determine soil pH in a 10:25 (w:vol) suspension of fresh soil in distilled water. Within eight hours of pot sampling, 10 g portions of fresh soil were extracted in 2 *M* KCl (1:10, w:vol), stirred on a horizontal shaker for one hour. The extracts were filtered through Whatman 40 filters and kept at -20 °C until NO_3^- and NH_4^+ were analysed colorimetrically on an autoanalyzer (Bran+Luebbe, Norderstedt, DE). Nitrogen-15 abundance of inorganic N was determined in extracts by the diffusion method, where NO_3^- and NH_4^+ are converted into NH_3, which is trapped on an acidified filter paper (Sørensen and Jensen 1991). The filters were subsequently analysed for ^{15}N as described for plant and soil samples.

Calculations

When calculating the N_2 fixation, the proportion of total N in plants derived from a $^{15}N_2$-enriched atmosphere (*P*) is determined as

$$P = N_L^*/N_P^*, \quad (1)$$

where N_L^* is the ^{15}N atom% excess enrichment of the legume after exposure to an atmosphere with a ^{15}N atom% excess enrichment of N_P^* (Warembourg 1993). Wood and McNeill (1993) show that this *P* value is independent of the plant N pool at the start of the labelling period, which makes the equation suitable for calculating N_2 fixation for the FIX pots. However, by extending their argumentation it can be established that the *P* value is also valid in cases where the plants accumulate N both before and after the labelling period. This makes the equation suitable for calculating N_2 fixation for the EMI pots as well. The equation is based on the assumptions that N supplied by soil, fertilizer and non-labelled atmosphere have the same ^{15}N abundance and that no $^{15}N_2$ remains during the last period. The amount of N originating from fixation during the labelling period (N_LP) is

$$N_LP = N_L N_L^*/N_P^*, \quad (2)$$

where N_L is the N accumulated by the plants during their full growth. For the FIX pots, the amount of N derived from fixation was calculated for each plant-soil fraction (*viz.* clover shoot, grass shoot, dead shoot, root and soil total N), provided that ^{15}N abundance of the fraction increased significantly between CONT and FIX pots. Total N_2 fixation was determined in the same way for the EMI pots. Additionally, the amount of fixed N_2 lost as N_2O during the eight days emission measurement was calculated, and included in the total N_2 fixation for the EMI pots.

Flux of N_2O was calculated from the linear increase in N_2O concentration in the headspace during the cover period. Emission of N_2O-N derived from biologically fixed N_2 was calculated from the emission of ^{15}N labelled N_2O, which was determined in two ways. If a significant N_2O emission (R^2 of N_2O concentration vs. time ≥ 0.7) and increase in ^{15}N abundance of N_2O (end-value ≥ 0.3689 atom%) were detected, then emission of ^{15}N labelled N_2O (CC^*) was calculated as

$$CC^* = C_t C_t^* - C_0 C_0^*, \quad (3)$$

where C_0 and C_t are the N_2O concentration calculated from the regression equation for the start and end of the cover period, respectively, and C_0^* and C_t^* are the atom% excess enrichment of N_2O at the start and end of the cover period, respectively. If only a significant increase in ^{15}N abundance of N_2O was detected then the emission of ^{15}N labelled N_2O was calculated as

$$CC^* = (C_t^* - C_0^*)C_0 \quad (4)$$

Emission of N_2O-N derived from fixed N_2 (*CP*) was then established as

$$CP = CC^*/N_P^*, \quad (5)$$

which corresponds to equation 2. The estimates were subsequently converted from concentration of N_2O to amount of N. The fraction of fixed N, which was emitted as N_2O (*FE*) was calculated as

$$FE = E_{N2O}/F_{tot}, \quad (6)$$

where E_{N2O} is the amount of fixed N emitted as N_2O per day and F_{tot} is the total N_2 fixation during the labelling period per day determined for the EMI pots.

Statistics

ANOVAs and Tukey's multiple comparison tests ($\alpha = 0.05$) were performed using SAS General Linear Model procedure (SAS Institute 1997). Homogeneity of variance was not obtained despite transformation when testing soil inorganic N for all grass-clover treatments. Hence, differences between median inorganic N content for each experiment were assessed using the Kruskal-Wallis test. The same constraint appeared for three fractions when testing ^{15}N abundance of grass-clover CONT pots against FIX and EMI pots, *viz.* soil of EMI pots at 26 weeks after emergence and clover shoot of FIX and EMI pots at 36 weeks after emergence. Thus, the medians were compared using the Mann-Whitney U-test. In some cases variation was indicated as coefficient of variance (CV), which is the standard deviation in percent of the mean.

Results

Labelling cabinet atmosphere

Nitrogen-15 abundance of N_2 in the labelling cabinet declined in an apparent exponential pattern (Figure 2), probably because of diffusion of N_2 through the 12 mm plexiglas window. This problem was also faced in other $^{15}N_2$ incubation studies (McNeill et al. 1994; Wood and McNeill 1993), and was compensated for by multiple additions of $^{15}N_2$. Mean $^{15}N_2$ enrichment over the labelling period was 0.4369, 0.4177 and 0.3724 atom% excess at 16, 26 and 36 weeks after emergence, respectively. Nitrogen-15 abundance of N_2O in the labelling cabinet gave no evidence for release of N_2O derived from biologically fixed N_2 during the labelling events (data not shown). During nighttime, the CO_2 concentration in the labelling cabinet increased to > 1200 ppm. After onset of light, the CO_2 decreased assisted by the CO_2 scrub (3-5 hours) to near ambient concentrations with a mean of 344, 530 and 468 ppm at 16, 26 and 36 weeks after emergence, respectively.

Biomass in grass-clover FIX pots

At 16 weeks of age, the clover component made up a significantly larger proportion of the living shoot biomass (82 %) than at 26 and 36 weeks after emergence (65 and 51 %). However, total living biomass including roots did not differ significantly between labelling events ($P = 0.6523$), and constituted 772 ± 38 g dry matter m^{-2} on average (Table 1). Despite the increased CO_2 level, conditions in the labelling cabinet had no significant effect on the growth of plants measured as living shoot and root biomass compared to the control ($P = 0.1176$) (Table 1).

Nitrogen fixation

Amount of N and ^{15}N abundance of the different plant-soil fractions appear in Table 2 and 3, respectively. The results on N_2 fixation revealed a significant effect of time ($P < 0.0001$). Accordingly, at 16 weeks after emergence N_2 fixation measured in grass-clover

FIX pots constituted 342 mg N m^{-2} d^{-1}, which declined to 38 and 67 mg N m^{-2} d^{-1} at 26 and 36 weeks, respectively (Figure 3). Overall the N_2 fixation differed between the FIX and the EMI pots (P = 0.0171), which mainly resulted from the higher N_2 fixation measured in the EMI pots at 16 weeks after emergence (Figure 3). The difference was probably due to variation in the clover biomass between pots randomly selected for the two treatments. Also, at 16 weeks, N_2 fixation calculated for the EMI pots includes fixed N found in the grass shoot and soil fractions, which had not yet reached a significant ^{15}N enrichment in the FIX pots (Table 3). Therefore, the fraction of fixed N_2 emitted as N_2O (*FE*, equation 6) was calculated using N_2 fixation measured in EMI pots. Fixed N accounted for 90 and 63 % of the N, which accumulated in clover shoots during the labelling period at 16 and 36 weeks after emergence, respectively, but the difference was insignificant (P = 0.2465). A percentage was not calculated for 26 week old plants because of negative N accumulation between START and FIX pots due to a severe aphid attack on the clover component (Table 2 B).

Distribution of fixed N

The majority of fixed N was found in clover shoot biomass (Figure 4). However, the proportion varied significantly between the labelling events, accounting for 96, 69 and 84 % of the fixed N at 16, 26 and 36 weeks after emergence, respectively. Transfer of fixed N from clover to companion grass determined in FIX pots was insignificant when the plants were 16 weeks old (Table 3 A). However, eight days after the labelling event when pots for determination of N_2O emission were sampled, a significant increase in ^{15}N abundance of grass shoots was detected, demonstrating that N transfer had taken place. At 26 and 36 weeks after emergence, the uncorrected transfer of fixed N from clover to grass shoots constituted 1.0 mg N m^{-2} d^{-1} (P = 0.9761), which represented 2.6 and 1.5 % of the fixed N (P = 0.0727) (Figure 4). An apparent N_2 fixation of 0.4 ± 0.1 mg N m^{-2} d^{-1} was detected in pots with grass. This apparent fixed N_2 was either supplied via free-living or associative N_2 fixing bacteria or an artefact due to absorption of ^{15}N-labelled ammonia ($^{15}NH_3$) through stomata (McNeill et al. 1994). Ammonia contamination of the $^{15}N_2$ gas cannot be excluded. Because of the relatively low importance, these two ^{15}N sources may only have introduced minor error in the calculated symbiotic N_2 fixation for the grass-clover pots. In contrast, they might have caused overestimation of the determined transfer of symbiotically fixed N_2. When the ^{15}N enrichment of grass shoots in mixture is corrected for ^{15}N enrichment of grass shoots in pure stand, then the transfer of fixed N amount to 0.7 ± 0.1 mg N m^{-2} d^{-1}, which represented 1.7 ± 0.3 % of the N accumulated in grass shoot during the labelling period.

N_2O emission and soil water content

Total N_2O emission changed significantly over time (P = 0.0004) and was 91, 416 and 259 µg N_2O-N m^{-2} d^{-1} at 16, 26 and 36 weeks after emergence, respectively (Figure 5 A). Emission of ^{15}N labelled N_2O was detected at 16 weeks after emergence only and could theoretically derive from 1) biological N_2 fixation in clover, 2) biological N_2 fixation by free-living or associative bacteria, or 3) $^{15}NH_3$ contamination of the $^{15}N_2$ gas. However, the two latter sources appeared to be minor as no ^{15}N labelled N_2O was detected at 26 and 36 weeks after emergence. At 16 weeks, emission of N_2O-N derived from biologically fixed N_2 constituted 1.6 ± 0.2 µg N_2O-N m^{-2} d^{-1}. Thus, 3.2 ± 0.5 ppm of the N_2 fixed by 16 week old clover was emitted as N_2O, which accounted for 2.1 ± 0.5 % of

the total N_2O emission. Loss of N_2O from grass pots measured at 36 weeks after emergence was 22 µg N_2O-N m^{-2} d^{-1} and did not differ significantly from the emission measured from grass-clover pots at 16 weeks. The advancement of the N_2O emission was found to be similar to that of the soil water content (Figure 5 A, B). The intention was to keep soil water content of the pots at 60-65 % of the water-holding capacity during N_2O emission measurements. However, because of excessive irrigation in the labelling cabinet prior to gas measurements, and low transpiration rate at 26 and 36 weeks, the mean soil water content of grass-clover pots was 65, 90 and 80 % of the water-holding capacity at 16, 26 and 36 weeks after emergence, respectively ($P = 0.0031$) (Figure 5 B). Soil water content of grass pots at 36 weeks was 64 %. Emission of $^{15}N_2$ from the grass-clover pots was assessed at 16 weeks after emergence, however it was found to be below the detection limit.

Soil inorganic nitrogen in grass-clover pots

The content of soil inorganic N varied between the experiments, *i.e.* the median of inorganic N was significantly lower at 16 weeks after emergence than at 36 weeks (Figure 6). The change was mainly a result of increased ammonium content. Soil inorganic N in the FIX pots corresponded to 75, 1582 and 327 mg N m^{-2} (0-17 cm soil layer) at 16, 26 and 36 weeks after emergence, respectively. At 26 weeks, the content in FIX pots was significantly higher than in the CONT and EMI pots. Attempt to determine the ^{15}N abundance of the inorganic N pool failed, since total amounts of inorganic N trapped from the KCl extracts were inadequate for a proper ^{15}N analysis.

Discussion

Nitrogen fixation

For 16 week old plants, the total N_2 fixation determined in grass-clover shoots and roots as well as in bulk soil constituted 342 mg N m^{-2} d^{-1}, which dropped dramatically to 38 and 67 mg N m^{-2} d^{-1} at 26 and 36 weeks after emergence, respectively (Figure 3). The main reason for this drop was a severe aphid attack on the clover component, which peaked during the experiment at 26 weeks and was still present 36 weeks after plant emergence. The aphids probably reduced the N_2 fixation by contributing to a decline in the clover content from 82 to 65 and 51 % of the total herbage dry weight. Clover content is known to be the major factor determining N_2 fixation in grass-clover swards (Kristensen et al. 1995). In field studies, N_2 fixation is usually determined in the harvested herbage only, e.g. N_2 fixation was reported between 206 and 235 kg N ha^{-1} y^{-1} in first year white clover-ryegrass swards having a mean clover content of about 50-60 % (Jørgensen et al. 1999; Vinther and Jensen 2000). Assuming a growth season of six months, the reported values correspond to daily N_2 fixation rates between 113 and 129 mg N m^{-2} d^{-1}. In conclusion, the determined N_2 fixation at 16 weeks after emergence was relatively high compared to annual field measurements. However, clover content and N_2 fixation vary over the growing season (e.g. Jørgensen et al. 1999; Vinther and Jensen 2000), thus results at 16 weeks represent N_2 fixation at optimal growth conditions. Furthermore, our studies estimate total amounts of fixed N in all pools in contrast to the field measurements.

N_2O emission

Total N_2O emission from the grass-clover pots was 91, 416 and 259 µg N_2O-N m^{-2} d^{-1} at 16, 26 ad 36 weeks after emergence, respectively (Figure 5 A), which is in the same order of magnitude as emissions determined for other extensively managed grasslands containing legumes (e.g. Carran et al. 1995; Wang et al. 1997). The increase in N_2O emission between 16 and 26 weeks after plant emergence might relate to the aphid-induced clover shoot death. The reason is that shoot death leads to decay of roots and nodules (Butler et al. 1959), which may act as a carbon source for denitrifying bacteria. Also, Beck and Christensen (1987) showed that N_2O emission increased when all above-ground ryegrass was removed or when grass leaves turned yellowish.

Soil inorganic N content tended to be higher at 26 and 36 weeks after emergence than at 16 weeks (Figure 6), which was probably due to increased mineralization of clover tissues combined with decreased clover N uptake. In addition to this, the elevated soil water content during the labelling at 26 weeks (Figure 5 B) may have caused the remarkably high soil inorganic N content in the FIX pots. During the following N_2O measurements, the soil was allowed to dry slightly, which enabled nitrification in aerobic microsites. In conclusion, at 26 and 36 weeks after plant emergence N_2O loss via denitrification was favoured by high availability of inorganic N, labile carbon compounds and anaerobic microsites.

Soil water content is often found to be a key factor influencing N_2O emissions (e.g. Carran et al. 1995; Ruz-Jerez et al. 1994). Comparing N_2O emission from grass and grass-clover pots (Figure 5 A) having the same soil water content (Figure 5 B – grass-clover at 16 weeks, grass at 36 weeks) reveals a tendency for higher N_2O emission from the grass-clover pots. This conforms with results obtained by Duxbury et al. (1982) indicating that legumes can increase N_2O emissions by factor two to three compared to unfertilized grass swards.

Transfer of fixed N

It is generally acknowledged that transfer of N from white clover to companion grass mainly involves the long-term mineralization of dead clover tissues taking place on a scale of months (e.g. Goodman 1988; Ledgard 1991). In addition to the long-term N release, a pool of relatively easily degradable clover residues (e.g. exudates, lysates, secretion and decaying fine roots) may contribute to soil inorganic N on a short-term scale, *viz.* within days or weeks after elimination from the clover plant. Consistent with this view, Laidlaw et al. (1996) observed high release of inorganic N from clover indicating a total turnover of clover root N within three months. In the present study, release of recently fixed N into the soil probably took place through this latter pathway.

