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## Sheep urine patch N<sub>2</sub>O emissions are lower from extensively-managed than intensively-managed grasslands



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### ABSTRACT

A large number of sheep graze extensively managed grasslands, including upland and hill areas. Excretal deposition of nitrogen (N) to upland soil is a potentially large source of the powerful greenhouse gas (GHG), nitrous oxide (N<sub>2</sub>O), however, few studies have assessed urine-patch N<sub>2</sub>O emissions from upland areas. Current default excretal N<sub>2</sub>O emission factors (EFs) are based on intensively managed lowland systems, with cattle excreta as the N source. We hypothesised that N<sub>2</sub>O emissions could differ substantially from those of lowland systems, due to differences in soil type, climate, topography, pasture composition and management factors along altitudinal and productivity gradients. We investigated N<sub>2</sub>O emission factors across two seasons (spring and autumn), for an extensive semi-improved, temperate grassland using IPCC-compliant and representative sheep urine patches (in terms of urine chemical composition, urine patch size and N loading rates). An automated GHG monitoring system provided high-frequency GHG data from sheep urine patches (756 and 1112 kg N ha<sup>-1</sup> applied in spring and autumn, respectively), reference artificial sheep urine patches (1066 and 1004 kg N ha<sup>-1</sup> applied in spring and autumn, respectively) and control treatments. In spring, urine patch N<sub>2</sub>O emission factors were  $-0.02 \pm 0.04$  (artificial sheep urine) and  $0.03 \pm 0.09\%$  (real sheep urine) of the applied N; in autumn emission factors were  $0.02 \pm 0.03$  (artificial sheep urine) and  $0.08 \pm 0.04\%$  (real sheep urine) of the applied N. These values are much lower than default inventory values (1% of applied N) for excreta deposited by grazing livestock. There was a greater pasture foliar N content following urine application in spring as opposed to autumn, and a significantly longer residence time of extractable mineral N in autumn. Our findings demonstrate the importance of generating country-specific N<sub>2</sub>O EFs based on altitude/productivity gradients of livestock production, with implications for national inventories and the accuracy of sustainability metrics of lamb produced in the UK uplands.

### 1. Introduction

There are over 87 million sheep in the EU (Eurostat, 2017), many of which graze land classed as ‘Less Favourable Areas’ (LFA), under EC Directive 75/276. These areas support rural economies and the provision of ecosystem services, largely representing farms situated on mountainous terrain, under poor production conditions (e.g. acidic soils, sloping land, high rainfall, cool climate and short growing season). The large numbers of livestock grazing on hill land globally, has been identified as a potentially large source of the greenhouse gas (GHG), nitrous oxide (N<sub>2</sub>O), via excretal deposition of nitrogen (N) to the soil (Luo et al., 2013). With a radiative forcing of ca. 296 times that of CO<sub>2</sub> and its contribution to the depletion of stratospheric ozone (Ravishankara et al., 2009), N<sub>2</sub>O is an important GHG associated with livestock production. Consumers of livestock products are becoming increasingly environmentally and ethically aware (Cantalapiedra-Hijar

et al., 2016; Porqueddu et al., 2017), yet the environmental implications of contrasting livestock production systems (e.g. intensive vs. extensive grasslands) are not clearly differentiated in terms of their GHG emissions.

The default value of 1% of excretal N deposited by grazing sheep emitted as N<sub>2</sub>O (IPCC, 2006), has recently been lowered to a UK country-specific value of 0.44% (Brown et al., 2017) based on new data from nation-wide field experiments (Barneze et al., 2015; Chadwick et al., 2018; Misselbrook et al., 2014). Assuming a 60%–40% split in excretion of N to urine and faeces respectively (Webb and Misselbrook, 2004), the urine only emission factor is closer to 0.69% (Chadwick et al., 2018). Although the country-specific value is an improvement in accuracy upon the default value, it is still derived from cattle excreta derived from livestock fed on lowland pasture and applied to intensively managed lowland pastures. These figures, therefore, do not take account of the potential variation in emissions which could occur

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due to differences in climate, soil, vegetation, topography and management factors (e.g. stocking density) along altitudinal and productivity gradients associated with hill grazing systems, nor does it apply specifically to sheep. The climate is generally cooler and wetter in the uplands, and inputs of N only occur via atmospheric deposition and livestock excreta. Extensively managed grassland soils are typically more acidic, contain greater amounts of organic matter compared to more intensively managed systems and have lower rates of mineralisation and nitrification (Williams et al., 1999).

Most studies which have measured N<sub>2</sub>O emissions from extensive grasslands have typically used synthetic sheep urine, or urine collected from lowland diets, presumably due to difficulties in collecting urine from sheep fed upland diets (e.g. Shand et al., 2002; Hoogendoorn et al., 2008). This does not take into account potential differences in the nitrogenous composition of sheep urine fed an upland diet, yet the composition of urine has been shown to alter N cycling and potential N<sub>2</sub>O emissions (Dijkstra et al., 2013; Luo et al., 2015). In addition, few studies use site-specific urine volumes and urine patch sizes, as reported in the meta-analysis of Selbie et al. (2015). Luo et al. (2013) found N<sub>2</sub>O emissions from sheep urine deposited to New Zealand hill land of low and medium slopes were lower than the current Intergovernmental Panel on Climate Change (IPCC) default value and suggested disaggregation of emissions based on slope class. Urine patch emission factors could also be disaggregated by grazing areas differing in primary productivity, yet limited data exist to underpin such an approach (Hopkins and Lobley, 2009).

In upland soils, low rates of nitrification following urea hydrolysis have been demonstrated following synthetic sheep urine addition (Thomas et al., 1988; Williams et al., 1999; Shand et al., 2002). Mahmood and Prosser (2006) reported a lag phase in nitrate production following synthetic sheep urine addition to upland soil microcosms. This was attributed to the structure of the initial ammonia oxidising microbial community in the extensively managed soil, with a lag phase being absent in intensively managed soils, due to a greater abundance of ammonia oxidisers (Webster et al., 2005). Reduced nitrification rates in upland soils could result in low N<sub>2</sub>O emissions from the urine patch, both from the process of nitrification itself and via reduced production of the substrate (NO<sub>3</sub><sup>-</sup>) for denitrification.

Here, we provide IPCC-compliant year-round measurements of N<sub>2</sub>O fluxes (including high frequency data during the period with the highest likelihood of N<sub>2</sub>O loss) from sheep urine applied to a semi-improved, extensively grazed (upland) grassland, under two contrasting periods of the grazing season. To best reflect emissions from such areas, sheep urine representative in chemical composition, volume and patch size were used. We hypothesise that due to low rates of nitrification, emissions from these areas will be lower than the N<sub>2</sub>O EF of approximately 0.69% for urine-N applied in the lowlands (Chadwick et al., 2018), indicating that emissions from such areas are currently over-estimated. Our data represent the first high frequency N<sub>2</sub>O data from sheep urine deposited to European hill grazing systems, which can contribute to national inventory N<sub>2</sub>O emission estimates, aid the development of more accurate sustainability metrics for upland lamb production (e.g. life cycle assessment and carbon footprint) and inform evidence-based policy decision making for the future of upland land management.

