Archived at http://orgprints.org/14150

Type of contribution:	Regular paper
Date of preparation:	5 March 2007
Number of text pages:	31
Number of tables:	5
Number of figures:	5
Instructions to the printer:	Figures 1, 3 and 5 are designed to cover two columns

Contribution of nitrification and denitrification to N_2O emissions from urine patches

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Prepared for submission to: Soil Biology & Biochemistry

Abstract

Urine deposition by grazing livestock causes an immediate increase in nitrous oxide (N_2O) emissions, but the responsible mechanisms are not well understood. A nitrogen-15 (^{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 ratio of nitrification (*i.e.* moles of N_2O-N produced per moles of nitrate produced) and denitrification (*i.e.* moles of N_2O produced per moles of $N_2O + N_2$ produced). 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 at 45 and 35% water-filled pore space (WFPS), respectively, and in each experiment a water control was included. 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 24 h, gross nitrification rates were measured in the field, whereas the denitrification rates were measured in soil cores in the laboratory. Compared with the water control, urine application increased the N_2O emission from 3.9 to 42.3 μg N_2O-N m^{-2} h^{-1}, whereas application of ammonium increased the emission from 0.9 to 6.1 μg N_2O-N m^{-2} h^{-1}. In the urine-affected soil, nitrification and denitrification contributed equally to the N_2O emission, and the increased N_2O loss resulted from a combination of higher rates and higher N_2O loss ratios of the processes. In the present study, an enhanced nitrification rate seemed to be the most important factor explaining the high initial N_2O emission from urine patches deposited on well-aerated soils.

Keywords

Denitrification; Grass-clover; Grassland; Gross nitrification; Loss ratio; ^{15}N; 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 nitrous oxide (N_2O) from these soils. Nitrous oxide is a well recognized greenhouse gas. Agricultural soils contribute approximately 50% of the World's anthropogenic N_2O emissions (IPCC, 2001) and currently this source of N_2O represents 2.4% of the European release of anthropogenic derived greenhouse gasses (EEA, 2006). 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 (Wrage et al., 2001) and the N_2O emission usually increases immediately after urine deposition (e.g. Allen et al., 1996; Koops et al., 1997; Yamulki et al., 1998). Williams et al. (1999) estimated that N_2O losses within the first 24 h after urine deposition accounted for approximately 8% of the annual N_2O emission from a grassland.

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, pH (Clough et al., 1998, 2004) and the amount of urine-nitrogen (N) deposited (Petersen et al., 2004). It is well-known that soil pH rises temporarily following urine deposition because alkaline products are formed during the rapid enzymatic hydrolysis of urea, which is expressed as

$$(NH_2)_2CO + 3\ H_2O \rightarrow 2\ NH_4^+ + OH^- + HCO_3^- \quad (1)$$

The raised pH in the urine-affected soil shifts the equilibrium between ammonium (NH_4^+) and dissolved ammonia ($NH_{3(aq)}$) towards $NH_{3(aq)}$ (Schmidt, 1982), which at high concentrations inhibits microbial activity. Sherlock and Goh (1983) applied urine, urea and ammonium corresponding to 20 g N m^{-2} dissolved in similar volumes of liquid and found that urine gave rise to the largest N_2O emission, especially immediately after application. Thus, elevated soil water content and availability of inorganic N only explain part of the urine-induced N_2O emission. Urine contains hippuric acid, which is known to accelerate the hydrolysis of urea and thereby also the formation of $NH_{3(aq)}$ (Whitehead et al., 1989). The findings of Sherlock and Goh (1983) could indicate that the $NH_{3(aq)}$ concentration in the soil solution plays an important roll for the initial N_2O production in urine patches.

The N_2O loss via nitrification and denitrification is influenced by four parameters (Firestone and Davidson, 1989; Granli and Bøckmann, 1994). These are the rate of gross nitrification ($\underline{N}$) and denitrification ($\underline{D}$), the N_2O loss ratio of nitrification, *i.e.* moles of N_2O-N lost per moles of nitrate (NO_3^-) produced ($\underline{L_N}$) and the N_2O loss ratio of denitrification, *i.e.* moles of N_2O lost per moles of $N_2 + N_2O$ produced ($\underline{L_D}$). Thus, the total loss of N_2O from nitrification and denitrification ($\underline{E}$) can be described as

$$\underline{E} = \underline{N} \times \underline{L_N} + \underline{D} \times \underline{L_D} \quad (2)$$

Any particular environmental factor may affect the four parameters differently. In urine patches, the rate of nitrification ($\underline{N}$) may be stimulated by the enhanced availability of the substrate, NH_4^+. However, as nitrite (NO_2^-) oxidation is more readily inhibited by $NH_{3(aq)}$ than the NH_4^+ oxidation (Harada and Kai, 1968), the N_2O formation

94 may rise due to chemical decomposition of NO_2^- or reduction of NO_2^- via nitrifier
95 denitrification (Wrage et al., 2001, 2004b). Thus, the first hypothesis is that in urine-
96 affected soil the N_2O loss from nitrification will increase mainly as a result of an
97 increase in the N_2O loss ratio of the process (L_N).