Transfer of recently fixed N from clover to companion grass was observed in the FIX pots at 26 and 36 weeks after emergence only. However, at 16 weeks recently fixed N was emitted as N_2O, demonstrating that recently fixed N was released from the clover component into the soil at that time. In line with this, an increase in ^{15}N abundance of grass shoots was detected eight days later in the EMI pots, indicating a slower transfer rate for 16 week old mixtures. The reason could be the high competition for light at 16 weeks (clover content 82 %), which seemed to suppress ryegrass growth and thereby N uptake (Table 2 A). On the other hand, the observed transfer at 26 and 36 weeks may be explained by improved light conditions (clover content 65 and 51 %), which tended to stimulate ryegrass growth and N uptake (Table 2 B, C). This conclusion is consistent

with results attained by Høgh-Jensen and Schjoerring (2000), showing highest N transfer in spring and autumn, where white clover growth is low and the growth of ryegrass is high.

Fixed N transferred from clover to grass constituted 1.7 ± 0.3 % of the N accumulated in grass shoots during the labelling period. In contrast, two other $^{15}N_2$ studies showed no transfer of fixed N from white clover to companion ryegrass in a 19 and 129 day experiment (McNeill et al. 1994; McNeill and Wood 1990). However, long-term field studies using the ^{15}N dilution technique have reported apparent transfer of fixed N from white clover to companion ryegrass in the range 0 to 80 % of the grass N content (Boller and Nösberger 1987; Ledgard 1991), with the percentage increasing over time after labelling. The transfer of fixed N found in the present study is low compared to the mentioned studies, which supports the general view that short-term N transfer via easily degradable clover residues is less important than the long-term transfer through decay of more recalcitrant clover tissues.

It is striking that recently fixed N was transferred to companion grass at 26 and 36 weeks after emergence, but was not detected in the emitted N_2O. This could indicate that ryegrass and the N_2O producing bacteria utilized different pools of labile N in the soil. One reason may be differences in the spatial distribution of grass roots and N_2O producing bacteria in relation to the zones of clover residue release. Another explanation may be that the fixed N was less available for the nitrifiers and denitrifiers, either due to amino acid uptake by the grass (Falkengren-Grerup et al. 2000) or because N was mostly transferred directly through common mycorrhizal mycelium (Frey and Schüepp 1992).

N_2O-N derived from fixed N_2

Recently fixed N released via easily degradable clover residues may be important in the flow from N_2 fixation to N_2O emission. However, the present study revealed that only 3.2 ± 0.5 ppm of the recently fixed N was emitted as N_2O on a daily basis. Furthermore, recently fixed N accounted for 2.1 ± 0.5 % of the emitted N_2O-N only. A large part of the remaining N_2O-N was most likely derived from previously fixed N_2, which indicates that long-term N release through decay of more recalcitrant clover tissues probably contributes considerably to the flow from N_2 fixation to N_2O emission.

The standard IPCC N_2O emission factor of 1.25 % is criticised by some authors for overestimating the N_2O emission from mineral fertilizer (e.g. Lægreid and Aastveit 2002) and by others for underestimating the long-term effect of manure and mineral fertilizer application (e.g. Schmid et al. 2001). According to the methodology currently recommended by IPCC, the national N_2O inventories should not include the contribution from biological N_2 fixation in grasslands (IPCC 1997). Some countries, e.g. Denmark and Switzerland, nonetheless include quantitative estimates of biological N_2 fixation in grasslands in their N_2O inventory, using an emission factor of 1.25 % as for other N inputs (Schmid et al. 2001; Mikkelsen et al. 2005).

However, the standard N_2O emission factor of 1.25 % might be considerably unrepresentative for biologically fixed N_2 as only a part of the fixed N is mineralized during the lifetime of the crop (Petersen and Olesen 2002), and because the mineralization occurs slowly (Velthof et al. 1998). The extent to which clover N released through mineralization will give rise to N_2O emission from the sward depends on several factors. First, it depends on the sinks for inorganic N, e.g. uptake by grass and clover, immobilization in microbial biomass and loss by leaching. Second, it depends on

whether the abiotic conditions favour N_2O production, e.g. temperature, carbon source and O_2 level, mainly regulated by the soil water content. Goodman (1991) showed that white clover primarily contributes to soil organic matter in autumn. In line with this, Garret et al. (1992) found that under mild conditions, 70 % of the annual N_2O emission from a white clover-ryegrass pasture occurred during autumn and winter. In the present study, the N_2O emission was measured under temperature and soil water regimes representative of summer conditions. However, even under conditions more favourable for N_2O emission, the contribution of recently fixed N to the N_2O emission would still be minor.

Via modelling, Schmid et al. (2001) estimated the N_2O emission factor for biologically fixed N_2 in permanent grasslands to be 0.22 %. However, after steady state in soil carbon and nitrogen was reached, the emission factor increased to 0.56 %. Obviously, these emission factors are associated with extremely large uncertainties. Ruz-Jerez et al. (1994) found that the annual N_2O emission represented about 1 % of the N input by legume fixation in grazed grass-clover swards. The N_2O loss often increased following a grazing period, mainly because of N return in animal excreta. Thus, the estimated emission factor includes the contribution from N_2 fixation as well as the effect of grazing. In contrast, the effect of grazing animals is accounted for separately in the IPCC methodology.

Biological N_2 fixation in grass-legume swards should not be neglected as a source of N_2O in the national greenhouse gas inventories, especially not when considering the large area of Europe covered by managed grasslands. However, based on the present study and data from the literature we find it unlikely that the N_2O emission factor for biologically fixed N_2 in grass-clover swards would reach the standard emission factor of 1.25 %.

Conclusions

Our results support the general view that recently fixed N contributes little to the N transfer from white clover to companion grass. Moreover, only a tiny fraction of the biologically fixed N_2 was lost as N_2O over the course of a few weeks, and this fraction represented about 2 % of the total N_2O-N emission. Thus, the long-term mineralization of dead clover tissues is probably more important than recently fixed N for the flow from N_2 fixation to N_2O emission.

Acknowledgements

This work was funded by the Danish Research Centre for Organic Farming (DARCOF) and the Danish Agricultural and Veterinary Research Council. We wish to thank Svend Danbæk for creating the temperature-controle-programme for the labelling cabinet, Anja Nielsen for her assistance with the isotopic analyses and Anders Priemé, Henning Høgh-Jensen and Daniel Carter for their useful comments on the manuscript.

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Table 1. Biomass (g dry matter m^{-2}) of the fractions clover shoot, dead shoot, grass shoot and root in grass-clover and grass pots at the start (START), for control (CONT), for determination of N_2 fixation (FIX) and for determination of N_2O emission (EMI), (A) 16 weeks, (B) 26 weeks and (C) 36 weeks after emergence; n = 4, means and SE (in brackets).

A. 16 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	402 (42)	548 (44)	493 (34)	645 (47)	–	–	–
Grass shoot	124 (8)	147 (10)	109 (5)	146 (4)	190 (13)	211 (8)	207 (3)
Root	431 (75)	322 (38)	221 (19)	257 (40)	235 (27)	190 (20)	218 (25)
Living biomass [a]	957 (116)	1017 (74)	823 (52)	1048 (78)	424 (34)	400 (23)	425 (25)

B. 26 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	392 (47)	341 (60)	302 (34)	271 (40)	–	–	–
Dead shoot	177 (16)	205 (25)	198 (16)	226 (20)	–	–	–
Grass shoot	127 (3)	183 (6)	158 (8)	193 (7)	312 (22)	293 (27)	266 (6)
Root	214 (11)	277 (29)	273 (13)	276 (36)	239 (29)	226 (30)	232 (22)
Living biomass [a]	734 (55)	802 (85)	733 (36)	740 (70)	551 (36)	518 (56)	499 (20)

C. 36 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	274 (27)	235 (62)	260 (75)	221 (55)	–	–	–
Dead shoot	334 (35)	327 (23)	330 (21)	424 (22)	–	–	–
Grass shoot	245 (16)	254 (5)	224 (9)	281 (14)	309 (8)	385 (12)	312 (14)
Root	238 (15)	314 (31)	275 (37)	207 (34)	194 (26)	245 (36)	184 (19)
Living biomass [a]	756 (23)	802 (86)	759 (102)	709 (100)	504 (26)	630 (40)	497 (15)

[a] Living biomass includes the fractions clover shoot, grass shoot and root.

Table 2. Amount of N (g N m^{-2}) in the fractions clover shoot, dead shoot, grass shoot and root in grass-clover and grass pots at the start (START), for control (CONT), for determination of N_2 fixation (FIX) and for determination of N_2O emission (EMI), (A) 16 weeks, (B) 26 weeks and (C) 36 weeks after emergence; n = 4, means and SE (in brackets).

A. 16 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	10.9 (0.6)	16.0 (1.3)	15.4 (0.4)	21.7 (1.8)	–	–	–
Grass shoot	1.58 (0.06)	1.71 (0.06)	1.57 (0.07)	1.95 (0.09)	1.69 (0.15)	1.94 (0.10)	1.85 (0.08)
Root	4.60 (0.67)	3.88 (0.68)	2.25 (0.11)	4.16 (0.46)	1.49 (0.15)	1.01 (0.06)	1.21 (0.15)
Living biomass [a]	17.1 (1.1)	21.6 (1.9)	19.3 (0.5)	27.8 (2.3)	3.18 (0.29)	2.96 (0.14)	3.06 (0.22)

B. 26 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	9.55 (1.50)	6.76 (1.14)	6.44 (0.81)	5.57 (0.60)	–	–	–
Dead shoot	3.81 (0.39)	4.49 (0.57)	3.96 (0.28)	4.84 (0.44)	–	–	–
Grass shoot	1.66 (0.05)	2.02 (0.09)	1.90 (0.08)	2.29 (0.13)	2.73 (0.16)	2.64 (0.16)	2.44 (0.04)
Root	3.48 (0.28)	4.95 (0.61)	4.85 (0.49)	4.47 (0.59)	1.49 (0.16)	1.43 (0.14)	1.58 (0.20)
Living biomass [a]	14.7 (1.7)	13.7 (1.7)	13.2 (1.0)	12.3 (1.0)	4.23 (0.21)	4.07 (0.30)	4.02 (0.18)

C. 36 weeks

	Grass-clover pots				Grass pots		
Fraction	START	CONT	FIX	EMI	START	CONT	FIX
Clover shoot	5.09 (0.64)	5.39 (1.42)	6.16 (1.45)	5.04 (1.25)	–	–	–
Dead shoot	6.52 (0.68)	7.12 (0.46)	7.84 (0.59)	9.56 (0.91)	–	–	–
Grass shoot	2.15 (0.11)	2.59 (0.10)	2.72 (0.07)	3.07 (0.09)	2.90 (0.04)	3.30 (0.13)	3.13 (0.11)
Root	3.52 (0.30)	4.45 (0.64)	3.50 (0.42)	2.82 (0.51)	1.19 (0.12)	1.56 (0.14)	1.39 (0.24)
Living biomass [a]	10.8 (0.7)	12.4 (2.1)	12.4 (1.8)	10.9 (1.8)	4.09 (0.11)	4.85 (0.27)	4.51 (0.33)

[a] Living biomass includes the fractions clover shoot, grass shoot and root.

Table 3. Nitrogen-15 abundance (atom%) of the fractions clover shoot, dead shoot, grass shoot, root and soil in pots for control (CONT), determination of N_2 fixation (FIX) and determination of N_2O emission (EMI) at 16, 26 and 36 weeks after emergence, (A) grass-clover pots, (B) grass pots; n = 4, means.[a] [b]

A. Grass-clover pots

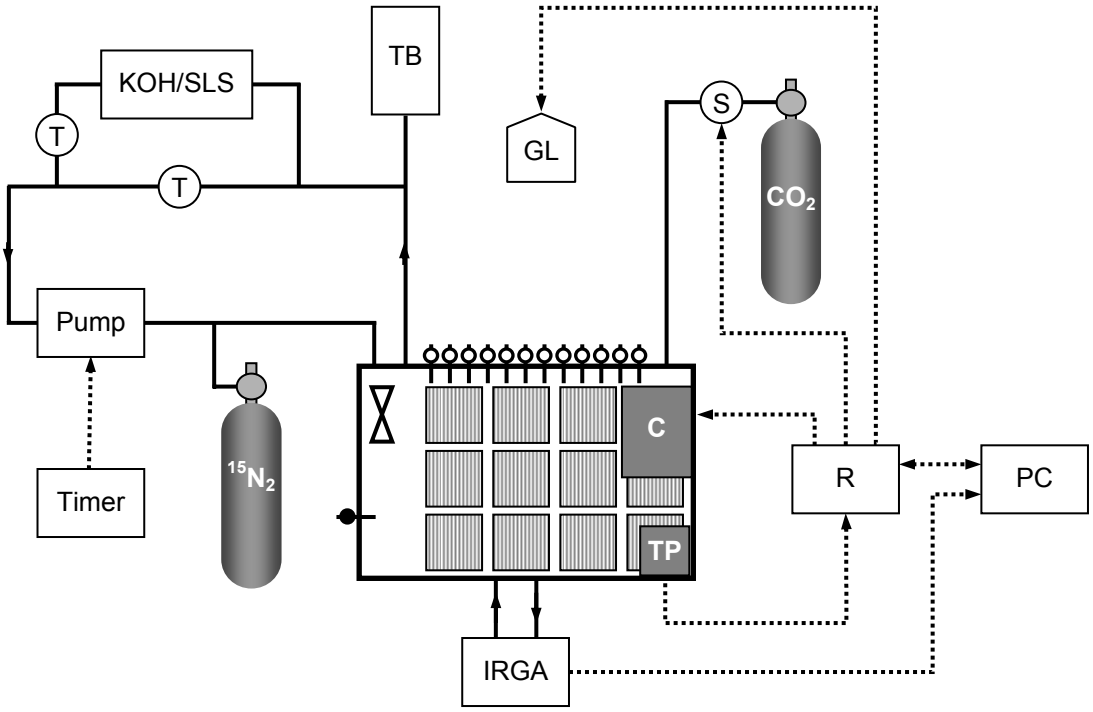
	16 weeks			26 weeks			36 weeks		
Fraction	CONT	FIX	EMI	CONT	FIX	EMI	CONT	FIX	EMI
Clover shoot	0.3661	**0.4787**	**0.4754**	0.3659	**0.3857**	**0.3783**	0.3660	**0.4077**	**0.4185**
Dead shoot	–	–	–	0.3662	**0.3697**	**0.3685**	0.3661	**0.3672**	**0.3669**
Grass shoot	0.3669	0.3675	**0.3689**	0.3659	**0.3683**	**0.3677**	0.3660	**0.3677**	**0.3672**
Root	0.3667	**0.3994**	**0.4054**	0.3666	**0.3742**	0.3696	0.3666	**0.3760**	**0.3771**
Soil	0.3688	0.3692	**0.3697**	0.3692	0.3692	0.3689	0.3691	0.3691	0.3692

B. Grass pots

	16 weeks		26 weeks		36 weeks	
Fraction	CONT	FIX	CONT	FIX	CONT	FIX
Shoot	0.3673	**0.3680**	0.3667	**0.3675**	0.3667	**0.3671**
Root	0.3677	**0.3684**	0.3676	0.3677	0.3675	0.3676
Soil	0.3694	0.3691	0.3693	0.3692	0.3692	0.3692

[a] Bold indicates significant increase in ^{15}N abundance from CONT pots to FIX and EMI pots.

[b] Coefficient of variance (CV) of ^{15}N abundance in the fractions clover shoot and root in grass-clover FIX and EMI pots was in the range 0.47-4.18 %. For all remaining fractions CV was in the range 0.01-0.36 %.



C	Compressor	T	Toggle valve
GL	Growth lamps	TP	Temperature probe
IRGA	Infra-red gas analyser	TB	Tedlar bag to prevent over-pressure
KOH	Potassium hydroxide scrub		Fan
PC	Computer		Tube for irrigation
R	Relays		Tube for collection of condensate
S	Solenoid valve		Pipe or tube
SLS	Soda lime scrub		Wiring and signal direction

Figure 1. The closed-system growth cabinet used for $^{15}N_2$-labelling.