## 2. Materials and methods

### 2.1. Study site

The study took place on an enclosed, semi-improved, upland grassland at the Henfaes Research Station, Abergwyngregyn, North Wales (270 m a.s.l.; 53°13'N, 4°0'W; Fig. S1). The field (11.5 ha) has been managed under Welsh Government agri-environment schemes for 10 years (previously Tir Gofal and currently Glastir), with low input grazed pasture and mechanical bracken control options. The field is

normally stocked at a density of approximately 4 ewes ha<sup>-1</sup> (ca. 0.32 Livestock Units ha<sup>-1</sup>), and has not been fertilised, limed or re-seeded in over 30 years. The soil is classified as an Orthic Podzol (FAO, 1981; Fig. S2), with the pasture comprising a mosaic of British NVC classifications U4 (*Festuca ovina* - *Agrostis capillaris* - *Galium saxatile* grassland) and M56 (*Lolium perenne* - *Cynosurus cristatus* grassland) (Rodwell, 2000).

Two experimental areas were established, one to receive a composite spring urine application in June, 2016, and another to receive a composite autumn urine application in October, 2016, hereafter referred to as spring and autumn, respectively. Both sites were situated on a slope of 13% and livestock were removed from all plots at least 3 months prior to treatment application. Treatments consisted of i) control (no urine), ii) artificial sheep urine, and iii) real sheep urine ( $n = 4$  for each treatment), laid out in a randomised block design. The vegetation in the plots were cut to a standard height (ca. 5 cm) 1 week prior to treatment application.

A meteorological station was installed at the experimental site (Skye Instruments Ltd., Llandrindod Wells, UK), recording weather data at half-hourly intervals. Missing data were gap-filled with meteorological data from the nearby COSMOS facility (Evans et al., 2016). The soil moisture probes ( $n = 2$ ; 10HS Moisture Sensor, Decagon Devices Inc., WA, USA) were calibrated for volumetric water content according to manufacturer's instructions (Cobos and Chambers, 2010), as outlined in Starr and Paltineanu (2002), with field soil packed to field bulk density values (0.65 g cm<sup>-3</sup>; mean of  $n = 8$  measurements). Total pore space (cm<sup>3</sup> cm<sup>-3</sup>) in the soil was calculated from the bulk density and the assumption of a particle density of 2.65 g cm<sup>-3</sup> for the mineral fraction and 1.4 g cm<sup>-3</sup> for the organic fraction (Rowell, 1994). Soil water-filled pore space (WFPS) was then calculated as a ratio of volumetric water content to soil porosity.

### 2.2. Soil characteristics

Soil characteristics ( $n = 4$ ; 0–10 cm; Table 1) of both study areas (spring, sampled on 08/06/16 and autumn, sampled on 29/09/16) were determined. Bulk density (0–5 cm) was determined by inserting 100 cm<sup>3</sup> metal rings into the soil, and removing the intact core. Cores were oven-dried (105 °C) and sieved (< 2 mm) to remove and weigh stones, correcting soil bulk density values for stone weight and volume. Gravimetric soil moisture was determined by drying soil in an oven

**Table 1**  
Characteristics of the Orthic Podzol ( $n = 4$ ; 0–10 cm) used in spring (treatments applied on 14/06/16) and autumn (treatments applied on 04/10/16) field studies. Results are expressed on a dry soil weight basis, as means  $\pm$  SEM, and letters denoting significant differences (two-sample t-test) between seasons of application.

Orthic Podzol properties	Spring	Autumn
Bulk density (g cm <sup>-3</sup> ) <sup>a</sup>	0.67 $\pm$ 0.03	0.64 $\pm$ 0.02
Gravimetric moisture content (%)	42.0 $\pm$ 3.4 a	63.7 $\pm$ 0.8 b
Organic matter (%)	16.9 $\pm$ 0.8	15.3 $\pm$ 0.6
pH	5.09 $\pm$ 0.08	4.98 $\pm$ 0.02
EC ( $\mu$ S cm <sup>-1</sup> )	38 $\pm$ 6 a	101 $\pm$ 7 b
Total C (g C kg <sup>-1</sup> )	81.2 $\pm$ 4.6	73.3 $\pm$ 7.0
Total N (g N kg <sup>-1</sup> )	6.1 $\pm$ 0.3	5.8 $\pm$ 0.5
C:N ratio	13.3 $\pm$ 0.2	12.6 $\pm$ 0.2
N mineralisation rate (mg N kg <sup>-1</sup> day <sup>-1</sup> )	30.0 $\pm$ 2.2	29.1 $\pm$ 1.7
Dissolved organic C (mg C kg <sup>-1</sup> )	352 $\pm$ 29.2	327 $\pm$ 9.84
Total dissolved N (mg N kg <sup>-1</sup> )	64.7 $\pm$ 4.7 b	51.5 $\pm$ 2.0 a
Microbial biomass C (g C kg <sup>-1</sup> )	2.61 $\pm$ 0.37	2.77 $\pm$ 0.08
Microbial biomass N (mg N kg <sup>-1</sup> )	402 $\pm$ 45	409 $\pm$ 5
Extractable NO <sub>3</sub> <sup>-</sup> (mg N kg <sup>-1</sup> )	8.48 $\pm$ 1.31	5.27 $\pm$ 0.81
Extractable NH <sub>4</sub> <sup>+</sup> (mg N kg <sup>-1</sup> )	18.9 $\pm$ 0.2 b	14.0 $\pm$ 1.0 a
Extractable P (mg P kg <sup>-1</sup> )	2.92 $\pm$ 1.43	0.97 $\pm$ 0.06
Exchangeable Na (mg kg <sup>-1</sup> )	19.8 $\pm$ 2.6 a	91.6 $\pm$ 12.3 b
Exchangeable K (mg kg <sup>-1</sup> )	14.9 $\pm$ 3.0 a	39.4 $\pm$ 8.4 b
Exchangeable Ca (mg kg <sup>-1</sup> )	231 $\pm$ 42	328 $\pm$ 86

<sup>a</sup> 0–5 cm.

(105 °C; 24 h) and organic matter was determined according to Ball (1964), by calculating the loss-on-ignition in a muffle furnace (450 °C; 16 h). Soil pH and electrical conductivity (EC) were determined on 1:2.5 (w/v) soil:distilled water suspensions (briefly shaken, and allowed to settle) using standard electrodes. Oven-dried and ground soils were used to determine the total soil C and N content and C:N ratio, on a TruSpec<sup>®</sup> Analyzer (Leco Corp., St. Joseph, MI). The N mineralisation rate of the upland soil was determined according to Waring and Bremner (1964), where soil NH<sub>4</sub><sup>+</sup> concentrations were measured by the method of Mulvaney (1996), before and after anaerobic incubation (40 °C; 7 d), on 1.0 M KCl soil extracts (1:10 w/v, soil: solution). Soils were also extracted using 0.5 M K<sub>2</sub>SO<sub>4</sub> (1:5 w/v, soil: solution), and the resulting extracts were analysed for NH<sub>4</sub><sup>+</sup>, as described previously, and NO<sub>3</sub><sup>-</sup> via the method of Miranda et al. (2001). The CHCl<sub>3</sub>-fumigation-extraction procedure of Voroney et al. (2008) was conducted, and the 0.5 M K<sub>2</sub>SO<sub>4</sub> extracts (before and after CHCl<sub>3</sub> fumigation) were measured on a Multi N/C 2100S analyser (AnalytikJena, Jena, Germany) to determine extractable soil dissolved organic C, total dissolved N and microbial biomass C and N (using  $K_{EC}$  and  $K_{EN}$  correction factors of 0.35 and 0.5, respectively). Soils were extracted with 1.0 M CH<sub>3</sub>COOH (1:5 w/v, soil: solution) to determine available P and exchangeable cations (Na, K and Ca). Extractable P was measured via the method of Murphy and Riley (1962) and cations were measured using a Sherwood Model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK).

