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INHIBITOR EFFECT OF HIPPURIC ACID ON SOIL N_2O EMISSIONS.

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SOIL NITROUS OXIDE EMISSIONS FROM GRASSLAND: POTENTIAL INHIBITOR EFFECT OF HIPPURIC ACID.

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Abstract

In grassland systems, cattle and sheep urine patches are recognized as nitrous oxide (N_2O) emission hot spots due to the high urinary nitrogen (N) concentrations. Hippuric acid (HA) is one of the constituents of ruminant urine that has been reported as a natural inhibitor of soil N_2O emissions. The aim of this study was to examine the potential for elevated ruminant urine HA concentrations to reduce N_2O emissions, in situ, on an acidic heavy clay soil under poorly drained conditions (WFPS > 85%). A randomized complete block design experiment with three replications and four treatments was conducted using the closed-static-flux chamber methodology. The four treatments were applied inside the chambers: control with no artificial urine application (C), control artificial urine (U), and enriched artificial urine containing two rates of HA (55.8 and 90 mM, U+HA1, U+HA2). Soil inorganic-N, soil dissolved organic carbon (DOC), soil pH as well as N_2O and methane (CH_4) fluxes were monitored over a 79-day period. Although N_2O emissions were not affected by the HA enriched urine treatments, U+HA2 positively affected the retention of N as NH_4^+ until day 3, when the soil pH dropped to values <5. Subsequently, as a consequence of rainfall events and soil acidification, it is likely that leaching or sorption onto clay reduced the efficacy of HA, masking any treatment differential effect on N_2O emissions. Moreover, CH_4 fluxes as well as DOC results reflected the soil anaerobic conditions which did not favour nitrification processes. Further research is needed to determine the fate of HA into the soil which might clarify the lack of an *in situ* effect of this compound.

1 Introduction

Up to 9% of the United Kingdom's greenhouse gas (GHG) emissions result from agriculture, with 55% of these GHG emissions in the form of nitrous oxide (N_2O) (*DEFR*A, 2011). In grassland systems, cattle and sheep urine patches are recognized N_2O emission hot spots due to the high urinary nitrogen (N) concentrations that may range from 3 to 20.5 g N L^{-1} urine (*Spek* et al., 2012; *Bristow* et al., 1992). In England and Wales, over 42% of the agricultural land area, is under permanent grassland (SEISMIC1 v.2.0.6. software 2000 dataset). Within this agricultural grassland, approximately 50% occurs on poorly drained soils with a shallow impermeable substrate where high levels of rainfall can lead to seasonal water logging when drainage systems have not been installed (*Granger* et al., 2010). This greatly reduces the soil aerobic status and favours the occurrence of anaerobic processes. Except for winter time, when cattle are usually removed from the land, such agricultural grasslands are permanently loaded during spring, summer and autumn with urine-N from ruminant depositions. Soil inorganic N, derived from ruminant urine, is prone to being lost as N_2O or N_2 via nitrifier-denitrification, denitrification, or codenitrification processes since increasing water-filled pore space (WFPS) enhances anaerobic conditions (*Linn* and *Doran*, 1984; *Balaine* et al. 2013; *Selbie* et al. 2015).

Studies performed under grazing conditions in soils of varying texture, and under varying WFPS, report N_2O emissions ranging from 0.02 to 2.33 % of ruminant urine-N applied (*Krol* et al., 2015; *Baral* et al., 2014; *Boon* et al., 2014; *Misselbrook* et al., 2014; *Zaman* et al., 2012; *Klein* et al., 2011; *de Kelly* et al., 2008; *Luo* et al., 2008; *Wachendorf* et al., 2008). This variability in N_2O emissions may be a consequence of variation in ruminant urine composition, which is controlled by the animal's diet (*Martin*, 1970 a, b; *Kreula* et al., 1978; *Van Vuuren* and *Simits*., 1997). In this sense, some of the constituents in the ruminant urine have been reported to affect subsequent soil N_2O emissions (*Van Groenigen* et al., 2005a, b; *Van Groenigen* et al., 2006; *Kool* et al., 2006). This is the case of hippuric acid (HA), a constituent naturally present in ruminant urine at concentrations between 0.37 and 0.70 g N L^{-1} (*Dijkstra* et al., 2013) depending on animal diet (*Kreula* et al., 1978). *In vitro*, HA has been

78 shown to mitigate N_2O emissions from soil (*Van Groenigen* et al., 2006; *Kool* et al., 2006; *Bertram* et
79 al. 2009) presumably due to the presence of benzoic acid (BA), a break-down product (*Bristow* et al.,
80 1992) which, along with its demonstrable antimicrobial activity in acidic mediums (*Marwan* and
81 *Nagel*, 1986), is known as a denitrification inhibitor (*Her* and *Huang*, 1995). Benzoic acid may be
82 adsorbed onto soil particles via van der Waal or hydrogen bonding and subsequently released as a
83 consequence of decreasing soil solution strength or as a result of competing ions (*Dalton*, 1999).
84 *Inderjit* and *Bhowmik* (2004) found that sorption of the BA onto soil particles is affected by clay
85 content, soil organic matter, pH, and the concentration of BA itself.
86 Hippuric acid has been reported to reduce soil N_2O emissions due to its inhibitory effect on both
87 nitrification and denitrification processes (*Bertram* et al., 2009). In addition, the concentration of HA
88 in urine has been reported to have a controlling effect on both the hydrolysis of urine-N and on NH_3
89 volatilization. Thus, HA may further affect N_2O emissions by altering substrate supply for microbial
90 mechanisms of N_2O production (*Van Groenigen*, et al., 2005).
91 Field studies carried out *in situ* on silt loam soils with WFPS ranging from 18% to 51% reported no
92 effect on N_2O emissions with increasing urine HA concentration (*Clough* et al., 2009). Similarly, *Krol*
93 et al., (2015) found no effect *in situ*, on a loam soil where WFPS ranged from 60% to 80%. By
94 contrast, the inhibitory effect of HA under anaerobic conditions (WFPS 92%) has been proven under
95 laboratory conditions (*Kool* et al., 2006). However, there are no reports on the *in situ* effects of
96 urinary HA concentration on N_2O emissions for heavy clay soils, with high values of WFPS (>85%), as
97 commonly found in grazed perennial pastures from the southwest of England.
98 The aim of this study was to examine the potential for elevated ruminant urine HA concentrations to
99 reduce *in situ* N_2O emissions on an acidic heavy clay soil under poorly drained soil conditions (WFPS
100 > 85%). Based on previous *in situ* studies (*Kool* et al., 2006; *Clough* et al., 2009; *Krol* et al., 2015) we
101 hypothesized that an increase in ruminant urine HA content could inhibit N_2O emissions when urine
102 was applied to acidic soils with a high clay content, due to the potential retention of HA by the clay

in the soil and due to the favourable pH conditions (<5.2) making viable the antimicrobial activity of benzoic acid (*Chipley*, 1983).