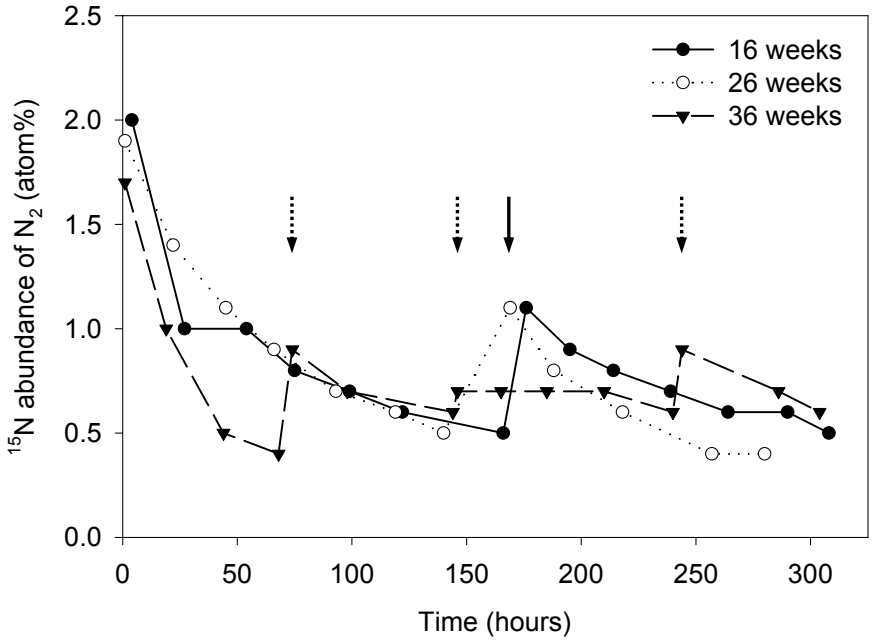


Figure 2. Changes in ^{15}N abundance of atmospheric N_2 in the labelling cabinet during the incubation at 16, 26 and 36 weeks after plant emergence. Arrows indicate time for additional $^{15}N_2$ supply (solid arrow = 16 and 26 weeks, dashed arrows = 36 weeks).

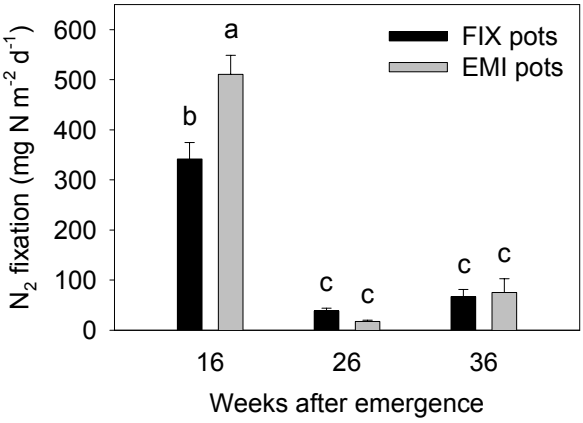


Figure 3. Biological N_2 fixation measured in grass-clover pots for determination of fixation (FIX) and for determination of N_2O emission (EMI) at 16, 26 and 36 weeks after emergence; n = 4, means ± SE. Bars with same letter are not significantly different.

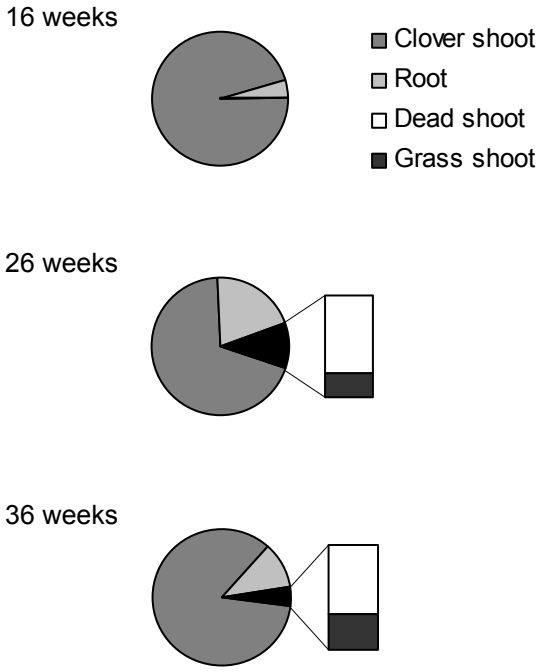


Figure 4. Distribution of biologically fixed N in the fractions clover shoot, root, dead shoot and grass shoot established in grass-clover pots for determination of fixation at 16, 26 and 36 weeks after emergence.

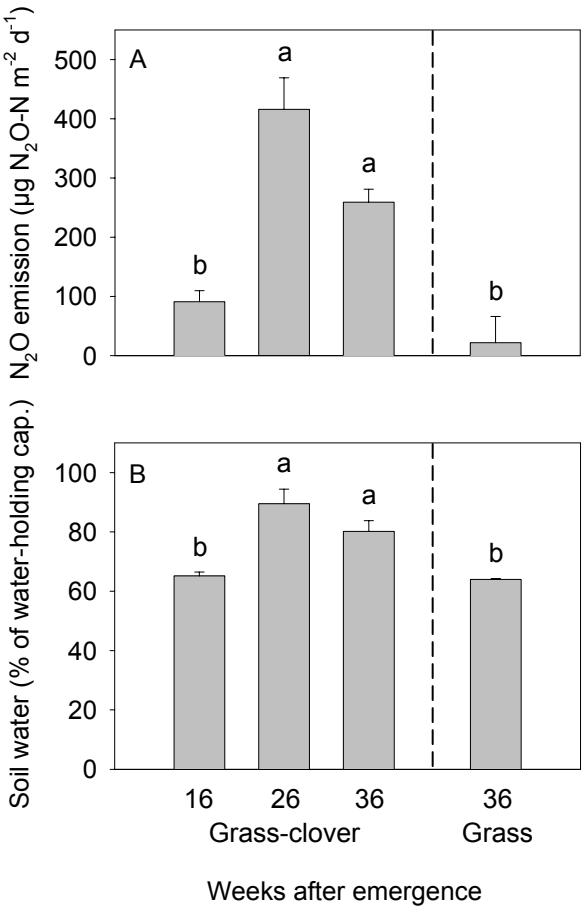


Figure 5. (A) Emission of N_2O and (B) soil water content (% of water-holding capacity) established for grass-clover pots for determination of emission at 16, 26 and 36 weeks after emergence and for grass pots at 36 weeks after emergence; n = 4, means ± SE. Bars with same letter are not significantly different.

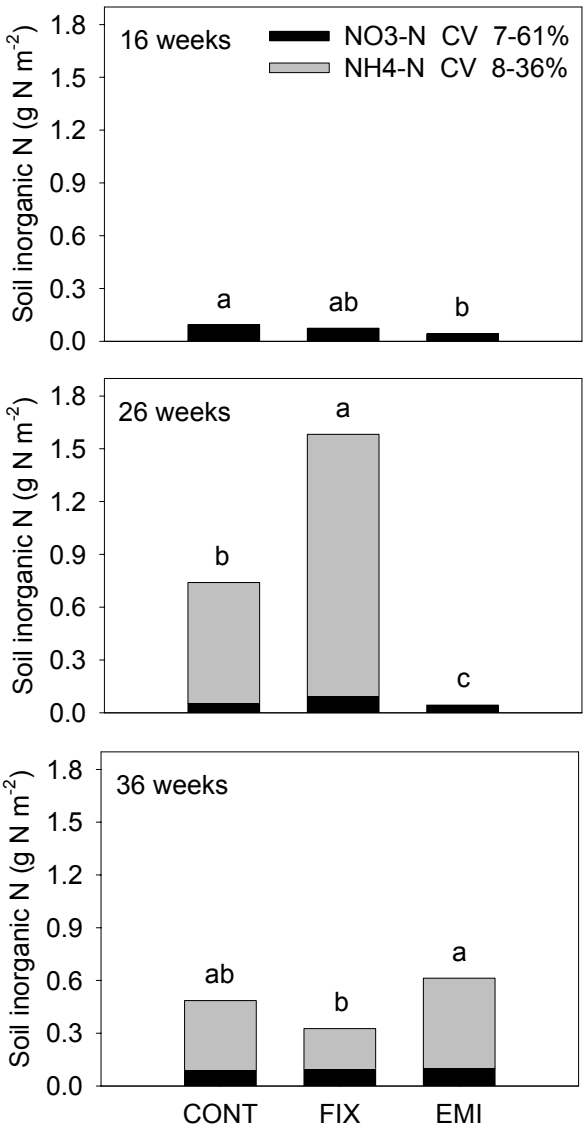


Figure 6. Content of soil nitrate and ammonium per square meter (0-17 cm soil layer) in grass-clover pots for control (CONT), determination of N_2 fixation (FIX) and determination of N_2O emission (EMI) at 16, 26 and 36 weeks after emergence; n = 4, means. Bars in each plot having same letter are not significantly different. Interval for coefficient of variance (CV) of N content in the nitrate and ammonium pool are indicated with legends.

Paper II

Is mineralization of root-derived C a key process explaining the high N_2O emission from low N-urine patches?

(Submitted to Nutrient Cycling in Agroecosystems)

Is mineralization of root-derived C a key process explaining the high N_2O emission from low N-urine patches?

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Abstract

Urine deposition on grassland causes significant N_2O losses, which is possibly partly because denitrification is stimulated by labile compounds released from scorched plant roots. Two 12-days experiments were conducted in ^{13}C-labelled grassland monoliths to investigate the link between N_2O production and carbon mineralization following urine application. Measurements of N_2O and CO_2 emissions from the monoliths as well as $\delta^{13}C$ signal of evolved CO_2 were done on day -4, -1, 0, 1, 2, 4, 5, 6 and 7 after application of urine corresponding to 3.1 and 5.5 g N m^{-2} in the first and second experiment, respectively. The $\delta^{13}C$ signal was also determined for soil organic matter (SOM), dissolved organic C (DOC) and CO_2 evolved by microbial respiration. In addition, denitrifying enzyme activity (DEA) and nitrifying enzyme activity (NEA) were measured on day -1, 2 and 7 after the first urine application event. Mean $\delta^{13}C$ signals appeared in the order DOC (-23 ‰) > SOM (-26 ‰) > microbial respiration (-27 ‰) > soil respiration (-29 ‰). Urine did not affect DEA, whereas NEA was enhanced 2 days after urine application. In the first experiment, urine had no significant effect on the N_2O flux, which was generally low (-8 to 14 μg N_2O-N m^{-2} h^{-1}). After the second application event, the N_2O emission increased significantly to 87 μg N_2O-N m^{-2} h^{-1} and the N_2O emission factor for the added urine-N was 0.18 %. However, the associated ^{13}C signal of soil respiration was unaffected by urine. Consequently, the increased N_2O emission following urine application was not caused by enhanced denitrification stimulated by labile compounds released from scorched plant roots.

Keywords

^{13}C, grassland, nitrous oxide, scorching, soil respiration, urine

Introduction

Urine deposited by grazing livestock is a major source of the nitrous oxide (N_2O) production in European grasslands. At present, N_2O emissions from agricultural soils account for 5 % of the European release of anthropogenic derived greenhouse gases (EEA 2002), and the main processes involved in the production are nitrification and denitrification (Firestone and Davidson 1989). Vertès et al. (1997) found that 90 % of the urine patches deposited by grazing heifers contained between 3 and 50 g N m^{-2}.

Previous studies have mainly focused on the N_2O emission from urine patches with high N content (e.g. Allen et al. 1996; Koops et al. 1997), however even a low rate of urine-N (6 g N m^{-2}) has been seen to significantly increase the N_2O emission from grassland (Williamson and Jarvis 1997).

In grassland soil, the urea component of urine is rapidly hydrolysed and ammonia (NH_3) is formed. Several studies have reported that plants were scorched by NH_3 following applications of high rates of urine-N (> 20-30 g N m^{-2}) (e.g. Petersen et al. 1998; Ritchey et al. 2003). Scorching was typically detected as shoot death; either caused by root death after exposure to dissolved ammonia ($NH_{3(aq)}$) (Richards and Wolton 1975) or because the shoot material had been in direct contact with NH_3 (Petersen et al. 1998). It is likely that low rates of urine-N (<< 20 g N m^{-2}) lead to damage of the root systems without affecting the shoot vitality.

The mechanism involved in the increased N_2O emission following urine deposition is complex and not well understood. However, as heterotrophic bacteria play a major role in denitrification, the process is strongly dependent on the supply of easily decomposable organic matter, particularly in urine patches where N availability is expected to be non-limiting. Root scorching due to $NH_{3(aq)}$ formed after urea hydrolysis may result in release of labile carbon compounds into the rhizosphere (Shand et al. 2002). Monaghan and Barraclough (1993) suggested that these labile compounds stimulate denitrification activity and thereby are part of the reason for the urine-induced N_2O emission.

In the present ^{13}C-labelling study, we examined the link between N_2O emission and carbon mineralization following urine application to soil under ^{13}C depleted grassland vegetation (*i.e.* grassland monoliths provided with depleted atmospheric CO_2 during 2 or 8 weeks). The synthetic urine applied simulated a urine patch with low N content (3.1 or 5.5 g N m^{-2}). We tested the hypothesis that increased N_2O emission after urine deposition is in part due to enhanced denitrification caused by ammonia-induced leaching of labile carbon compounds from the plant roots. Because the plant material was ^{13}C depleted in the grassland monoliths studied, the N_2O emission should be paralleled by a decrease in the $\delta^{13}C$ signal of CO_2 evolved by soil respiration.

Materials and methods

Grassland monoliths

The experiment was conducted in grassland monoliths placed in a ^{13}C-labelling facility at Institut National de la Recherche Agronomique (INRA), Clermont-Ferrand, France. The former management practise and the experimental facility were described in detail by Klumpp (2004). Briefly, in June 2002 the monoliths (50 cm × 50 cm × 40 cm deep) were taken from an intensively managed semi-natural grassland dominated by perennial

ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.) and Yorkshire fog grass (*Holcus lanatus* L.). The slightly acidic sandy soil contained 4.1 % C and 0.42 % N, and the pH_{H2O} was 6.6. Monoliths were placed in temperature controlled transparent enclosures kept under natural daylight. The enclosures were part of an open flow ^{13}C-labelling system, where ambient CO_2 was scrubbed and replaced by fossil fuel derived CO_2, which is depleted in ^{13}C. Starting 22 April 2003, plants were provided with CO_2 having a $\delta^{13}C$ signal of about -21.5 ‰. The external climate (PAR, temperature and humidity) and temperature of each enclosure was monitored continuously.

Urine treatment

To simulate grazing, the vegetation of six monoliths was cut to a height of 6 cm on 22 April and 9 June. Two weeks after the first cut (*viz*. on 7 May) and one week after the second cut (*viz*. on 16 June) urine was evenly applied on three of the monoliths using a watering can (Day 0). The three remaining monoliths were controls and received urine at the end of each experiment. The artificial urine was prepared using the recipe described by Doak (1952). The urine had a total N content of 0.7 g N l^{-1} and consisted of urea (1.12 g l^{-1}), hippuric acid (0.42 g l^{-1}), allantoin (0.18 g l^{-1}) and creatinine (0.09 g l^{-1}) and pH was adjusted to 7 with NaOH. Delta ^{13}C of each urine component was determined on an elemental analyser (EA1110, Carlo Erba, Milano, IT) coupled in continuous flow mode to an isotope ratio mass spectrometer (IRMS; FinniganMAT Delta plus, Bremen, DE). The amount of N applied via urine represented 90 % of the N removed in the harvested herbage, and corresponded to 3.1 g N m^{-2} at the first application event and 5.5 g N m^{-2} at the second event. To keep soil moisture constant, the monoliths were irrigated every evening with a total amount of 108 and 128 mm water during the first and the second 12-days experiment, respectively.

