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In situ N_2O emissions are not mitigated by hippuric and benzoic acids under denitrifying conditions

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Abstract

Ruminant urine patches deposited onto pasture are a significant source of greenhouse gas nitrous oxide (N_2O) from livestock agriculture. Increasing food demand is predicted to lead to a rise in ruminant numbers globally, which, in turn will result in elevated levels of urine-derived N_2O. Therefore mitigation strategies are urgently needed. Urine contains hippuric acid and together with one of its breakdown products, benzoic acid, has previously been linked to mitigating N_2O emissions from urine patches in laboratory studies. However, the sole field study to date found no effect of hippuric and benzoic acids concentration on N_2O emissions. Therefore the aim of this study was to investigate the *in situ* effect of these urine constituents on N_2O emissions under conditions conducive to denitrification losses. Unadulterated bovine urine (0 mM of hippuric acid, U) was applied, as well as urine amended with either benzoic acid (96 mM, U+BA) or varying rates of hippuric acid (8 and 82 mM, U+HA1, U+HA2). Soil inorganic nitrogen (N) and N_2O fluxes were monitored over a 66 day period. Urine application resulted in elevated N_2O flux for 44 days. The largest N_2O fluxes accounting for between 13% (U) and 26% (U+HA1) of total loss were observed on the day of urine application. Between 0.9 and 1.3% of urine-N was lost as N_2O. Cumulative N_2O loss from the control was 0.3 kg N_2O-N ha^{-1} compared with 11, 9, 12, and 10 kg N_2O-N ha^{-1}

for the U, U+HA1, U+HA2, and U+BA treatments, respectively. Incremental increases in urine HA or increase in BA concentrations had no effect on N_2O emissions. Although simulation of dietary manipulation to reduce N_2O emissions through altering individual urine constituents appears to have no effect, there may be other manipulations such as reducing N content or inclusion of synthetic inhibitory products that warrant further investigation.

Keywords: benzoic acid, hippuric acid, N_2O mitigation, greenhouse gas, urine constituents, urine patches, denitrification

1. Introduction

Nitrous oxide (N_2O), a greenhouse gas (GHG) with a global warming potential of 298 over a 100 year period, is one of the main GHGs contributing to global climate change (IPCC, 2013). Rising concentrations also contribute to the destruction of the stratospheric ozone layer (Ravishankara et al., 2009). During the last century, atmospheric N_2O concentrations have increased by approximately 20% and are still increasing by 0.2-0.3% yr^{-1} (Thomson et al., 2012). Agriculture contributes over 40% of global N_2O emissions (Denman et al., 2007), with soil-based emissions in pastoral systems having a proportionately large impact. In Ireland, 32% of national GHG emissions originate from agriculture (Duffy et al., 2013) where the predominant system is pastoral based production from ruminant livestock (Breen et al., 2010). Grazing ruminant livestock deposit 75 – 90% of their nitrogen (N) intake onto pasture as dung and urine. These pasture, range and paddock (PRP) emissions comprise over 40% of the N_2O emitted from these production systems (Oenema et al., 2005). A typical urine patch has a surface area of 0.2 m^2 and receives 2 L of urine with an N rate of 10 g N L^{-1}, which corresponds to an N rate of 1000 kg N ha^{-1} (Haynes and Williams, 1993), although significant variation around these values is to be expected. The Irish national dairy herd of 1.08 million

cows deposits approximately 21.65 million litres of urine to Irish grassland soils on a daily basis (CSO, 2013, Duffy et al. 2014). This represents an N load to the soil of approximately 216.5 Mg day^{-1}. Between 50 and 90% of the urinary-N is in the form of urea (Doak, 1952; Bristow et al., 1992). Urea rapidly hydrolyses to ammonium (NH_4^+), and is then nitrified to nitrate (NO_3^-) which may be subsequently denitrified through a series of enzyme-catalysed, microbial processes. Nitrous oxide can be produced during both nitrification and denitrification processes, as well as nitrifier denitrification (Wrage et al., 2001, Zhu et al., 2013).