98 The rate of denitrification (D) in urine-affected soil may be stimulated by a
99 urine-induced rise in dissolved organic carbon (DOC), either via solubilization of soil
100 organic carbon (C), or because labile compounds are released from scorched plant roots
101 (Monaghan and Barraclough, 1993). However, the N_2O loss ratio of denitrification (L_D)
102 decreases when pH increases and when the NO_3^-/labile C ratio decreases (Hutchinson
103 and Davidson, 1993; Simek and Cooper, 2002), *i.e.* denitrification is more complete
104 with more N_2O reduction. Thus, the second hypothesis is that in urine-affected soil the
105 N_2O loss from denitrification will increase due to an increase in the rate of the process
106 (D).

107 The objective of the present study was to investigate the mechanisms involved
108 in the initial N_2O production following urine deposition. More specifically, the
109 objective was to assess changes in the four parameters that influence the N_2O
110 production (*viz.* N, D, L_N and L_D) in urine patches deposited on a sward having
111 intermediate soil water content (about 45% water-filled pore space, WFPS). Artificial
112 urine (52.9 g N m^{-2}) was used and a water treatment was included as control. In
113 addition, an experiment with an ammonium solution and water was conducted at lower
114 soil water content (about 35% WFPS) to study the effect of soil moisture on the four
115 parameters. The nitrogen-15 (^{15}N) labelling and acetylene inhibition techniques were
116 used to quantify the parameters. Separate labelling of the NH_4^+ and NO_3^- pools with ^{15}N
117 has been used frequently to assess the contribution of nitrification and denitrification to

118 the N_2O production in soil, but was only involved in a few studies on urine-affected soil
119 (e.g. Monaghan and Barraclough, 1993).
120
121 **2. Materials and methods**
122
123 *2.1. Field site*
124 The study was conducted in a sward consisting of white clover (*Trifolium repens*
125 L.), red clover (*Trifolium praténse* L.) and perennial ryegrass (*Lolium perenne* L.)
126 during the second production year. The grass-clover sward was part of an organic crop
127 rotation, which represented a dairy system, and was situated in Taastrup, 18 km west of
128 Copenhagen (55º 40′N, 12º 18′E). The soil was a loamy sand with a total N content of
129 0.21%, total C content of 2.1% and pH in water of 7.9. Microplots were established in
130 December 2002 by pushing 56 PVC cylinders (30 cm i.d. by 30 cm long) into the soil to
131 a depth of approximately 22.5 cm. Grazing was simulated during the summer of 2003
132 by regularly mowing the sward to 15 cm high. Soil temperature at a depth of 10 cm was
133 registered continuously and measurements of daily rainfall were obtained from a local
134 meteorological station (CWB, 2003).
135 The effect of ammonium (52.9 g N m^{-2}) on the nitrification and denitrification
136 processes was examined in the first experiment, which took place from 26 to 30 August
137 2003. Two weeks later, the effect of artificial urine (52.9 g N m^{-2}) on the processes was
138 assessed in the second experiment. In each experiment, a set of 28 microplots was used.
139

2.2. *Solutions for ^{15}N field measurements*

Nitrogen-15 techniques were used in the field to determine the rate of gross nitrification and the amount of N_2O produced via nitrification or denitrification. Five solutions were prepared for the first experiment, *viz.* water only, water/$^{15}NH_4^+$, water/$^{15}NO_3^-$, ammonium/$^{15}NH_4^+$ and ammonium/$^{15}NO_3^-$. 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. 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}. Details of the content, ^{15}N labelling, total N concentration and specific purpose of each of the solutions are given in Table 1. Table 1

2.3. *^{15}N field measurements*

For practical reasons, 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 h of solution application. Briefly, each microplot was sealed with a PVC lid (5 cm inner height) fitted with a rubber septa to allow gas sampling. The lid had an EPDM

(Ethylene Propylene Diene Monomer) 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 enrichment, 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 min of cover period, a sample of the headspace gas was taken with a syringe through the rubber septa and stored in a 3.5 ml vial for later analysis of N_2O concentration. At the end of the cover period (150 min), a 120 ml sample was taken to determine the ^{15}N enrichment of N_2O.

Following gas sampling a soil sample, consisting of four soil cores (0-10 cm depth, 2 cm diameter) was collected from each microplot. 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 h, soil sampling was repeated in these microplots to determine the rate of gross 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 (100-120 mesh) 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 enrichment 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).

188

189 *2.5. Soil analyses*

190 Coarse roots and pebbles (> 4 mm) were removed by tweezers. Within 7 h of
191 soil sampling, 20 g portions of each 'root free' soil sample were extracted in 1 M KCl
192 (1:5, w:vol), stirred on a horizontal shaker for 1 h at 140 rpm. The extracts were filtered
193 through Whatman 40 filters and kept at -20 °C until further analysis.

