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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 with 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-d 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.

Key words: bovine urine / heavy clay soil / N_2O emissions / natural nitrification inhibition

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) (*DEFRA*, 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 (*Bristow* et al., 1992; *Spek* et al., 2012). 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 waterlogging 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 (*de Klein*

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et al., 2011; *Kelly* et al., 2008; *Luo* et al., 2008; *Wachendorf* et al., 2008; *Zaman* and *Nguyen*, 2012; *Baral* et al., 2014; *Boon* et al., 2014; *Misselbrook* et al., 2014; *Krol* et al., 2015). This variability in N$_2$O emissions may be a consequence of variation in ruminant urine composition, which is controlled by the animal's diet (*Martin*, 1970a, 1970b; *Kreula* et al., 1978; *van Vuuren* and *Smits*., 1997). In this sense, some of the constituents in the ruminant urine have been reported to affect subsequent soil N$_2$O emissions (*van Groenigen* et al., 2005a, 2005b, 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 shown to mitigate N$_2$O emissions from soil (*van Groenigen* et al., 2006; *Kool* et al., 2006; *Bertram* et al., 2009) presumably due to the presence of benzoic acid (BA), a break-down product (*Bristow* et al., 1992) which, along with its demonstrable antimicrobial activity in acidic mediums (*Marwan* and *Nagel*, 1986), is known as a denitrification inhibitor (*Her* and *Huang*, 1995). Benzoic acid may be adsorbed onto soil particles *via* Van der Waals or hydrogen bonding and subsequently released as a consequence of decreasing soil solution strength or as a result of competing ions (*Dalton*, 1999). *Inderjit* and *Bowhmik* (2004) found that sorption of the BA onto soil particles is affected by clay content, soil organic matter, pH, and the concentration of BA itself.

Hippuric acid has been reported to reduce soil N$_2$O emissions due to its inhibitory effect on both nitrification and denitrification processes (*Bertram* et al., 2009). In addition, the concentration of HA in urine has been reported to have a controlling effect on both the hydrolysis of urine-N and on NH$_3$ volatilization. Thus, HA may further affect N$_2$O emissions by altering substrate supply for microbial mechanisms of N$_2$O production (*van Groenigen*, et al., 2005a, 2005b).

Field studies carried out *in situ* on silt loam soils with WFPS ranging from 18% to 51% reported no effect on N$_2$O emissions with increasing urine HA concentration (*Clough* et al., 2009). Similarly, *Krol* et al. (2015) found no effect *in situ* on a loam soil where WFPS ranged from 60% to 80%. By contrast, the inhibitory effect of HA under anaerobic conditions (WFPS 92%) has been proven under laboratory conditions (*Kool* et al., 2006). However, there are no reports on the *in situ* effects of urinary HA concentration on N$_2$O emissions for heavy clay soils with high values of WFPS (> 85%), as commonly found in grazed perennial pastures from the southwest of England.

The aim of this study was to examine the potential for elevated ruminant urine HA concentrations to reduce *in situ* N$_2$O emissions on an acidic heavy clay soil under poorly drained soil conditions (WFPS > 85%). Based on previous *in situ* studies (*Kool* et al., 2006; *Clough* et al., 2009; *Krol* et al., 2015) we hypothesized that an increase in ruminant urine HA content could inhibit N$_2$O emissions when urine 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 Material 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 (*Köppen*, 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 (CEC) 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 waterlogged 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 is presented in Tab. 1. Meteorological data, consisting of air temperature and precipitation, were 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 × 2.8 m) with a 1 m separation as buffer. Five chambers were installed within each replicate plot (*i.e.*, 60 chambers in total) and an area of 1 m^2 (1 m × 1 m) was delineated next to each replicate plot for soil sampling.

The closed static chamber technique was used (*Rochette* and *Erisksen-Hamel*, 2008) for determining soil gas fluxes. Each chamber comprised a white polyvinyl chloride (PVC) open ended box with a 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 order to ensure a good seal between the chamber and soil, the boxes were inserted into the soil to a depth of 0.1 m more

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

than 24 h before the flux measurements began (*Parkin* and *Venterea*, 2010). The effective height of each chamber was recorded internally at the centre of each wall and in the centre of the chamber to use in the calculation of the fluxes. The resultant chamber effective height was the weighted mean of the 5 points taken (including two times the centre height) and ranged between 0.09 and 0.18 m. The lid was fitted with a sampling port with a three-way valve and placed on top of the box at the beginning of each gas sampling day.

2.3 Treatments

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

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-d period. Gas samples were taken between 11:00 am and 2:00 pm 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

Table 2: Synthetic urine composition by treatment.

