

1 **Lack of increased availability of root-derived C may explain the low**  
2 **N<sub>2</sub>O emission from low N-urine patches**

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20 **Abstract**

21 Urine deposition on grassland causes significant N<sub>2</sub>O losses, which in some cases may  
22 result from increased denitrification stimulated by labile compounds released from  
23 scorched plant roots. Two 12-day experiments were conducted in <sup>13</sup>C-labelled  
24 grassland monoliths to investigate the link between N<sub>2</sub>O production and carbon  
25 mineralization following application of low rates of urine-N. Measurements of N<sub>2</sub>O  
26 and CO<sub>2</sub> emissions from the monoliths as well as δ<sup>13</sup>C signal of evolved CO<sub>2</sub> were  
27 done on day -4, -1, 0, 1, 2, 4, 5, 6 and 7 after application of urine corresponding to 3.1  
28 and 5.5 g N m<sup>-2</sup> in the first and second experiment, respectively. The δ<sup>13</sup>C signal was  
29 also determined for soil organic matter, dissolved organic C and CO<sub>2</sub> evolved by  
30 microbial respiration. In addition, denitrifying enzyme activity (DEA) and nitrifying  
31 enzyme activity (NEA) were measured on day -1, 2 and 7 after the first urine  
32 application event. Urine did not affect DEA, whereas NEA was enhanced 2 days after  
33 urine application. In the first experiment, urine had no significant effect on the N<sub>2</sub>O  
34 flux, which was generally low (-8 to 14 μg N<sub>2</sub>O-N m<sup>-2</sup> h<sup>-1</sup>). After the second  
35 application event, the N<sub>2</sub>O emission increased significantly to 87 μg N<sub>2</sub>O-N m<sup>-2</sup> h<sup>-1</sup>  
36 and the N<sub>2</sub>O emission factor for the added urine-N was 0.18 %. However, the  
37 associated <sup>13</sup>C signal of soil respiration was unaffected by urine. Consequently, the  
38 increased N<sub>2</sub>O emission from the simulated low N-urine patches was not caused by  
39 enhanced denitrification stimulated by labile compounds released from scorched plant  
40 roots.

41

42 **Keywords**

43 <sup>13</sup>C, denitrification, grassland, nitrification, nitrous oxide, root scorching, soil  
44 respiration, urine

45

46 **Introduction**

47 Urine deposited by grazing livestock is a major source of the nitrous oxide (N<sub>2</sub>O)  
48 production in European grasslands. At present, N<sub>2</sub>O emissions from agricultural soils  
49 account for 5 % of the European release of anthropogenic derived greenhouse gases  
50 (EEA 2002), and the main processes involved in the production are nitrification and  
51 denitrification (Firestone and Davidson 1989). The mechanism responsible for the  
52 increased N<sub>2</sub>O emission following urine deposition is complex and not well  
53 understood. Vertès et al. (1997) found that 90 % of the urine patches deposited by  
54 grazing heifers contained between 3 and 50 g N m<sup>-2</sup>. Urea (NH<sub>2</sub>CONH<sub>2</sub>) is the  
55 predominant component of urine and typically accounts for over 70 % of the urine-N  
56 content (Oenema et al. 1997). In the soil, urea is rapidly hydrolysed to NH<sub>4</sub><sup>+</sup>, OH<sup>-</sup> and  
57 HCO<sub>3</sub><sup>-</sup>, which makes urea-N available for the nitrifying bacteria. However, recent  
58 studies revealed that urea-derived N only constituted a minor part of the N<sub>2</sub>O-N  
59 emitted during the days after urine application (Bol et al. 2004; Clough et al. 2004).  
60 The major part of N<sub>2</sub>O-N originated from other sources, e.g. from soil N. Nonetheless,  
61 studies have shown that the N<sub>2</sub>O emission increases almost linearly with the amount  
62 of urine-N deposited (Van Groeningen et al. 2005a, b). Thus, the amount of urine-N  
63 appears to have an indirect effect on the rate of N<sub>2</sub>O emission.