2 Materials and methods

2.1 Site location

The field trial was carried out in 2015 on a permanent grassland, dominated by ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.), from September 29th to December 16th at Rothamsted Research, North Wyke, Devon, UK (50:46:10N, 3:54:05W). The climate is a temperate maritime climate (*Koppen*, 1931), typical of South-West England. The soil used for the experiment is defined by the British soil classification (*Avery*, 1980) as a clayey typical non-calcareous pelosol of the Halstow series and as either a stagnivertic cambisol, or as an aeric haplaquept by the FAO and USDA taxonomic classification systems, respectively. The soil has a brownish clay loam A horizon while the B horizon is clayey with marked gleying confined below 40 cm (*Harrod* and *Hogan*, 2008). It is characterized, with an unusually low cation exchange capacity (C.E.C.) relative to clay content, which is partly an expression of the micaceous nature of its clay minerals and partly of the relatively coarse size and therefore small surface area of the clay (*Harrod* and *Hogan*, 2008).

This soil is water-logged for considerable periods of the year. The impermeable nature is confirmed by the low fraction of drainable pores and it has very slow hydraulic conductivity (*Harrod* and *Hogan*, 2008).

Initial analysis of the upper 10 cm of the soil profile are presented in Table 1. Meteorological data, consisting of air temperature and precipitation, was collected from a station located 500 m away from the field site.

2.2 Experimental and chamber design

A randomized complete block design experiment was set up with three replicate plots per each of four treatments. Blocks were 3 m apart and replicate plots were 5.6 m^2 (2 m x 2.8 m) with a 1 m

127 separation as buffer. Five chambers were installed within each replicate plot (i.e., 60 chambers in
128 total) and an area of 1 m^2 (1 m x 1 m) was delineated next to each replicate plot for soil sampling.

129 The closed static chamber technique was used (*Rochette* and *Erisksen-Hamel*, 2008) for determining
130 soil gas fluxes. Each chamber comprised a white polyvinyl chloride (PVC) open ended box with a
131 volume of 0.032 m^3 (length 0.4 m, width 0.4 m, height 0.25 m; *Cardenas* et al., 2010) and a lid. In
132 order to ensure a good seal between the chamber and soil, the boxes were inserted into the soil to a
133 depth of 0.1 m more than 24 h before the flux measurements began (*Parkin* and *Venterea*, 2010).
134 The effective height of each chamber was recorded internally at the centre of each wall and in the
135 centre of the chamber to use in the calculation of the fluxes. The resultant chamber effective height
136 was the weighted mean of the 5 points taken (including two times the centre height), and ranged
137 between 0.09 and 0.18 m. The lid was fitted with a sampling port with a three-way valve and placed
138 on top of the box at the beginning of each gas sampling day.

139 *2.3 Treatments*

140 On September 30th, four treatments were applied inside the chambers and in the 1-m^2-plot
141 delineated for soil sampling. Treatments consisted on: control with no artificial urine application (C),
142 control artificial urine containing HA 37 mM (U), enriched artificial urine containing HA 55.8 mM
143 (U+HA1), and enriched artificial urine containing HA 90 mM (U+HA2). The respective N application
144 rates for the C, U, U+HA1, and U+HA2 were 0, 516, 528, and 552 kg N ha^{-1}. Treatments were
145 prepared the day before the application using the recipe described by *Doak* (1952) (Table 1), and
146 stored at 4°C overnight. HA concentrations were defined based on previous published studies (Table
147 2). Urine was applied using a watering can at a rate of 5 L m^{-2} and when applied its average
148 temperature was 16.4 °C.

149 *2.4 Greenhouse gas measurements*

Greenhouse gases, including N_2O and methane (CH_4), were monitored one day before treatment application and on 22 occasions after treatment application over a 79-day period. Gas samples were taken between 11:00 a.m and 2:00 p.m on each sampling day, four times a week for the first two weeks, twice weekly for the next five weeks, and weekly thereafter (*Misselbrook* et al., 2014). Sampling was conducted according to *Chadwick* et al. (2014). Atmospheric samples were collected at the start (T0) and at the end (three at each time) of the sampling run to provide background ambient values. Chamber lids were placed on the chambers sequentially across the paddocks and after 40 min a gas sample was collected from each closed chamber (T40) via a sampling port fixed in the lid using a plastic 50 mL syringe fitted with a 3-way luer-lok tap. The sample was then transferred to a pre-evacuated (-1 atm.) *22* mL vial, using a hypodermic needle, that had a chloro-butyl rubber septum (Chromacol). Samples were analysed within two days by gas chromatography on a Perkin Elmer Clarus 500 GC and TurboMatrix 110 auto headspace sampler equipped with an electron capture detector (ECD) and a flame ionization detector (FID). The separation column employed was a Perkin Elmer EliteQ PLOT megabore capillary (30 m long, 0.53 mm i.d.), operated at 35 °C. The ECD detector was set at 300°C and the carrier gas was N_2. Gas fluxes were calculated based on the linear increase in the gas concentration inside the chamber in 40 minutes, i.e. increase in gas concentration from T0 to T40 (Smith and Dobbie, 2001). Confirmation of the linearity of the gas flux was confirmed by taking four gas samples from one of the chambers that received urine at T0, T20, T40 and T60 on every sampling occasion. Soil surface temperature was measured at the beginning and at the end on each sampling day.