To characterise the microbial community structure, soil was collected ( $n = 5$ ) from non-urine treated areas of the experimental plots and frozen at -80 °C. The soil microbial community was characterised via phospholipid fatty acid (PLFA) analysis following high throughput PLFA extraction, according to Buyer and Sasser (2012).

### 2.3. Sheep urine treatments, chemical composition and application

To apply a consistent treatment between both the spring and autumn experiments, an artificial sheep urine treatment was included, as the composition of the real sheep urine collected at different times of the year was an uncontrollable variable in terms of total N and other chemical constituents. Artificial sheep urine was made up according to that of Lucas and Jones (2006), but modified by increasing the urea content of the urine to achieve 6 g N l<sup>-1</sup>. This was conducted to achieve an N concentration close to the mid-point of urine N concentrations reported for sheep and cattle (2–12 g N l<sup>-1</sup>; Selbie et al., 2015). Real sheep urine was collected from Welsh Mountain ewes ( $n = 6$ ), using urine collection pens (Fig. S3) described in Marsden et al. (2017), approved by Bangor University's College of Natural Sciences Ethics Committee (Ethics approval code CNS2016DC01). This breed of sheep was chosen as it is representative of the breed utilised for grazing within the study area.

The sheep were allowed to graze (ad libitum) the same vegetation present in the experimental plots, at a similar time of year to both treatment application dates: 23/05/16 to 01/06/16 and 05/09/16 to 16/09/16 for spring and autumn, respectively. The sheep urine treatment comprised a bulked sample of all individual urine events collected from each seasonal urine collection trial ( $n = 56$  events for spring and  $n = 43$  events for autumn). During urine collection the volumes of individual urine events were recorded. The mean urine volume from each seasonal urine collection trial was used to determine the volume of urine to apply to the plots in each season. The area of soil to apply the urine volume to (i.e. the urine patch size) was determined by applying the seasonal mean volumes ( $n = 3$ ; for both spring and autumn) as water containing Brilliant Blue dye to the soil (Fig. S4), overlaying a sheet of acetate, and tracing the extent of the simulated urine patch 'wetted area' with a marker pen. This approach was taken to ensure that urine of a homogenous composition would be applied to the plots and that the urine would be representative in chemical composition, and the urine volume and patch size were specific to the season of application and the soil/vegetation types under study.

Prior to application to the field, each urine type was mixed thoroughly in a large container and subsequently bottled (into individual 'patches') for field application. Plots (1.5 m by 1.5 m) consisted of a chamber for monitoring GHG emissions, where one urine patch (real or artificial) was applied to the centre of each chamber (see Figs. S5 and S6 for details of experimental and plot layouts). In spring, an additional 9 urine patches (real or artificial) were applied in the area of soil surrounding the chambers, to provide enough patches to conduct routine soil sampling for a full year following application. In autumn, 9 artificial urine patches were applied to the soil around the chambers, however, only 7 urine patches were applied to the soil around the real urine treatment chambers. This was due to a larger volume applied for the real urine treatment in autumn, resulting in a greater patch size and a greater area for soil sampling (thus requiring fewer additional urine patches).

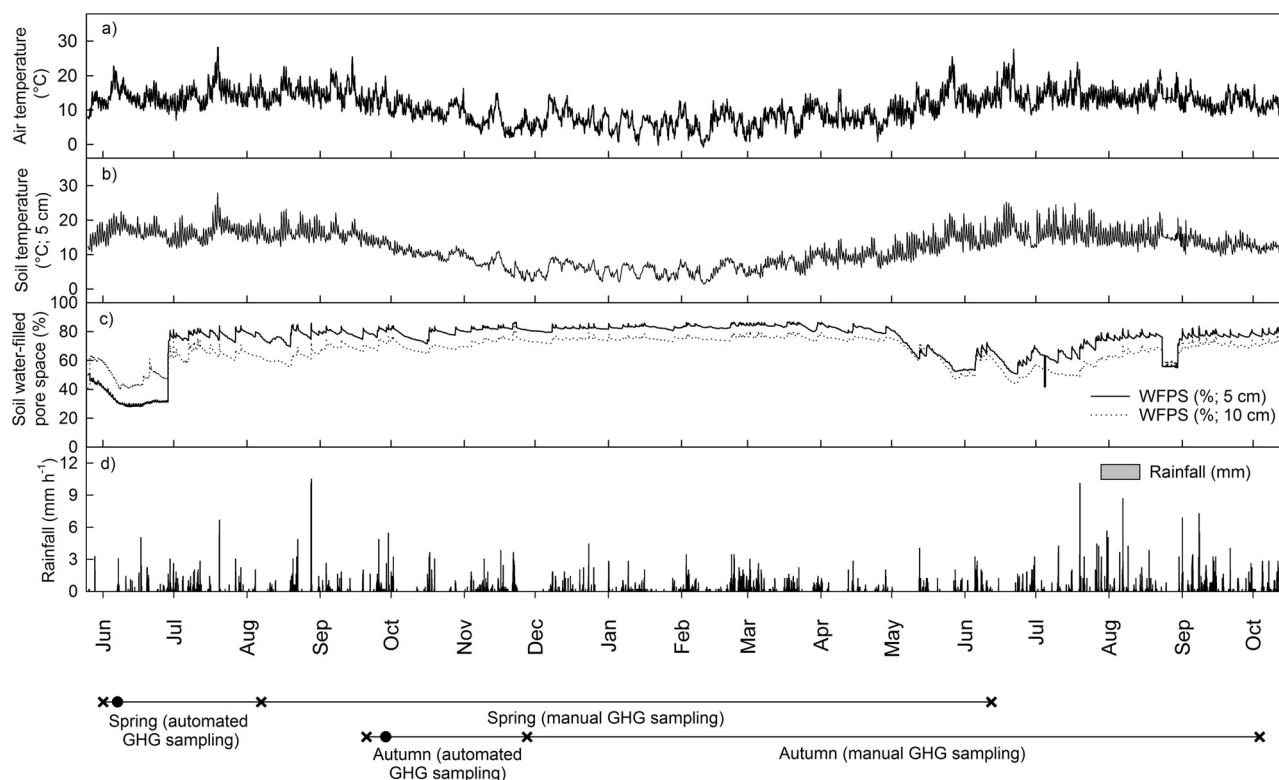
The total urine N and C was determined on the Multi N/C 2100S analyser, where a subsample of the urine from each bottle was analysed, resulting in  $n = 40$  measurements for all urine types, except the autumn real urine, which had  $n = 28$ . The pH and EC were measured on a subsample of the bulked urine treatments (real and artificial), using standard electrodes. Urea, NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> were determined on three analytical replicates of each bulked urine type. Urea content was measured via the method of Orsenneau et al. (1992), and the NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> were measured as described previously. Real sheep urine samples were analysed for allantoin, creatinine, uric acid, hippuric acid and benzoic acid using a Varian Pro Star 310 HPLC system (Varian Inc., Palo Alto, CA) using a C18 HyperClone<sup>®</sup> 5 μm 12 nm ODS column (250 × 4.6 mm) column (Phenomenex Inc., Cheshire, UK). Briefly, the variable wavelength detection was set at 218 nm, with a flow rate of 0.7 ml min<sup>-1</sup>, pumping mobile phase A (KH<sub>2</sub>PO<sub>4</sub>; 17 g l<sup>-1</sup>; adjusted to pH 4) or mobile phase B (60% mobile phase A and 40% HPLC-grade methanol). Urine samples were diluted in mobile phase A as necessary, prior to analysis.

