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

D.J. Krol *, P.J. Forrestal, G.J. Lanigan, K.G. Richards

*Teagasc, Johnstown Castle Environmental Research Centre, Wexford, Ireland*

## HIGHLIGHTS

- $N_2O$ emissions on day of urine application ranged 13–26% of total $N_2O$ loss.
- Hippuric and benzoic acids do not reduce $N_2O$ in situ under high WFPS conditions.
- $N_2O$ urine emission factor ranged 0.9–1.3% over 66 days.

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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 acid 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.



## 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., 2014) 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

* Corresponding author.
*E-mail address:* dominika.krol@teagasc.ie (D.J. Krol).

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

In light of the conflicting results from previous lab studies (Kool et al., 2006a; van Groenigen et al., 2006; Bertram et al., 2009) and the single in situ field study (Clough et al., 2009), the current experiment provides an in situ evaluation of the effects of HA and BA on $N_2O$ emissions from real urine applied to pasture. Timing of the experiment was chosen to coincide with WFPS values conducive to denitrification and high $N_2O$ fluxes (Dobbie and Smith, 2001; Smith et al., 2003). The specific objectives of this study were: 1) to evaluate the effect of incremental increases in HA, and an increase in BA concentration on urine $N_2O$ emissions, 2) to quantify potential reduction in $N_2O$ emissions from urine as affected by BA or HA composition, and 3) to assess the differences between HA and BA amended urine on $N_2O$ emissions.

## 2. Materials and methods

### 2.1. Site characteristics

The present in situ experiment was conducted on a loam soil (13.9% clay, 33.2% silt, 52.9% sand; N content 0.3%, C content 3.16%, organic C content 3.14%, pH 5.7) classified as a Eutric Cambisol (FAO-Unesco, 1988) at the Teagasc Johnstown Castle Environmental Research Centre, Co. Wexford, Ireland (52°18′N; 6°30′W). Pasture at the study site consisted of perennial ryegrass (*Lolium perenne* L.) reseeded in 2010, which had a history of replacement stock grazing. Previous fertilisation consisted of a combination of urea and calcium ammonium nitrate (CAN) at a mean rate of 84 kg N $ha^{-1}$ $yr^{-1}$ over the previous four years. Animals were excluded from the experimental site for six months prior to the 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 minimise 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 1 M $H_2SO_4$ to reduce the pH to 3 and the other sub-sample was preserved by adding 100 μL $L^{-1}$ 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