2.5 Soil sampling and analysis

Soil samples were taken at 10 cm depth on every gas sampling occasion from each of the 1-m^2-plot delineated next to each treatment replicate plot. Samples were dried for 48 h at 105 °C to determine gravimetric water content (θ_g). Soil BD was calculated after treatment application in each plot. Then WFPS was calculated using the BD, an assumed soil particle density (2.65 g cm^{-3}) and θ_g. Average

175 WFPS between the four treatments for every sampling date was calculated. Soil mineral N was
176 determined weekly by extracting soil in 2 M KCl (20 g of fresh soil: 40 mL 2 M KCl, shaken for 1 h).
177 The extracts were analysed with colorimetric analysis, using an Aquakem 600 discrete analyser, for
178 NH_4^+-N and for NO_3^--N.
179 Soil samples were collected for pH determination on seven occasions within the experimental period
180 in a 1:2.5 (vol/vol) fresh soil-water suspension shaken for 15 minutes (*Ministry of Agriculture*
181 *Fisheries and Food*, 1986) using a pH meter fitted with a general-purpose combination electrode.

182 The same soil samples were analyzed for dissolved organic carbon (DOC) by shaking 50 g of soil (dry
183 weight) in 200 mL of ultrapure water at 120 revolutions per minute, for 60 minutes at room
184 temperature. Extracts were then centrifuged for 15 minutes at 4600 g and filtered through 0.45-µm
185 cellulose acetate filter papers (*Guigue* et al., 2014) before analyzing them on a total organic carbon
186 analyser (Shimadzu TOC-L).

187 *2.6 Data analysis*

188 The N_2O flux data had a skewed distribution so it was log transformed as ln (N_2O flux + 1). A one-way
189 analysis of variance (ANOVA) was performed to determine the effect of the treatments on the
190 transformed N_2O fluxes, on CH_4 fluxes as well as on soil NH_4^+-N, soil NO_3^--N, pH, and DOC for each
191 sampling date. Also, an ANOVA was performed to determine treatment effect on cumulative N_2O
192 emissions. All statistical analysis was done using the R software (*Fox*, 2005).

193 **3 Results**

194 *3.1 Meteorological data*

195 Total precipitation over the experimental period was 170.8 mm with the highest event (13.6 mm) in
196 November 29th (Figure 1). Initially, WFPS was 85% and steadily increased until the soil was saturated,
197 with an average of 97.9% for the experiment, with values > 100% when water was lying on the soil

surface (Fig.1). Soil surface temperature averaged 14°C with a steady decrease from a maximum of 18 °C to a minimum of 10 °C on day 79 (Figure 1).

((Figure 1))

3.2 Soil nitrogen content, DOC and pH

Soil NH_4^+-N concentration in all urine treatments was significantly higher ($p<0.01$) than the control throughout the experiment and increased up to 379.5 mg NH_4^+-N kg dry $soil^{-1}$ by day 3 after treatment application (Figure 2a). On day 3, the U+HA2 treatment showed significantly higher soil NH_4^+-N concentration ($p<0.05$), than either the U and U+HA1 treatments but after day 3 soil NH_4^+-N concentrations did not differ among treatments and declined over time to about 50 mg NH_4^+-N kg dry $soil^{-1}$.

Soil NO_3^--N concentrations ranged from 0 to 10 mg NO_3^--N kg dry $soil^{-1}$ and there were no significant differences between urine treatments and the control, except for days 35 and 64 when the soil NO_3^--N concentration in the control was lower ($p<0.05$) than in the urine treatments (Figure 2b).

((Figure 2))

Soil DOC ranged from 11 to 61 mg kg dry $soil^{-1}$ during the study. The U and the U+HA2 treatment peaked (59 and 61 mg DOC kg dry $soil^{-1}$, respectively) three days after treatment application with a second peak, < 44 mg DOC kg dry $soil^{-1}$, on day 22 (Figure 3). Meanwhile, DOC concentrations in the U+HA1 treatment were ≤ 30 mg DOC kg dry $soil^{-1}$ throughout the study. The control DOC concentrations ranged from 19 to 39 mg DOC kg dry $soil^{-1}$, following a similar trend as described for the U and U+HA2 treatments. After day 35, all treatments had average DOC concentrations < 25 mg DOC kg dry $soil^{-1}$.

((Figure 3))

Soil pH averaged 5.11 (± 0.15) prior to treatment application. On day 3, after the urine treatments were applied, pH values decreased to 4.84, 4.85, and 4.98 for the U, U+HA1 and U+HA2 treatments, respectively, and did not differ significantly. The pH remained < 5.0 until the end of the experiment,

with the lowest pH values measured on day 35. These values were lower ($p<0.05$) than the pH from the control which averaged 5.26 during the experiment.

3.3 Nitrous oxide emissions

During the first 20 days of the experiment, daily N_2O fluxes showed no significant differences between the control and the urine treatments with fluxes < 20 g of N_2O-N ha^{-1} day^{-1} with a small peak, five days after application (Figure 2c). The highest fluxes from the urine treatments appeared on day 22, with other peaks on days 38, 45 and 56 in all urine treatments. Emissions from the control were up to 1.79 g N_2O-N ha^{-1} day^{-1} while N_2O emissions from U, U+HA1 and U+HA2 were up to 28.13, 41.71 and 24.57 g N_2O-N ha^{-1} day^{-1}, respectively. On days 22, 28, 35, 45 and 50 the emissions from the urine treatments were higher ($p<0.05$) than that from the control. However, there were no significant differences between the U and the U+HA treatments on these sampling days with the three treatments having similar N_2O-N fluxes trends.

Cumulative emissions from the U, U+HA1 and U+HA2 treatments were 660 (±187), 757 (±377), and 564 (±289) g N_2O-N ha^{-1}, respectively, and did not differ significantly. These values were higher ($p<0.05$) than the cumulative emissions from the control which averaged 5.89 g N_2O-N ha^{-1}. As a percentage of the urine-N applied, the cumulative N_2O-N fluxes for the urine treatments averaged 0.13% (± 0.03).