Gas and soil sampling in monoliths

About four weeks prior to urine application, small white chambers consisting of two 3-cm diameter PVC pipes (80 ml) and three 5-cm diameter PVC pipes (112 ml) were installed in each monolith between the plants. Every pipe was connected to a three-way sampling valve on the outside of the enclosure via a 75 cm silicon tube. The sampling valve was fitted with a 5 ml syringe and a needle. Starting four days before urine application (Day -4), below-ground production of N_2O and CO_2 as well as the $\delta^{13}C$ of the evolved CO_2 were determined by use of the static chamber method. Measurement of CO_2 emission was done between 11 am and 1 pm by briefly lifting the enclosures to seal the 3-cm pipes with rubber stoppers for 40 minutes. One 2-ml gas sample was collected via the external valves after 0, 20 and 40 minutes of incubation. To measure N_2O emission, the 5-cm pipes were sealed by rubber stoppers for 90 minutes between 1:30 and 4:30 pm. Two 2-ml gas samples were taken at the beginning and at the end of the incubation period. A volume of N_2 equal to the sample volume was added to the pipe before each gas sampling to maintain atmospheric pressure. All samples were stored in 2-ml crimp-seal vials, which had been evacuated before use. After penetration by the needle the vials were sealed with Terostat IX to allow long-term storage. Determination of gas emissions from urine-treated and control monoliths was done on day -4, -1, 0, 1, 2, 4, 5, 6 and 7 after urine application. Furthermore, $\delta^{13}C$ of the evolved CO_2 was measured once before the labelling started.

Two days after urine application, two soil cores (0-10 cm depth, 2 cm diameter) were collected in each monolith for chemical analyses and determination of microbial respiration. Sampling holes were closed with cement filled PVC tubes to prevent aeration of the soil and drainage of water. The two soil samples from each monolith were pooled. Roots and stubbles were removed by tweezers during a period of one hour per sample.

Destructive harvest of monoliths

To measure the $\delta^{13}C$ signal of unlabelled and ^{13}C-labelled plant material**,** four monoliths were harvested on 5 May (unlabelled) and 10 June 2003 (^{13}C-labelled). Root samples from the 0-10 cm soil layer were obtained by wet sieving of air-dried soil slices (40 cm × 6 cm × 10 cm). Root samples and plant shoot samples were oven-dried at 60 °C for 48 h, ground and analysed for $\delta^{13}C$ on the elemental analyser and IRMS. On 5 May, samples of 40 g fresh 'root free' soil were obtained by sieving (2 mm) and removing roots by tweezers for 40 minutes per sample in order to measure $\delta^{13}C$ of unlabelled soil C pools.

Soil analyses

Within 36 hours of soil sampling or destructive harvest, two 10 g portions of each fresh 'root free' soil sample were extracted in 1 M KCl (1:5, w:vol), stirred on a rotary shaker for one hour (only one portion on 5 May). The extracts were filtered through Whatman 40 filters and kept at -20 °C until further analysis. Concentration of ammonium (NH_4^+) and nitrate (NO_3^-) in the extracts were analysed colorimetrically on an autoanalyzer (Bran+Luebbe, Norderstedt, DE). Dissolved organic carbon (DOC) in the extracts was measured on a TOC/TN analyzer (Formacs, Skalar, Breda, NL). To determine $\delta^{13}C$ of DOC, 10 ml of each extract was freeze-dried for 2 days, and the solid residue was then analysed for $\delta^{13}C$ on the elemental analyser and IRMS.

To establish the $\delta^{13}C$ of CO_2 evolved by microbial respiration, 10 g portions of fresh 'root free' soil were incubated for 24 h at 25 °C in 250 ml screw capped serum bottles mounted with rubber stoppers. Empty bottles were included as controls. Gas samples for determination of CO_2 concentration and $\delta^{13}C$ of CO_2 were taken after 0 (ambient), 1, 3, 10 and 24 hours of incubation and stored in 2-ml vials. A volume of N_2 equal to the sample volume was added to the bottle before each gas sampling.

Soil pH was determined in a 10:25 (w:vol) suspension of fresh soil in distilled water (not soil from 5 May). The remaining of the 'root free' soil was air-dried and analysed for $\delta^{13}C$ on the elemental analyser and IRMS. In addition, total C and total N was measured on soil samples from 5 May.

Gas analyses

To measure N_2O concentrations, the vials were pressurized by adding 2 ml N_2 before analysis by gas chromatography (GC-14B, Shimadzu, Kyoto, JP). The samples for CO_2 determination were added 0.5 ml N_2 and the concentrations were established by gas chromatography (HP 6890, Agilent, Palo Alto, US). The $\delta^{13}C$ of CO_2 was determined following condensation in two successive cool traps (liquid N_2) and chromatographically separation of CO_2 on a trace gas preparation-concentration unit (PreCon FinniganMAT,

Bremen, DE) coupled in continuous flow mode to the IRMS. Gas samples were analysed for CO_2, N_2O and $\delta^{13}C$ of CO_2 within 26, 36 and 61 days of sampling, respectively.

Denitrifying and nitrifying enzyme activities

To determine denitrifying and nitrifying enzyme activities, two soil cores (0-10 cm depth, 2 cm diameter) were collected in each monolith on day -1, 2 and 7 after the first urine application. The two soil samples from each monolith were pooled and the soil was sieved (2 mm). Denitrifying enzyme activity (DEA) was measured on the fresh soil samples over a short period according to Smith and Tiedje (1979) (for details, see Patra et al. 2005). Briefly, 10 g (equivalent oven-dried) soil was placed into 150 ml flasks, and KNO_3 (200 µg NO_3-N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) was added. The atmosphere of each flask was evacuated and replaced by a 90:10 He-C_2H_2 mixture. During incubation at 26 °C, gas samples (200 µl) were taken after 4 and 6 hours and immediately analyzed for N_2O by chromatography (Varian, STAR 3400 CX, Walnut Creek, US).

Nitrifying enzyme activity (NEA) was measured according to Lensi et al. (1986). For each fresh soil sample, two sub samples (equivalent to 10 g oven-dried) were placed in 150 ml flasks. One sub sample was used to estimate the initial soil NO_3^- content. This sub sample was supplied with 6 ml of a suspension containing a denitrifying organism (*Pseudomonas fluorescens*, O.D. 580 nm = 2) in a solution of glucose and glutamic acid (final soil C concentration for each: 0.5 mg C g^{-1} dry soil). The atmosphere in the flask was replaced by a He-C_2H_2 mixture (90-10) and N_2O accumulation was measured until soil NO_3^- was converted fully to N_2O. The other sub sample was used to determine potential NO_3^- accumulation. In this case, 4 ml of a $(NH_4)_2SO_4$ solution was added (final concentration 200 µg N g^{-1} dry soil). Water was added to achieve 70 % water holding capacity. After aerobic incubation (7 h at 26 °C), which allows nitrate to accumulate, the soil samples were enriched with *Pseudomonas fluorescens* and incubated as described above. Nitrous oxide was analyzed on a Varian STAR 3400 gas chromatograph.

Calculations and statistics

Nitrifying enzyme activity was computed by subtracting the nitrate initially present in the soil from that present after aerobic incubation. All results on $^{13}C/^{12}C$ ratios are reported using the $\delta^{13}C$ notation, *i.e.*:

$$\delta^{13}C\ (‰) = 1000 \times (R_{sample} / R_{standard} - 1),$$

where $R = {}^{13}C/{}^{12}C$. Internal standards were used to check and correct for changes in N_2O, CO_2 and $\delta^{13}C$ of CO_2 between time of sampling and analysis. The emission of N_2O and CO_2 were calculated using linear regression and the $\delta^{13}C$ of the evolved CO_2 was established by Keeling plots (Keeling 1958). To simplify, $\delta^{13}C$ of CO_2 from soil respiration and microbial respiration are referred to as $\delta^{13}C$ of soil respiration and microbial respiration, respectively.

In general, the mean of the results obtained in each monolith was used, which gives 3 replicates. Some data are reported as the overall mean ± standard error. Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Tukey's multiple comparison tests ($\alpha = 0.05$) were performed using SAS General Linear Model procedure (SAS Institute 1997). Furthermore, ANCOVAs were performed with SAS Mixed Model

procedure on the repeated measurements of N_2O, CO_2 and $\delta^{13}C$ of CO_2 using means of the measurements on day -4 and -1 before urine application as covariate ($\delta^{13}C$ of CO_2 after the first application, covariate not included). The ANCOVAs for CO_2 emission after the first application and N_2O emission after the second application were performed on log transformed data.

Results

Irrigation and temperature

The distributions of the 108 and 128 mm water given in the first and the second experiment, respectively, appear from Figure 1. Air temperature in the enclosures during gas measurement ranged between 15 and 27 ºC in the first experiment (data not shown). In the second experiment, the temperature was on average 12 ºC higher ($P < 0.0001$) and varied between 26 and 40 ºC. Air temperature did not differ between time of CO_2 and N_2O measurements ($P = 0.14$).

N_2O emission

Prior to the first urine application, there was no significant difference in the emission of N_2O and CO_2 from the control and the urine monoliths ($P \geq 0.32$; Fig. 2 A, 3 A). However, the initial emission of N_2O and CO_2 in the first experiment tended to be higher from the control monoliths, which is in line with results for NEA (see below). Homogeneity of variance was not obtained despite transformation when testing the emission of N_2O after the first urine application. Thus, no statistical analysis was performed. Application of urine, however, seemed to have a slight effect on the N_2O flux from the urine monoliths in the first experiment (3.1 g N m^{-2}; Fig. 2 A). Overall the flux of N_2O was very low during the first experiment, varying between -8 and 14 μg N_2O-N m^{-2} h^{-1}.

In contrast, urine application equivalent to 5.5 g N m^{-2} in the second experiment had a significant effect on the N_2O emission ($P = 0.047$), which increased to 87 ± 57 μg N_2O-N m^{-2} h^{-1} (Fig. 2 B). The emission remained elevated for at least 8 days, but declined gradually with time ($P = 0.049$).

Respiration and $\delta^{13}C$ of respiration

Urine application had a significant effect on the amount of CO_2 emitted from the grassland monoliths in the two experiments ($P \leq 0.040$; Fig. 3). A peak in the CO_2 emission took place on the day of application, which was probably mainly caused by the hydrolysis of urea (NH_2CONH_2), resulting in formation of NH_4^+, OH^- and HCO_3^-. The rate of microbial respiration measured on 'root free' soil samples in the laboratory did not change over the course of the study or between treatments ($P \geq 0.79$) and the mean rate was 3.4 ± 0.2 μg CO_2-C g^{-1} dry soil h^{-1} (data not shown).

Mean $\delta^{13}C$ of soil respiration determined in the grassland monoliths during the study was -28.5 ‰. No decline in $\delta^{13}C$ of soil respiration was observed following urine application (Fig. 4) and urine had no significant effect on the ^{13}C signal ($P \geq 0.16$). The CO_2 peak on the day of urine application that partly derived from hydrolysis of urea ($\delta^{13}C$ -34 ‰) did only affect the $\delta^{13}C$ of soil respiration in the first experiment, where $\delta^{13}C$ of CO_2 from

the urine treatment dropped significantly below that of the control on the day of application (Fig. 4 A).

The ^{13}C signal of soil respiration increased following days with high irrigation, *viz.* day -1 and 5 in the first experiment (Fig. 1, 4 A). On day 5 after the first application event, the CO_2 emission increased as well (Fig. 3 A). Delta ^{13}C of microbial respiration established on 'root free' soil samples did not change during the study ($P = 0.38$; Fig. 5) and the urine treatment had no significant effect on the ^{13}C signal ($P = 0.72$).

Delta ^{13}C of other C pools

Delta $\delta^{13}C$ of plant shoot and root measured just before the second experiment revealed that the vegetation had been significantly labelled ($P < 0.0001$; Fig. 5). However, the shoot material was much more depleted than the roots, which still consisted primarily of 'old' unlabelled C. Delta ^{13}C of DOC tended to decrease over the course of the study ($P = 0.078$; Fig. 5). Furthermore, $\delta^{13}C$ of soil organic matter (SOM) declined significantly during the period from the start of labelling to the first experiment ($P = 0.05$). The urine treatment had no effect on $\delta^{13}C$ of DOC and SOM ($P \geq 0.22$). In general, $\delta^{13}C$ of DOC differed from $\delta^{13}C$ of soil respiration measured at the start of labelling and on day 2 after urine application ($P = 0.05$), whereas $\delta^{13}C$ of SOM and microbial respiration was rather similar ($P > 0.05$).

Denitrifying and nitrifying enzyme activities

Measurements of DEA (Fig. 6 A) and NEA (Fig. 6 B) revealed that the monoliths used for the urine treatment and the control in the first experiment differed significantly even before urine was applied ($P \leq 0.020$). This difference was accounted for in the statistical analyses by including the measurements before urine application as covariate. Urine had no effect on DEA when measured on day 2 and 7 after application ($P = 0.88$). In contrast, NEA appeared to increase following urine application. However, due to the number of replicates (two or three), the effect of urine on NEA was not statistically significant ($P = 0.17$).

Inorganic N, DOC and soil pH

The content of soil inorganic N in the 0-10 cm soil layer measured on day 2 after urine application did not differ between the two experiments ($P = 0.76$) and was significantly higher in the urine treatment (1.46 g N m^{-2}) than in the control (0.49 g N m^{-2}; $P = 0.042$) (data not shown). The increased level of inorganic N in the urine treatment was almost exclusively caused by a rise in the NH_4^+ content. The NO_3^- content was below the detection limit in the first experiment and had a mean value of 0.05 g N m^{-2} (0-10 cm soil layer) in the second experiment.

The content of DOC in the upper 0-10 cm of the soil was similar in the urine treatment and the control (17.8 g C m^{-2}; $P = 0.88$). Despite urea hydrolysis, no pH increase was observed in the urine treated soil when measured on day 2 after application ($P = 0.23$; data not shown).

Discussion

Increased N_2O emission after urine application

According to the peaks in CO_2 emission (Fig. 3), urea hydrolysis was completed within 24 hours, which is in line with results obtained in other studies (Petersen et al. 1998; Bol et al. 2004). In the first experiment in May, urine application corresponding to 3.1 g N m^{-2} did not lead to a significant increase of the N_2O emission (Fig. 2 A). In contrast, the N_2O emission increased significantly following urine application equivalent to 5.5 g N m^{-2} in the second experiment in June (Fig. 2 B).

A possible reason for the larger N_2O emission in the second experiment compared to the first could be the temperature, which was about 12 ºC higher in the second experiment (mean 34 ºC). Christensen (1983) found that the Q_{10} value for N_2O production in soil was 2-3. The emission on the day of urine application was 6-fold higher in the second experiment than it was in the first, which suggests that the difference in N_2O production between the two experiments was too big to be accounted for by a temperature effect only. The different response at the two application events may be explained partly by the larger amount of N added in the second experiment (5.5 vs. 3.1 g N m^{-2}). Furthermore, nitrifying bacteria may compete with plants for NH_4^+ (Verhagen et al. 1995; Kaye and Hart 1997), and the N demand of the plants was probably higher during the first experiment in May, as this was a period associated with rapid plant growth. In addition, the elevated temperature during the second experiment in June possibly stressed the plants, which may have slowed down plant growth and thereby reduced N uptake compared to the first experiment. Thus, in the second experiment, a relatively slowly growing plant biomass was present to assimilate a larger amount of inorganic N. More inorganic N was therefore left for the nitrifying and denitrifying microorganisms, which enabled increased N_2O production in the second experiment.

Maximum N_2O emission measured was 87 ± 57 μg N_2O-N m^{-2} h^{-1}. In comparison, Williamson and Jarvis (1997) measured emission of 600 μg N_2O-N m^{-2} h^{-1} after application of similar amounts of urine-N (6 g N m^{-2}) to a grassland on poorly drained silty clay loam in November. However, the sandy soil and moderate soil moisture in the present study offered less favourable conditions for N_2O production, which largely explains the lower emission.

Assuming an N loss of 20 % due to NH_3 volatilization and nitric oxide (NO) emission (IPCC 1997), the N_2O emission factor for the added urine-N measured over the 8 days was 0.18 ± 0.08 %. In the study by Williamson and Jarvis (1997), where a similar amount of urine-N was applied, the N_2O emission factor measured over 37 days was 5 % (De Klein et al. 2001). According to the guidelines issued by the Intergovernmental Panel on Climate Change (IPCC 1997), the N_2O emission from urine deposited by grazing livestock should be calculated as 2 % of the N remaining after NH_3 volatilization and NO emission, which are assumed to account for 20 % of the total N content. However, a review of 10 field studies showed that median N_2O emission factor of real urine was 0.9 % (Van Groenigen et al. 2005), and the present study supports a reduction of the IPCC default emission factor as well.