64 As heterotrophic bacteria play a major role in denitrification, the process is  
65 strongly dependent on the supply of easily decomposable organic matter, particularly  
66 in urine patches where N availability is expected to be non-limiting. Root scorching  
67 due to NH<sub>3(aq)</sub> formed after urea hydrolysis may result in release of labile carbon  
68 compounds into the rhizosphere (Shand et al. 2002). Monaghan and Barraclough  
69 (1993) suggested that these labile compounds stimulate denitrification activity and

70 thereby are part of the reason for the urine-induced N<sub>2</sub>O emission. However, the  
71 degree of scorching depends on the amount of NH<sub>3</sub> formed (Ritchey et al. 2003),  
72 which is influenced by the amount of urea-N applied, soil pH and the cation exchange  
73 capacity of the soil (Bolan et al. 2004). The low N<sub>2</sub>O emission from low N-urine  
74 patches may, in part, be caused by the lack of root scorching and thereby low  
75 availability of labile carbon compounds for the denitrifying bacteria.

76 In the present <sup>13</sup>C-labelling study, we examined the link between N<sub>2</sub>O  
77 emission and carbon mineralization following urine application to soil under <sup>13</sup>C  
78 depleted grassland vegetation (*i.e.* grassland monoliths provided with depleted  
79 atmospheric CO<sub>2</sub> during 2 or 8 weeks). The artificial urine applied simulated a urine  
80 patch with low N content (3.1 or 5.5 g N m<sup>-2</sup>). We tested the hypothesis that the low  
81 N<sub>2</sub>O emission from low N-urine patches is caused by the lack of root scorching, and  
82 thus, the lack of increased availability of root-derived C for the denitrifying bacteria.  
83 Because the plant material was <sup>13</sup>C depleted in the grassland monoliths studied, our  
84 hypothesis implies that the N<sub>2</sub>O emission should be paralleled by a constant δ<sup>13</sup>C  
85 signal of CO<sub>2</sub> evolved by soil respiration.

86

## 87 **Materials and methods**

88

### 89 *Grassland monoliths*

90 The study was conducted in grassland monoliths placed in a <sup>13</sup>C-labelling facility at  
91 Institut National de la Recherche Agronomique (INRA), Clermont-Ferrand, France.  
92 The former management practise and the experimental facility were described in  
93 detail by Klumpp (2004). Briefly, in June 2002 the monoliths (50 cm × 50 cm × 40  
94 cm deep) were taken from an intensively managed semi-natural grassland dominated

95 by perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.) and  
96 Yorkshire fog grass (*Holcus lanatus* L.). The slightly acidic sandy soil contained 4.1  
97 % C and 0.42 % N, and the  $\text{pH}_{\text{H}_2\text{O}}$  was 6.6. Monoliths were placed in temperature  
98 controlled transparent enclosures kept under natural daylight. The enclosures were  
99 part of an open flow  $^{13}\text{C}$ -labelling system, where ambient  $\text{CO}_2$  was scrubbed and  
100 replaced by fossil fuel derived  $\text{CO}_2$ , which is depleted in  $^{13}\text{C}$ . Starting 22 April 2003,  
101 plants were provided with  $\text{CO}_2$  having a  $\delta^{13}\text{C}$  signal of about -21.5 ‰. The external  
102 climate (PAR, temperature and humidity) and temperature of each enclosure was  
103 monitored continuously.

104

#### 105 *Urine treatment*

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

119 108 and 128 mm water during the first and the second 12-day experiment,  
120 respectively.

121

### 122 *Gas and soil sampling in monoliths*

123 About four weeks prior to urine application, small white chambers consisting of two  
124 3-cm diameter PVC pipes (80 ml) and three 5-cm diameter PVC pipes (112 ml) were  
125 installed in each monolith between the plants. Every pipe was connected to a three-  
126 way sampling valve on the outside of the enclosure via a 75 cm silicon tube. The  
127 sampling valve was fitted with a 5 ml syringe and a needle. Starting four days before  
128 urine application (Day -4), below-ground production of N<sub>2</sub>O and CO<sub>2</sub> as well as the  
129  $\delta^{13}\text{C}$  of the evolved CO<sub>2</sub> were determined by use of the static chamber method.