The N_2O emissions from urinary N vary widely with reported emission factors (E.F.) ranging between 0.3 (van der Weerden et al., 2011) and 13.3% (Kool et al., 2006a). On a national scale, the N_2O emissions from urine patches are estimated using a default EF value of 2% from the current Intergovernmental Panel on Climate Change (IPCC) guidelines (IPCC, 2006). An increase in ruminant numbers globally driven by a rise in demand for dairy and meat could lead to elevated levels of urine-derived N_2O. Therefore mitigation strategies are urgently needed (Oenema et al., 2005). Possible mitigation technologies can be divided into three categories: a) soil management, b) animal interventions, and c) animal breeding (de Klein and Eckard, 2008; Luo et al, 2010). Specific technologies include manipulation of NO_3^- availability, soil aeration, fertiliser management, effluent management, nitrification inhibitors, irrigation or drainage, reducing wet season grazing, altered diet, feed additives, and improving herd genetics.

Nitrogen intake is a principal driver of N losses from cattle (Dijkstra et al., 2013), thus optimizing N intake by animals is a strategy for mitigating N_2O losses associated with N deposition to pasture in dung and urine. Another strategy of interest is dietary amendment to

90 manipulate the composition and/or the partitioning of animal excreta with the major focus
91 being on urinary N, which is most vulnerable to losses. Hippuric acid (HA) concentration can
92 be manipulated by adjusting the protein content of cattle diets (Kreula et al., 1978, Dijkstra et
93 al., 2013). Kool et al. (2006a) found that increasing HA content of synthetic urine from 3% to
94 9% of total N decreased N_2O emissions from 7.2% to 4.5%. Similarly, the study of van
95 Groenigen et al. (2006) showed that increasing HA concentration in synthetic urine from 0.4
96 to 5.6 mM kg^{-1} of soil decreased N_2O by over 50%. In urine, HA breaks down to glycine and
97 benzoic acid (BA) (Bristow et al., 1992). The latter inhibit enzymes and general microbial
98 activity (Fenner et al., 2005) and these antimicrobial properties have led to the use of BA in
99 food preservation (Chipley, 1983). Microbial inhibition of BA is performed through
100 disrupting microbial cell membrane permeability which affects substrate transport and
101 oxidative phosphorylation from the electron transport system (Fresse et al., 1973, Brul and
102 Coote, 1999). Kool et al. (2006b) suggested that N_2O inhibition occurred in the presence of
103 BA. This hypothesis was confirmed by van Groenigen et al. (2006) who found that both HA
104 and BA inhibit denitrification and N_2O emissions. A study by Bertram et al. (2009) found a
105 65% reduction in N_2O emissions from real urine treatments with increased HA or BA
106 concentrations. Bertram et al. (2009) noted that both nitrification and denitrification were
107 affected by the treatments. Although the effect of HA and BA was confirmed in the
108 laboratory experiments of Kool et al. (2006a), van Groenigen et al. (2006), and Bertram et al.
109 (2009), the sole *in situ* study to date found no effect of HA and BA concentration on N_2O
110 emissions (Clough et al. 2009). It was argued that the environmental conditions during the
111 study did not favour N_2O loss due to low water-filled pore space (WFPS), on average 32%.
112 Furthermore, the authors pointed out that the lack of N_2O response to HA and BA may be
113 related to differences in soil pH, microbial communities, and the presence of vegetation. The

114 authors suggested a comprehensive *in situ* examination of the effect of HA and BA on N_2O
115 and microbial sub-populations.
116
117 In light of the conflicting results from previous lab studies (Kool et al., 2006a, van Groenigen
118 et al., 2006, Bertam et al., 2009,) and the single *in situ* field study (Clough et al., 2009), the
119 current experiment provides an *in situ* evaluation of the effects of HA and BA on N_2O
120 emissions from real urine applied to pasture. Timing of the experiment was chosen to
121 coincide with WFPS values conducive to denitrification and high N_2O fluxes (Dobbie and
122 Smith, 2001; Smith et al., 2003). The specific objectives of this study were: 1) to evaluate the
123 effect of incremental increases in HA, and an increase in BA concentration on urine N_2O
124 emissions, 2) to quantify potential reduction in N_2O emissions from urine as affected by BA
125 or HA composition, and 3) to assess the differences between HA and BA amended urine on
126 N_2O emissions.
127