### 2.4. Monitoring urine patch GHG emissions

A mobile automated GHG monitoring system (Queensland University of Technology, Institute for Future Environments, Brisbane, Australia), as previously described in Marsden et al. (2016), was used to monitor emissions from the urine patch and control treatments (Fig. S7). Stainless steel chamber bases (0.25 m<sup>2</sup> basal area) were dug into the plots (10 cm depth) four weeks prior to treatment application in both spring and autumn and chambers (50 cm × 50 cm × 15 cm) were clipped onto the bases. Emissions were monitored for one week prior to treatment application and nine weeks following treatment application with the automated system, where emissions were expected to be highest. After this period, slightly smaller chambers (40 cm × 40 cm × 20 cm) were inserted inside the previous chamber area. These chambers were then used to complete monthly manual gas sampling, using the static chamber technique, to provide year-round measurements of N<sub>2</sub>O emissions (Fig. S8).

In the automated system, chamber headspace samples were pumped (ca. 200 ml min<sup>-1</sup>) through Teflon tubing to a LI-COR LI-820 (Licor, St Joseph, MI, USA) to measure CO<sub>2</sub>, before passing through an Ascarite filter (periodically changed to remove moisture and CO<sub>2</sub>), before introduction to a gas chromatograph (SRI 8610C, Torrance, USA). The gas chromatograph contained a <sup>63</sup>Ni electron capture detector (ECD) to measure N<sub>2</sub>O and a flame ionization detector (FID) to measure CH<sub>4</sub>. Briefly, each block of chambers closed for 1 h, where four chamber headspace samples were taken from each chamber (once every 15 min), with a calibration standard analysed after every fourth gas sample. This resulted in eight flux measurements per 24 h period, during uninterrupted measurements. An SC100 V air compressor (SGS Engineering Ltd., Derby, UK) was used to fill compressed air lines linked to pneumatic actuators on the chambers, to open and close chamber lids automatically. A 6000E Silence generator (DMSO, Brest, France), supplied





**Fig. 1.** Weather data across the two year-long field studies, displaying a) air temperature, b) soil temperature (5 cm), c) soil water-filled pore space (WFPS) at 5 and 10 cm depth, and d) rainfall (mm). Legends apply to respective panels only. Lines at the bottom of the figure represent the experimental periods, for either spring (treatments applied on 14/06/16) or autumn (04/10/16). Crosses on each line differentiate between high frequency GHG monitoring and manual GHG sampling and dots represent the point of treatment application.

Classic 6 OPvS 660 2 V Solar Liquid Cells (GNB Industrial Power, UK) connected to an RCC-03 Control Module and a Studer Xtender XTM 3500-24 unit combining inverter, battery charger and transfer system (Struder, Sion, Switzerland), which provided power to the automated system.

During static chamber measurements, gas samples were taken with a syringe into 20 ml evacuated glass vials, (once every 15 min for 45 min) upon closure of the chamber lids, to match the sampling frequency of the automated GHG monitoring system. Gas samples were measured on a Perkin Elmer 580 Gas Chromatograph (GC), served with a Turbo Matrix 110 auto sampler (Perkin Elmer Inc., Beverly, CT, USA). The gas samples passed through two Elite-Q mega bore columns via a split injector, with one connected to an ECD for  $N_2O$  determination, and the other to an FID for  $CO_2$  and  $CH_4$  determination.

### 2.5. Urine patch soil sampling and analysis

Soils were sampled (0–10 cm) from the additional urine patches surrounding the chambers, or from the control plots, using a 1.3 cm diameter auger. Within each urine patch, soil was sampled in triplicate, sampling from the upper edge, to middle, to lower edge of the patch moving down the slope gradient. Triplicate cores were bulked to provide a representative urine patch soil sample ( $n = 4$ ). In spring, soils were sampled four days prior to treatment application, three times within the first week after treatment application, twice in the second week, once per week for two weeks, then once every two weeks for four weeks, following which the plots were sampled monthly until one year after treatment application. The same frequency of sampling was conducted for autumn, however, the initial sampling took place eight days prior to treatment application. Urine patch pH and EC were measured and extractions were performed within 24 h of sample collection. Samples were homogenised by hand, removing stones and roots, before

extracting with 0.5 M  $K_2SO_4$ , and the resulting extracts analysed for  $NO_3^-$ ,  $NH_4^+$ , extractable dissolved organic C and total dissolved N. All mentioned extractions and analyses were conducted in the same manner as described in Section 2.2.

### 2.6. Above ground biomass, foliar N content and foliar C:N ratio

Plots were cut four times throughout the year following the spring and three times throughout the year following the autumn urine applications, in order to simulate grazing and to measure the plant biomass and foliar C and N content. The biomass from within the chambers were collected and dried in an oven (80 °C; 24 h). The vegetation was ground and analysed for total N and C content, as described for the soil in Section 2.2.

### 2.7. Data processing and statistical analysis

Differences in the chemical properties of the Orthic Podzol, in either spring or autumn were compared, to confirm the similarities between the plots and to note any differences between seasons. This was conducted via two sample t-tests, using the ‘stats’ package within R Studio version 1.0.153 (R Core Team, 2017), after confirming the data conformed to normality (Shapiro Wilk test) and homogeneity of variance assumptions (F-test). Differences between collected sheep urine volumes during the spring and autumn were compared as above, after  $\ln$  transformation. Cumulative  $N_2O$  and  $CH_4$  fluxes were determined through calculating the area under the curves via integration in SigmaPlot® (v13.0, Systat Software Inc., Hounslow, UK). The urine patch  $N_2O$  emission factors (% of applied urine-N released as  $N_2O$ -N) were determined after correcting for the control emissions and the area under the chamber that urine was not applied to (Marsden et al., 2016). The artificial urine  $N_2O$  emission factors were compared between

spring and autumn by a two sample t-test, as these treatments were targeted to have the same N application rate. To assess the duration of perturbation to the levels of extractable soil  $\text{NH}_4^+$  and  $\text{NO}_3^-$  following urine application (and hence increased potential for  $\text{N}_2\text{O}$  emissions), each urine treatment was compared to the control in both seasons of application (spring or autumn) via t-tests, and differences in plant biomass, foliar N content and C:N ratio were compared by t-tests, for each harvest date.

### 3. Results

#### 3.1. Atmospheric and soil climate data

The weather data for the 12 months following the spring and autumn urine applications are displayed in Fig. 1. The mean air temperature over the year following urine application was 10.1 °C in spring and 10.0 °C in autumn (Fig. 1a). The total rainfall over the year-long monitoring period was 1452 mm in spring and 1607 mm in autumn. When comparing the mean temperature and rainfall over the high intensity GHG monitoring period the spring was warmer and wetter (14.0 °C; 257 mm; 60 days after treatment application) compared to autumn (8.1 °C; 209 mm; 60 days after treatment application). In spring, the mean soil WFPS across the year was 77% at 5 cm depth, and 69% at 10 cm depth. During the high intensity monitoring period the mean and range of the soil WFPS was 66 (28–84)% at 5 cm depth and 60 (42–75)% at 10 cm depth, experiencing a drier period at the beginning of the trial (Fig. 1d). Across the full year in autumn, the mean soil WFPS was 76% at 5 cm depth and 69% at 10 cm depth. In the high intensity monitoring period in autumn, the mean and range of the soil WFPS was 80 (65–86)% at 5 cm depth and 71 (22–100)% at 10 cm depth.