130 Measurement of CO<sub>2</sub> emission was done between 11 am and 1 pm by briefly lifting  
131 the enclosures to seal the 3-cm pipes with rubber stoppers for 40 minutes. One 2-ml  
132 gas sample was collected via the external valves after 0, 20 and 40 minutes of  
133 incubation. To measure N<sub>2</sub>O emission, the 5-cm pipes were sealed by rubber stoppers  
134 for 90 minutes between 1:30 and 4:30 pm. Two 2-ml gas samples were taken at the  
135 beginning and at the end of the incubation period. A volume of N<sub>2</sub> equal to the sample  
136 volume was added to the pipe before each gas sampling to maintain atmospheric  
137 pressure. All samples were stored in 2-ml crimp-seal vials, which had been evacuated  
138 before use. After penetration by a needle the vials were sealed with Terostat IX to  
139 allow long-term storage. Determination of gas emissions from urine-treated and  
140 control monoliths was done on day -4, -1, 0, 1, 2, 4, 5, 6 and 7 after urine application.  
141 Furthermore,  $\delta^{13}\text{C}$  of the evolved CO<sub>2</sub> was measured once before the labelling started.

142 Two days after urine application, two soil cores (0-10 cm depth, 2 cm diameter)  
143 were collected in each monolith for chemical analyses and determination of microbial

144 respiration. Sampling holes were closed with cement filled PVC tubes to prevent  
145 aeration of the soil and drainage of water. The two soil samples from each monolith  
146 were pooled. Roots and stubbles were removed by tweezers during a period of one  
147 hour per sample.

148

#### 149 *Destructive harvest of monoliths*

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

158

#### 159 *Soil analyses*

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

169 To establish the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  evolved by microbial respiration, 10 g portions of  
170 fresh 'root free' soil were incubated for 24 h at 25 °C in 250 ml screw capped serum  
171 bottles mounted with rubber stoppers. Empty bottles were included as controls. Gas  
172 samples for determination of  $\text{CO}_2$  concentration and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  were taken after 0  
173 (ambient), 1, 3, 10 and 24 hours of incubation and stored in 2-ml vials. A volume of  
174  $\text{N}_2$  equal to the sample volume was added to the bottle before each gas sampling.

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

179

#### 180 *Gas analyses*

181 To measure  $\text{N}_2\text{O}$  concentrations, the vials were pressurized by adding 2 ml  $\text{N}_2$  before  
182 analysis by gas chromatography (GC-14B, Shimadzu, Kyoto, JP). The samples for  
183  $\text{CO}_2$  determination were added 0.5 ml  $\text{N}_2$  and the concentrations were established by  
184 gas chromatography (HP 6890, Agilent, Palo Alto, US). The  $\delta^{13}\text{C}$  of  $\text{CO}_2$  was  
185 determined following condensation in two successive cool traps (liquid  $\text{N}_2$ ) and  
186 chromatographically separation of  $\text{CO}_2$  on a trace gas preparation-concentration unit  
187 (PreCon FinniganMAT, Bremen, DE) coupled in continuous flow mode to the IRMS.  
188 Gas samples were analysed for  $\text{CO}_2$ ,  $\text{N}_2\text{O}$  and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  within 26, 36 and 61 days  
189 of sampling, respectively.

190

#### 191 *Denitrifying and nitrifying enzyme activities*

192 To determine denitrifying and nitrifying enzyme activities, two soil cores (0-10 cm  
193 depth, 2 cm diameter) were collected in each monolith on day -1, 2 and 7 after the

194 first urine application. The two soil samples from each monolith were pooled and the  
195 soil was sieved (2 mm).

196 Denitrifying enzyme activity (DEA) was measured on the fresh soil samples  
197 over a short period according to Smith and Tiedje (1979) (for details, see Patra et al.  
198 2005). Briefly, 10 g (equivalent oven-dried) soil was placed into 150 ml flasks, and  
199  $\text{KNO}_3$  (200  $\mu\text{g NO}_3\text{-N g}^{-1}$  dry soil), glucose (0.5 mg C  $\text{g}^{-1}$  dry soil) and glutamic acid  
200 (0.5 mg C  $\text{g}^{-1}$  dry soil) was added. The atmosphere of each flask was evacuated and  
201 replaced by a 90:10 He- $\text{C}_2\text{H}_2$  mixture. During incubation at 26 °C, gas samples (200  
202  $\mu\text{l}$ ) were taken after 4 and 6 hours and immediately analysed for  $\text{N}_2\text{O}$  by  
203 chromatography (Varian, STAR 3400 CX, Walnut Creek, US).