128 **2. Materials and Methods**

129 *2.1 Site characteristics*

130 The present *in situ* experiment was conducted on a loam soil (13.9% clay, 33.2% silt, 52.9%
131 sand; N content 0.3%, C content 3.16%, organic C content 3.14%, pH 5.7) classified as a
132 Eutric Cambisol (FAO-Unesco, 1988) at the Teagasc Johnstown Castle Environmental
133 Research Centre, Co. Wexford, Ireland (52°18'N; 6° 30'W). Pasture at the study site
134 consisted of perennial ryegrass (*Lolium perenne* L.) reseeded in 2010, which had a history of
135 replacement stock grazing. Previous fertilisation consisted of a combination of urea and
136 calcium ammonium nitrate (CAN) at a mean rate of 84 kg N ha^{-1} yr^{-1} over the previous four
137 years. Animals were excluded from the experimental site for six months prior to the
138 beginning of the experiment with grass being harvested for silage in order to minimise

potential confounding effects of urine patches resulting from prior grazing. Grass was cut to a 5 cm height and allowed to regrow to a height of approximately 7-8 cm prior to commencement of the experiment. Rainfall, air and soil temperature were recorded at the meteorological station 1 km from the experimental site.

2.2 Treatments

Urine was collected directly from lactating Holstein-Friesian dairy cows which had been grazing at pasture. Urine was collected into 25 L containers, sealed to minimize N loss by volatilization, and refrigerated. The required volume of refrigerated urine for the experiment was homogenised by mixing in a 220 L barrel. This was sub-sampled for N content determination and then rapidly returned to 25 L drums, sealed, and refrigerated. The urinary-N was content was 4.5 g N L^{-1}. The N content was adjusted to approximately 8.0 g N L^{-1} by adding urea to the urine to approach the upper bound of urine-N content for dairy cows reported by Haynes and Williams (1993). Urine was amended to specific concentrations of HA or BA by spiking with either or both acids. A control urine treatment received no HA or BA addition. Urine was stored at 4°C, and for the two days prior to treatment applications urine temperature was increased to 30°C, the capacity of the available incubation facilities, prior to application to approximate *in vivo* urination, at body temperature. The experimental treatments associated with urine constituent concentrations are summarized in Table 1.

To verify HA and BA concentrations samples were collected at application. Two 30 mL sub-samples of urine from every treatment were taken. Urine samples were diluted 1:3 with high-performance liquid chromatography (HPLC) grade deionised water and one sub-sample was preserved by adding 1M H_2SO_4 to reduce the pH to 3 and the other sub-sample was preserved by adding 100 uL L^{-1} chloroform. Samples were labelled and stored at -20°C until analysis. The concentrations were determined using HPLC at the Agri Food Biosciences Institute,

Belfast. Urinary-N content was determined in a 1:500 dilution of urine samples on an Aquakem 600 discreet analyser (Cabrera and Beare, 1993).

Urine treatments were applied in the morning of 14th October 2013. The experimental design was a complete randomized block with six replicates. A volume of 2 L of urine was applied uniformly inside each 0.16 m^3 chamber equivalent to an N loading of approximately 1000 kg N ha^{-1} creating N_2O sampling urine patch. Paired with each N_2O sampling urine patch was an adjacent urine patch of a same size that was used for soil sampling. A 0.16 m^3 N_2O chamber collar was used as a template for urine application to the soil sampling plot. These paired soil sampling urine patches were used to measure soil pH, gravimetric water content and mineral N concentrations as noted below.

2.3 N_2O sampling and analysis

Nitrous oxide fluxes were measured on 19 occasions over ten weeks following application of treatments using the closed static chamber technique (Mosier, 1989; de Klein and Harvey, 2012). Square stainless steel collars for N_2O flux measurements with dimensions of 40 cm by 40 cm were inserted at a minimum depth of 5 cm into the soil before urine application. Stainless steel non-insulated, non-vented covers (10 cm high) were used to form a headspace chamber for measurement of N_2O, with a total headspace volume of approximately 16 L. Chamber collars were covered with a neoprene strip and a 10 L container filled with water was placed on top of chamber cover upon sampling to ensure airtight seal of the headspace. A 10 mL sample was drawn 40 minutes after chamber closure through a rubber septum (Becton Dickinson, UK) using a 10 mL polypropylene syringe (BD Plastipak, Becton Dickinson, UK) fitted with a hypodermic needle (BD Microlance 3, Becton Dickinson, UK). The 10 mL air sample was injected into a pre-evacuated (to -1000mbar) 7 mL screw-cap septum glass vials