3.3 Methane emissions

Soil CH_4 emissions for all treatments, including the control, were < 5 g ha^{-1} d^{-1} until day 28. After this time, CH_4 emissions steadily increased in all treatments, including the control, peaking at 40 g CH_4 ha^{-1} day^{-1} at the end of the experiment (Figure 2d). Cumulative CH_4 emissions did not significantly differ among the four treatments and averaged 623.5 g CH_4 ha^{-1}.

4 Discussion

4.1 Effect of HA on soil variables

247 Synthetic urine treatments resulted in changes on soil inorganic N, pH and DOC explained by the
248 hydrolysis of the urea. Particularly, the U+HA2 treatment showed an inhibitory effect on nitrification
249 as soil NH_4^+-N remained as NH_4^+-N until day 3 showing significantly higher soil NH_4^+-N concentration
250 (>379 NH_4^+-N kg dry $soil^{-1}$) and lower NO_3^--N (<1.7 mg) than the other treatments. However, this
251 pattern was not observed for the remainder of the experiment which might be explained by the
252 leaching of the HA as a consequence of the rainfall events recorded on days 6, 7, and 8 (Figure 1)
253 when 22.2 mm of rainfall occurred. Alternatively, the sorption of benzoic acid onto soil particles may
254 explain the lack of a continued HA effect. In this sense, the decrease in soil pH after day 3 might have
255 favoured the adsorption of benzoic acid to clay through weak physical forces (*Indejirt* and *Prasanta*,
256 2004). Thus, it seems probable that both, HA leaching and benzoic acid sorption onto clay, were
257 responsible for the lack of HA inhibitory effect on soil NH_4^+-N nitrification after day 3. Indeed, the
258 decline in soil NH_4^+-N and the increases in NO_3^- concentrations after day 3 indicate the occurrence of
259 nitrification processes. However, NO_3^--N concentrations were much lower than previously reported
260 in similar studies (e.g. *Clough* et al., 2009). The lower NO_3^--N concentrations in this study might be
261 explained either by pasture N uptake or by the high WFPS, that provided conditions for promoting
262 the development of anaerobic microsites suitable for denitrification. The rate of nitrification also
263 appeared slow when compared to prior studies where the nitrification is often complete within a
264 month under urine patches on pasture soil (e.g. *Clough* et al., 2009).

265 The DOC values increased as a result of urea hydrolysis increasing soil pH but then decreased to < 25
266 mg DOC kg $soil^{-1}$ when WFPS was > 100%. Such changes in DOC with increasing WFPS are indicative
267 of anaerobic heterotrophic processes such as denitrification consuming DOC. This indicates a low or
268 negligible supply of oxygen, which would also have slowed or prevented nitrification processes,
269 further explaining the relatively prolonged and slow decline in soil NH_4^+-N concentrations.

270 Nitrification processes would have also promoted the observed decrease in soil pH due to the
271 release of free H^+, as similarly reported by *Krol* et al. (2015). Moreover, the formation of BA from HA

might have also contributed to the decrease in soil pH. The observed acidification that occurred in this study (pH= 4.6 after HA application) might have favoured the sorption of BA onto clay preventing not only its antimicrobial action but also its inhibition effect on denitrification.

4.2 Effect of HA on nitrous oxide emissions

The lack of a HA effect on N_2O fluxes after day 3 under our field conditions ratifies previous results reported under more aerobic conditions (*Krol* et al., 2015; *Clough* et al., 2009) in terms of potential *in situ* effects of HA. As previously stated, the highest U+HA treatment inhibited nitrification as soil NH_4^+-N remained as NH_4^+-N until day 3. However, N_2O emission was not inhibited, which means that N_2O was not the result of the nitrification from the added NH_4^+-N, but possibly from denitrification from the soil NO_3^--N. On day 3, WFPS was ~80% so the soil was not saturated and nitrification did occur. Indeed, soil NO_3^--N concentration was higher in the U and U+HA treatments compared to the control indicating NO_3^--N formation.

The percentage of N applied subsequently emitted as N_2O reported in this study was similar to that reported by *Di* and *Cameron* (2006) and by *Taghizadeh-Toosi* et al. (2012) but lower than that reported by *Clough* et al. (2009) and *Krol* et al. (2015). This lower percentage of N emitted might be explained by the occurrence of the higher values of WFPS registered when compared to *Clough* et al. (2009) and *Krol* et al. (2015). High WFPS reduces relative soil gas diffusivity increasing soil anaerobic conditions, which leads to higher losses of N as N_2 instead of N_2O (*Balaine* et al. 2016). Alternatively, the acidic soil pH (< 5.0) could have favoured chemodenitrification processes as a result of nitrite, formed as a consequence of nitrification or denitrification, producing nitrous acid and reacting with soil organic matter (*Heil* et al., 2016), and thus further reducing the substrate available for N_2O production. However, the percentage of N applied emitted as N_2O (0.13 %) was considerably lower than that reported in the laboratory study conducted by *Kool* et al. (2006) under similar anaerobic conditions (2.1 % for the high HA treatment; WFPS=97 %). Although such experiment was conducted on a different soil type, the difference in the percentage of N applied emitted as N_2O may be a

297 consequence of plant uptake of mineral N in our study, which might decrease N susceptible of being
298 emitted as N_2O. However, values of soil NH_4^+-N were similar to those reported by *Kool* et al. (2006).
299 Nevertheless, the effect of HA on N_2O emissions appears not to be related to the amount of mineral
300 N present in the soil (*Koo*l et al., 2006).
301 *Van Groenigen* et al. (2006) reported that the HA inhibition effect occurred at a concentration of 3.9
302 mmol HA kg^{-1} soil, which is a similar concentration as in the U+HA2 treatment in the current study.
303 However, the permanent soil water logging conditions after day 3 (WFPS > 85%) may have resulted
304 in leaching of the HA and the formed BA after treatments application.
305 Therefore, our results showed that the manipulation of ruminant urine via diet selection will not
306 have a mitigation effect on N_2O emissions. In this sense, our findings suggested that there is no point
307 in introducing changes in the diet of the ruminants in order to increase the concentration of HA in
308 their urine to reduce N_2O emissions under high soil WFPS conditions.