204 Nitrifying enzyme activity (NEA) was measured according to Lensi et al.  
205 (1986). For each fresh soil sample, two sub samples (equivalent to 10 g oven-  
206 dried) were placed in 150 ml flasks. One sub sample was used to estimate the  
207 initial soil  $\text{NO}_3^-$  content. This sub sample was supplied with 6 ml of a suspension  
208 containing a denitrifying organism (*Pseudomonas fluorescens*, O.D. 580 nm = 2)  
209 in a solution of glucose and glutamic acid (final soil C concentration for each: 0.5  
210 mg C  $\text{g}^{-1}$  dry soil). The atmosphere in the flask was replaced by a He- $\text{C}_2\text{H}_2$  mixture  
211 (90-10) and  $\text{N}_2\text{O}$  accumulation was measured until soil  $\text{NO}_3^-$  was converted fully  
212 to  $\text{N}_2\text{O}$ . The other sub sample was used to determine potential  $\text{NO}_3^-$  accumulation.  
213 In this case, 4 ml of a  $(\text{NH}_4)_2\text{SO}_4$  solution was added (final concentration 200  $\mu\text{g}$   
214 N  $\text{g}^{-1}$  dry soil). Water was added to achieve 70 % water holding capacity. After  
215 aerobic incubation (7 h at 26 °C), which allows nitrate to accumulate, the soil  
216 samples were enriched with *Pseudomonas fluorescens* and incubated as described  
217 above. Nitrous oxide was analysed on a Varian STAR 3400 gas chromatograph.

218

219 *Calculations and statistics*

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

223 
$$\delta^{13}\text{C} (\text{‰}) = 1000 \times (R_{\text{sample}} / R_{\text{standard}} - 1),$$

224 where  $R = ^{13}\text{C}/^{12}\text{C}$ . Internal standards were used to check and correct for changes in  
225  $\text{N}_2\text{O}$ ,  $\text{CO}_2$  and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  between time of sampling and analysis. The emission of  
226  $\text{N}_2\text{O}$  and  $\text{CO}_2$  were calculated using linear regression and the  $\delta^{13}\text{C}$  of the evolved  $\text{CO}_2$   
227 was established by Keeling plots (Keeling 1958). To simplify,  $\delta^{13}\text{C}$  of  $\text{CO}_2$  from soil  
228 respiration and microbial respiration are referred to as  $\delta^{13}\text{C}$  of soil respiration and  
229 microbial respiration, respectively.

230 In general, the mean of the results obtained in each monolith was used, which  
231 gives 3 replicates. Some data are reported as the overall mean  $\pm$  standard error.  
232 Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Tukey's  
233 multiple comparison tests ( $\alpha = 0.05$ ) were performed using SAS General Linear  
234 Model procedure (SAS Institute 1997). Furthermore, ANCOVAs were performed  
235 with SAS Mixed Model procedure on the repeated measurements of  $\text{N}_2\text{O}$ ,  $\text{CO}_2$  and  
236  $\delta^{13}\text{C}$  of  $\text{CO}_2$  using means of the measurements on day -4 and -1 before urine  
237 application as covariate ( $\delta^{13}\text{C}$  of  $\text{CO}_2$  after the first application, covariate not  
238 included). The ANCOVAs for  $\text{CO}_2$  emission after the first application and  $\text{N}_2\text{O}$   
239 emission after the second application were performed on log transformed data.

240

241 **Results**

242

243 *Irrigation and temperature*

244 The distributions of the 108 and 128 mm water given in the first and the second  
245 experiment, respectively, appear from Figure 1. Air temperature in the enclosures  
246 during gas measurement ranged between 15 and 27 °C in the first experiment (data not  
247 shown). In the second experiment, the temperature was on average 12 °C higher ( $P <$   
248 0.0001) and varied between 26 and 40 °C. Air temperature did not differ between time  
249 of CO<sub>2</sub> and N<sub>2</sub>O measurements ( $P = 0.14$ ).