188 (Labco, UK). The syringe was flushed once with ambient air before collecting sample from
189 the chamber. Eight samples of ambient air were collected on each gas sampling occasion and
190 were used as T_0 for the N_2O concentration for the flux calculations from the chambers.
191 Linearity of headspace N_2O concentrations was checked at each sampling occasion by
192 collecting five headspace samples per chamber from five various treatments throughout 60
193 minutes closed period (Chadwick et al., 2014). Nitrous oxide concentrations were analysed
194 using a gas chromatograph (GC) (Varian CP 3800 GC, Varian, USA) fitted with a ^{63}Ni
195 electron capture detector (ECD) with high purity helium as a carrier gas. Samples were
196 returned to ambient pressure immediately before analysis and fed into the system by a
197 Combi-Pal automatic sampler (CTC Analysis, Switzerland). The temperatures of column,
198 injector and detector were 60, 60 and 300 °C, respectively. The GC was calibrated daily and a
199 reference gas standard of known concentration was analysed every eight unknown samples.
200 Areas under N_2O peaks were integrated using Star Chromatography Workstation (Varian,
201 USA). Hourly N_2O emissions were calculated based on the rate of change in N_2O
202 concentration within the chamber during the measurement period. These emissions accounted
203 for air temperature, atmospheric pressure, and the ratio of surface area to chamber volume.
204 Samples were collected between 10 am and 12 am and therefore hourly N_2O flux was
205 assumed to be representative of the average hourly flux of the day and was subsequently used
206 to calculate daily emissions (Blackmer et al. 1982; de Klein et al. 2003). Cumulative
207 emissions were calculated by integrating the calculated daily fluxes and linear interpolation
208 between measurement points (de Klein and Harvey, 2012).
209
210 *2.4 Soil sampling and analysis*
211 Soil was sampled on 12 occasions over ten weeks following application of treatments. Three
212 replicate paired soil sampling patches were sampled for each treatment with a soil corer (3

cm diameter x 10 cm depth). The cores were placed in plastic sample bags, sealed, and placed in a coolbox for the transport into the laboratory. Samples were sieved using a 4 mm sieve and sub-samples were analysed for gravimetric moisture content, mineral N content, and pH. Gravimetric moisture content was determined by drying the samples for 24 h at 105°C. Volumetric soil moisture content was determined using Theta Probe (type ML2, Delta-T-Devices, UK) during each measurement in order to calculate WFPS (Maljanen et al., 2007). Mineral N was determined by extraction in 2 M KCl (20 g of fresh soil: 100 mL 2 M KCl, shaken for 1 h), the extracts were analysed for NH_4^+-N and total oxidised N (TON) which is a sum of nitrite (NO_2^--N) and NO_3^- by colorimetric analysis using an Aquakem 600 discrete analyser. Soil pH was determined in a 1:2 suspension of deionised water with a digital pH meter (In Lab Routine, Mettler Toledo) with glass and calomel electrodes.

2.5 Statistical analyses

Repeated measures ANOVA was conducted using proc MIXED procedure of SAS 9.3 (© 2002-2010, SAS Institute Inc., Cary, NC, USA) was used to output lsmeans by treatment and day for N_2O-N, soil mineral N: NH_4^+-N and TON, and soil pH. This was a blocked, two way factorial test with both day and treatment as factors. Daily N_2O-N flux was log-transformed log(flux + 10) prior to the test in order to overcome non normal distribution of data. Differences between treatments in terms of cumulative N_2O-N flux over the study period were determined using the proc MIXED procedure of SAS and the F-protected Least Significant Difference (L.S.D.) test. The F-protected L.S.D. test, which is a liberal test, was chosen to protect against Type I error i.e. to guard against incorrect rejection of a true null hypothesis.

3. Results

3.1 Environmental variables

The field site received 253 mm of rainfall during the 66 days of the experiment. Within two weeks of urine application, 155.3 mm rainfall was recorded, approximately 130% of the 30 year average rainfall for October. Approximately 50% of the rain fell within the first ten days post-application (Fig. 1). Soil temperature generally decreased throughout the field experiment, falling from a daily average of 14°C to below 7°C (Fig. 1). Between October and December there was approximately 9% more rainfall compared with the 30 year average and the soil temperature was on average 11% higher. The WFPS averaged 71.4% (S.E.M 1.3, n=342) for the experimental period and ranged between 60.1% and 80.3%.