Assessment of the possible link between N_2O production and mineralization of root-derived C

Soil respiration is composed partly of heterotropic respiration by SOM decomposing organisms, mycorrhizal fungi and rhizosphere microorganisms, and partly of root respiration. The urine-induced N_2O emission was not linked to an increase of soil respiration. The same result appears from the study by Bol et al. (2004), whereas Lovell and Jarvis (1996) found that soil respiration increased significantly following urine application. Results from the second experiment revealed that $\delta^{13}C$ of soil respiration was intermediary compared to $\delta^{13}C$ of SOM (-26 ‰) and $\delta^{13}C$ of the roots (-32 ‰), whereas $\delta^{13}C$ of microbial respiration established on 'root free' soil samples (-27 ‰) was generally close to $\delta^{13}C$ of SOM, indicating the source of microbial respiration.

Urine deposition by grazing livestock is known to trigger significant N_2O production, but the mechanisms involved are very complex and not well understood. It has been suggested that labile compounds released from scorched plant roots stimulate denitrification activity, and thus is part of the reason for the increased N_2O emission following urine deposition (Monaghan and Barraclough 1993). The aim of the present study was to test this hypothesis by assessing the source of CO_2 emitted following urine application. If the increased N_2O emission were a result of higher denitrifying activity due to a supply of labile compounds released from scorched plant roots (more depleted than other soil C pools in the monoliths studied), then $\delta^{13}C$ of soil respiration would be expected to decline. Contrary to the hypothesis, the increased N_2O emission in the second experiment was not related to increased mineralization of plant-derived C, *viz*. $\delta^{13}C$ of soil respiration was unaffected by the urine application (Fig. 4 B). The plant material may have been inadequately ^{13}C-labelled in order to trace plant-derived C in other C pools. However, the result may indicate that no significant root scorching occurred following urea hydrolysis. Lack of urine-effect on other soil C measures (*i.e.* $\delta^{13}C$ of microbial respiration, soil content and $\delta^{13}C$ of DOC) supported that root scorching was probably negligible. The urine compounds remaining after urea hydrolysis (hippuric acid, creatinine and allantoin; $\delta^{13}C$ -26 ‰) did not affect the results on $\delta^{13}C$ of DOC because of their low amount (< 3 g C m^{-2} vs. 17.8 g DOC-C m^{-2} in the 0-10 cm soil layer). The degree of scorching depends on the amount of NH_3 formed (Ritchey et al. 2003). Our results show that application of 5.5 g urine-N m^{-2} gave rise to $NH_{3(aq)}$ concentration in the soil solution that was too low to cause significant scorching of the roots. In contrast, a related study demonstrated that urine application equivalent to 50.9 g N m^{-2} significantly increased the mineralization of plant derived C, possibly as a result of root damage due to scorching (P. Ambus, pers. comm.).

Alternative processes explaining the urine-induced N_2O emission

The concentration of N in livestock urine may vary between 1 and 20 g N l^{-1} (Oenema et al. 1997), thus the concentration used in the present study (0.7 g N l^{-1}) was in the lower end of this range. A nitrogen concentration of urine above 16 g N l^{-1} leads to microbial stress due to $NH_{3(aq)}$ and low osmotic potential, and thereby to inhibition of nitrification (Monaghan and Barraclough 1992; Bol et al. 2004). The low urine-N concentration in the present study means that nitrification most likely occurred, and the process might play a major role in the increase in N_2O emission. This view is supported by the apparent increase of NEA in the urine treated soil during the first experiment (Fig. 6) and the presence of soil NO_3^- on day 2 after the second application event. Hence, the elevated

N_2O emission immediately following urine application was probably caused by a rapid nitrification-denitrification turnover of urea-derived N. This mechanism is different from that following application of higher rates of urine-N, where nitrification is typically inhibited for a couple of days (Monaghan and Barraclough 1992; Bol et al. 2004).

In conclusion, the increased N_2O emission following urine application at rates up to 5.5 g N m^{-2} was not caused by enhanced denitrification stimulated by an increased availability of labile plant compounds. Furthermore, strong competition for inorganic N between plants and microorganisms combined with low urine-N rates limited the N_2O loss from this semi-natural grassland.

Acknowledgements

This work was funded by the Danish Research Centre for Organic Farming (DARCOF) and the Danish Agricultural and Veterinary Research Council. The first author thanks for the 'Short-term Scientific Mission' funding from the EU COST Action 627 and the Exchange Grant from European Science Foundation, Programme on 'Stable Isotopes in Biospheric-Atmospheric Exchange', which enabled the experimental work at INRA, Clermont-Ferrand, France. We wish to thank Nadine Guillaumaud for help during enzyme activity assays, Per Ambus and Liselotte Meltofte for their assistance with the gas analyses as well as Daniel Carter and Per Ambus for their useful comments on the manuscript.

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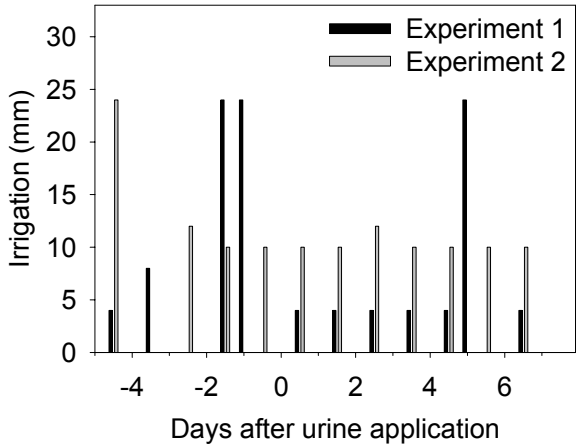

Figure 1. Distribution of irrigation during the first and second experiment.

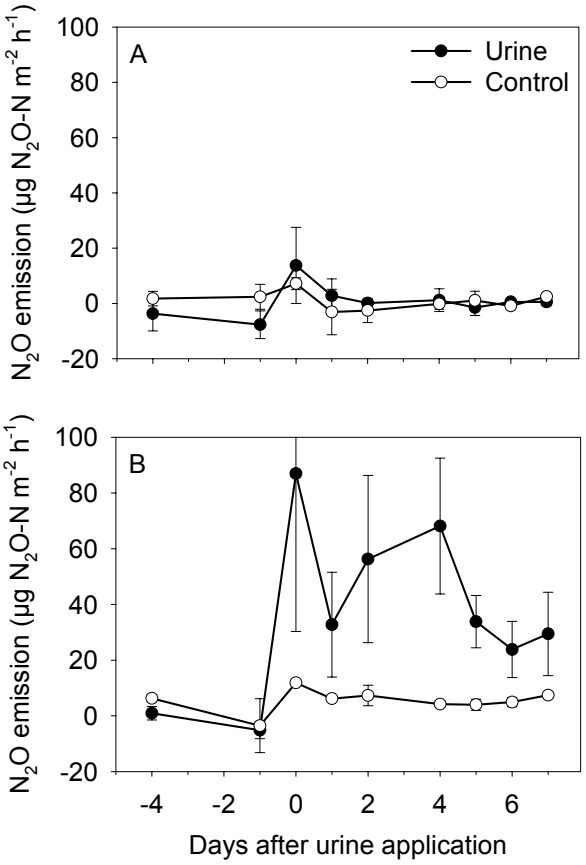

Figure 2. Emission of N_2O from the urine treatment and the control during (A) the first experiment and (B) the second experiment; n = 3; means ± SE.

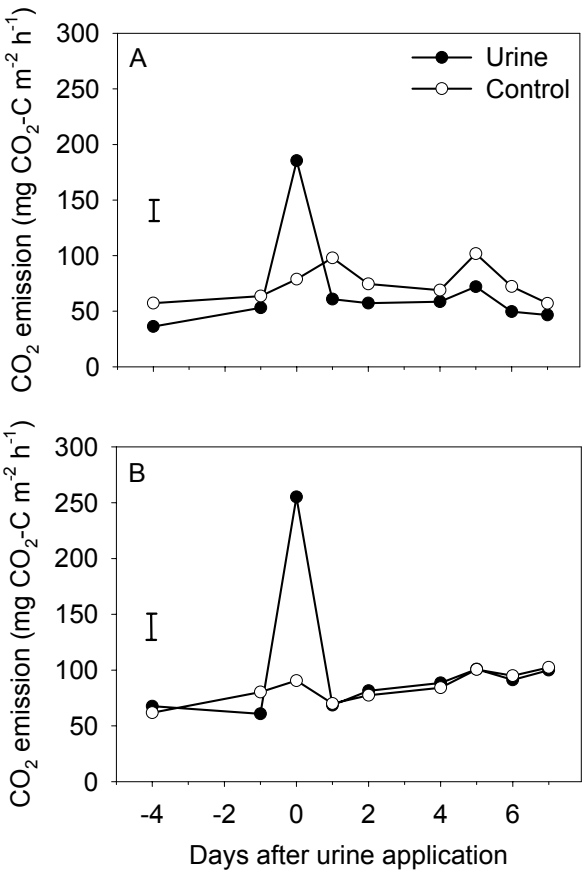

Figure 3. Emission of CO_2 from the urine treatment and the control during (A) the first experiment and (B) the second experiment; n = 3; means; the bars indicate the Minimum Significant Difference.

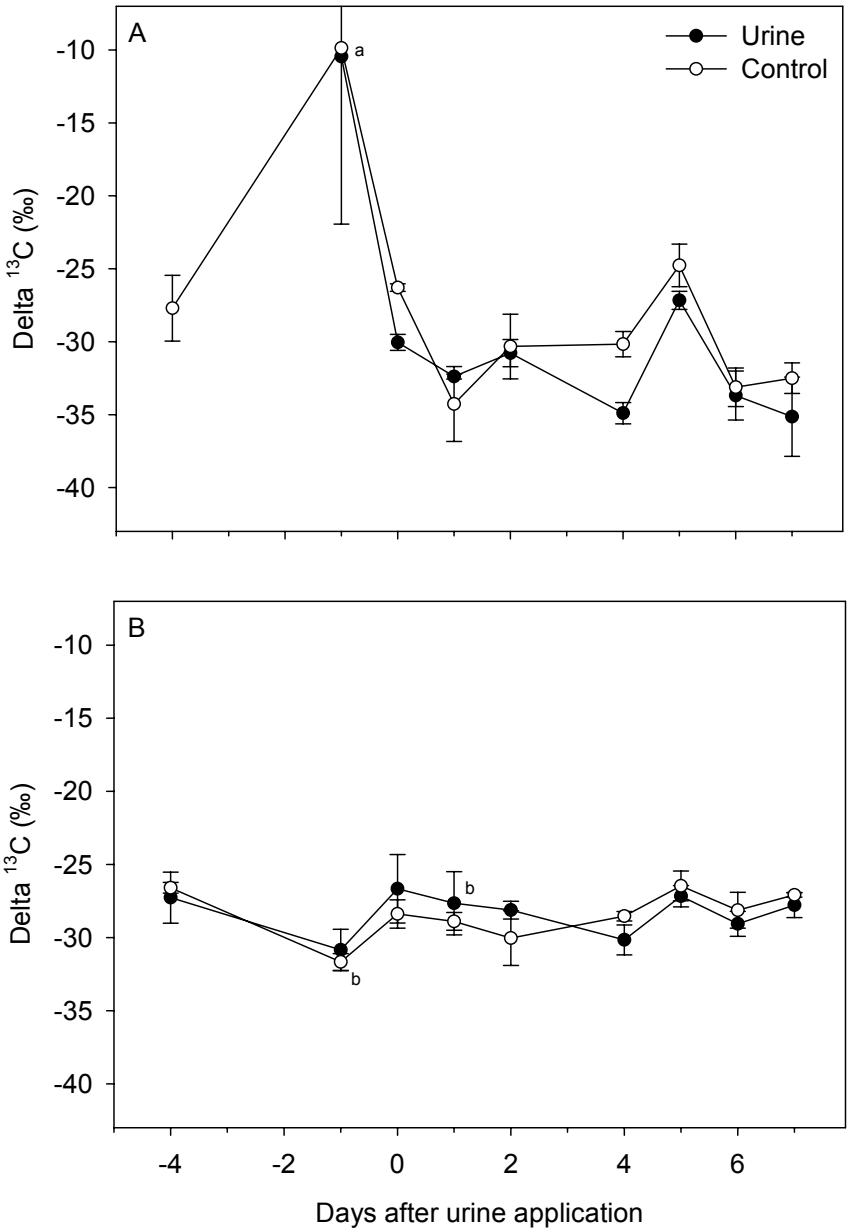

Figure 4. Delta ^{13}C of CO_2 evolved by soil respiration in the urine treatment and the control during (A) the first experiment and (B) the second experiment; n = 3; means ± SE. [a] *Urine, n = 1; control, n = 2.* [b] *One outlying sub measurement was not included.*

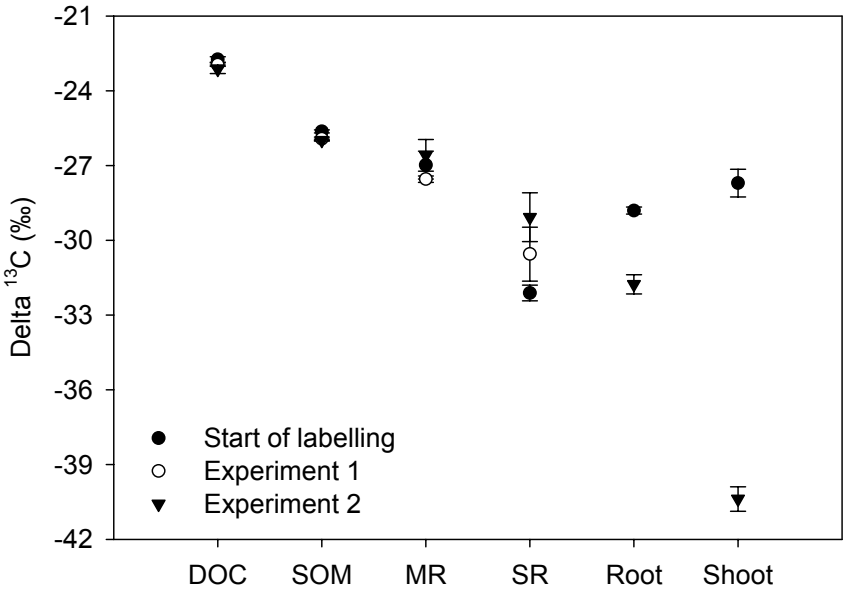

Figure 5. Delta ^{13}C of dissolved organic C (DOC), soil organic matter (SOM) and CO_2 evolved by microbial respiration (MR) and soil respiration (SR) determined at the start of labelling and on day 2 of the first and second experiment, as well as $\delta^{13}C$ of root and shoot determined at the start of labelling and on day -6 of the second experiment; n = 4-6; means ± SE.

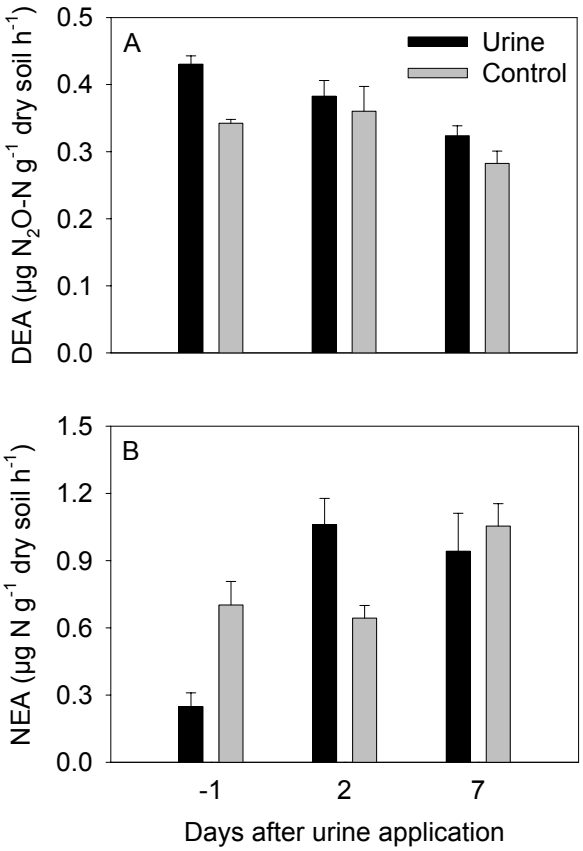

Figure 6. (A) Denitrifying enzyme activity, DEA, and (B) nitrifying enzyme activity, NEA, in the urine treatment and the control on day -1, 2 and 7 after the first urine application event; n = 2-3; means ± SE.