250

251 *N<sub>2</sub>O emission*

252 Homogeneity of variance was not obtained despite transformation when testing the  
253 emission of N<sub>2</sub>O after the first urine application. Thus, no statistical analysis was  
254 performed on the N<sub>2</sub>O data from the first experiment. Application of urine appeared to  
255 have no significant effect on the N<sub>2</sub>O flux from the grassland monoliths in the first  
256 experiment (3.1 g N m<sup>-2</sup>; Fig. 2 A). Overall the flux of N<sub>2</sub>O was very low during the  
257 first experiment, varying between -8 and 14 µg N<sub>2</sub>O-N m<sup>-2</sup> h<sup>-1</sup>.

258 In contrast, urine application equivalent to 5.5 g N m<sup>-2</sup> in the second experiment  
259 had a significant effect on the N<sub>2</sub>O emission ( $P = 0.047$ ), which increased to  $87 \pm 57$   
260 µg N<sub>2</sub>O-N m<sup>-2</sup> h<sup>-1</sup> (Fig. 2 B). The emission remained elevated for at least 8 days, but  
261 declined gradually with time ( $P = 0.049$ ).

262

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

264 Urine application had a significant effect on the amount of CO<sub>2</sub> emitted from the  
265 grassland monoliths in the two experiments ( $P \leq 0.040$ ; Fig. 3). A peak in the CO<sub>2</sub>

266 emission took place on the day of application, which was probably mainly caused by  
267 the hydrolysis of urea, resulting in formation of  $\text{HCO}_3^-$ .

268 Mean  $\delta^{13}\text{C}$  of soil respiration determined in the grassland monoliths during the  
269 study was  $-28.5\text{‰}$ . No decline in  $\delta^{13}\text{C}$  of soil respiration was observed following  
270 urine application (Fig. 4) and urine had no significant effect on the  $^{13}\text{C}$  signal ( $P \geq$   
271  $0.16$ ). The  $\text{CO}_2$  peak on the day of urine application that partly derived from  
272 hydrolysis of urea ( $\delta^{13}\text{C} -34\text{‰}$ ) did only affect the  $\delta^{13}\text{C}$  of soil respiration in the first  
273 experiment, where  $\delta^{13}\text{C}$  of  $\text{CO}_2$  from the urine treatment dropped significantly below  
274 that of the control on the day of application (Fig. 4 A). The  $^{13}\text{C}$  signal of soil  
275 respiration increased following days with high irrigation, viz. day -1 and 5 in the first  
276 experiment (Fig. 1, 4 A). On day 5 after the first application event, the  $\text{CO}_2$  emission  
277 increased as well (Fig. 3 A).

278 The rate of microbial respiration measured on 'root free' soil samples in the  
279 laboratory did not change over the course of the study or between treatments ( $P \geq$   
280  $0.79$ ) and the mean rate was  $3.4 \pm 0.2 \mu\text{g CO}_2\text{-C g}^{-1} \text{ dry soil h}^{-1}$  (data not shown).  
281 Delta  $^{13}\text{C}$  of microbial respiration established on the 'root free' soil samples was  
282 stable during the study ( $P = 0.38$ ; Fig. 5) and the urine treatment had no significant  
283 effect on the  $^{13}\text{C}$  signal ( $P = 0.72$ ).

284

#### 285 *Delta $^{13}\text{C}$ of other C pools*

286 Delta  $\delta^{13}\text{C}$  of plant shoot and root measured just before the second experiment  
287 revealed that the vegetation had been significantly labelled ( $P < 0.0001$ ; Fig. 5).  
288 However, the shoot material was more depleted than the roots. Delta  $^{13}\text{C}$  of DOC  
289 tended to decrease over the course of the study ( $P = 0.078$ ; Fig. 5). Furthermore,  $\delta^{13}\text{C}$   
290 of soil organic matter (SOM) declined significantly during the period from the start of

291 labelling to the first experiment ( $P = 0.05$ ). The urine treatment had no effect on  $\delta^{13}\text{C}$   
292 of DOC and SOM ( $P \geq 0.22$ ). In general,  $\delta^{13}\text{C}$  of DOC differed from  $\delta^{13}\text{C}$  of soil  
293 respiration measured at the start of labelling and on day 2 after urine application ( $P =$   
294  $0.05$ ), whereas  $\delta^{13}\text{C}$  of SOM and microbial respiration was rather similar ( $P > 0.05$ ).