194 Dissolved organic carbon in the extracts from $^{15}NO_3^-$ labelled microplots was
195 measured on a TOC-5000A total organic C analyzer (Shimadzu, Kyoto, JP). The
196 content of NH_4^+, NO_3^- and NO_2^- in extracts were analysed colorimetrically on an
197 autoanalyzer (Bran+Luebbe, Norderstedt, DE). Nitrogen-15 enrichment of NH_4^+ and
198 NO_3^- were determined in extracts by the diffusion method (Sørensen and Jensen, 1991).
199 Briefly, NH_4^+ in the extract was converted into NH_3, which was trapped on an acidified
200 filter paper. Subsequently, NO_3^- was converted via NH_4^+ into NH_3, which was trapped
201 on another filter. The filters were analysed for ^{15}N using an elemental analyser (EA
202 1110, Carlo Erba, Milano, IT) coupled in continuous flow mode to the IRMS. Some
203 carry-over of NH_4^+ was detected on the NO_3^- filters from the ammonium and urine
204 treatments, which was corrected for via the autoanalyzer measurements.

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

209

210 *2.6. Acetylene inhibition technique*

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

218 Incubation with C_2H_2 was initiated using two soil cores from each microplot.
219 Thus, 4 ml C_2H_2 (acetone free, AGA A/S, Copenhagen, DK) was injected along the
220 length of each core using a veterinary needle connected to a 5 ml syringe and a C_2H_2
221 reservoir via a three-way valve (Ambus and Christensen, 1993). The two cores were
222 placed in a 2 litre glass jar, which was closed with a rubber-sealed lid fixed with
223 clamps. A volume (180 ml) of headspace air was extracted from the jar and then
224 replaced with 180 ml of C_2H_2 using 60 ml syringes and a rubber septa mounted in the
225 lid of the jar. The resulting C_2H_2 concentration in soil and headspace atmosphere was
226 9%, which inhibits nitrification and is above the 5% needed to block the reduction of
227 N_2O to N_2 (Okereke, 1984). Subsequently, control incubations were initiated on the
228 other two soil cores, using pure N_2 instead of C_2H_2. The glass jars were then incubated
229 at 15ºC. After 2, 5 and 20 h of incubation, a 30 ml sample of headspace gas was taken
230 through the rubber septa and transferred to a 3.5 ml N_2-flushed Venoject vial using a
231 syringe. A volume of 30 ml N_2 was added to the jar before each gas sampling to
232 maintain atmospheric pressure. Soil dry matter was determined after the last gas
233 sampling (oven drying at 105 °C for 24 h).

The 3.5 ml gas samples had 2 ml of N_2 added 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

Fluxes of N_2O in the field and laboratory were calculated from the increase in N_2O concentration in the headspace during the incubation periods. 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 ($\underline{C}^*$) could be determined as

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

where $\underline{C_0}$ and $\underline{C_t}$ are the N_2O concentration calculated from the regression equation at the start and at the end of the cover period, respectively, and $\underline{C_0}^*$ and $\underline{C_t}^*$ are the ^{15}N atom% excess enrichment of N_2O at the start and at the end of the cover period, respectively.

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 ($\underline{CC}^*$) was established as

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

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

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

Gas measurements were initiated within 2.5 h of solution application and therefore it may be assumed that the unlabelled N pool (*i.e.* NH_4^+ in $^{15}NO_3^-$ labelled microplots and NO_3^- in $^{15}NH_4^+$ labelled microplots) had not yet been labelled via transformation of labelled N (Panek et al., 2000). Furthermore, the added solutions were assumed to affect the 0-2 cm soil layer, as this was the mean penetration depth.
As a result, emission of N_2O-N derived from nitrification (*CP*) was established as

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

where $\underline{N_i^{*}}$ is the calculated ^{15}N atom% excess enrichment of NH_4^+ in the 0-2 cm soil layer. The estimates were subsequently converted from concentrations of N_2O to amounts 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 mixes

282 homogeneously with the soil pool (Stevens et al., 1997), but in field trials it may be
283 difficult to obtain completely uniform labelling. However, when the addition of labelled
284 N by far exceeds the native soil N, there is initially only one significant pool, which is
285 practically uniform (Bergsma et al., 1999).
286 Gross nitrification rates were calculated according to Mosier and Schimel (1993)
287 using the isotopic dilution of ^{15}N labelled NO_3^- in the 0-10 cm soil layer measured
288 during 24 h. The rate of nitrification and denitrification in the 0-2 cm soil layer was
289 estimated as $^1/_5$ of the activity in the 0-10 cm layer. Gravimetric water content was
290 converted to water-filled pore space using measured soil bulk density and assuming a
291 particle density of 2.65 g cm^{-3}.
292 Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and
293 Tukey's multiple comparison tests ($\alpha = 0.05$) were performed using SAS General
294 Linear Model procedure (SAS Institute, 1997). Statistics on net N_2O emissions and
295 denitrification rates were performed on square root and log transformed data. Some
296 results are reported as the mean ± standard error.
297
298 **3. Results**
299
300 *3.1. Soil moisture, temperature and pH*
301 Water-filled pore space of the soil increased between the two experiments due to
302 rains during the intervening period ($P < 0.0001$; Fig. 1). Mean soil temperature at 10 cm Figure 1
303 depth during the experimental periods was 15.3 °C and did not vary significantly
304 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). In the water treatment, the major part of the inorganic N was found as NH_4^+ ($P < 0.0001$) (Table 2). 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. 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. In all treatments the soil content of NO_2^- was below the detection limit of about 0.1 μg N g^{-1} dry soil.