3.2 N_2O fluxes

Nitrous oxide fluxes increased immediately following treatment application. The largest daily fluxes were observed on the day of application for all three amended urine treatments (Fig. 2). Throughout the experiment, fluxes from the control treatment ranged between 1.1 and 12.0 g N_2O-N ha^{-1} d^{-1} (Fig. 2). Mean daily N_2O fluxes from the urine treatments over the experimental period were greater ($P<0.05$) than fluxes from the control ranging from 20 to 702, 10 to 958, 19 to 1160, and 15 to 757 g N_2O-N ha^{-1} d^{-1} for the U, U+HA1, U+HA2, and U+BA treatments, respectively. Nitrous oxide fluxes decreased to control treatment values by Day 44 in the U+HA2 treatment and Day 51 in the U, U+HA1 and U+BA treatments. Effects of urine constituents manipulation were only seen on the day of urine application when fluxes from the U+HA1, U+HA2, and U+BA treatments were higher ($P<0.05$) than those from unadulterated urine. Fluxes of N_2O from the U, U+HA1, U+HA2, and U+BA treatments were not different throughout the remainder of the experiment.

Cumulative N_2O loss for the control treatment over the duration of the experiment was 0.3 kg N_2O-N ha^{-1} compared with 10.6, 9.2, 11.6, and 9.9 kg N_2O-N ha^{-1} for the U, U+HA1, U+HA2, U+BA treatments respectively (Fig. 3). Urine deposition increased cumulative N_2O emissions ($P<0.05$) compared to the control (Fig. 3), however, there were no effects of urine manipulation on cumulative N_2O emissions. The cumulative N_2O-N fluxes as a percentage of applied urine-N equated to 1.0(±0.13), 0.9(±0.16), 1.3(±0.28), and 0.9(±0.10) for the U, U+HA1, U+HA2, and U+BA treatments, respectively (S.E.M. in brackets).

Linearity analysis of N_2O data showed that 14% of all measured values represented no flux, and of valid fluxes 87% represented linear and 13% quadratic increase over time.

3.3 Soil inorganic N and soil pH

Mean soil NH_4^+-N concentrations in the control plots ranged between 3.1 and 22.5 kg N ha^{-1}. Concentrations of NH_4^+-N in the soil under urine treatments ranged from 9.4 to 443.5, 6.3 to 389.8, 4.6 to 325.2, and 5.2 to 385.9, kg N ha^{-1} for the U, U+HA1, U+HA2, and U+BA treatments, respectively. Soil NH_4^+-N concentrations were highest immediately following urine application and ranged between 325.2 and 443.5 kg ha^{-1} for both the U+HA2 and U treatments on Day 0. Soil NH_4^+-N levels from urine treatments were tenfold higher than those of the control until Day 18 and steadily declined over the experiment, returning to similar levels as in the control by Day 44. Higher ($P<0.05$) soil NH_4-N levels were observed in the U treatment on day 10 compared with the U+HA1 treatment, and on Day 0 compared with the U+HA2 treatment (Fig. 4a). There were no differences in soil NH_4^+-N concentrations between the U+HA1, U+HA2, and U+BA treatments throughout the experiment and concentrations declined at a similar rate for all urine treatments (Fig. 4a).

Concentrations of soil TON in the control were low throughout the measurement period with mean concentrations between 0 and 11.6 kg N ha^{-1} (Fig. 4b). As the NH_4^+-N pool in the urine

286 treatments nitrified, soil TON levels increased over time with the U+BA and U+HA1
287 treatments different (*P<0.05*) from the control from Day 15, the U treatment from Day 18
288 and the U+HA2 treatment on Day 24 before returning to levels found in the control by Day
289 66. Soil TON concentration in the U treatment was greater (*P<0.05*) than in the HA2
290 treatment between Days 51 and 58, and was also greater (*P<0.05*) in the U+BA than in the
291 U+HA1 and U+HA2 treatments on Day 58. Soil TON concentrations under the urine
292 treatments ranged from 5.8 to 102.6, 7.7 to 86.3, 3.0 to 66.4, and 4.0 to 104.3, kg N ha^{-1} for
293 the U, U+HA1, U+HA2, and U+BA treatments, respectively.