295

#### 296 *Denitrifying and nitrifying enzyme activities*

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

305

#### 306 *Inorganic N, DOC and soil pH*

307 The content of soil inorganic N in the 0-10 cm soil layer measured on day 2 after  
308 urine application did not differ between the two experiments ( $P = 0.76$ ) and was  
309 significantly higher in the urine treatment ( $1.46 \text{ g N m}^{-2}$ ) than in the control ( $0.49 \text{ g N}$   
310  $\text{m}^{-2}$ ;  $P = 0.042$ ) (data not shown). The increased level of inorganic N in the urine  
311 treatment was almost exclusively caused by a rise in the  $\text{NH}_4^+$  content. The  $\text{NO}_3^-$   
312 content was below the detection limit in the first experiment and had a mean value of  
313  $0.05 \text{ g N m}^{-2}$  (0-10 cm soil layer) in the second experiment.

314 The content of DOC in the upper 0-10 cm of the soil was similar in the urine  
315 treatment and the control ( $17.8 \text{ g C m}^{-2}$ ;  $P = 0.88$ ). Despite urea hydrolysis, no pH

316 increase was observed in the urine treated soil when measured on day 2 after  
317 application ( $P = 0.23$ ; data not shown).

318

## 319 **Discussion**

320

### 321 *Increased N<sub>2</sub>O emission after urine application*

322 According to the peaks in CO<sub>2</sub> emission (Fig. 3), urea hydrolysis was completed  
323 within 24 hours, which is in line with results obtained in other studies (Petersen et al.  
324 1998; Bol et al. 2004). In the first experiment in May, urine application corresponding  
325 to 3.1 g N m<sup>-2</sup> did not lead to a significant increase of the N<sub>2</sub>O emission (Fig. 2 A). In  
326 contrast, the N<sub>2</sub>O emission increased significantly following urine application  
327 equivalent to 5.5 g N m<sup>-2</sup> in the second experiment in June (Fig. 2 B).

328 A possible reason for the larger N<sub>2</sub>O emission in the second experiment  
329 compared to the first could be the temperature, which was about 12 °C higher in the  
330 second experiment (mean 34 °C). Christensen (1983) found that the Q<sub>10</sub> value for N<sub>2</sub>O  
331 production in soil was 2-3. The emission on the day of urine application was 6-fold  
332 higher in the second experiment than it was in the first, which suggests that the  
333 difference in N<sub>2</sub>O production between the two experiments was too big to be  
334 accounted for by a temperature effect only. The different responses at the two  
335 application events may be explained partly by the larger amount of N added in the  
336 second experiment (5.5 vs. 3.1 g N m<sup>-2</sup>). Furthermore, nitrifying bacteria may  
337 compete with plants for NH<sub>4</sub><sup>+</sup> (Verhagen et al. 1995; Kaye and Hart 1997). Compared  
338 to plant growth in May, the growth rate was reduced during the second experiment in  
339 June. Thus, probably the plants left more inorganic N for the nitrifying and

340 denitrifying bacteria in the second experiment, which enabled increased N<sub>2</sub>O  
341 production.