#### 3.2. Soil and sheep urine characteristics

The characteristics of the soil between the seasons were mostly similar (see Table 1), however, the soil was significantly wetter ( $p < 0.01$ ) with a higher EC ( $p < 0.001$ ) at the beginning of autumn, compared to spring. The total extractable dissolved N and  $\text{NH}_4^+$  were higher in spring compared to autumn ( $p < 0.05$  for both). Levels of exchangeable Na and Ca were also significantly greater in autumn compared to spring ( $p < 0.01$  and  $p < 0.05$ , respectively). The PLFA data can be found in supplementary information (Table S1). The soil displayed microbial community characteristics typical of extensive grazing systems, with a high microbial biomass ( $607 \pm 23$  nmol PLFA  $\text{g}^{-1}$  soil DW), a diverse microbial community indicated by a Gram-negative: Gram-positive ratio close to 1 ( $0.91 \pm 0.02$ ), and a high fungal: bacterial ratio ( $0.13 \pm 0.01$ ).

The mean individual urine event volume was significantly larger ( $p < 0.001$ ) in the autumn urine collection trial ( $385 \pm 18$  ml;  $n = 43$ ) compared to the spring urine collection trial ( $214 \pm 22$  ml;  $n = 56$ ). Values close to the mean urine volume of each season were chosen for use in the Brilliant Blue dye studies, and for the final volumes used for the field trial (200 ml for spring and 350 ml for autumn). 200 ml of Brilliant Blue dye resulted in a mean simulated urine patch area of  $113 \pm 12$  cm<sup>2</sup>, and 350 ml Brilliant Blue dye resulted in simulated urine patch areas of  $200 \pm 46$  cm<sup>2</sup>. The artificial urine volume and area were kept constant between spring and autumn, at 200 ml and 113 cm<sup>2</sup>. Details of the treatment application and the chemical properties of the real and artificial sheep urine, used in spring and autumn, can be found in Table 2. Despite the artificial urine being made up to a standard recipe, the artificial urine had a slightly lower N content (and thus a lower N loading rate upon application) in autumn compared to spring.

#### 3.3. Urine patch GHG emissions

Patterns of  $\text{N}_2\text{O}$  emissions from the control, artificial and real sheep urine treatments in spring are shown in Fig. 2a, c and e, respectively. For the same treatments in autumn, the  $\text{N}_2\text{O}$  emissions are displayed in Fig. 2b, d and e. The magnitude of mean  $\text{N}_2\text{O}$  emissions arising from the semi-improved upland field site were generally low in both seasonal trials, rarely exceeding  $100 \mu\text{g N}_2\text{O-N m}^{-2} \text{h}^{-1}$ . Cumulative  $\text{N}_2\text{O}$  emissions from the control, artificial sheep urine and real sheep urine treated chambers in spring were  $3.06 \pm 0.23$ ,  $2.81 \pm 0.43$  and  $3.22 \pm 0.71$  mg  $\text{N}_2\text{O-N m}^{-2}$  over the year following treatment applications, respectively. In autumn, cumulative  $\text{N}_2\text{O}$  emissions were  $2.95 \pm 0.43$ ,  $3.09 \pm 0.37$ ,  $3.80 \pm 0.49$  mg  $\text{N}_2\text{O-N m}^{-2}$  over the year from the control, artificial sheep urine and real sheep urine, respectively. After correcting for the area of the chamber that was not influenced by either artificial or real urine, the  $\text{N}_2\text{O}$  emission factors in spring were  $-0.02 \pm 0.04\%$  of the artificial sheep urine-N and  $0.03 \pm 0.09\%$  of the real sheep urine-N. In autumn, the  $\text{N}_2\text{O}$  emission factors were  $0.02 \pm 0.03\%$  of the artificial sheep urine-N and  $0.08 \pm 0.04\%$  of the real sheep urine-N. The artificial urine patch  $\text{N}_2\text{O}$  emission factors were not significantly different ( $p > 0.05$ ) between seasons.

The  $\text{CO}_2$  and  $\text{CH}_4$  fluxes for the automated measurement period can be found in the supplementary information (Figs. S9 and S10, respectively). The  $\text{CO}_2$  emissions followed a diurnal trend, with a higher magnitude of flux in spring compared to autumn. Mean peak emission rates ranged between  $248$ – $322$  mg  $\text{CO}_2 \text{m}^{-2} \text{h}^{-1}$  across all treatments in spring, whereas in autumn mean peak emission rates ranged between  $150$ – $188$  mg  $\text{CO}_2 \text{m}^{-2} \text{h}^{-1}$  across all treatments. The semi-improved grassland was a sink for  $\text{CH}_4$  throughout the automated measurement period. In spring, cumulative  $\text{CH}_4$  uptake rates were  $1.92 \pm 0.25$ ,  $2.24 \pm 0.22$  and  $1.83 \pm 0.24$  mg  $\text{CH}_4 \text{m}^{-2}$  over 60 days from the control, artificial sheep urine and real sheep urine treatments, respectively. In autumn, the cumulative  $\text{CH}_4$  uptake rates were  $1.23 \pm 0.85$ ,  $1.85 \pm 0.35$  and  $1.75 \pm 0.15$  mg  $\text{CH}_4 \text{m}^{-2}$  over 60 days from the control, artificial sheep urine and real sheep urine treatments, respectively.

#### 3.4. Mineral N dynamics in the urine patch

The soil extractable mineral N dynamics following treatment application can be seen in Fig. 3a and c for spring, and Fig. 3b and d for autumn. The soil extractable  $\text{NH}_4^+$  concentration peaked one day following real urine application in spring at  $40 \pm 8$  mg  $\text{NH}_4^+ \text{-N kg}^{-1}$  soil DW. The artificial urine application in spring peaked at a later time of three days following application, at a concentration of  $71 \pm 15$  mg  $\text{NH}_4^+ \text{-N kg}^{-1}$  soil DW. The differences here are likely to be due to the higher total N application in the artificial urine compared to the real urine. In spring, there were no discernible peaks in  $\text{NO}_3^-$  following application of either real or artificial urine (Fig. 3c). The extractable  $\text{NH}_4^+$  concentrations reached higher concentrations in autumn compared to spring, despite the similar N application rates for the artificial urine. Specifically, the  $\text{NH}_4^+$  concentration peaked one day after application to soil at  $306 \pm 70$  mg  $\text{NH}_4^+ \text{-N kg}^{-1}$  soil DW in the artificial urine treatment and  $205 \pm 18$  mg  $\text{NH}_4^+ \text{-N kg}^{-1}$  soil DW in the real urine treatment. In autumn, the  $\text{NO}_3^-$  peaked 21 days after artificial urine application, at  $57 \pm 8$  mg  $\text{NO}_3^- \text{-N kg}^{-1}$  soil DW. Following real urine application the  $\text{NO}_3^-$  peaked on day 27 at  $18 \pm 9$  mg  $\text{NO}_3^- \text{-N kg}^{-1}$  soil DW, however, a similar sized peak also occurred 7 days after urine application at  $17 \pm 2$  mg  $\text{NO}_3^- \text{-N kg}^{-1}$  soil DW.