309 *4.3 Effect of HA on methane emissions*

310 It has previously been shown that CH_4 production in rice paddies and soil suspensions occurs under
311 much stronger reducing conditions than observed for N_2O emissions (*Yu* et al., 2001; 2003). The
312 steady increase of CH_4 emissions for all treatments after day 35 coincided with WFPS greater than
313 100% and a decline in DOC concentrations. Such anaerobic conditions would have favoured the
314 decomposition process of soil organic material through which CH_4 was produced, via DOC
315 fermentation catalyzed by methanogenic microorganisms (Rizzo et al, 2013). Thus, the CH_4 emissions
316 further demonstrate the favourable soil conditions for denitrification.
317

318 **5 Conclusions**

319 The results of this study show that an inhibitor effect was observed for the highest U+HA treatment
320 just until day 3, as soil NH_4^+-N remained as NH_4^+-N more than the other treatments. However, such
321 inhibitor effect was not reflected neither on soil NO_3^- concentration nor on N_2O emissions. After day

3, it seems likely that a combination of HA leaching under the permanent soil water logging conditions and a sorption of BA into clay under optimal soil pH may explain the lack of an inhibitor HA effect on N_2O emissions.

Therefore, we have ratified the lack of a mitigation effect *in situ* under strongly reducing conditions. Our study showed that the potential manipulation of ruminant urine, via diet selection, to optimise HA concentration will not mitigate N_2O emissions. Further studies using ^{13}C-labelled benzoic acid or HA should be performed to determine the residence time and fate of HA in soil.

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References

Avery, B. W. (1980): Soil Classification for England and Wales (Higher Categories). Soil Survey of England and Wales. Soil Survey Technical Monograph No. 14, Harpenden, UK.

Balaine, N., Clough, T. J., Beare, M. H., Thomas, S. M., Meenken, E. D., Ross, J. G. (2013): Changes in Relative Gas Diffusivity Explain Soil Nitrous Oxide Flux Dynamics. *Soil Sci. Soc. Am. J.* 77, 5, 1496-1505.

345 *Balaine, N., Clough, T. J., Beare, M. H., Thomas, S. M., Meenken, E. D.* (2016): Soil Gas Diffusivity
346 Controls N_2O and N_2 Emissions and their Ratio. *Soil Sci. Soc. Am. J.* 80, 529-540.
347 *Baral, K. R., Thomsen, A. G., Olesen, J. E., Peterson, S. O.* (2014): Controls of nitrous oxide emission
348 after simulated cattle urine deposition. *Agric. Ecosyst. Environ*. 188, 103-110.
349 *Bertram, J. E., Clough, T. J., Sherlock, R. R., Condron, L. M., O' Callaghan, M., Wells, N. S., Ray, J. L.*
350 (2009): Hippuric acid and benzoic acid inhibition of urine derived N_2O emissions from soil. *Glob.*
351 *Change Bio.* 15, 2067-2077.
352 *Boon, A., Robinson, J. S., Chadwick, D. R., Cardenas, L. M.* (2014): Effect of cattle urine addition on
353 the surface emissions and subsurface concentrations of greenhouse gases in a UK peat grassland.
354 *Agric. Ecosyst. Environ.* 186, 23-32.
355 *Bristow, A. W., Whitehead, D. C., Cockburn, J.E.* (1992): Nitrogenous constituents in the urine of
356 cattle, sheep and goats. *J. Sci. Food Agr.* 59, 387–394.
357 *Cardenas, L. M., Thorman, R., Ashlee, N., Butler, M., Chadwick, D., Chambers, B., Cuttle, S., Donovan,*
358 *N., Kingston, H., Lane, S., Dhanoa, M. S., Scholefield, D.* (2010): Quantifying annual N_2O emission
359 fluxes from grazed grassland under a range of inorganic fertiliser nitrogen inputs. *Agric. Ecosyst.*
360 *Environ.* 136, 218–26.
361 *Clough, T. J., Ray, J. L., Buckthought, L. E., Calder, J., Baird, D., O'Callaghan, M., Sherlock, R. R.,*
362 *Condron, L. M.* (2009): The mitigation potential of hippuric acid on N_2O emissions from urine
363 patches: An in situ determination of its effect. *Soil Biol. Biochem.* 41, 2222-2229.
364 *Chadwick, D. R., Cardenas, L., Misselbrook, T. H., Smith, K. A., Rees, R. M., Watson, C. J., McGeough,*
365 *K. L., Williams, J. R., Cloy, J. M., Thorman, R. E., Dhanoa, M. S.* (2014): Optimizing chamber methods
366 for measuring nitrous oxide emissions from plot- based agricultural experiments. *Eur. J. Soil Sci.* 65,
367 295–307.
368 *Chipley, J.R.* (1983): Sodium benzoate and benzoic acid. In: Branen, A.L., Davidson, P.M. (Eds.),
369 Antimicrobials in Foods. M. Decker, New York, pp. 11–35.

Dalton, B.R. (1999*)*: The occurrence and behavior of plant phenolic acids in soil environment and their potential involvement in allelochemical interference interactions: methodological limitations in establishing conclusive proof of allelopathy. In: Inderjit, Dakshini KMM, Foy CL (eds) Principles and practices in plant ecology: allelochemical interactions. CRC, BocaRaton, Fla., pp. 57–74.

DEFRA. (2011): Greenhouse Gas Emission Projections for UK Agriculture to 2030. http://www.defra.gov.uk/corporate/evidence/economics/ (accessed 15.11.20).

de Klein, C. A. M., Cameron, K. C., Di, H. J., Rys, G., Monaghan, R. M., Sherlock, R. R. (2011): Repeated annual use of the nitrification inhibitor dicyandiamide (DCD) does not alter its effectiveness in reducing N_2O emissions from cow urine. *Animal Feed Sci. Technol.* 166, 480–491.