342 Maximum N<sub>2</sub>O emission measured was  $87 \pm 57 \mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$ . In  
343 comparison, Williamson and Jarvis (1997) measured emission of  $600 \mu\text{g N}_2\text{O-N m}^{-2}$   
344  $\text{h}^{-1}$  after application of similar amounts of urine-N ( $6 \text{ g N m}^{-2}$ ) to a grassland on poorly  
345 drained silty clay loam in November. However, the sandy soil and moderate soil  
346 moisture in the present study offered less favourable conditions for N<sub>2</sub>O production,  
347 which largely explains the lower emission. More generally, relative N<sub>2</sub>O emission at  
348 peak emission date (*i.e.* N<sub>2</sub>O emission expressed per unit of applied urine-N)  
349 computed from published data (Allen et al. 1996; Clough et al. 1996; Yamulki et al.  
350 1998; Bol et al. 2004; Van Groenigen et al. 2005b) varied between 2 and  $123 \mu\text{g N}_2\text{O-}$   
351  $\text{N h}^{-1} \text{ g}^{-1}$  urine-N. The median of these observations ( $n = 15$ ) is  $17 \mu\text{g N}_2\text{O-N h}^{-1} \text{ g}^{-1}$   
352 urine-N, which is close to the relative N<sub>2</sub>O emission of  $16 \mu\text{g N}_2\text{O-N h}^{-1} \text{ g}^{-1}$  urine-N  
353 observed in our study.

354 Assuming an N loss of 20 % due to NH<sub>3</sub> volatilization and nitric oxide (NO)  
355 emission (IPCC 1997), the N<sub>2</sub>O emission factor for the added urine-N measured over  
356 the 8 days was  $0.18 \pm 0.08 \%$ . In the study by Williamson and Jarvis (1997), where a  
357 similar amount of urine-N was applied, the N<sub>2</sub>O emission factor measured over 37  
358 days was 5 % (De Klein et al. 2001). According to the guidelines issued by the  
359 Intergovernmental Panel on Climate Change (IPCC 1997), the N<sub>2</sub>O emission from  
360 urine deposited by grazing livestock should be calculated as 2 % of the N remaining  
361 after NH<sub>3</sub> volatilization and NO emission, which are assumed to account for 20 % of  
362 the total N content. However, a review of 10 field studies showed that median N<sub>2</sub>O  
363 emission factor of real urine was 0.9 % (Van Groenigen et al. 2005a), and the present  
364 study supports a reduction of the IPCC default emission factor as well.

365

366 *Assessment of the possible link between N<sub>2</sub>O production and availability of root-*  
367 *derived C in urine patches*

368 Urine deposition by grazing livestock is known to trigger significant N<sub>2</sub>O production,  
369 but the mechanisms involved are very complex and not well understood. It has been  
370 suggested that labile compounds released from scorched plant roots stimulate  
371 denitrification activity, and thus is part of the reason for the increased N<sub>2</sub>O emission  
372 following urine deposition (Monaghan and Barraclough 1993). We propose that the  
373 small increase in N<sub>2</sub>O emission from low N-urine patches partly is caused by the lack  
374 of root scorching and associated release of labile carbon compounds. The aim of the  
375 present study was to test this hypothesis by assessing the source of CO<sub>2</sub> emitted  
376 following application of a low rate of urine-N. If the increased N<sub>2</sub>O emission were a  
377 result of higher denitrifying activity due to a supply of labile compounds released  
378 from scorched plant roots (more depleted than other soil C pools in the monoliths  
379 studied), then  $\delta^{13}\text{C}$  of soil respiration would be expected to decline after urine  
380 application. In line with the hypothesis, the increased N<sub>2</sub>O emission in the second  
381 experiment was not related to increased mineralization of plant-derived C, *viz.*  $\delta^{13}\text{C}$  of  
382 soil respiration was unaffected by the urine application (Fig. 4 B). The plant material  
383 may have been inadequately <sup>13</sup>C-labelled in order to trace plant-derived C in other C  
384 pools. However, the result may indicate that no significant root scorching occurred  
385 following urea hydrolysis. Lack of urine-effect on other soil C measures (*i.e.*  $\delta^{13}\text{C}$  of  
386 microbial respiration, soil content and  $\delta^{13}\text{C}$  of DOC) supported that root scorching  
387 was probably negligible. The urine compounds remaining after urea hydrolysis  
388 (hippuric acid, creatinine and allantoin;  $\delta^{13}\text{C}$  -26 ‰) did not affect the results on  $\delta^{13}\text{C}$

389 of DOC because of their low amount ( $< 3 \text{ g C m}^{-2}$  vs.  $17.8 \text{ g DOC-C m}^{-2}$  in the 0-10  
390 cm soil layer).