294 Soil pH for the control treatment ranged between 5.4 and 5.7 (0-10 cm) during the
295 experimental period (Fig. 5). Following application of urine treatments soil pH increased
296 (*P<0.01*) to between 6.4 and 6.8 (Fig. 5), after which soil pH declined over time. The rate of
297 decline in soil pH was very similar to the decreasing NH_4^+-N concentrations (Fig. 4a) during
298 the initial 16 days. On Day 18 soil pH values for the urine treatments dropped below the
299 control (*P<0.05*) (Fig. 5). On Day 37 soil pH values of the U+BA and U+HA1 treatments
300 were lower (*P<0.05*) than the control. However, by Day 44 there were no differences in soil
301 pH values between the urine treatments and the control.

302

303 **4. Discussion**

304 Urine deposition stimulates N_2O emissions and in the current experiment these fluxes spiked
305 rapidly following urine application. The largest N_2O fluxes, which accounted for between
306 13% (Urine) and 26% (Urine + HA1) of the cumulative emissions, were observed on the day
307 of urine application. This effect was also reported by Williams et al. (1999) who found the
308 highest N_2O fluxes occurred 6 hours following urine application. Maljanen et al. (2007) also
309 reported maximum N_2O emissions immediately after urine application in summer and

310 autumn. We hypothesise that an increase in water soluble carbon content, possibly due to the
311 lysing of microbial cell membranes in soil following urine application or soil aggregate
312 slaking could have been responsible for stimulating denitrification activity leading to high
313 N_2O emissions (Lambie et al., 2012). However, many studies either do not measure N_2O
314 emissions on the day of urine application, or only point out measurement frequency without
315 indicating the starting point (van Groenigen et al., 2005, Clough et al., 2009, van der Weeden
316 et al., 2011). Wachendorf et al. (2008) suggested that the maximum N_2O emissions might
317 have been missed in their study as a result of long intervals between gas sampling. Finding
318 the highest N_2O flux on the day of urine application underpins the importance of gas
319 measurement immediately following treatment application.

320 Fluxes of N_2O remained elevated in the current study for a minimum of 44 days which is
321 comparable with other studies that have found emissions elevated for 30-70 days (Allen et al.,
322 1997; van Groenigen et al., 2005; van der Weerden et al., 2011). Other studies, however,
323 report longer emission periods, with de Klein et al. (2003) reporting that four months after
324 urine application the N_2O fluxes were still significantly higher than background levels. It is
325 possible that in this study, carried out on a well-draining loam soil, leaching was a significant
326 N loss pathway (Clough et al., 1998). The findings of this study are in agreement, however,
327 with the work of Selbie et al. (2014) that was conducted on a sandy loam soil from the same
328 location as the current experiment. The emission factors for the urine treatments over the 66
329 day period were comparable to previously reported values of 0.0% to 2.3% following urine
330 application in autumn-winter and spring-summer (Allen et al., 1996), 0.3 to 0.9% over 103
331 days (van Groenigen et al., 2005), and 0.29% over 125-133 days following autumn
332 application and for 166-173 days following spring application (van der Weerden et al., 2011).
333 Other studies reported emission factors of 0.3 to 2.5% over 80, 150, and 182 days from the
334 Waikato organic, the Waikato mineral and the Otago soil, respectively (de Klein et al., 2003),

335 or <1% over 150 days (Clough et al. 1996) and <2% over 406 days (Clough et al., 1998).
336 Previous studies found in Ireland reported emission factors below 0.4% over 80 days in year
337 one and 360 days in year two (Selbie et al., 2014). These reported emission factors are
338 substantially lower than the IPCC EF of 2% per annum (IPCC, 2006).

339 Simulation of dietary manipulation of ruminant urine by adding HA or BA had no significant
340 effect on N_2O emissions under conditions of high WFPS (60 - 80%) favouring loss via
341 denitrification. Similarly, Clough et al. (2009), the only *in situ* experiment to date to examine
342 the effect of urine HA and BA addition, also reported no effect of HA and BA manipulation
343 on N_2O emission. However, Clough et al. (2009) stated that their results were potentially
344 inconclusive because of low WFPS (<35%) and high soil pH (>6.4) throughout the
345 experiment. Antimicrobial activity of BA is only effective in acidic conditions, as the
346 undissociated molecule is responsible for antimicrobial activity (Chipley, 1983). Both HA
347 and BA are weak organic acids with pKa of 3.62 and 4.19, respectively, therefore over 50%
348 of these compounds would have been present in their dissociated form in this study. In basic
349 conditions, BA dissociates to the benzoate form, which is not as toxic to bacteria as its acid
350 form. After the initial rise following urine application, soil pH at 0-10 cm in this experiment
351 oscillated around pH 5, which should allow for some of the compound to remain in the
352 undissociated acid form and to exhibit its antimicrobial properties. However this effect was
353 not seen and it can be speculated that microbial community was not affected by the
354 compounds. Additionally, the dissociated forms of HA and BA carry a negative charge and,
355 as such, are liable to leaching.