Dijkstra, J., Onema, O., van Groenigen, J. W., Spek, J. W., van Vuuren, A. M., Bannink, A. (2013): Diet effects on urine composition of cattle and N_2O emissions. *Animal* 7, 292-302.

Doak, B. W. (1952): Some chemical changes in the nitrogenous constituents of urine when voided on pasture. *J. Agric. Sci.* 42, 162–171.

Fox, J. (2005): The R Commander: a basic-statistics graphical user interface to R. *J. Stat. Softw.* 14, 9.

Granger, S. J., Bol, R., Meier-Augenstein, W., Leng, M. J., Kemp H. F., Heaton, T. H. E., White, S. M. (2010): The hydrological response of heavy clay grassland soils to rainfall in south-west England using δ2H. *Rapid Commun. Mass Spectrom.* 24, 475–482.

Guigue, J., Mathieu, O., Lévêque, J., Mounier, S., Laffont, R., Maron, P. A., Navarro, N., Chateau, C., Amiotte-Suchet, P., Lucas, Y. (2014): A comparison of extraction procedures for water-extractable organic matter in soils. *Eur. J. Soil Sci.* 65, 520–530.

Harrod, T. R., Hogan, D. V. (2008): The Soils of North Wyke and Rowden. Unpublished Report to North Wyke Research, Revised Edition of Original Report by TR Harrod, *Soil Survey of England and Wales*.

Heil, J., Vereecken, H., Brüggemann, N. (2016): A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *Eur. J. Soil Sci.* 67, 23–39

396 *Her J., Huang, J., (1995):* Influences of carbon source and C/N ratio on nitrate/nitrite denitrification
397 and carbon breakthrough. *Bioresource Technol.* 54, 45–51.
398 *Inderjit, Bowhmik, P. C.* (2004): Sorption of benzoic acid onto soil colloids and its implications for
399 allelopathy studies. *Biol. Fertil. Soils* 40, 345–348.
400 *Kelly, K. B., Phillips, F. A., Baigent, R.* (2008): Impact of dicyandiamide application on nitrous oxide
401 emissions from urine patches in northern Victoria, Australia. *Aust. J. Exp. Agric.* 48, 156-159.
402 *Kool, D. M., Hoffland, E., Hummelink, E. W. J., Van Groenigen, J. W.* (2006): Increased hippuric acid
403 content of urine can reduce soil N_2O fluxes. *Soil Biol. Biochem.* 38, 1021–1027.
404 *Kreula, M., Rauramaa, A., Ettala, T.* (1978): The effect of feeding on the hippuric acid content of
405 cow's urine. *J. Agr. Sci. Finland* 50, 372–377.
406 *Krol, D. J., Forrestal, P. J., Lanigan, G. J., Richards, K. G.* (2015): In situ N_2O emissions are not
407 mitigated by hippuric and benzoic acids under denitrifying conditions. *Sci. Total Environ*. 511, 362-
408 368.
409 *Linn, D. M., Doran J. W.* (1984): Effect of water-filled pore space on carbon dioxide and nitrous oxide
410 production in tilled and non-tilled soils. *Soil Sci. Soc. Am. J.* 48, 1267-1272.
411 *Luo, J., Lindsey, S. B., Ledgard, S. F.* (2008): Nitrous oxide emissions from animal urine application on
412 a New Zealand pasture. *Biol. Fertil. Soils* 44, 463–470.
413 *Martin, A.K.* (1970a): The urinary aromatic acids excreted by sheep given S24 perennial ryegrass cut
414 at six stage of maturity. *Br. J. Nutr.* 24, 943–959.
415 *Martin, A.K.* (1970b): Effect of stage of maturity of perennial ryegrass on its content of some organic
416 acids and phenolic compounds. *J. Sci. Food Agr*. 21, 496–501.
417 *Marwan, A. G., Nagel, C. W.* (1986): Quantitative determination of infinite inhibition concentrations
418 of antimicrobial agents. *Appl. Environ. Microb.* 51, 559–561.
419 *Ministry of Agriculture, Fisheries and Food*. (1986): The Analysis of Agricultural Materials, 3rd edn
420 Reference book 427. HMSO, London.

421 *Misselbrook, T. H., Cardenas, L. M., Camp, V., Thorman, R. E., Williams, J. R., Rollett, A. J., Chambers,*
422 *B. J.* (2014): An assessment of nitrification inhibitors to reduce nitrous oxide emissions from UK
423 agriculture. *Environ. Res. Lett.* 9, 115006.
424 *Parkin, T. B., Venterea, R. T.* (2010): USDA-ARS GRACEnet Proyect Protocols. In: Follet, R.F (Eds),
425 Chapter 3. Chamber-Based Traze Gas Flux Measurements. Sampling Protocols, Beltsville, MD, 1–39.
426 *Rizzo, A., Boano, F., Revelli, R., Ridolfi, L.* (2013): Role of water flow in modelling methane emissions
427 from flooded paddy soils. *Adv. Water Resour.* 52, 261–274.
428 *Rochette, P., Erisksen-Hamel, N. S.* (2008): Chamber measurements of soil nitrous oxide flux: are
429 absolute values reliable? *Soil Sci. Soc. Am. J.* 72,331–342.
430 *Selbie, D. R., Lanigan, G. J., Laughlin, R. J., Di, H. J., Moir, J. L., Cameron, K. C., Clough, T. J., Watson, C.*
431 *J., Grant, J., Somers, C., Richards, K. G.* (2015): Confirmation of co-denitrification in grazed grassland.
432 *Sci. Rep.* 5, 17361.
433 *Smith, K. A., Dobbie, K. E.* (2001): The impact of sampling frequency and sampling times on chamber-
434 based measurements of N_2O emissions from fertilized soils. *Global Change Biol*. 7, 933–945.
435 *Spek, J. W., Bannink A., Gort G., Hendriks W. H., Dijkstra J*. (2012): Effect of sodium chloride intake on
436 urine volume, urinary urea excretion, and milk urea concentration in lactating dairy cattle. *J. Dairy*
437 *Sci.* 95, 7288–7298.
438 *Taghizadeh-Toosi, A., Clough, T. J., Sherlock, R. R., Condron, L. M.* (2012): A wood based low-
439 temperature biochar captures NH_3-N generated from ruminant urine-N, retaining its bioavailability.
440 *Plant Soil* 353, 73-84.
441 *Van Groenigen, J. W., Kuikman, P. J., De Groot, W. J. M., Velthof, G. L.* (2005a): Nitrous oxide
442 emission from urine-treated soil as influenced by urine composition and soil physical conditions. *Soil*
443 *Biol. Biochem.* 37, 463–473.
444 *Van Groenigen, J. W., Velthof, G. L., Van der Bolt, F. J. E., Vos, A., Kuikman, P. J.* (2005b): Seasonal
445 variation in N_2O emissions from urine patches: effects of urine concentration, soil compaction and
446 dung. *Plant Soil* 273, 15–27.