Table 2

The soil content of DOC showed an effect of the added solutions ($P = 0.0003$), which resulted 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 ($P = 0.032$).

Figure 2

3.3. N_2O emission

The N_2O emission and the final ^{15}N enrichment of N_2O in the chambers are shown in Table 3 for each individual treatment. 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

Table 3

329 = 0.36; Table 3). Compared to the water control, ammonium application increased the
330 N_2O emission from 0.9 to 6.1 μg N_2O-N m^{-2} h^{-1} (P = 0.011), whereas application of
331 urine increased the emission from 3.9 to 42.3 μg N_2O-N m^{-2} h^{-1} (P < 0.0001) (Fig. 3). Figure 3
332 The increased N_2O emission from the water treated microplots between the two
333 experiments was probably a result of increased WFPS (P = 0.0021). Using N_2O
334 emission from the water treatments as a covariate revealed a significantly higher N_2O
335 emission from the urine treatment compared to the ammonium treatment (P = 0.013).
336
337 *3.4. ^{15}N of inorganic N*
338 For soil sampled 5 h after solution application, the ^{15}N enrichment of NH_4^+ and
339 NO_3^- in the paired treatments of water and ammonium or urine deviated more than
340 could be explained by the initial soil content of NH_4^+ and NO_3^- (Table 4). This Table 4
341 suggested that the ^{15}N enrichment had changed over the 12-hour period that separated
342 application of solutions and KCl extraction of the soil, and was perhaps a result of
343 microbial activity or due to exchange of $^{15}NH_4^+$ with $^{14}NH_4^+$ adsorbed to soil colloids.
344 Furthermore, the ^{15}N enrichment of NH_4^+ in the water/$^{15}NO_3^-$ treatments showed that
345 some NO_3^- had been converted into NH_4^+, possibly via immobilization followed by
346 mineralization or via dissimilatory nitrate reduction to ammonium (DNRA). Recovery
347 of the added ^{15}N in the inorganic N pool of the 0-10 cm soil layer ranged between 8 and
348 118%, with the highest recovery occurring in the ammonium/$^{15}NH_4^+$ and urine/$^{15}NH_4^+$
349 treatments. Low recovery was probably because of N uptake by plants or loss via
350 denitrification.
351

352 *3.5. Source of N_2O produced in the 0-2 cm soil layer*

353 In the labelled microplots, the ^{15}N enrichment of the emitted N_2O often
354 exceeded the enrichment of soil NH_4^+ or NO_3^- measured 5 h after solution application in
355 the 0-10 cm soil layer (Fig. 4; Table 4). In some cases it also exceeded the initial Figure 4
356 enrichment calculated for the 0-10 cm soil layer. Thus, the ^{15}N enrichment of the
357 labelled pool being nitrified or denitrified appeared to be higher than the mean
358 enrichment in the 0-10 cm soil layer. During gas measurement, the added solutions were
359 therefore assumed to affect the 0-2 cm soil layer only, as this was the mean penetration
360 depth. The ^{15}N enrichment of N_2O emitted from the labelled microplots and the
361 calculated ^{15}N enrichment of soil NH_4^+ and NO_3^- in the 0-2 cm soil layer are shown in
362 Figure 4. Missing values are (1) N_2O from the water/$^{15}NH_4^+$ treatment in the first
363 experiment, where no net N_2O emission took place (Table 3), and (2) NH_4^+ from the
364 urine/$^{15}NH_4^+$ treatment, because the progression of urea hydrolysis was unknown.

365 Based on the emission of ^{15}N labelled N_2O and the enrichment of NH_4^+ and
366 NO_3^- in the labelled soil layer, it is possible to calculate the contribution of NH_4^+
367 oxidation and NO_3^- reduction in the soil layer in question to the total N_2O emission (Fig.
368 3). The responsible process for N_2O formation via NO_3^- reduction was most likely
369 denitrification. As regards the water treatment, the emission of N_2O derived from
370 nitrification or denitrification in the 0-2 cm soil layer was rather similar ($P \geq 0.053$) and
371 did not change significantly between the two experiments ($P = 0.060$) (Fig. 3). In the
372 ammonium treatment, the N_2O loss from denitrification in the 0-2 cm soil layer rose to
373 1.1 μg N_2O-N m^{-2} h^{-1} ($P = 0.014$), whereas the N_2O loss from nitrification in the 0-2 cm
374 layer increased to 5.7 μg N_2O-N m^{-2} h^{-1} ($P = 0.0061$), leading to a considerably higher
375 N_2O loss from nitrification than from denitrification ($P = 0.015$). In the urine treatment,