356 All four urine treatments produced a similar pattern of N_2O emissions, although differences
357 in N_2O emission were observed on the day of application, where the U+HA2 treatment
358 emitted more N_2O than other urine treatments ($P<0.05$) . However, increased concentrations
359 of HA or BA consistently resulted in a small, but not significant elevation rather than

reduction of N_2O emissions. It had been hypothesised by van Groenigen et al. (2006) and Bertram et al. (2009) that 96 mM HA and 50 mM BA would mitigate N_2O loss, but this was not observed in the current study. Effects of urine composition on N_2O emissions were estimated using a liberal statistical testing but even this approach revealed no significant reduction in emissions due to amending HA and BA concentrations. Overall, HA or BA addition in this study had no *in situ* effect on cumulative N_2O emissions.

Previous studies reporting HA or BA mitigation effects were conducted in laboratory conditions on soil cores with no vegetation present (Bertram et al., 2009; van Groenigen et al., 2006). Mechanical disturbance to soil, such as sieving and drying, can alter microbial community size, structure and function (Garcia-Orenes et al., 2013). The studies of both van Groenigen et al. (2006) (sods from underneath grass cover) and Bertram et al. (2009) (sieved soil packed into cores) used disturbed soil communities. This could have subsequently affected residence and metabolism of HA and BA in soil in these studies. Therefore microbial activity responsible for degradation of HA/BA could have been higher in the *in situ* experiment resulting in rapid decrease of HA/BA concentrations. Moreover, laboratory experiments are performed in controlled conditions and in some cases on homogenised soils, therefore greatly reducing spatial variability associated with *in situ* experimentation, and making it more likely to confirm treatment effects. Finally, laboratory studies do not simulate leaching which can be a significant loss pathway, especially in sandy textured, well drained soils (Clough et al., 1998).

Benzoic acid naturally occurs in the soil through root exudates (Bertin et al., 2003) and can be broken down by soil bacteria (Siciliano and Germida, 1998; Deubel et al., 2000). Clough et al. (2009) hypothesised that if the pasture species used in their study exuded BA through root rhizo-deposition, then the soil microbial community could have been pre-dosed to this compound and its toxicity subsequently reduced. Therefore BA could have been degraded

385 rapidly after urine application. Furthermore, a large proportion of microbial biomass in
386 temperate soils consists of fungi (Ruzicka et al., 2000), especially in permanent grasslands
387 (Frey et al., 1999). A previous study conducted on Irish grassland observed that soil mineral
388 N transformations and denitrification were dominated by fungi (Laughlin & Stevens 2002). It
389 is possible that BA does not affect fungal denitrification and thus would not affect soils
390 where fungi-derived N_2O emissions predominate.

391 In the current experiment, urine treatments were applied to pasture, directly simulating
392 deposition of excreta by grazing ruminants. Our results lend weight to the lack of *in situ* N_2O
393 mitigation potential of both HA and BA observed by Clough et al. (2009). This study
394 speculated that the lack of observed effect in their experiment may have been due to
395 conditions which did not favour N_2O loss i.e. low WFPS (18 - 51%) and pH between 6.4 and
396 8.5. These conditions do not encourage denitrification and antimicrobial activity of BA
397 occurring predominantly in acidic environment, therefore could have served as a possible
398 explanation for low emissions and uniform response to treatments. The current study
399 addresses the knowledge gap of N_2O mitigation potential under environmental conditions
400 which were favourable for N_2O loss i.e. high WFPS (60 - 80%) (Smith et al., 2003), and
401 where the antimicrobial activity of BA is viable i.e. acidic pH (Chipley, 1983).