Urine compound	Treatment		
	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
$CaCl_2 \cdot 2\,H_2O$	0.40	0.40	0.40
$MgCl \cdot 5\,H_2O$	1.20	1.20	1.20
Na_2SO_4	3.70	3.70	3.70

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 min, *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 at 105°C for 48 h 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 WFPS between the four treatments for every sampling date was calculated. Soil mineral N was determined weekly by extracting soil in 2 M KCl (20 g of fresh soil : 40 mL 2 M KCl, shaken for 1 h). The extracts were analysed with colorimetric analysis, using an Aquakem 600 discrete analyser, for NH_4^+-N and for NO_3^--N.

Soil samples were collected for pH determination on seven occasions within the experimental period in a 1:2.5 (v/v) fresh soil–water suspension shaken for 15 min (*Ministry of Agriculture, Fisheries and Food*, 1986) using a pH meter fitted with a general-purpose combination electrode. The same soil samples were analyzed for dissolved organic carbon (DOC) by shaking 50 g of soil (dry weight) in 200 mL of ultrapure water at 120 revolutions per minute for 60 min at room temperature. Extracts were then centrifuged for 15 min at 4600 *g* and filtered through 0.45-μm cellulose acetate filter papers (*Guigue* et al., 2014) before analyzing them on a total organic carbon analyser (Shimadzu TOC-L).

2.6 Data analysis

The N_2O flux data had a skewed distribution so it was log transformed as ln (N_2O flux + 1). A one-way analysis of variance (ANOVA) was performed to determine the effect of the treatments on the 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 sampling date. Also, an ANOVA was performed to determine treatment effect on cumulative N_2O emissions. All statistical analysis was done using the R software (*Fox*, 2005).

3 Results

3.1 Meteorological data

Total precipitation over the experimental period was 170.8 mm with the highest event (13.6 mm) in November 29th (Fig. 1). Initially, WFPS was 85% and steadily increased until the soil was saturated, 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 (Fig. 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 (Fig. 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 (Fig. 2b).
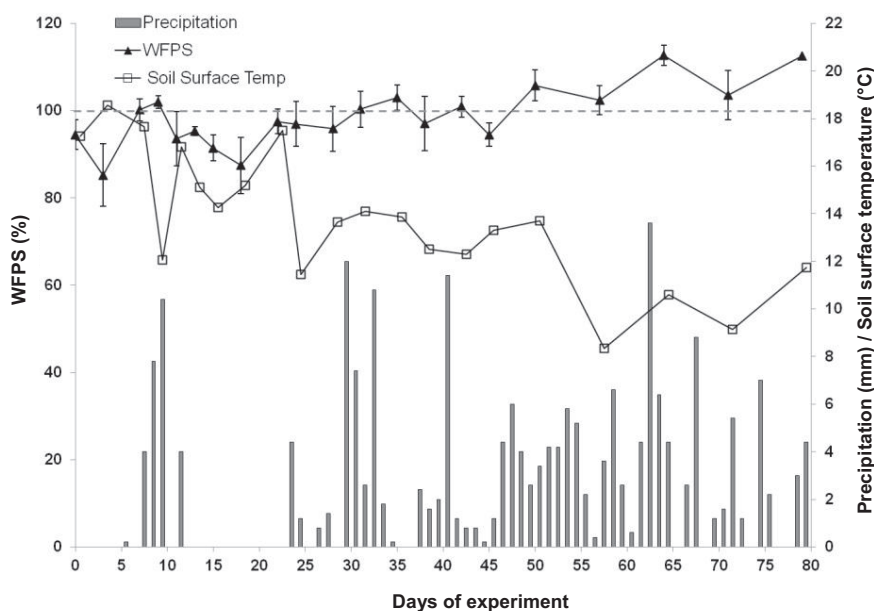
Soil DOC ranged from 11 to 61 mg kg dry soil⁻¹ during the study. The U and the U+HA2 treatment peaked (59 and 61 mg DOC kg dry soil⁻¹, respectively) three days after treatment application with a second peak, < 44 mg DOC kg dry soil⁻¹, on day 22 (Fig. 3). Meanwhile, DOC concentrations in the U+HA1 treatment 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.

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 (Fig. 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 d⁻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 d⁻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).