376 the N_2O loss from denitrification in the 0-2 cm soil layer rose to 21.0 μg N_2O-N m^{-2} h^{-1}
377 ($P < 0.0001$). The N_2O emission derived from nitrification could not be calculated in the
378 urine treatment because the ^{15}N enrichment of NH_4^+ was unknown. However, it is likely
379 that nitrification was the source of the urine-induced N_2O emission, which was not
380 accounted for by denitrification in the 0-2 cm soil layer. Thus, the N_2O loss from
381 nitrification in the 0-2 cm soil layer was calculated to be 20.1 ± 1.2 μg N_2O-N m^{-2} h^{-1},
382 and consequently, nitrification and denitrification contributed equally to the N_2O
383 production in the urine treatment ($P = 0.63$).
384
385 *3.6. Rate of gross nitrification and denitrification*
386 The rate of gross nitrification in the water treatment measured via $^{15}NO_3^-$ pool
387 dilution declined between the two experiments ($P = 0.0066$; Table 5). In the urine Table 5
388 treatment, the nitrification rate increased by factor 9 compared to the water control ($P <$
389 0.0001). A rate could not be determined for the ammonium/$^{15}NO_3^-$ treatment, because
390 the ^{15}N enrichment of NO_3^- apparently increased between the two measurements (Table
391 4). This was probably caused by difficulties in collecting a representative soil sample,
392 combined with increased uncertainty in the determinations, induced by the correction
393 for carry-over of NH_4^+.
394 During the first experiment, the glass jar incubation with and without acetylene
395 revealed a high N_2O reductase activity, which in some cases caused the soil to be a sink
396 of atmospheric N_2O (Fig. 5 A). Production of N_2O via nitrification was detected in the Figure 5
397 ammonium treatment, however net N_2O emission stopped after 5 h, probably because
398 N_2O reductase was induced (Fig. 5 C vs. D). The denitrification rate, determined via the
399 acetylene incubation, demonstrated that the denitrifying activity in the water treatment

400 increased between the two experiments (P = 0.0002), and was 7-fold higher in the urine
401 treatment compared to the water control (P = 0.0014) (Table 5). No denitrifying activity
402 was detected in the ammonium treatment. In the urine treatment, the N_2O loss ratios of
403 nitrification ($\underline{L_N}$) seemed to increase substantially compared to the water control and the
404 loss ratios of denitrification ($\underline{L_D}$) also appeared to increase (Table 5).
405
406 **4. Discussion**
407
408 *4.1. Effect of urine on the N_2O production*
409 Immediately after application of urine corresponding to 52.9 g N m^{-2} the
410 emission of N_2O was 42.3 μg N_2O-N m^{-2} h^{-1} (Fig. 3), which is comparable to the initial
411 rates determined in other field studies, where similar amounts of urine-N were applied
412 (Allen et al., 1996; Yamulki et al., 1998). Nitrification and denitrification seemed to
413 contribute equally to the N_2O production in the urine-affected soil. This could relate to
414 the intermediate soil water content of about 45% WFPS, which provided both aerobic
415 and anaerobic microsites, enabling nitrification and denitrification to occur
416 simultaneously. In contrast, Koops et al. (1997) found that initial N_2O from urine
417 applied on very dry top-soil was mainly produced via nitrification, however, in moist
418 soil (75-90% WFPS) denitrification was reported to be the dominant source of the initial
419 N_2O (Monaghan and Barraclough, 1993; De Klein and Van Logtestijn, 1994). In all the
420 mentioned studies urine was applied at rates between 40 and 55 g N m^{-2}, thus in
421 amounts comparable to the present study.
422 Concerning the N_2O produced during nitrification, the hypothesis was that the
423 N_2O production would increase mainly as a result of an increase in the N_2O loss ratio of

424 the process ($\underline{L_N}$). This hypothesis turned out only to be partly right as both the gross
425 nitrification rate ($\underline{N}$) and the N_2O loss ratio of nitrification ($\underline{L_N}$) increased substantially
426 in the urine treatment compared to the water control. More specifically, the simulated
427 urine deposition lead to a 9-fold increase of the nitrification rate and calculated on soil
428 weight basis the rate corresponded to 6.3 ± 0.3 μg N g^{-1} soil d^{-1}. Studies reporting gross
429 nitrification rates in urine-affected soil are very rare. For comparison, however, gross
430 nitrification was measured to be 2.4 μg N g^{-1} soil d^{-1} in a laboratory study on soil at 50%
431 WFPS and fertilized with 20 g N m^{-2} (Bateman and Baggs, 2005). Application of urine
432 in the field at rates below 53 g N m^{-2} has been seen to inhibit nitrification for 2-14 d,
433 possibly as a result of microbial stress (Thomas et al., 1988; Bol et al. 2004). Monaghan
434 and Barraclough (1992) found that inhibition of nitrification due to $NH_{3(aq)}$ toxicity and
435 salt-induced stress only occurred when the urine-N concentration exceed 16 g N l^{-1}.
436 Thus, the urine-N concentration of 15.6 g N l^{-1} in the present study may explain why the
437 nitrifying bacteria were not adversely affected in the urine-treated soil.
438 The increased NH_4^+ availability was most likely a part of the reason for the
439 higher nitrification rate in the urine treatment than in the water control. Furthermore,
440 soil pH_{H2O} rose from 7.9 in the water control to 8.3 in the urine treatment because of the
441 alkaline products formed during the hydrolysis of urea. A recent field study
442 demonstrated that the rate of nitrification increased with soil pH_{H2O} in the range from 6
443 to 8, which supported indications found in earlier studies (Kyveryga et al., 2004).
444 Hence, the higher soil pH in the urine treatment could be part of the reason for the
445 increased nitrification rate, indicating that the higher soil pH, in part, caused the greater
446 N_2O loss from nitrification. In line with this, Yoshida and Alexander (1970) showed

447 that the N_2O production by an ammonia-oxidizing bacterium strongly increased when
448 pH was raised from 6 to 8.