402 The range of concentrations of HA and BA used in this work was comparable to that of
403 previous studies. However, the infiltration depth of the urine solutions applied to the soil
404 surface differs hugely. Previous laboratory studies used cores with soil depths of 5 cm and 6
405 cm for van Groenigen et al. (2006) and Bertram et al. (2009), respectively. Clough et al.
406 (2009) assumed urine movement to a depth of 10 cm, but the study of Monaghan et al. (1999)
407 demonstrated the potential of urine to infiltrate further. In their case, 68% of urine moved
408 below 20 cm within 6 hours of application. Additionally, Pakrou and Dillon (1995) found that
409 rainfall promoted downward movement of urine by macropore flow causing urine to infiltrate

410 to a depth of 30 cm in their experiment. Williams and Haynes (1994) reported an irregular
411 pattern of urine infiltration to a maximum depth of 40 cm. These reports suggest that the
412 effective concentration of both compounds in urine affected soil could have reduced
413 dramatically under *in situ* conditions compared with the cores used in the lab experiments.
414 Little is known about the fate and longevity of these compounds in soils. Clough et al. (2009)
415 hypothesised that the efficacies of the HA/BA could have been reduced due to preferential
416 leaching of the acids away from urine. Selbie et al. (2013) hypothesised that the fate of minor
417 urine constituents such as HA/BA is likely to be similar to that of urea, but with some delay,
418 probably due to a longer decomposition process. The effective threshold concentration of BA
419 for inhibition of N_2O producing microorganisms is unclear. It is commonly determined based
420 on factors including pH, temperature, genus and species of the microorganisms in question
421 (Chipley, 1983). However, if higher concentrations of HA or BA in urine are needed to
422 mitigate N_2O emissions, these could be difficult to achieve due to the low solubility of both
423 compounds. Solubility of HA and BA in cold water is 22 mM and 25 mM, respectively
424 (Fischer Scientific, 2014), and is higher in urine (McGeough, personal communication). The
425 mean concentration of HA in unaltered ruminant urine found by Kreula et al. (1978) was 66
426 mM, whereas concentrations of both HA and BA in previous studies were amended to a
427 maximum of 96 mM and 50 mM, respectively (Bertram et al., 2009). In this experiment, the
428 highest concentrations for HA and BA were 82 mM and 96 mM, respectively, which for HA
429 is comparable to previous studies, and for BA is the highest achieved concentration to date. It
430 was not possible to achieve higher concentrations in natural urine collected from dairy cows.

431 Mineral N in urine-affected soils followed established patterns over time (Wachendorf et al.,
432 2008). Ammonium concentrations were highest immediately after urine application, which is
433 consistent with hydrolisation of urea, and declined at a similar rate regardless of urine
434 treatment, whereas TON concentrations increased over time which is consistent with

435 nitrification of ammonium. As nitrification occurs there is a corresponding decline in soil pH
436 which is consistent with the release of free H^+ ions during nitrification (Whitehead, 1995;
437 Cookson and Cornforth, 2002; Clough et al., 2009). Contrary to Bertram et al. (2009),
438 mineral N dynamics were unaffected by the composition of the applied urine.

439 Although simulation of dietary manipulation to reduce N_2O emissions through altering
440 individual urine constituents appears to have no effect, there may be other urine
441 manipulations, such as reducing N content or inclusion of novel inhibitory products that
442 warrant further investigation.

443

444 **5. Conclusions**

445 N_2O loss from urine deposition on pasture was measured under conditions favourable for
446 both denitrification and the antimicrobial activity of benzoic acid. N_2O fluxes were observed
447 to increase immediately post-urine application, possibly due to mobilisation of labile carbon
448 pools in the soil. Despite this, the cumulative N_2O emission factors over the experimental
449 period ranged from 0.9 – 1.3%, substantially below the 2% IPCC default emission factor.
450 Simulating manipulation of ruminant urine by adding incremental levels of hippuric and/or
451 benzoic acids had no effect on N_2O emissions indicating that these acids do not affect
452 denitrification *in situ*. Similarly, no treatment effect on nitrification was observed as soil
453 mineral N following application of urine was not affected. Although promising N_2O loss
454 mitigation responses to increasing levels of benzoic and hippuric acid in urine were observed
455 in the lab these acids are not effective at mitigation N_2O loss *in situ*.

456

457

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631

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