Paper III

Mechanisms involved in N_2O production via nitrification and denitrification in urine patches

(Prepared for submission to Soil Biology & Biochemistry)

Mechanisms involved in N_2O production via nitrification and denitrification in urine patches

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Abstract

Urine deposition by grazing livestock causes an immediate rise of the nitrous oxide (N_2O) emission, but the responsible mechanisms are not well understood. A ^{15}N-labelling study was conducted in an organic grass-clover sward to examine the initial effect of urine on the rates and N_2O loss ratios of nitrification and denitrification, and to evaluate the importance of the chemical conditions arising in urine-affected soil. The effect of artificial urine (52.9 g N m^{-2}) and ammonium solution (52.9 g N m^{-2}) was examined in separate experiments, where water controls were included. In each experiment, the N_2O loss derived from nitrification or denitrification was determined in the field immediately after application of ^{15}N-labelled solutions. During the next about 24 hours, the nitrification rate was established in the field, whereas the denitrification rate was measured in soil cores in the laboratory. Compared with the water control, urine application increased the N_2O emission 11-fold to 42.3 µg N_2O-N m^{-2} h^{-1}, whereas application of ammonium increased the emission 7-fold to 6.1 µg N_2O-N m^{-2} h^{-1}. The enhanced N_2O emission from the urine treatment compared to the water control was a result of increased N_2O loss from both nitrification and denitrification caused by a combination of higher rates and higher loss ratios of the processes. The effect on denitrification of the ammonium and urine treatments could not be compared because the soil water content rose between the two experiments. However, the N_2O loss from nitrification was greater following the urine treatment compared to the ammonium treatment, which seemed to result from a higher nitrification rate. The rate of nitrification in the urine treatment appeared to be stimulated, not only by the high NH_4^+ availability, but also by the raised soil pH following urea hydrolysis. In the present study, an enhanced nitrification rate seemed to be the most important factor explaining the high initial N_2O emission from simulated urine patches.

Keywords

denitrification, grass-clover, grassland, loss ratio, ^{15}N, nitrification, nitrous oxide, pH, urine

1 Introduction

Grazed grasslands cover about 40% of the agricultural area in Europe (FAO, 2004), and urine deposited by grazing livestock has a large impact on the emission of N_2O from these soils. Williams et al. (1999) estimated that N_2O losses within the first 24 hours after urine deposition accounted for approximately 8% of the annual N_2O emission from a grassland. Agricultural soils contribute with about half of the anthropogenic N_2O emission (IPCC, 2001) and currently this source of N_2O represents 5% of the European release of anthropogenic derived greenhouse gasses (EEA, 2002). Furthermore, N_2O is involved in the depletion of the stratospheric ozone layer (Crutzen, 1981).

Nitrous oxide is mainly produced by nitrifying and denitrifying bacteria in the soil and the production is influenced by four parameters (Firestone and Davidson, 1989; Granli and Bøckmann, 1994). These are the rate of nitrification (N) and denitrification (D), the N_2O loss ratio of nitrification, *i.e.* N_2O-N lost per NO_3^- produced (L_N) and the N_2O loss ratio of denitrification, *i.e.* N_2O-N lost per N_2+N_2O produced (L_D). Thus, the total loss of N_2O from nitrification and denitrification (E) can be described as

$$E = N \times L_N + D \times L_D \quad (1)$$

An environmental factor may affect the four parameters differently.

The N_2O emission usually increases immediately after urine deposition (e.g. Allen et al., 1996; Yamulki et al., 1998). Sherlock and Goh (1983) found that more N_2O was emitted after application of urine-N than after equivalent amounts (20 g N m^{-2}) of urea-N or ammonium-N dissolved in similar volume of liquid, and the difference was mainly related to the initial effect on the N_2O emission. Thus, elevated soil water content and availability of inorganic N only explain part of the urine-induced N_2O emission. The mechanisms responsible for the high N_2O loss from urine patches are not well understood and may vary according to abiotic factors, e.g. soil type, moisture and pH (Clough et al., 1998, 2004), and the amount of urine-N deposited (Petersen et al., 2004).

The objective of the present study was to investigate the mechanisms involved in the initial N_2O production following urine deposition. More specifically, the objective was to assess changes in the four parameters that influence the N_2O production (*viz.* N, D, L_N and L_D) and evaluate the impact of the chemical conditions arising in urine-affected soil. The effect of artificial urine (52.9 g N m^{-2}) with 80% of N as urea was compared with the effect of an ammonium solution (52.9 g N m^{-2}) and a water treatment was included as control. It is well-known that soil pH rises temporarily following urine deposition because alkaline products are formed during the rapid hydrolysis of urea, which is expressed as

$$(NH_2)_2CO + 3\ H_2O \rightarrow 2\ NH_4^+ + OH^- + HCO_3^- \quad (2)$$

The high N_2O emission from urine patches may result from a raised soil pH and NH_4^+ content combined with reduced O_2 concentrations. Thus, the first hypothesis was that the N_2O emission from the treatments would increase in the order: urine > ammonium > water.

The rate of nitrification may be stimulated by the enhanced availability of the substrate, NH_4^+. However, the higher pH in the urine treatment compared to the ammonium treatment shifts the $NH_4^+ - NH_{3(aq)}$ equilibrium towards $NH_{3(aq)}$ (Schmidt, 1982). Nitrite oxidation is more readily inhibited by $NH_{3(aq)}$ than the NH_4^+ oxidation (Harada and Kai, 1968), which may lead to increased N_2O formation due to chemical decomposition of

NO_2^- or reduction of NO_2^- via nitrifier denitrification (Wrage et al., 2001, 2004). Thus, the second hypothesis was that the rate of nitrification (*N*) in the urine treatment would be lower than in the ammonium treatment, whereas the N_2O loss ratio of the process (L_N) would be highest in the urine treatment.

The rate of denitrification (*D*) in the urine treatment may be stimulated by a urine-induced rise in dissolved organic carbon (DOC), either via solubilization of soil organic C, or because labile compounds are released from scorched plant roots (Monaghan and Barraclough, 1993). However, the N_2O loss ratio of denitrification (L_D) decreases when pH increases and when the NO_3^-/labile C ratio decreases (Hutchinson and Davidson, 1993; Simek and Cooper, 2002). Thus, the third hypothesis was that the rate of denitrification in the urine treatment would be higher than in the ammonium treatment, whereas L_D would be lowest in the urine treatment.

The ^{15}N labelling and acetylene inhibition techniques were used to test these hypotheses. Separate labelling of the NH_4^+ and NO_3^- pools with ^{15}N has been used frequently to assess the contribution of nitrification and denitrification to the N_2O production in soil, but was only involved in a few studies on urine-affected soil (e.g. Monaghan and Barraclough, 1993).

2 Materials and methods

2.1 Field site

The study was conducted in a sward consisting of white clover (*Trifolium repens* L.), red clover (*Trifolium praténse* L.) and perennial ryegrass (*Lolium perenne* L.) during the second production year. The grass-clover sward was part of an organic crop rotation, which represented a dairy system, and was situated in Taastrup, 18 km west of Copenhagen (55º 40′N, 12º 18′E). The soil was a loamy sand with total N content of 0.21%, total C content of 2.1% and pH in water of 7.9. Microplots were established in December 2002 by pushing 56 PVC cylinders (30 cm i.d. by 30 cm long) into the soil to a depth of approximately 22.5 cm.

The effect of ammonium (52.9 g N m^{-2}) on the nitrification and denitrification processes was examined in the first experiment, which took place from 26 to 30 August 2003. Two weeks later, the effect of artificial urine (52.9 g N m^{-2}) on the processes was assessed in the second experiment. In each experiment, a set consisting of 28 microplots was used. To simulate grazing, the sward was mown to approximately 15 cm height every second week from the end of May 2003. The two last cuts took place four days prior to each experiment, and here 42 mm water was also applied to the microplots for the coming experiment to ensure comparable soil water content. Soil temperature at a depth of 10 cm was registered continuously and measurements of daily rainfall were obtained from a local meteorological station (CWB, 2003).

2.2 Solutions for ^{15}N field measurements

Nitrogen-15 technique was used in the field to determine the rate of nitrification and the amount of N_2O produced via nitrification or denitrification. Five solutions were prepared for the first experiment, *viz.* water, water/$^{15}NO_3^-$, ammonium/$^{15}NO_3^-$, water/$^{15}NH_4^+$ and ammonium/$^{15}NH_4^+$. The total N concentrations of the ammonium and water solutions

were 15.6 g N l^{-1} and ≤ 0.02 g N l^{-1}, respectively. Ammonium was supplied as NH_4Cl, $^{15}NO_3^-$ as 99 atom% $K^{15}NO_3$ and $^{15}NH_4^+$ as 99 atom% $^{15}NH_4Cl$. The content of $^{15}NO_3$-N or $^{15}NH_4$-N in the five solutions was 0, 1.6, 1.9, 0.37 and 48 mmol ^{15}N l^{-1}, respectively.

In the second experiment, ammonium in the solutions was replaced by artificial urine consisting of urea (28.5 g l^{-1}), hippuric acid (11.9 g l^{-1}), creatinine (0.3 g l^{-1}), allantoin (0.6 g l^{-1}), uric acid (0.2 g l^{-1}), NH_4Cl (1.4 g l^{-1}), $KHCO_3$ (22.9 g l^{-1}) and KCl (16.9 g l^{-1}) (De Klein and Van Logtestijn, 1994), giving a total N concentration in the solutions of 15.6 g N l^{-1}. In the second experiment, the content of $^{15}NO_3$-N or $^{15}NH_4$-N in the five solutions (*viz.* water, water/$^{15}NO_3^-$, urine/$^{15}NO_3^-$, water/$^{15}NH_4^+$ and urine/$^{15}NH_4^+$) was 0, 1.6, 1.9, 0.37 and 42 mmol ^{15}N l^{-1}, respectively.

2.3 ^{15}N field measurements

The two $^{15}NH_4^+$ labelled solutions were applied on day 1 of each experiment (*viz.* 26 August and 9 September), whereas the remaining three solutions were applied the following day. More specifically, 240 ml of each solution was carefully applied on the soil surface in four microplots using a 60 ml Plastikpak syringe fitted with a veterinary injection needle. Subsequently, 180 ml distilled water was added using the same technique, which altogether resulted in a mean penetration depth of about 2 cm.

Measurement of N_2O emission by a static chamber method was initiated within 2.5 hours of solution application. Briefly, each microplot was closed by a PVC lid (5 cm inner height) fitted with a rubber stopper to allow gas sampling. The lid had an EPDM rubber gasket on the sealing edge and was weighed down to ensure a complete seal. For analysis of initial N_2O concentration and ^{15}N abundance, three 3.5 ml N_2-flushed Venoject vials and three evacuated 120 ml serum bottles were filled with samples of ambient air using a 60 ml syringe. After 50, 100 and 150 minutes of cover period, a sample of the headspace gas was removed through the rubber stopper and stored in a 3.5 ml vial for later analysis of N_2O concentration. At the end of the cover period (150 minutes), a 120 ml sample was taken for analysis of ^{15}N abundance of N_2O.

Following gas measurement, a soil sample consisting of four soil cores (0-10 cm depth, 2 cm diameter) was collected in each microplot, and the samples were kept cold in an insulated box during transport to the laboratory. In the microplots labelled with $^{15}NO_3^-$, the sampling holes were closed with 50 ml screw capped test tubes to prevent aeration of the soil and drainage of water. After about 24 hours, soil sampling was repeated in these microplots to determine the rate of nitrification via $^{15}NO_3^-$ pool dilution.

2.4 Analysis of gas samples from the field

The 3.5 ml gas samples were pressurized by adding 2 ml N_2 before they were analysed for N_2O in a gas chromatograph (GC-14B, Shimadzu, Kyoto, JP) fitted with a HaySep Q column and an electron capture detector (column and detector temperature were 30 °C and 300 °C, respectively). The 120 ml samples were analysed for ^{15}N abundance of N_2O following removal of H_2O and CO_2 as well as cryogenic focusing of N_2O on a trace gas concentration unit (PreCon, Thermo Corporation, Bremen, DE) coupled in continuous flow mode to an isotope-ratio mass spectrometer (IRMS; Finnigan MAT Delta Plus, Bremen, DE).

2.5 Soil analyses

Roots and pebbles (> 4 mm) were removed by tweezers during a period of 10 minutes per soil sample. Within seven hours of soil sampling, 20 g portions of each 'root free' soil sample were extracted in 1 *M* KCl (1:5, w:vol), stirred on a horizontal shaker for one hour. The extracts were filtered through Whatman 40 filters and kept at -20 °C until further analysis.

Dissolved organic carbon in the extracts from $^{15}NO_3^-$ labelled microplots was measured on a TOC-5000A total organic C analyzer (Shimadzu, Kyoto, JP). The content of NH_4^+, NO_3^- and NO_2^- in extracts were analysed colorimetrically on an autoanalyzer (Bran+Luebbe, Norderstedt, DE). Nitrogen-15 abundance of NH_4^+ and NO_3^- were determined in extracts by the diffusion method (Sørensen and Jensen, 1991). Briefly, NH_4^+ in the extract was converted into NH_3, which was trapped on an acidified filter paper. Subsequently, NO_3^- was converted via NH_4^+ into NH_3, which was trapped on another filter. The filters were analysed for ^{15}N using an elemental analyser (EA 1110, Carlo Erba, Milano, IT) coupled in continuous flow mode to the IRMS. Some carry-over of NH_4^+ was detected on the NO_3^- filters from the ammonium and urine treatments, which was corrected for via the autoanalyzer measurements.

Soil pH was determined in a 10:25 (w:vol) suspension of fresh soil in distilled water using soil sampled on day 3 of each experiment. Samples of air-dried soil from the water-only treatment in the first experiment were finely ground and analysed for total C and total N on the elemental analyser.

2.6 Acetylene inhibition technique

The rate of denitrification was determined on soil cores in the laboratory using acetylene (C_2H_2) inhibition of the bacterial reduction of N_2O to N_2 (Ryden et al., 1987). On day 4 of the experiments, unlabelled solutions of water and ammonium or urine were applied in four microplots each, using the same technique as described above. From each microplot, four soil cores were then collected between the plants in PVC tubes of 10 cm by 4.4 cm inner diameter. The tubes were sealed at the bottom and brought to the laboratory, where the degree of soil compaction was registered.

Incubation with C_2H_2 was initiated using two soil cores from each microplot. Thus, 4 ml C_2H_2 (acetone free, AGA A/S, Copenhagen, DK) was injected along the length of each core using a veterinary needle connected to a 5 ml syringe and a C_2H_2 reservoir via a three-way valve (Ambus and Christensen, 1993). The two cores were placed in a 2 l glass jar, which was closed with a rubber-sealed lid fixed by clamps. About 180 ml headspace air was evacuated from the jar and then replaced with 180 ml C_2H_2 using 60 ml syringes and a rubber stopper mounted in the lid of the jar. The resulting C_2H_2 concentration in soil and headspace atmosphere was about 9%, which inhibits nitrification and is above the 5% needed to block the reduction of N_2O to N_2 (Okereke, 1984). Subsequently, control incubations were initiated on the other half of the soil cores, using pure N_2 instead of C_2H_2. The glass jars were then placed at 15ºC. After 2, 5 and 20 hours of incubation, a 30 ml sample of headspace gas was taken through the rubber stopper and transferred to a 3.5 ml N_2-flushed Venoject vial using a syringe. A volume of 30 ml N_2 was added to the jar before each gas sampling to maintain atmospheric pressure. Soil dry matter was determined after the last gas sampling (oven drying at 105 °C for 24 h).