In spring, levels of  $\text{NH}_4^+$  were significantly higher than the control up to 14 days after artificial urine application (days 0, 1, 3, 10 and 14 were significantly higher at  $p < 0.01$ ), but day 7 was not ( $p > 0.05$ ). The real urine treatment  $\text{NH}_4^+$  concentrations in spring were also significantly higher than the control for up to 14 days after treatment application (days 0, 1, 3 and 14 were significantly higher at  $p < 0.01$ ),

**Table 2**

Treatment application details and chemical properties of the bulked Welsh Mountain ewe urine and artificial sheep urine applied to either spring (treatment application on 14/06/16) or autumn (treatment application on 04/10/16) plots. Values represent means  $\pm$  SEM of  $n = 3$  analytical replicates, unless otherwise stated.

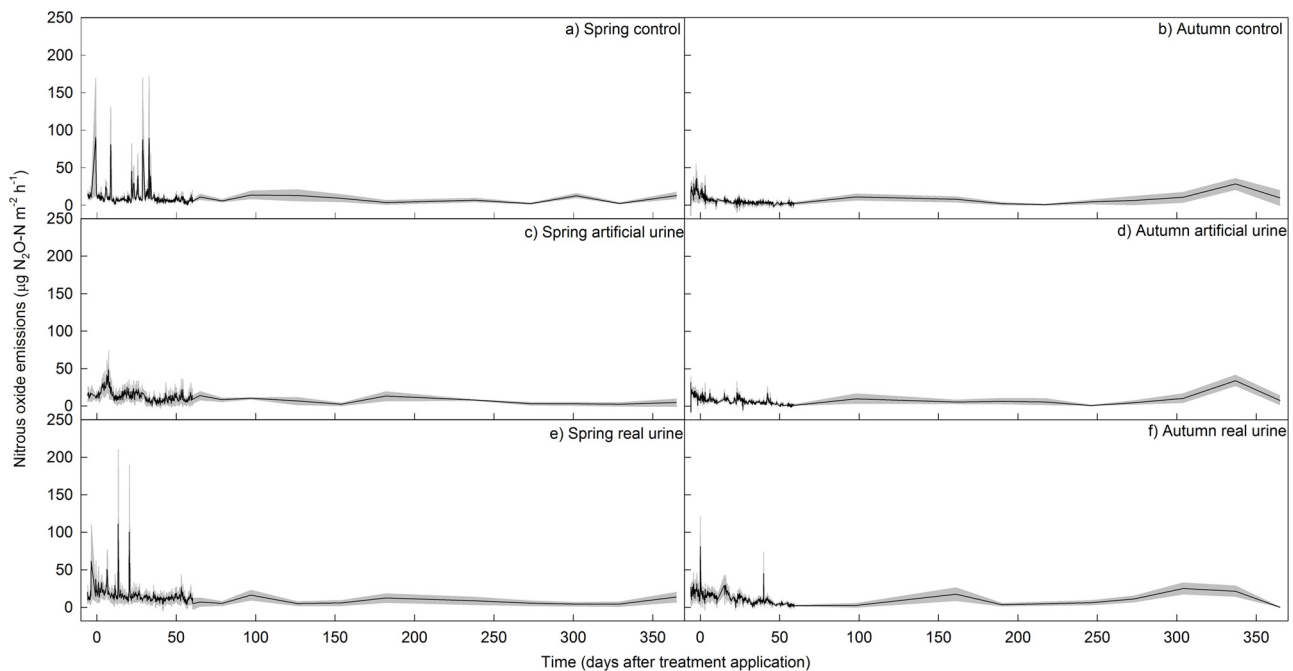
Sheep urine characteristics	Real sheep urine (applied in spring)	Artificial sheep urine (applied in spring)	Real sheep urine (applied in autumn)	Artificial sheep urine (applied in autumn)
Volume applied (ml)	200	200	350	200
Urine patch size (cm <sup>2</sup> )	113	113	200	113
N loading rate (kg N ha <sup>-1</sup> )	756	1066	1112	1004
pH	8.03	7.45	8.48	8.18
EC (mS cm <sup>-1</sup> )	10.9	10.3	13.8	10.1
Total N (g N l <sup>-1</sup> )	4.27 $\pm$ 0.15 <sup>a</sup>	6.02 $\pm$ 0.06 <sup>a</sup>	6.35 $\pm$ 0.22 <sup>b</sup>	5.67 $\pm$ 0.16 <sup>a</sup>
Total C (g C l <sup>-1</sup> )	7.96 $\pm$ 0.24 <sup>a</sup>	3.81 $\pm$ 0.04 <sup>a</sup>	10.1 $\pm$ 0.40 <sup>b</sup>	3.99 $\pm$ 0.11 <sup>a</sup>
Urea (g N l <sup>-1</sup> )	3.72 $\pm$ 0.55	4.89 $\pm$ 0.41	4.58 $\pm$ 0.30	5.62 $\pm$ 0.22
NO <sub>3</sub> <sup>-</sup> (mg N l <sup>-1</sup> )	0.99 $\pm$ 0.18	< l.o.d	0.61 $\pm$ 0.04	< l.o.d
NH <sub>4</sub> <sup>+</sup> (mg N l <sup>-1</sup> )	10.1 $\pm$ 0.02	3.05 $\pm$ 0.50	3.75 $\pm$ 0.06	3.80 $\pm$ 0.21
Hippuric acid (g l <sup>-1</sup> )	12.4 $\pm$ 0.2	1.85 <sup>c</sup>	6.80 $\pm$ 0.25	1.85 <sup>c</sup>
Allantoin (mg l <sup>-1</sup> )	116 $\pm$ 11	600 <sup>c</sup>	118 $\pm$ 9	600 <sup>c</sup>
Creatinine (mg l <sup>-1</sup> )	41.0 $\pm$ 2.8	15 <sup>c</sup>	21.0 $\pm$ 0.3	15 <sup>c</sup>
Benzoic acid (mg l <sup>-1</sup> )	22.1 $\pm$ 1.8	–	14.3 $\pm$ 1.2	–
Uric acid (mg l <sup>-1</sup> )	76.1 $\pm$ 6.5	5 <sup>c</sup>	66.0 $\pm$ 5.7	5 <sup>c</sup>

l.o.d = limit of detection.

<sup>a</sup> ( $n = 40$ ).

<sup>b</sup> ( $n = 28$ ).

<sup>c</sup> the concentration in the artificial urine (not measured).