447 *Van Groenigen, J. W., Palermo, V., Kool, D. M, Kuikman, P. J.* (2006): Inhibition of denitrification and
448 N_2O emission by urine-derived benzoic and hippuric acid. *Soil Biol. Biochem.* 38, 2499-2502.
449 *Van Vuuren, A. M., Smits, M. C. J.* (1997): Effect of nitrogen and sodium chloride intake on
450 production and composition of urine in dairy cows. In Gaseous nitrogen emissions from grasslands
451 (ed. SC Jarvis and BF Pain), 195–199. CAB International, Wallingford, UK.
452 *Wachendorf, C., Lampe, C., Taube, F., Dittert, K.* (2008): Nitrous oxide emissions and dynamics of soil
453 nitrogen under ^{15}N-labeled cow urine and dung patches on a sandy grassland soil. *J. Plant Nutr. Soil*
454 *Sci.* 171, 171-180.
455 *Yu, K., Wang, Z., Vermoesen, A., Patrick Jr., W., Van Cleemput, O.* (2001): Nitrous oxide and methane
456 emissions from different soil suspensions: effect of soil redox status. *Biol. Fertil. Soils* 34, 25–30.
457 *Yu, K., Patrick Jr., W. H.* (2003): Redox range with minimum nitrous oxide and methane production in
458 a rice soil under different pH. *Soil Sci. Soc. America J.* 67, 1952-1958.
459 *Zaman, M., Nguyen, M. L.* (2012): How application timings of urease and nitrification inhibitors affect
460 N losses from urine patches in pastoral system. *Agric. Ecosyst. Environ.* 156, 37–48.
461

462 **Table 1.** Soil initial conditions of the experiment

Soil variables					
Bulk density	NH_4^+-N	NO_3^--N	DOC	pH	WFPS
mg m^{-3}	--------g N kg soil $^{-1}$ --------		mg C kg $soil^{-1}$		%
1.11	5.78	2.03	18.94	5.11	91.23

463

464 **Table 2.** Synthetic urine composition by treatment

	Treatment		
Urine compound	**U**	**U+HA1**	**U+HA2**
	---------- g L^{-1} ----------		
Urea	16.9	16.9	16.99
Hippuric Acid	6.78	9.98	16.00
Allantoin	4.12	4.12	4.12
Uric Acid	0.24	0.24	0.24
Creatinine	0.89	0.89	0.89
$KHCO_3$	14.00	14.00	14.00
KCl	10.50	10.50	10.50
$CaCl_2 \cdot 2H_2O$	0.40	0.40	0.40
$MgCl \cdot 5H_2O$	1.20	1.20	1.20
Na_2SO_4	3.70	3.70	3.70

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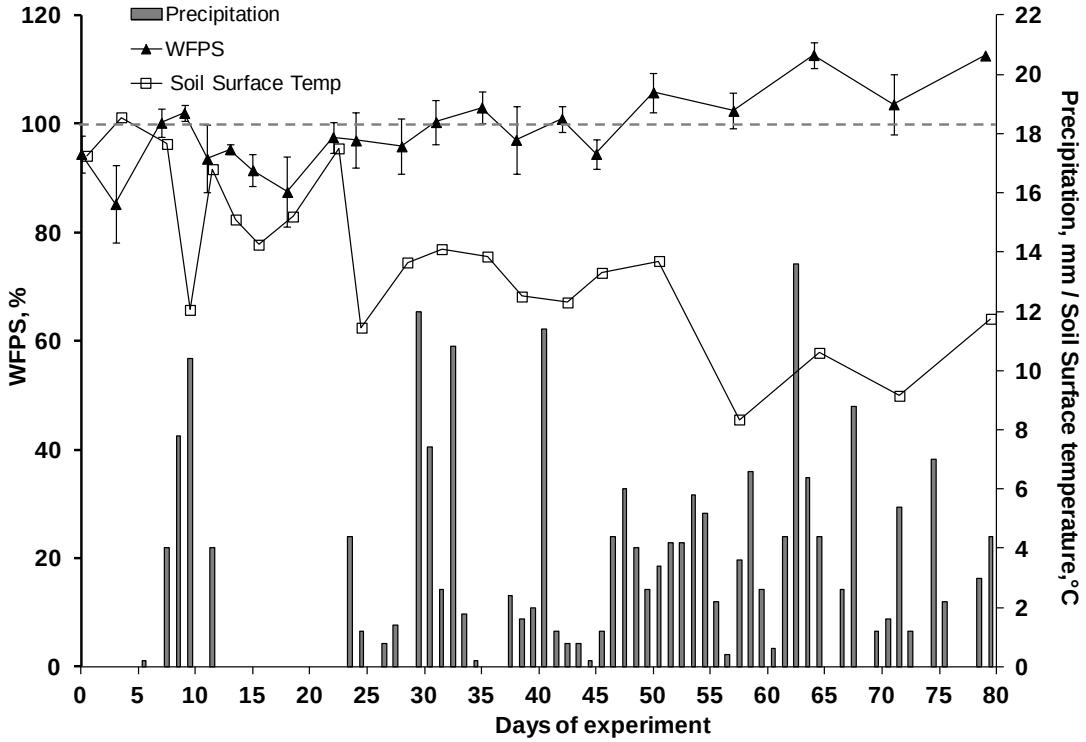
466

Figure 1. Precipitation (mm), WFPS (%) and soil surface temperature (°C) over the experimental period.