The 3.5 ml gas samples were added 2 ml N_2 before they were analysed for N_2O in a gas chromatograph (Chrompack-9001, Chrompack, Middelburg, NL) fitted with two HayeSep Q columns (60-80 and 80-100 mesh, respectively) and an electron capture detector (column and detector temperature were 60 °C and 325 °C, respectively).

2.7 Calculations and statistics

Flux of N_2O in the field and laboratory was calculated from the linear increase in N_2O concentration in the headspace during the incubation periods. For the urine/$^{15}NO_3^-$ treatment, however, data was fitted to an exponential equation and the N_2O emission was calculated for t = 0. The rate of denitrification in the 0-10 cm soil layer was established from the N_2O-N formation in the glass jars with C_2H_2.

When significant N_2O emission was detected from the microplots (*i.e.* R^2 of N_2O concentration vs. time ≥ 0.65), then the ^{15}N enrichment of the emitted N_2O (C^*) could be determined as

$$C^* = (C_t C_t^* - C_0 C_0^*) / (C_t - C_0), \quad (3)$$

where C_0 and C_t are the N_2O concentration calculated from the regression equation at the start and at the end of the cover period, respectively, and C_0^* and C_t^* are the atom% excess enrichment of N_2O at the start and at the end of the cover period, respectively. Nitrogen-15 abundance of N_2O-N emitted from unlabelled microplots did not deviate from the natural abundance of ^{15}N at 0.3663 atom%.

For the labelled microplots, the ^{15}N enrichment of the emitted N_2O often exceeded the enrichment of soil NH_4^+ or NO_3^- measured about 5 hours after solution application in the 0-10 cm soil layer. In some cases it also exceeded the initial enrichment calculated for the 0-10 cm soil layer. Thus, the ^{15}N enrichment of the labelled pool being nitrified or denitrified appeared to be higher than the mean enrichment in the 0-10 cm soil layer. During gas measurement, the added solutions were therefore assumed to affect the 0-2 cm soil layer, as this was the mean penetration depth.

The emission of N_2O derived from nitrification was calculated from the ^{15}N labelled N_2O emitted from the $^{15}NH_4^+$ labelled microplots, which was determined in two ways. If a significant N_2O emission took place, then emission of ^{15}N labelled N_2O (CC^*) was established as

$$CC^* = C_t C_t^* - C_0 C_0^* \quad (4)$$

If only a significant increase in ^{15}N abundance of N_2O was detected, then the emission of ^{15}N labelled N_2O was calculated as

$$CC^* = (C_t^* - C_0^*) C_0 \quad (5)$$

Gas measurements were initiated within 2.5 hours of solution application and therefore it may be assumed that the unlabelled N pool (*viz.* NH_4^+ or NO_3^-) had not yet been labelled via transformation of labelled N (Panek et al., 2000). As a result, emission of N_2O-N derived from nitrification (CP) was established as

$$CP = CC^*/N_i^*, \quad (6)$$

where N_i^* is the calculated atom% excess enrichment of NH_4^+ in the 0-2 cm soil layer. The estimates were subsequently converted from concentration of N_2O to amount of N. Likewise, emission of N_2O derived from denitrification was determined from the

emission of ^{15}N labelled N_2O from the $^{15}NO_3^-$ labelled microplots, using equation 4 and 5, and the atom% excess enrichment of NO_3^- in the 0-2 cm soil layer, using equation 6. Panek et al. (2000) made similar calculations. The ^{15}N tracer technique is based on the assumption that the ^{15}N labelled compound mix homogeneously with the soil pool (Stevens et al., 1997), but in field trials it may be difficult to obtain completely uniform labelling. However, when the addition of labelled N by far exceeds the native soil N, there is initially only one significant pool, which is practically uniform (Bergsma et al., 1999).

The rate of gross nitrification (N) was established from the isotopic dilution of ^{15}N labelled NO_3^- in the 0-10 cm soil layer measured during 24 hours, using the following equation

$$N = (B - B_0) \log[(I_0 B) / (B_0 I)] / \log(B/B_0), \quad (7)$$

where B_0 and B are the total amount of $^{14+15}NO_3$-N in mg g^{-1} dry soil at the start and at the end of the period, respectively, and I_0 and I are the amount of $^{15}NO_3$-N in mg g^{-1} dry soil at the start and at the end, respectively (Mosier and Schimel, 1993). This equation also requires that the $^{15}NO_3^-$ mixes homogeneously with the soil NO_3^- pool. The rate of nitrification and denitrification in the 0-2 cm soil layer was estimated as $\frac{1}{5}$ of the activity in the 0-10 cm layer. Gravimetric water content was converted to water filled pore space (WFPS) using measured soil bulk density and assuming a particle density of 2.65 g cm^{-3}.

Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Tukey's multiple comparison tests ($\alpha = 0.05$) were performed using SAS General Linear Model procedure (SAS Institute, 1997). Statistics on net N_2O emissions and denitrification rates were performed on square root and log transformed data, respectively. Some results are reported as the mean ± standard error.

3 Results

3.1 WFPS, soil temperature and pH

Water filled pore space of the soil increased between the two experiments due to rains during the intervening period ($P < 0.0001$; Fig. 1). Furthermore, data on WFPS revealed an effect of day ($P < 0.0001$). This primarily related to the denitrification measurements conducted on day 4 of each experiment, where WFPS in the soil cores was about 11%-points higher than in the microplots the previous day. The reason was partly lack of plant transpiration and reduced drainage because the soil core was isolated from the tension of the soil profile.

Mean soil temperature at 10 cm depth during the experimental periods was 15.3 °C and did not vary significantly between day and night or between the two experiments ($P \geq 0.29$; data not shown). Soil pH was 7.9 in the water treatment, decreased to 7.4 in the ammonium treatment and increased to 8.3 in the urine treatment ($P < 0.05$; data not shown).

3.2 Inorganic N and DOC

The small amounts of ^{15}N label added in the water treatments had no significant effect on the content of soil NH_4^+ and NO_3^- in the microplots ($P \geq 0.057$; data not shown). Soil

NH_4^+ in the water treatment was higher in the first experiment than in the second experiment ($P \leq 0.041$), whereas the NO_3^- level was similar in the two experiments ($P \geq 0.063$) (Table 1). Overall, data on soil NO_3^- showed an effect of the added solutions ($P < 0.0001$), which derived from high net nitrification in the urine treatment. Thus, on the day of application, soil NO_3^- in the urine treatment was twice the amount in the water and ammonium treatments, and the following day the difference had increased to a factor 11. The increase in soil inorganic N in the urine treatment compared to the water control indicated that 84 ± 4% of the urea was hydrolysed before the first soil extraction. Soil content of NO_2^- was below the detection limit in all treatments.

The soil content of DOC showed an effect of the added solutions ($P = 0.0003$), which derived from a higher content in the urine treatment compared to the water control in the second experiment ($P = 0.0028$; Fig. 2). However, relatively, the NO_3^- content increased more than the DOC content, and therefore the NO_3^-/DOC ratio increased in the urine treatment compared to the water control in the second experiment ($P = 0.032$).

3.3 N_2O emission

The $^{15}NH_4^+$ and $^{15}NO_3^-$ label added in the water treatments had no effect on the amount of N_2O emitted from the microplots ($P = 0.36$; data not shown). Emission of N_2O from the water treated microplots increased from 0.9 to 3.9 μg N_2O-N m^{-2} h^{-1} between the two experiments, probably as a result of increased WFPS ($P = 0.0021$; Fig. 3). Using N_2O emission from the water treatments as covariate revealed a significantly higher N_2O emission from the urine treatment compared to the ammonium treatment ($P = 0.013$). Compared to the water control, ammonium application increased the emission 7-fold to 6.1 μg N_2O-N m^{-2} h^{-1}, whereas application of urine increased the emission 11-fold to 42.3 μg N_2O-N m^{-2} h^{-1}.

3.4 ^{15}N of inorganic N

The ^{15}N enrichment of NH_4^+ and NO_3^- in soil from the 0-10 cm soil layer appeared to change during the up to 12-hour period that separated application of solutions and KCl extraction of the soil sampled about 5 hours after application (Table 2). For example, the enrichment in the paired treatments of water and ammonium or urine deviated more than could be explained by the initial soil content of NH_4^+ and NO_3^-. This suggested that the ^{15}N enrichment had changed, perhaps as a result of microbial activity or due to exchange of $^{15}NH_4^+$ with $^{14}NH_4^+$ adsorbed to soil colloids. Furthermore, the ^{15}N enrichment of NH_4^+ in the water/$^{15}NO_3^-$ treatments indicated that dissimilatory nitrate reduction to ammonium (DNRA) was taking place. Recovery of the added ^{15}N in the inorganic N pool of the 0-10 cm soil layer ranged between 8 and 118%, with the highest recovery occurring in the ammonium/$^{15}NH_4^+$ and urine/$^{15}NH_4^+$ treatments. Low recovery was probably because of N uptake by plants or loss via denitrification.

3.5 Source of N_2O produced in the 0-2 cm soil layer

The ^{15}N enrichment of N_2O emitted from the microplots and the calculated ^{15}N enrichment of soil NH_4^+ and NO_3^- in the 0-2 cm soil layer appear from Figure 4. Missing values are (1) N_2O from the water/$^{15}NH_4^+$ treatment in the first experiment, where no net N_2O emission took place, and (2) NH_4^+ from the urine/$^{15}NH_4^+$ treatment, because the progression of urea hydrolysis was unknown. Based on the ^{15}N enrichment of the emitted

N_2O and the enrichment of NH_4^+ and NO_3^- in the labelled soil layer, it is possible to calculate the contribution of NH_4^+ oxidation and NO_3^- reduction in the soil layer in question to the total N_2O emission (Fig. 3).

As regards the water treatment, the emission of N_2O derived from oxidation of NH_4^+ or reduction of NO_3^- in the 0-2 cm soil layer did not change significantly between the two experiments ($P = 0.060$), and the contribution of the two sources was rather similar ($P \geq 0.053$) (Fig. 3). The responsible process for N_2O formation via NO_3^- reduction was most likely denitrification. The loss of N_2O originating from denitrification in the 0-2 cm soil layer rose 7-fold in the ammonium treatment ($P = 0.014$) and 39-fold in the urine treatment ($P < 0.0001$) compared to the respective water control. In the ammonium treatment, the N_2O loss from nitrification in the 0-2 cm soil layer increased 48-fold compared to the water control ($P = 0.0061$), leading to a considerably higher N_2O loss from nitrification than from denitrification ($P = 0.015$). In the urine treatment, the N_2O emission derived from nitrification could not be calculated because the ^{15}N enrichment of NH_4^+ was unknown. However, it is likely that nitrification was the source of the urine-induced N_2O emission, which was not accounted for by denitrification in the 0-2 cm soil layer. Thus, the N_2O loss from nitrification in the 0-2 cm soil layer was calculated to be 20.1 ± 1.2 μg N_2O-N m^{-2} h^{-1}. When holding this emission against the ^{15}N enrichment of N_2O emitted from the urine/$^{15}NH_4^+$ treatment, it turned out that 28% of the urea should have been hydrolysed by the time of gas measurement in order to estimate the same N_2O loss from nitrification in the $^{15}NO_3^-$ and $^{15}NH_4^+$ labelled microplots. This progress of urea hydrolysis is not unrealistic. Consequently, in the urine treatment, nitrification and denitrification contributed equally to the N_2O production ($P = 0.63$), and the N_2O loss from nitrification appeared to be greater than in the ammonium treatment.

3.6 Rate of nitrification and denitrification

The rate of gross nitrification in the water treatment measured via $^{15}NO_3^-$ pool dilution declined between the two experiments ($P = 0.0066$; Table 3). In the urine treatment, the nitrification rate increased by factor 9 compared to the water control ($P < 0.0001$). A rate could not be determined for the ammonium/$^{15}NO_3^-$ treatment, because the ^{15}N enrichment of NO_3^- apparently increased between the two measurements (Table 2). This was probably caused by difficulties in collecting a representative soil sample, combined with increased uncertainty in the determinations, induced by the correction for carry-over of NH_4^+.

During the first experiment, the glass jar incubation with and without acetylene revealed a high N_2O reductase activity, which in some cases caused the soil to be a sink of atmospheric N_2O (Fig. 5 A). Production of N_2O via nitrification was detected in the ammonium treatment, however net N_2O emission stopped after 5 hours, probably because N_2O reductase was induced (Fig. 5 C vs. D). The denitrification rate, determined via the acetylene incubation, demonstrated that the denitrifying activity in the water treatment increased between the two experiments ($P = 0.0002$), and was substantially higher in the urine treatment compared to the water control ($P = 0.0014$) (Table 3). Mean compaction of the soil cores was 1.1 ± 0.3%. The N_2O loss ratios of nitrification (L_N) and denitrification (L_D) appear in Table 3.

4 Discussion

4.1 Effect of urine on the four parameters that influence the N_2O production

Immediately after application of urine corresponding to 52.9 g N m^{-2} the emission of N_2O was 42.3 µg m^{-2} h^{-1} (Fig. 3). This is comparable to the initial rates determined in other field studies, where similar amounts of urine-N were applied (Allen et al., 1996; Yamulki et al., 1998). The measured N_2O formation via NO_3^- reduction was presumably due to denitrification. Additionally, DNRA could have produced a part of the N_2O originating from NO_3^- in the water treatments (Tiedje, 1988). The enhanced N_2O emission from the urine treatment compared to the water control seemed to be a result of increased N_2O loss from both nitrification and denitrification, caused by a combination of higher rates and higher loss ratios of the processes (Table 3). Thus, the four parameters that influence the N_2O production all appeared to rise following urine application (Equation 1).

The increased NH_4^+ availability was a part of the reason for the higher nitrification rate in the urine treatment than in the water control. This is in contrast to other studies where similar amounts of urine-N led to inhibition of nitrification for a couple of days, probably due to microbial stress caused by $NH_{3(aq)}$ and low osmotic potential (e.g. Bol et al., 2004). A study on pure cultures of an ammonia-oxidizing bacterium showed that the N_2O loss ratio of nitrification rose with increasing NH_4^+ concentration up to about 1 g NH_4-N l^{-1} (Yoshida and Alexander, 1970). Hence, the greater soil content of NH_4^+ may explain the increased N_2O loss ratio of nitrification in the urine treatment.

The rate of denitrification in the urine treatment was stimulated by the enhanced supply of NO_3^- from nitrification, the higher pH and possibly by the increased soil content of DOC. The organic compounds in the added urine largely explained the observed rise of DOC in the urine treatment, thus there was no evidence for release of DOC due to root scorching or solubilization of soil organic C. When denitrifying bacteria have much greater access to oxidant than to reductant, the nitrogen oxide may be incompletely reduced, resulting in a high N_2O/N_2 ratio of end products (Hutchinson and Davidson, 1993). Hence, the higher N_2O loss ratio of denitrification in the urine treatment compared to the water control might be a result of increased NO_3^-/DOC ratio (Fig. 2). The determined N_2O loss ratios of denitrification were consistent with results from three ryegrass field studies, where the N_2O loss ratio of denitrification varied between 4 and 27% (Stevens and Laughlin, 1998).

4.2 Evaluating the impact of the chemical conditions arising in urine-affected soil

The ammonium treatment was included in the present study to evaluate the importance of the chemical conditions for the N_2O production in urine-affected soil, e.g. the raised soil pH following urea hydrolysis. However, the comparison between the urine and ammonium treatments was complicated by the fact that WFPS rose between the two experiments (Fig. 1). Results from the water treatments revealed improved conditions for N_2O production in the second experiment. Apparently, this was related to an enhanced denitrification rate stimulated by a more frequent occurrence of anaerobic microsites following the rise in WFPS (Table 3). Compared to the water control, urine application

increased the N_2O emission by a factor 11, whereas application of ammonium increased the emission by a factor 7. Thus, urine appeared to have a greater impact on the N_2O production than addition of equivalent amounts of NH_4-N did. This result verifies the first hypothesis regarding the relative amount of N_2O emitted from the three treatments (*viz.* urine > ammonium > water) and furthermore supports the findings of Sherlock and Goh (1983). Consequently, the chemical conditions that arose in the urine-affected soil seemed to influence the N_2O formation.