449 In well-aerated soils, the N_2O loss ratio of nitrification is usually below 0.5%
450 (Ambus, 2005; Mathieu et al. 2006). In the second experiment, the ratio seemed to
451 increase from 0.02% in the water control to 0.29% in the urine-treated soil. To my
452 knowledge this is the first time the N_2O loss ratio of nitrification has been determined in
453 urine patches. In the study of Bateman and Baggs (2005), which was conducted at
454 equivalent soil water content (50% WFPS) but with lower N addition (20 g N m^{-2} as
455 NH_4NO_3), the N_2O loss ratio of nitrification was determined to be 0.18%. A study on
456 pure cultures of an ammonia-oxidizing bacterium showed that the N_2O loss ratio rose
457 with increasing NH_4^+ concentration up to about 1 g NH_4-N l^{-1} (Yoshida and Alexander,
458 1970). Thus, the gradual increase in the N_2O loss ratio of nitrification from the water
459 control via the study of Bateman and Baggs (2005) to the urine treatment may relate to
460 the increase in NH_4^+ availability.

461 The hypothesis for denitrification was that the N_2O loss from the process would
462 increase solely as a result of an increase in the rate of the process ($\underline{D}$). The N_2O loss
463 ratio of denitrification was predicted to decline. This hypothesis also turned out only to
464 be partly right as both the denitrification rate ($\underline{D}$) and the N_2O loss ratio of
465 denitrification ($\underline{L_D}$) appeared to increase in the urine treatment compared to the water
466 control. Calculated as diurnal value, denitrification in the 0-10 cm soil layer constituted
467 49 ± 10 mg N m^{-2} d^{-1}. This rather low rate was expected as high rates of denitrification
468 are usually associated with soil water contents above 60% WFPS (Davidson, 1991; De
469 Klein and Van Logtestijn, 1996). The rate is in the same order of magnitude at rates
470 measured by Koops et al. (1997) in urine patches on peat soil.

471 The denitrification rate in the urine treatment was stimulated by the enhanced
472 supply of NO_3^- from nitrification and possibly also by the higher pH and the increased
473 soil content of DOC (Weier et al., 1993; Simek and Cooper, 2002). The organic
474 compounds in the added urine largely explained the observed rise of DOC in the urine
475 treatment, thus there was no evidence for release of DOC due to root scorching or
476 solubilization of soil organic C as observed in some studies (e.g. Monaghan and
477 Barraclough, 1993; Shand et al., 2002).
478 A recent study showed that the hippuric acid component of urine inhibited
479 denitrification via the breakdown product benzonic acid, and thereby decreased the N_2O
480 emission (Van Groenigen et al., 2006). However, the denitrifying activity was only
481 affected when the concentration of hippuric acid exceeded 3.9 mmol kg^{-1} soil. These
482 findings are supported by the present study where a hippuric acid content of 3.4 mmol
483 kg^{-1} soil in the 0-5 cm layer did not seem to hinder denitrification.
484 The N_2O loss ratio of denitrification appeared to increase from 0.9% in the water
485 control to 5.1% in the urine treatment. When denitrifying bacteria have much greater
486 access to oxidant than to reductant they tend to reduce nitrogen oxide incompletely,
487 resulting in a high N_2O/N_2 ratio of end products (Hutchinson and Davidson, 1993;
488 Weier et al., 1993). Hence, the higher N_2O loss ratio of denitrification in the urine
489 treatment compared to the water control might be a result of increased NO_3^-/DOC ratio
490 (Fig. 2). The measured N_2O loss ratio of denitrification in the urine treatment is much
491 lower than ratios, which can be calculated from the data presented by De Klein and Van
492 Logtestijn (1994), giving N_2O loss ratios during denitrification of 54 and 80% following
493 application of 40 g urine-N m^{-2}. However, my ratio is with in the range of 4 and 27%