391 More generally, the urine-induced rise in  $\text{N}_2\text{O}$  emission was not linked to an  
392 increase of soil respiration. The same result appears from a study by Bol et al. (2004),  
393 where urine was applied corresponding to 23 or  $40 \text{ g urea-N m}^{-2}$ . In contrast, Lovell  
394 and Jarvis (1996) found that soil respiration increased significantly following  
395 application of urine equivalent to about  $20 \text{ g N m}^{-2}$ .

396 Our results show that application of  $5.5 \text{ g urine-N m}^{-2}$  gave rise to a  $\text{NH}_{3(\text{aq})}$   
397 concentration in the soil solution that did not cause significant scorching of the roots  
398 and, thus, that root scorching could not be responsible for the urine-induced  $\text{N}_2\text{O}$   
399 emission from the simulated low N-urine patch. In contrast, a related study  
400 demonstrated that application of a high rate of urine-N ( $50.9 \text{ g N m}^{-2}$ ) significantly  
401 increased the mineralization of plant-derived C, possibly as a result of root damage  
402 due to scorching (P. Ambus, personal communication).

403

#### 404 *Alternative processes explaining the urine-induced $\text{N}_2\text{O}$ emission*

405 The concentration of N in livestock urine may vary between 1 and  $20 \text{ g N l}^{-1}$  (Oenema  
406 et al. 1997), thus the concentration used in the present study ( $0.7 \text{ g N l}^{-1}$ ) was in the  
407 lower end of this range. A nitrogen concentration of urine above  $16 \text{ g N l}^{-1}$  leads to  
408 microbial stress due to  $\text{NH}_{3(\text{aq})}$  and low osmotic potential, and thereby to inhibition of  
409 nitrification (Monaghan and Barraclough 1992; Bol et al. 2004). The low urine-N  
410 concentration in the present study means that nitrification most likely occurred, and  
411 the process might play a major role in the increase in  $\text{N}_2\text{O}$  emission. This view is  
412 supported by the apparent increase of NEA in the urine treated soil during the first  
413 experiment (Fig. 6) and the presence of soil  $\text{NO}_3^-$  on day 2 after the second

414 application event. Hence, the elevated N<sub>2</sub>O emission immediately following urine  
415 application was probably caused by a rapid nitrification-denitrification turnover of  
416 urea-derived N. This mechanism is different from that following application of higher  
417 rates of urine-N, where nitrification is typically inhibited for a couple of days  
418 (Monaghan and Barraclough 1992; Bol et al. 2004).

419 In conclusion, the increased N<sub>2</sub>O emission following urine application at rates  
420 up to 5.5 g N m<sup>-2</sup> was not caused by enhanced denitrification stimulated by an  
421 increased availability of labile plant compounds. Furthermore, strong competition for  
422 inorganic N between plants and microorganisms combined with low urine-N rates  
423 limited the N<sub>2</sub>O loss from this semi-natural grassland.

424

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435

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

523

524 *Figure 2.* Emission of N<sub>2</sub>O from the urine treatment and the control during (A) the  
525 first experiment and (B) the second experiment; n = 3; means ± SE.

526

527 *Figure 3.* Emission of CO<sub>2</sub> from the urine treatment and the control during (A) the  
528 first experiment and (B) the second experiment; n = 3; means; the bars indicate the  
529 Minimum Significant Difference.

530

531 *Figure 4.* Delta <sup>13</sup>C of CO<sub>2</sub> evolved by soil respiration in the urine treatment and the  
532 control during (A) the first experiment and (B) the second experiment; n = 3; means ±  
533 SE. <sup>a</sup>Urine, n = 1; control, n = 2. <sup>b</sup>One outlying sub measurement was not included.

534

535 *Figure 5.* Delta <sup>13</sup>C of dissolved organic C (DOC), soil organic matter (SOM) and  
536 CO<sub>2</sub> evolved by microbial respiration (MR) and soil respiration (SR) determined at  
537 the start of labelling and on day 2 of the first and second experiment, as well as δ<sup>13</sup>C  
538 of root and shoot determined at the start of labelling and on day -6 of the second  
539 experiment; n = 4-6; means ± SE.

540

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