4.3 Source of N_2O in the urine and ammonium treatments

Soil water content is a major factor controlling the contribution of nitrification and denitrification to the N_2O production in urine patches. Koops et al. (1997) found that initial N_2O from urine applied on very dry top-soil was mainly produced via nitrification, however, in moist soil denitrification was reported to be the dominant source of the initial N_2O (Monaghan and Barraclough, 1993; De Klein and Van Logtestijn, 1994). In the present study, N_2O was primarily formed during nitrification following the ammonium treatment (Fig. 3, 5), whereas nitrification and denitrification contributed equally to the N_2O emission in the urine treatment. The greater N_2O loss from denitrification in the urine treatment compared to the ammonium treatment, may partly be explained by the higher WFPS, although WFPS never exceeded 60%, which is the limit known to trigger substantial denitrification activity (Davidson, 1991). For this reason, the third hypothesis, which concerned denitrification, could not be tested.

The urine treatment gave rise to the largest N_2O loss from nitrification, which could also relate to the higher WFPS as the maximum nitrification rate was reported to occur at 50% WFPS in a sandy loam soil (Grundmann et al., 1995). However, the water treatments provided no evidence for a general increase of nitrification between the two experiments (Table 3). Thus, the greater N_2O loss from nitrification in the urine treatment compared to the ammonium treatment, mainly appeared to be an effect of the treatments and not an effect of the change in WFPS. Furthermore, the rate of nitrification seemed to be lower in the ammonium treatment than in the urine treatment, as no NO_3^- accumulation was detected, although the denitrification rate was minor (Table 1). This observation led to rejection of the second hypothesis regarding the effect on the nitrification rate of the two treatments.

4.4 Possible mechanisms involved in the urine-induced N_2O loss from nitrification

Soil pH_{H2O} was 7.9 in the water treatment and declined to 7.4 in the ammonium treatment because NH_4^+ is a weak acid. In the urine treatment, however, pH increased to 8.3 due to the alkaline products formed during the enzymatic hydrolysis of urea. A recent field study demonstrated that the rate of nitrification increased with soil pH_{H2O} in the range from 6 to 8, which supported indications found in earlier studies (Kyveryga et al., 2004). Hence, the higher soil pH most likely was the reason for the greater nitrification rate in the urine treatment compared to the ammonium treatment, indicating that the higher soil pH, in part, caused the greater N_2O loss from nitrification. In line with this, Yoshida and Alexander (1970) showed that the N_2O production by an ammonia-oxidizing bacterium strongly increased when pH was raised from 6 to 8. Furthermore, studies conducted under conditions favouring nitrification revealed a higher N_2O

production in slightly alkaline soils (pH_{H2O} 7.8 to 8.2) compared to neutral soils (pH 6.8) (Bremner and Blackmer, 1978, 1980).

4.5 Conclusions

The increase in pH following urea hydrolysis seemed to be a key factor influencing the N_2O loss from nitrification, but the responsible mechanism was unexpected. It appeared that the rate of nitrification was stimulated, not only by the high NH_4^+ availability, but also by the increased soil pH following urine application. In the urine treatment, nitrification and denitrification contributed equally to the N_2O production. However, the present study did not provide knowledge on the impact of the chemical conditions on the N_2O loss from denitrification in urine-affected soil, because of the change in WFPS between the ammonium and urine experiments.

In itself a high nitrification rate does lead to raised N_2O losses, and furthermore, it enables a high denitrification rate with associated N_2O losses. Based on the present study, an increased nitrification rate therefore appeared to be the most important factor explaining the high initial N_2O emission from urine patches.

5 Acknowledgements

This work was funded by the Danish Research Centre for Organic Farming (DARCOF) and the Danish Agricultural and Veterinary Research Council. I wish to thank Per Ambus for his qualified advice, Liselotte Meltofte for her assistance with the ^{15}N diffusion as well as Sten Struwe, Finn P. Vinther and Jane Hansen for their valuable comments on the manuscript.

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Table 1. Content of soil NH_4^+ and NO_3^- (g N m^{-2}) in the 0-10 cm soil layer of the microplots determined about 5 and 31 hours after application of water, ammonium solution and urine; n = 4-12, means and SE (in brackets).

		5 hours after application		31 hours after application	
Experiment	Treatment	NH_4^+	NO_3^-	NH_4^+	NO_3^-
1	Water	0.29 (0.03)	0.14 (0.01)	0.47 (0.06)	0.12 (0.00)
1	Ammonium	54.94 (4.72)	0.14 (0.04)	57.75 (5.35)	0.09 (0.01)
2	Water	0.19 (0.03)	0.11 (0.01)	0.27 (0.03)	0.08 (0.02)
2	Urine	39.09 (1.91)	0.24 (0.04)	28.15 (2.12)	1.05 (0.05)

Table 2. Nitrogen-15 enrichment of soil NH_4^+ and NO_3^- (atom% excess) in the 0-10 cm soil layer measured about 5 and 31 hours after application of water, ammonium solution or urine labelled with $^{15}NH_4^+$ or $^{15}NO_3^-$; n = 1-4, means.

		5 hours		31 hours	
Experiment	Treatment	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NH_4^+$	$^{15}NO_3^-$
1	Water/$^{15}NH_4^+$	0.2631	0.7915	ND	ND
1	Ammonium/$^{15}NH_4^+$	4.6710	0	ND	ND
2	Water/$^{15}NH_4^+$	0.2908	0.8711	ND	ND
2	Urine/$^{15}NH_4^+$	5.3603	0	ND	ND
1	Water/$^{15}NO_3^-$	0.6058	9.5903	1.0128	2.4571
1	Ammonium/$^{15}NO_3^-$	0.0053	2.7122	0.0117	3.2492
2	Water/$^{15}NO_3^-$	0.7360	3.8859	0.5722	1.1632
2	Urine/$^{15}NO_3^-$	0.0336	2.0624	0.0414	0.1059

Table 3. Rates of nitrification and denitrification, N_2O-N lost per NO_3^- produced via nitrification (L_N) and N_2O-N lost per N_2+N_2O produced via denitrification (L_D) in the 0-2 cm soil layer of microplots treated with water, ammonium solution and urine; n = 4, means and SE (in brackets).

Experiment	Treatment	Nitrification mg NO_3-N m^{-2} h^{-1}	L_N ‰	Denitrification µg N m^{-2} h^{-1}	L_D %
1	Water	1.3 (0.1)	0.11	0.60 (0.37)	26
1	Ammonium	ND	ND	0	ND
2	Water	0.8 (0.1)	0.18	62 (16)	0.9
2	Urine	6.9 (0.3)	2.91	412 (81)	5.1

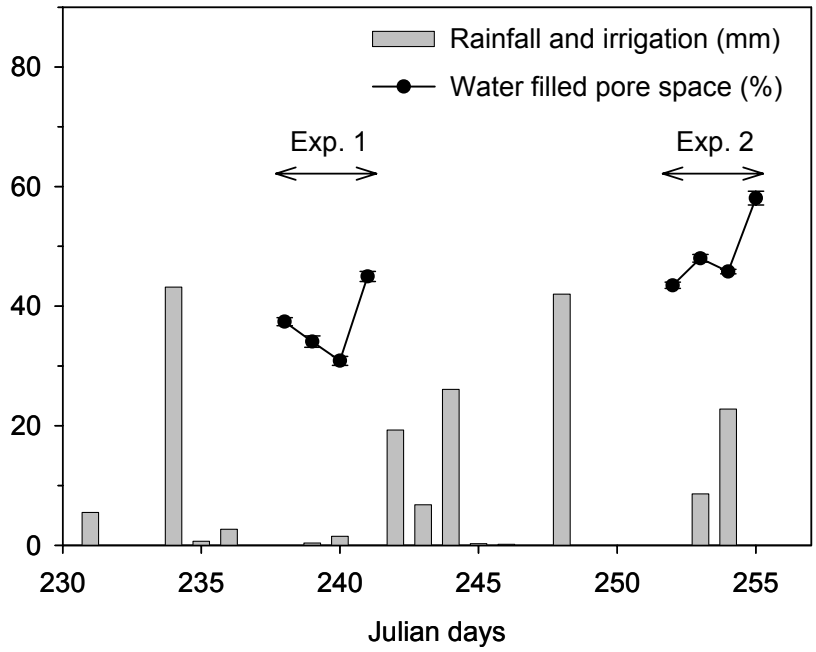

Figure 1. Summed rainfall and irrigation (mm) as well as water filled pore space in the 0-10 cm soil layer (%; n = 4, means ± SE) during the experimental period.

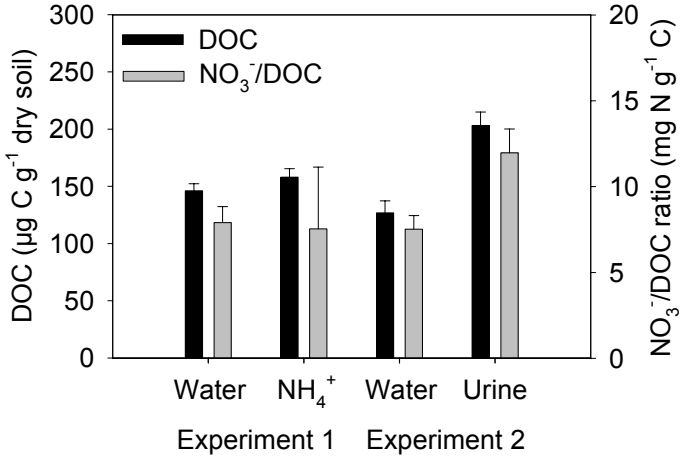

Figure 2. Soil content of dissolved organic carbon (DOC) and the NO_3^-/DOC ratio in the 0-10 cm soil layer of microplots treated with water, ammonium solution and urine; n = 4; means ± SE.

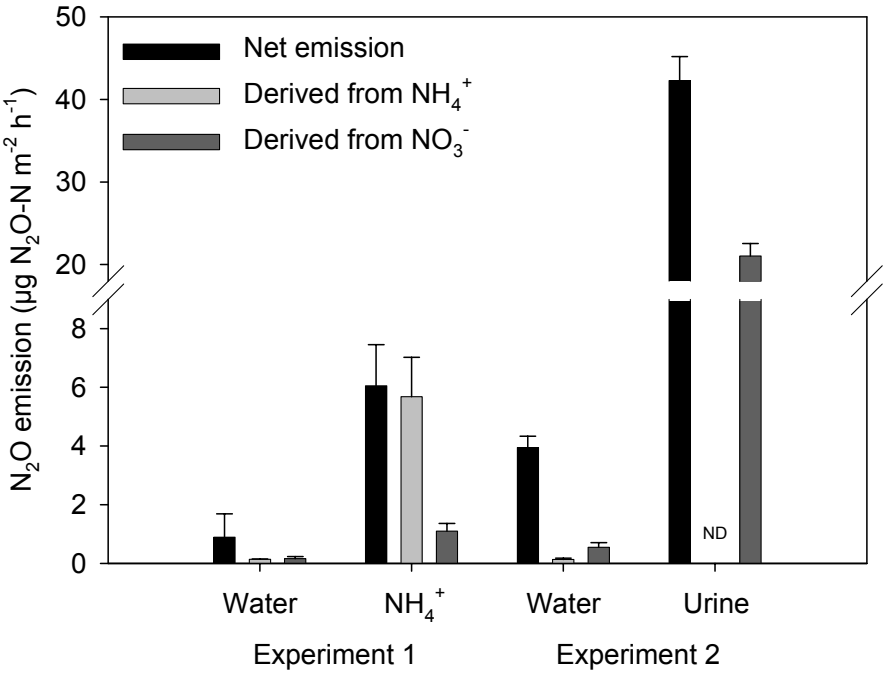

Figure 3. Net N_2O emission (n = 6-12) as well as N_2O derived from oxidation of NH_4^+ in the 0-2 cm soil layer determined in $^{15}NH_4^+$ labelled microplots (n = 4) and N_2O derived from reduction of NO_3^- in the 0-2 cm soil layer determined in $^{15}NO_3^-$ labelled microplots (n = 4) for the water, ammonium and urine treatment; means ± SE. Please, note the break on the y-axis.

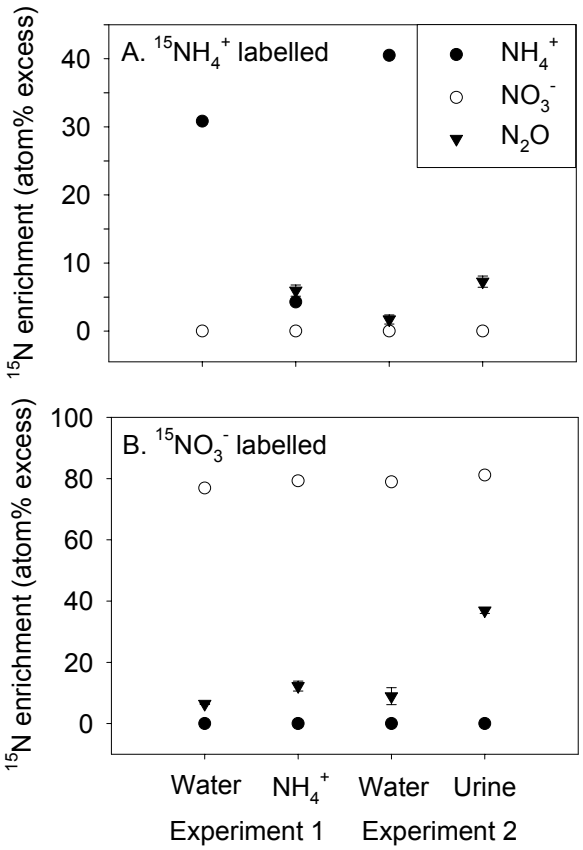

Figure 4. Calculated ^{15}N enrichment of soil NH_4^+ and NO_3^- in the 0-2 cm soil layer as well as measured ^{15}N enrichment of emitted N_2O (n = 4, means ± SE) in microplots treated with water, ammonium solution or urine labelled with (A) $^{15}NH_4^+$ or (B) $^{15}NO_3^-$.

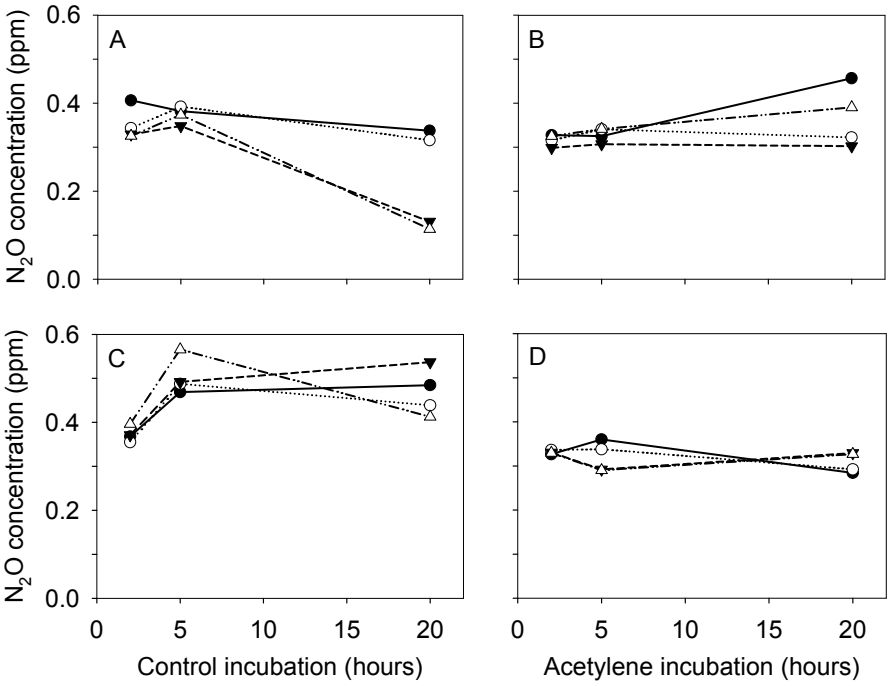

Figure 5. Headspace concentration of N_2O in four glass jars during control or acetylene incubation of soil cores taken during the first experiment from microplots treated with (A, B) water or (C, D) ammonium solution; n = 1.

Mission

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