494 reported for ryegrass swards fertilized with about 300 kg N ha^{-1} (Kester et al., 1997;
495 Stevens and Laughlin, 1998).
496
497 *4.2. Effect of soil moisture on the N_2O production*
498 The rise in the soil water content between the two experiments enabled an
499 evaluation of the effect of soil moisture on the N_2O production via nitrification and
500 denitrification based on the results for the water treatment. The soil water content
501 increased from about 35% WFPS in the first experiment to about 45% WFPS in the
502 second experiment (Fig. 1). The net emission of N_2O from the water treatment rose
503 between the two experiments, which seemed to be a result of increased denitrification
504 rate (Fig. 3; Table 5). Presence of O_2 is most often the limiting factor for denitrification
505 (Tiedje, 1988; Robertson, 1989), and the increased rate was probably caused by the
506 more frequent occurrence of anaerobic microsites following the rise in WFPS.
507 In most cases the measured N_2O formation via NO_3^- reduction was probably due
508 to denitrification. Furthermore, the ^{15}N data indicated that DNRA possibly occurred in
509 the water treatment (Table 4) and therefore this process could have produced a part of
510 the N_2O originating from NO_3^-. Occurrence of DNRA was also reported in a study on
511 soil from permanent grassland (Stevens et al., 1998). The N_2O loss ratio for
512 denitrification in the water treatment appeared to be highest in the first experiment
513 (Table 5), which is in line with Tiedje (1988) stating that the N_2O/N_2 ratio generally
514 increases with increasing O_2 concentrations.
515 The rate of nitrification dropped as the soil water content increased from 35 and
516 45% WFPS. This is contrary to the study of Grundmann et al. (1995), where maximum
517 nitrification rate in a sandy loam soil was found at 50% WFPS. The N_2O loss ratio of

518 the process appeared to increase slightly (Table 5), leading to an unchanged N_2O loss
519 from nitrification (Fig. 3).
520 At 35% WFPS during the first experiment, simultaneous emission and
521 consumption of N_2O by the soil sometimes took place, e.g. in the water/$^{15}NH_4^+$
522 treatment emission of ^{15}N labelled N_2O was detected without net N_2O emission (Table
523 3). In some cases the soil acted as a net sink of atmospheric N_2O (e.g. Fig. 5A), which
524 was also observed in other studies on grassland (Glatzel and Stahr, 2001; Wrage et al.,
525 2004a). The responsible process for the reduction of N_2O could be denitrification,
526 nitrifier denitrification or DNRA (Poth, 1986; Conrad, 1996).
527
528 *4.3. Effect of ammonium on the N_2O production*
529 Application of an ammonium solution (52.9 g N m^{-2}) to the pasture soil at about
530 35% WFPS resulted in a rather low N_2O emission of 6.1 µg N_2O-N m^{-2} h^{-1}, which was
531 primarily formed during nitrification (Fig. 3, 5). The rate of nitrification seemed to be
532 low as no NO_3^- accumulation was detected, although the denitrification rate was minor
533 (Table 2, 5). In many ways the results for the ammonium treatment deviated from the
534 results obtained in the urine treatment during the second experiment. The retarded
535 processes in the ammonium treatment could relate to the lower soil water content, the
536 lower pH_{H2O} of 7.4 and a slightly lower osmotic potential compared to the urine
537 treatment.
538
539 *4.4. Conclusions*
540 Nitrification and denitrification contributed equally to the enhanced N_2O
541 emission from the simulated urine patches and the N_2O loss was caused by a

542 combination of higher rates and higher loss ratios of the processes. The study shows that
543 even though denitrification rates are low at soil water contents under 60% WFPS, the
544 process may account for a considerable part of the N_2O produced in urine patches
545 deposited on these soils. The nitrification rate was stimulated by the high NH_4^+
546 availability and possibly also by the increased soil pH following urea hydrolysis. In
547 itself a high nitrification rate does lead to raised N_2O losses, and furthermore, it enables
548 denitrification with associated N_2O losses. Based on the present study, an increased
549 nitrification rate therefore appears to be the most important factor explaining the high
550 initial N_2O emission from urine patches deposited on well-aerated soils. The study
551 delivers new information on the mechanisms responsible for the N_2O formation in urine
552 patches, and the results are suitable for incorporation into process-based modelling of
553 N_2O emissions from grazed grasslands.
554

555 **Acknowledgements**

556 This work was funded by the Danish Research Centre for Organic Farming
557 (DARCOF) and the Danish Agricultural and Veterinary Research Council. I wish to
558 thank Dr. Per Ambus for his qualified advice, Liselotte Meltofte for her assistance with
559 the ^{15}N diffusion as well as Dr. Sten Struwe, Dr. Finn P. Vinther and Jane Hansen for
560 their valuable comments on the manuscript.
561

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Table 1. Contents of the solutions prepared for the two experiments, *i.e.* the ^{15}N labelled compound and its amount, other contents (*viz.* unlabelled NH_4Cl or urine), final ^{15}N enrichment of NH_4^+ or NO_3^- and total N concentration. The solutions were applied on microplots and the N_2O emission was measured from all treatments. Additional purposes of the individual treatments are given in the table.