Figure 2. Soil NH_4^+ - N content (mg NH_4^+ - N kg^{-1}) (a), soil NO_3^--N content (mg NO_3^--N kg^{-1}) (b), daily mean N_2O flux (g N_2O-N ha^{-1} d^{-1}) (c) and Ddaily mean CH_4 flux (g CH_4 ha^{-1} d^{-1}) (d) for all treatments over the experimental period. Vertical bars show standard error of the treatment means (n=3). Significant differences ($\alpha<0.05$) from the control are marked with an asterisk

Figure 3. Dissolved organic carbon (DOC, mg C kg $soil^{-1}$) per treatment over the experimental period. Vertical bars show standard error of the treatment means (n=3)**.**

476 **Figure 1**



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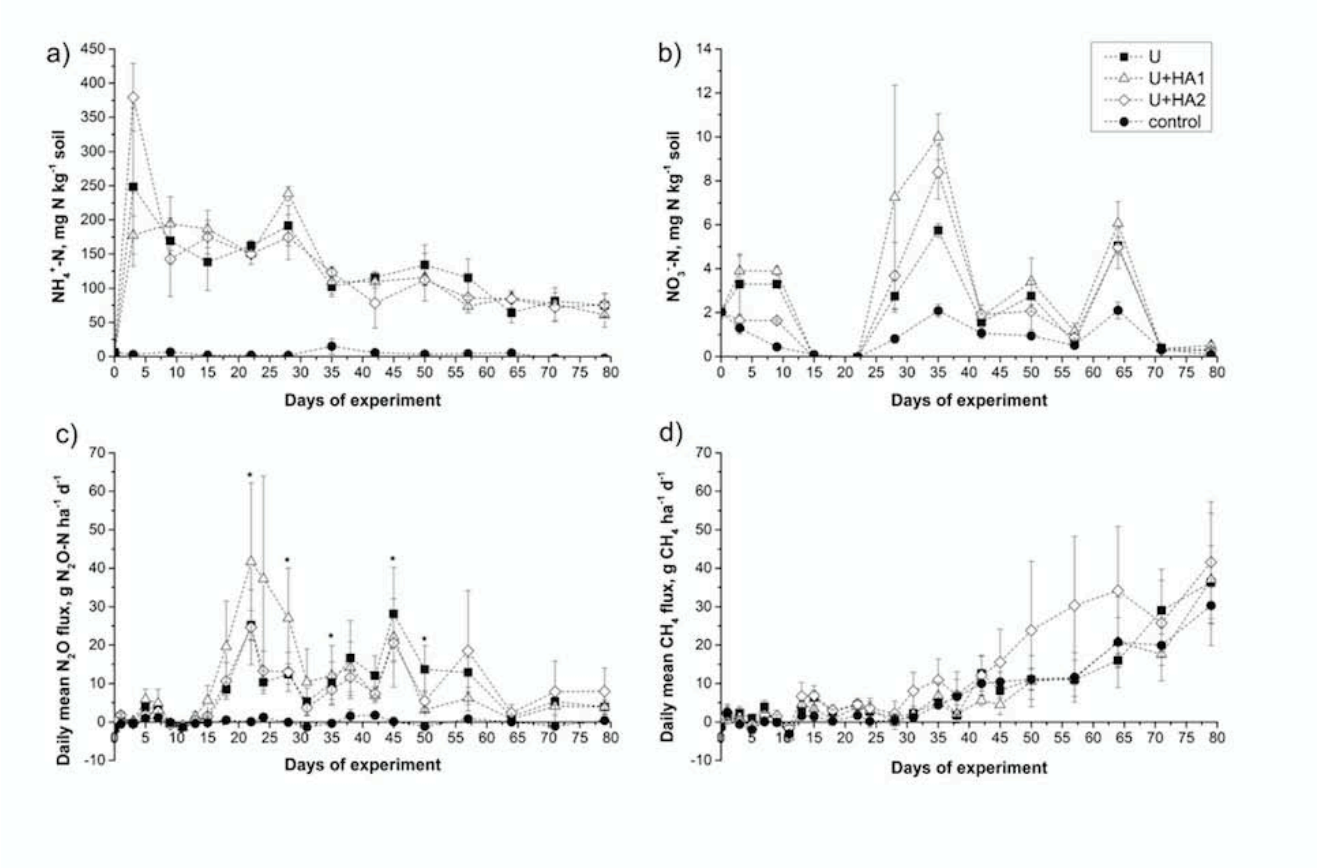
479 **Figure 2.**



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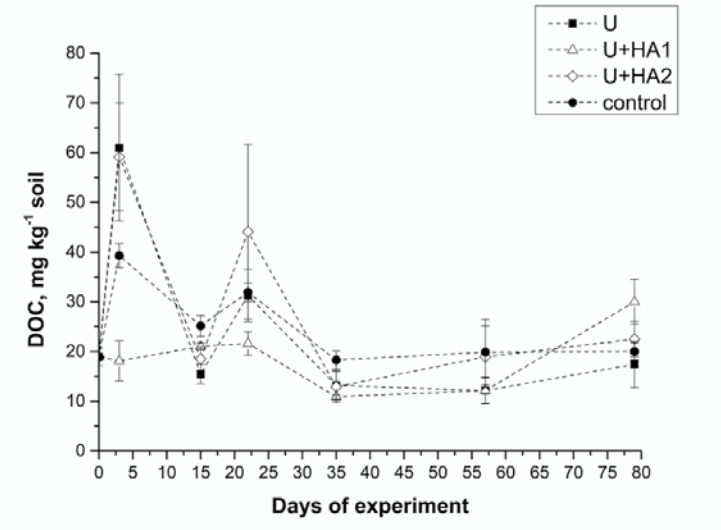
484 **Figure 3.**



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