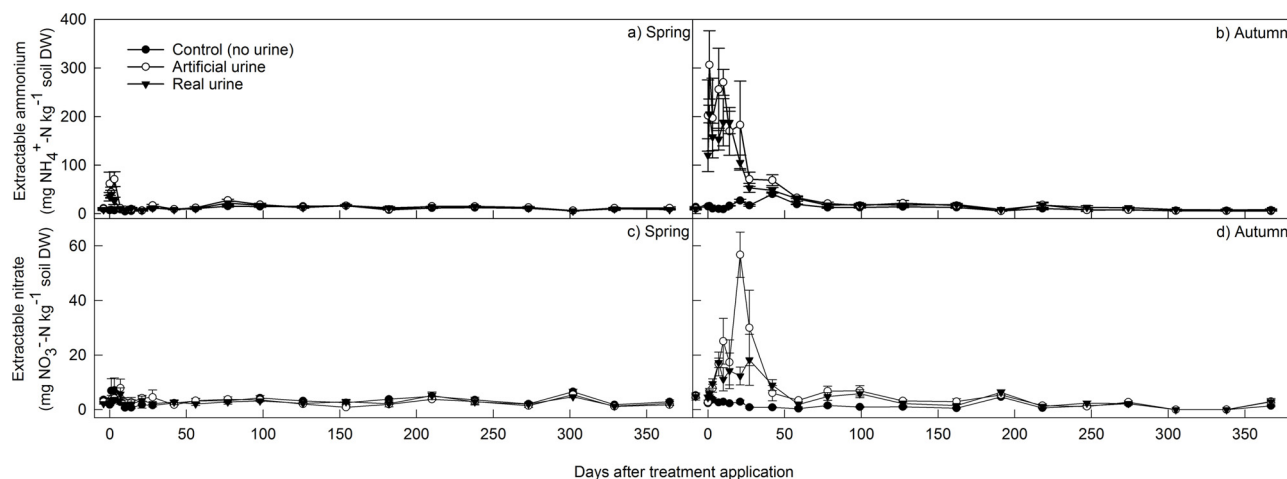


**Fig. 2.** Soil nitrous oxide emissions from a semi-improved upland pasture, with treatments applied on 14/06/16 (spring) or 04/10/16 (autumn). Emissions are displayed from the control treatments (panels a and b), the artificial sheep urine treatment (panels c and d) and the real sheep urine treatment (panels e and f). Black lines represent mean treatment emissions ( $n = 4$ ), with the shaded areas representing the upper and lower bounds of the SEM.

but days 7 and 10 were not ( $p > 0.05$ ). The NH<sub>4</sub><sup>+</sup> concentrations were significantly greater than control values for a longer period in autumn compared to spring. In the real urine treatment NH<sub>4</sub><sup>+</sup> concentrations were significantly higher than the control on days 0, 1, 3, 7, 10, 14, 21 and 27 (all at  $p < 0.01$ ), but had returned to control values by day 42 ( $p > 0.05$ ). In the artificial urine treatment in autumn, the NH<sub>4</sub><sup>+</sup> concentration was significantly higher on days 0, 1, 3, 7, 10, 14, 21, 27, 58 and 78 (all at  $p < 0.05$ ), and had returned to control values 99 days after treatment application.

Levels of extractable NO<sub>3</sub><sup>-</sup> were not significantly greater than control values at any time point ( $p > 0.05$ ) following artificial or real sheep urine application in spring. In autumn, however, extractable soil NO<sub>3</sub><sup>-</sup> concentrations following artificial urine application were

significantly higher than the control on days 7, 10, 14, 21, 27, and 42 ( $p < 0.05$ ), returning to control values on days 58 and 78 ( $p > 0.05$ ), and then becoming significantly higher than the control on days 99, 127 and 162 ( $p < 0.05$ ). Following real urine application in autumn, the extractable soil NO<sub>3</sub><sup>-</sup> levels were significantly higher than the control on days 3, 7, 10, 14, 21, 27, 42, 58 ( $p < 0.05$ ), returning to control values on day 78 ( $p > 0.05$ ), but was significantly higher again 99 days after treatment application ( $p < 0.05$ ), following which the NO<sub>3</sub><sup>-</sup> levels were not significantly greater than the control for the duration of the year ( $p > 0.05$ ).



**Fig. 3.** Extractable soil ammonium (panels a and b) and nitrate (panels c and d), following treatment application (control, artificial sheep urine or real sheep urine) to a semi-improved upland pasture on 14/06/16 (spring) or 04/10/16 (autumn). Symbols represent means ( $n = 4$ ), bars represent SEM, and figure legends apply to all panels.

### 3.5. Soil analyses

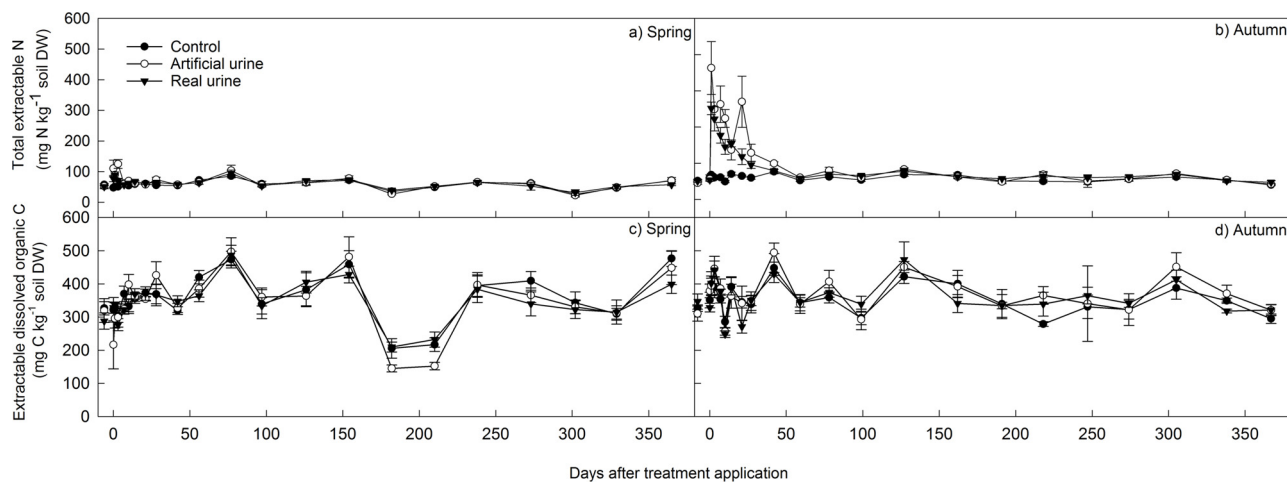
The levels of soil extractable dissolved N can be found in Fig. 4a and b, for spring and autumn, respectively. Extractable dissolved organic C can be found in Fig. 4c and d, for spring and autumn, respectively. In spring, the total extractable N increased in comparison to the control following treatment application, but the effect was shorter-lived and of a smaller magnitude in comparison to autumn (Fig. 4a and b). In spring, when comparing to the control, the total extractable soil N increased by a maximum of  $37.6 \text{ mg N kg}^{-1}$  soil DW in the real sheep urine treatment and  $54.5 \text{ mg N kg}^{-1}$  soil DW in the artificial sheep urine treatment, within the first three days after treatment application. In autumn, when comparing to the control, the total extractable N increased by  $184 \text{ mg N kg}^{-1}$  soil DW in the real sheep urine treatment and  $295 \text{ mg N kg}^{-1}$  soil DW in the artificial sheep urine treatment, the day after treatment application. A clear response of extractable dissolved organic C to urine application was not evident in either season of treatment application (Fig. 4c and d), with treatment responses being similar to the control.

The soil pH and EC following urine application can be found in supplementary information (Fig. S11). There was not a particularly strong increase in soil pH after urine application, considering the alkalinity of the applied urine (ranging from 7.5 to 8.5), indicating a high

buffering capacity of this soil. The soil pH in both spring and autumn (Fig. S11a and b), typically ranged between pH 4.5 and 5.5. In autumn, the artificial urine treatment appeared to have acidified slightly in comparison to the control towards the second half of the year. In spring, no discernible increase in soil pH following either type of urine application was observed, in comparison to the control. The soil pH reached a maximum on the day of urine application in autumn at  $\text{pH } 5.88 \pm 0.13$  and  $5.44 \pm 0.14$  in the artificial and real sheep urine treatments, respectively, although the control values were similar at  $\text{pH } 5.42 \pm 0.04$ . Soil EC values increased compared to the control following application of either urine type, in both seasons. In spring, the EC peaked one week after urine application ( $63 \pm 14 \mu\text{S cm}^{-1}$ ) and artificial urine application ( $106 \pm 56 \mu\text{S cm}^{-1}$ ), compared to the control ( $39 \pm 6 \mu\text{S cm}^{-1}$ ). In autumn, the EC of the soil increased sharply three days after real urine application, reaching  $218 \pm 41 \mu\text{S cm}^{-1}$ , and peaked one week after artificial urine application ( $177 \pm 24 \mu\text{S cm}^{-1}$ ) and steadily returned to control values. The soil EC remained elevated for a longer time period in autumn compared to spring.