		Labelled compound		Other contents	Final ^{15}N enrichment	Total N concentration	This treatment specifically provided data on
		$^{15}NH_4Cl$ (99 atom%)	$K^{15}NO_3$ (99 atom%)				
Experiment	Treatment	mmol ^{15}N l^{-1}	mmol ^{15}N l^{-1}		atom%	g N l^{-1}	
1+2	Water only	0	0	None	–	0	Background ^{15}N enrichment of emitted N_2O, soil NH_4^+ and NO_3^- in microplots treated with water
1+2	Water/$^{15}NH_4^+$	0.37	0	None	99	0.005	The N_2O loss from nitrification in microplots treated with water
1+2	Water/$^{15}NO_3^-$	0	1.6	None	99	0.023	The N_2O loss from denitrification and the nitrification rate in microplots treated with water
1	Ammonium/$^{15}NH_4^+$	48	0	NH_4Cl (14.9 g N l^{-1})	4.6	15.6	The N_2O loss from nitrification in microplots treated with an ammonium solution
1	Ammonium/$^{15}NO_3^-$	0	1.9	NH_4Cl (15.6 g N l^{-1})	99	15.6	The N_2O loss from denitrification and the nitrification rate in microplots treated with an ammonium solution
2	Urine/$^{15}NH_4^+$	42	0	Artificial urine [a]	61	15.6	The N_2O loss from nitrification in microplots treated with artificial urine
2	Urine/$^{15}NO_3^-$	0	1.9	Artificial urine [a]	99	15.6	The N_2O loss from denitrification and the nitrification rate in microplots treated with artificial urine

[a] 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 (adjusted to make a total concentration of 1.4 g l^{-1}), $KHCO_3$ (22.9 g l^{-1}) and KCl (16.9 g l^{-1})

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

		5 h after application		31 h 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)

731

732 *Table 3.* Net N_2O emission and final ^{15}N enrichment of headspace N_2O for all

733 treatments measured in microplots; n = 1-4, means and SE (in brackets).

Experiment	Treatment	Net N_2O emission μg N_2O-N m^{-2} h^{-1}	^{15}N enrichment of headspace N_2O atom%
1	Water only	0 (0)	0.3694 (0.0008)
1	Water/$^{15}NH_4^+$	0 (ND)	0.5651 (0.0132)
1	Water/$^{15}NO_3^-$	1.8 (1.5)	0.9266 (0.2275)
1	Ammonium/$^{15}NH_4^+$	5.5 (1.8)	1.4005 (0.0943)
1	Ammonium/$^{15}NO_3^-$	6.5 (2.2)	3.4536 (0.6615)
2	Water only	3.6 (0.9)	0.3676 (0.0005)
2	Water/$^{15}NH_4^+$	3.3 (0.4)	0.5881 (0.0545)
2	Water/$^{15}NO_3^-$	5.0 (0.4)	2.2738 (0.6671)
2	Urine/$^{15}NH_4^+$	38.5 (4.8)	5.0307 (0.4215)
2	Urine/$^{15}NO_3^-$	46.1 (2.6)	34.0064 (0.9668)

734

735 The treatments are described in Table 1.

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

		5 h after application		31 h after application	
Experiment	Treatment	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NH_4^+$	$^{15}NO_3^-$
1	Water/$^{15}NH_4^+$	0.2631 (ND)	0.7915 (ND)	ND	ND
1	Ammonium/$^{15}NH_4^+$	4.6710 (0.0277)	0 (ND)	ND	ND
2	Water/$^{15}NH_4^+$	0.2908 (ND)	0.8711 (ND)	ND	ND
2	Urine/$^{15}NH_4^+$	5.3603 (0.1208)	0 (ND)	ND	ND
1	Water/$^{15}NO_3^-$	0.6058 (0.0337)	9.5903 (ND)	1.0128 (0.1005)	2.4571 (ND)
1	Ammonium/$^{15}NO_3^-$	0.0053 (0.0012)	2.7122 (ND)	0.0117 (0.0014)	3.2492 (ND)
2	Water/$^{15}NO_3^-$	0.7360 (ND)	3.8859 (ND)	0.5722 (ND)	1.1632 (ND)
2	Urine/$^{15}NO_3^-$	0.0336 (0.0022)	2.0624 (0.1477)	0.0414 (0.0048)	0.1059 (0.0537)

739

740 *Table 5.* Rates of gross nitrification and denitrification, moles of N_2O-N lost per moles
741 of NO_3^- produced via nitrification ($\underline{L_N}$) and moles of N_2O lost per moles of $N_2 + N_2O$
742 produced via denitrification ($\underline{L_D}$) in the 0-2 cm soil layer of microplots treated with
743 water, ammonium solution and urine; n = 4, means and SE (in brackets).

Experiment	Treatment	Gross nitrification mg NO_3-N m^{-2} h^{-1}	$\underline{L_N}$ %	Denitrification µg N m^{-2} h^{-1}	$\underline{L_D}$ %
1	Water	1.3 (0.1)	0.01	0.60 (0.37)	26
1	Ammonium	ND	ND	0 (ND)	ND
2	Water	0.8 (0.1)	0.02	62 (16)	0.9
2	Urine	6.9 (0.3)	0.29	412 (81)	5.1

744

745 **Figure captions**

746

747 *Figure 1.* Summed rainfall and irrigation (mm) as well as water-filled pore space in the
748 0-10 cm soil layer (%; n = 4, means ± SE) during the experimental period.

749

750 *Figure 2.* Soil content of dissolved organic carbon (DOC) and the NO_3^-/DOC ratio in
751 the 0-10 cm soil layer of microplots treated with water, ammonium solution and urine; n
752 = 4; means ± SE.

753

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

759

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

763

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