3.4 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 d⁻¹ at the end of the ex-
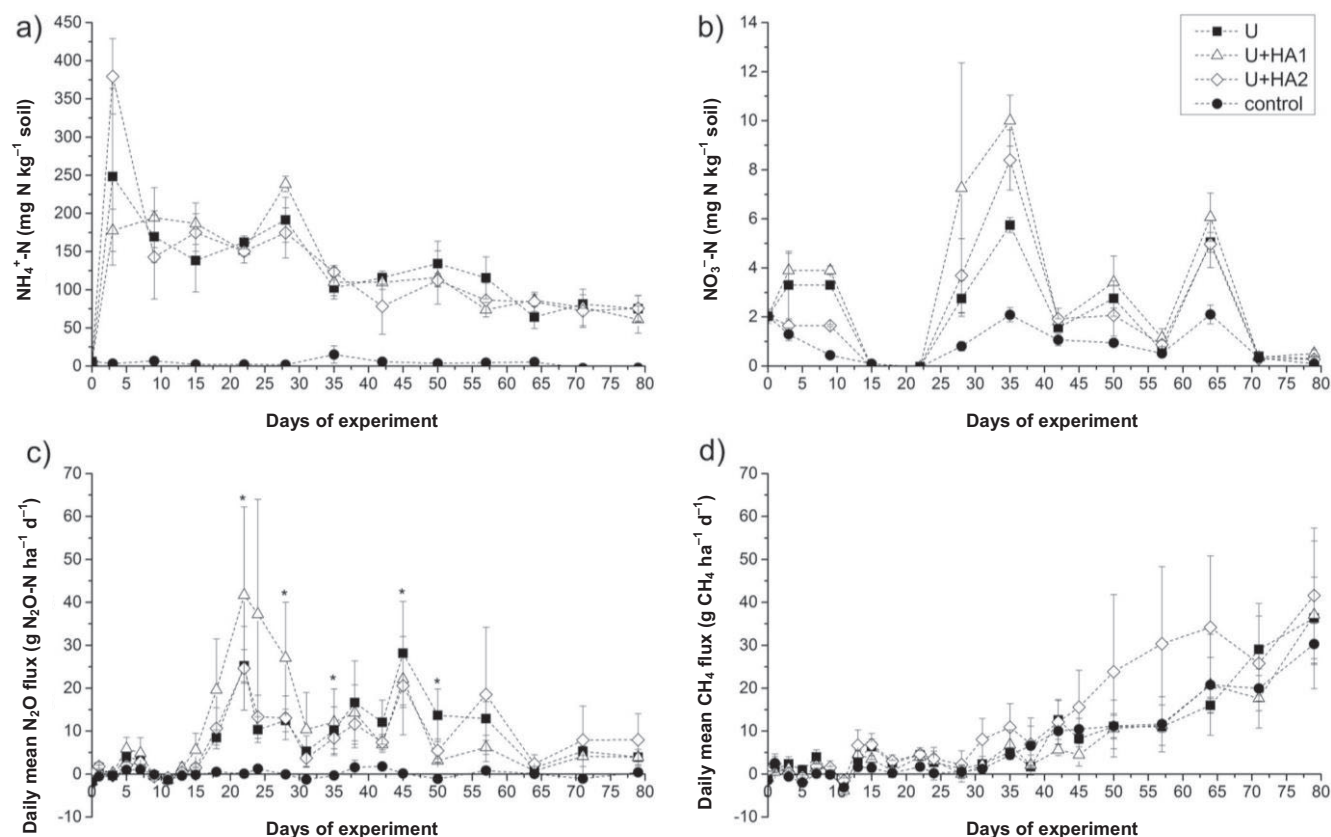

Figure 1: Precipitation (mm), WFPS (%), and soil surface temperature (°C) over the experimental period.

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 daily 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 (α < 0.05) from the control are marked with an asterisk.
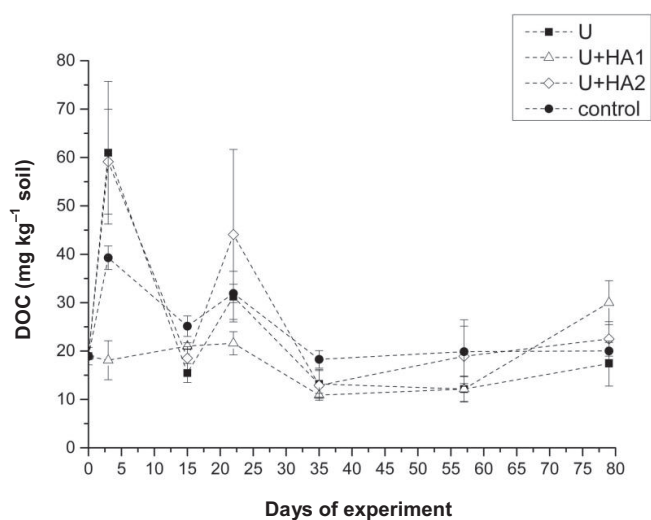

Figure 3: Dissolved organic carbon (DOC, mg C kg soil^{-1}) per treatment over the experimental period. Vertical bars show standard error of the treatment means (*n* = 3).

periment (Fig. 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

Synthetic urine treatments resulted in changes on soil inorganic N, pH, and DOC explained by the hydrolysis of the urea. Particularly, the U+HA2 treatment showed an inhibitory effect on nitrification as soil NH_4^+-N remained as NH_4^+-N until day 3, showing significantly higher soil NH_4^+-N concentration (> 379 NH_4^+-N kg dry soil^{-1}) and lower NO_3^--N (<1.7 mg) than the other treatments. However, this pattern was not observed for the remainder of the experiment which might be explained by the leaching of the HA as a consequence of the rainfall events recorded on days 6, 7, and 8 (Fig. 1) when 22.2 mm of rainfall occurred. Alternatively, the sorption of benzoic acid onto soil particles may explain the lack of a continued HA effect. In this sense, the decrease in soil pH after day 3 might have favoured the adsorption of benzoic acid to clay through weak physical forces (*Inderjit* and *Bowhmik*, 2004). Thus, it seems probable that both, HA leaching and benzoic acid sorption onto clay, were responsible for the lack of HA inhibitory effect on soil NH_4^+-N nitrification after day 3. Indeed, the decline in soil NH_4^+-N and the increases in NO_3^- concentrations after day 3 indicate the occurrence of nitrification processes. However, NO_3^--N concentrations were much lower than previously reported in similar studies (*e.g.*, *Clough* et al., 2009). The lower NO_3^--N concentrations in this study might

be explained either by pasture N uptake or by the high WFPS that provided conditions for promoting the development of anaerobic microsites suitable for denitrification. The rate of nitrification also appeared slow when compared to prior studies where the nitrification is often complete within a month under urine patches on pasture soil (*e.g.*, *Clough* et al., 2009).

The DOC values increased as a result of urea hydrolysis increasing soil pH but then decreased to < 25 mg DOC kg soil^{-1} when WFPS was > 100%. Such changes in DOC with increasing WFPS are indicative of anaerobic heterotrophic processes such as denitrification consuming DOC. This indicates a low or negligible supply of oxygen, which would also have slowed or prevented nitrification processes, further explaining the relatively prolonged and slow decline in soil NH$_4^+$-N concentrations.

Nitrification processes would have also promoted the observed decrease in soil pH due to the 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$_2$O fluxes after day 3 under our field conditions ratifies previous results reported under more aerobic conditions (*Clough* et al., 2009; *Krol* et al., 2015) 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$_2$O emission was not inhibited, which means that N$_2$O 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$_2$O 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$_2$O (*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$_2$O production. However, the percentage of N applied emitted as N$_2$O (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$_2$O may be a consequence of plant uptake of mineral N in our study, which might decrease N susceptible of being emitted as N$_2$O. However, values of soil NH$_4^+$-N were similar to those reported by *Kool* et al. (2006). Nevertheless, the effect of HA on N$_2$O emissions appears not to be related to the amount of mineral N present in the soil (*Kool* et al., 2006).

van Groenigen et al. (2006) reported that the HA inhibition effect occurred at a concentration of 3.9 mmol HA kg $^{-1}$ soil, which is a similar concentration as in the U+HA2 treatment in the current study. However, the permanent soil waterlogging conditions after day 3 (WFPS > 85%) may have resulted in leaching of the HA and the formed BA after treatments application.

Therefore, our results showed that the manipulation of ruminant urine *via* diet selection will not have a mitigation effect on N$_2$O emissions. In this sense, our findings suggested that there is no point in introducing changes in the diet of the ruminants in order to increase the concentration of HA in their urine to reduce N$_2$O emissions under high soil WFPS conditions.

4.3 Effect of HA on methane emissions

It has previously been shown that CH$_4$ production in rice paddies and soil suspensions occurs under much stronger reducing conditions than observed for N$_2$O emissions (*Yu* et al., 2001; *Yu* and *Patrick*, 2003). The steady increase of CH$_4$ emissions for all treatments after day 35 coincided with WFPS greater than 100% and a decline in DOC concentrations. Such anaerobic conditions would have favoured the decomposition process of soil organic material through which CH$_4$ was produced, *via* DOC fermentation catalyzed by methanogenic microorganisms (*Rizzo* et al., 2013). Thus, the CH$_4$ emissions further demonstrate the favourable soil conditions for denitrification.

5 Conclusions

The results of this study show that an inhibitor effect was observed for the highest U+HA treatment just until day 3, as soil NH$_4^+$-N remained as NH$_4^+$-N more than the other treatments. However, such inhibitor effect was not reflected neither on soil NO$_3^-$ concentration nor on N$_2$O emissions. After day 3, it seems likely that a combination of HA leaching under the permanent soil waterlogging conditions and a sorption of BA into clay under optimal soil pH may explain the lack of an inhibitor HA effect on N$_2$O 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$_2$O 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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