### 3.6. Above-ground plant biomass, foliar N content and C:N ratio

A summary of the data for the plant biomass harvests from within



**Fig. 4.** Total extractable nitrogen (panels a and b) and total extractable dissolved organic carbon (panels c and d), following treatment application (control, artificial sheep urine or real sheep urine) to a semi-improved upland pasture on 14/06/16 (spring) or 04/10/16 (autumn). Symbols represent means ( $n = 4$ ), bars represent SEM, and figure legends apply to all panels.



the chambers, the foliar N content and C:N ratio can be seen in Supplementary information (Table S2). After the first biomass cut in spring (three weeks after urine application), the plant biomass was not significantly greater than the control ( $p > 0.05$ ) in either the artificial or real sheep urine treatments. Compared to the control foliar N content ( $2.56 \pm 0.07\%$ ), foliar N content was significantly higher in the artificial urine ( $3.14 \pm 0.08\%$ ;  $p < 0.01$ ) and the real urine treatment ( $2.87 \pm 0.08\%$ ;  $p < 0.05$ ). The foliar C:N ratio was significantly lower than the control in the artificial urine ( $14.3 \pm 0.5$ ;  $p < 0.01$ ) and the real urine treatment ( $15.6 \pm 0.4$ ;  $p < 0.5$ ). No significant differences ( $p > 0.05$ ) were detected compared to the control values for either artificial or real sheep urine, in terms of plant biomass, foliar N content or foliar C:N ratio following the second and third biomass harvests. In the final biomass harvest, the artificial urine treatment had a greater plant biomass ( $315 \pm 6 \text{ g DM m}^{-2}$ ;  $p < 0.05$ ) compared to the control ( $268 \pm 18 \text{ g DM m}^{-2}$ ), a lower foliar N content ( $1.50 \pm 0.03\%$ ;  $p < 0.05$ ) compared to the control ( $1.65 \pm 0.04\%$ ), and a higher foliar C:N ratio ( $60.4 \pm 0.7$ ;  $p < 0.05$ ) compared to the control ( $27.6 \pm 0.8$ ). No significant differences ( $p > 0.05$ ) were found when comparing the urine (artificial or real) treatments to the control in terms of above-ground biomass, foliar N content or foliar C:N ratio, in any of the three harvests from autumn.

## 4. Discussion

### 4.1. Upland urine patch parameters

This study contributes a novel data-set for sheep urine patch  $\text{N}_2\text{O}$  emissions on an extensively grazed, upland pasture, across two contrasting seasons. The mean urine event volumes are within the ranges of individual urine event volumes (19–397 ml;  $n = 40$  events) reported by Marsden et al. (2017) for the same breed of sheep fed a lowland diet. In this study, the mean individual sheep urine event volume in spring (214  $\pm$  22 ml) was similar to values reported in Haynes and Williams (1993) of 100–200 ml, but was higher in autumn (385  $\pm$  18 ml). The greater volume of urine between seasons resulted in an increased patch size in the urine patch area simulations with Brilliant Blue dye. Our resulting patch sizes were smaller than that reported in other studies, resulting in high urine volume-to-area ratios (ca.  $171 \text{ m}^{-2}$ ), as opposed to the commonly used 4–5  $\text{m}^{-2}$  for typical sheep urine events (Haynes and Williams, 1993; Hoogendoorn et al., 2008). This resulted in high N loading rates (756–1112  $\text{kg N ha}^{-1}$ ) across treatments for our study, despite the urine-N content not being at the higher end of reported values (ranging from 1.4 to 17.8  $\text{g N l}^{-1}$  in Hoogendoorn et al., 2010). The large disparity between our values for the urine patch wetted area and literature values, suggests that site-specific measurements of the urine patch wetted area are important to establish.

The breed of sheep utilised in this study was representative to this study area, however, breeds with differing N use efficiencies and selective grazing preferences may be utilised across other extensively grazed grasslands. This has the potential to influence urine patch parameters and N cycling within the urine patch. The N contents of the bulked spring and autumn sheep urine were lower in this study (ranging from 4 to 6  $\text{g N l}^{-1}$ ) than that reported for the same breed of sheep fed a lowland diet (14.5  $\text{g N l}^{-1}$ ; Marsden et al., 2017). The N content of the measured purine derivatives (allantoin, creatinine, and uric acid) and hippuric acid made up 25% of the total urinary N content of the bulked spring urine and 9% of the bulked autumn urine, which is within the range of 3–28% of the N content of cattle urine reported by Chadwick et al. (2018). Benzoic and hippuric acids are excreted from precursor phenolic constituents within the diet (Martin, 1982; Dijkstra et al., 2013) and the hippuric acid concentrations in spring and autumn ranged from 6 to 12  $\text{g compound l}^{-1}$  in this study, which is slightly higher than the range of 1–9  $\text{g compound l}^{-1}$  in ewes fed a ryegrass and white clover pasture (Bristow et al., 1992). Our values for allantoin were slightly lower than reported for ewes in Bristow et al. (1992), but

the values for creatinine and uric acid were similar. We have shown that urine patch parameters can differ seasonally (e.g. volume and N constituents), which has the potential to influence the temporal dynamics of N cycling e.g. other minor N constituents degrade more slowly than urea (Dijkstra et al., 2013). The urinary hippuric acid concentration were higher in spring compared to autumn in this study. Hippuric acid has been investigated as a natural nitrification inhibitor which could reduce  $\text{N}_2\text{O}$  emissions from urine patches, although contrasting evidence of its effectiveness is reported in the literature (Kool et al., 2006; Bertram et al., 2009; Clough et al., 2009; Krol et al., 2015).

### 4.2. Urine patch $\text{N}_2\text{O}$ emissions from extensive compared to intensive grasslands

By considering urine N content and composition, volume and patch size, we believe to have captured variation in urine patch parameters which could vary due to season and the upland location of this study, providing a robust urine-patch  $\text{N}_2\text{O}$  emission dataset for extensively grazed grassland, which can be compared with that of intensively managed lowland systems (which current IPCC default excretal  $\text{N}_2\text{O}$  emission factors are based on). As hypothesised, our reported  $\text{N}_2\text{O}$  emission factors are below the updated UK country-specific IPCC urine emission factor (ca. 0.69% for urine-N; Chadwick et al., 2018), and some replicates even produced small negative emission factors. The  $\text{N}_2\text{O}$  fluxes following urine application did not produce a sustained peak of  $\text{N}_2\text{O}$  above baseline levels, characteristic of urine deposited to lowland fertile grasslands (Bell et al., 2015; Cardenas et al., 2016). The magnitude of  $\text{N}_2\text{O}$  fluxes were lower than those reported in other studies of urine application to lowland grassland soils e.g. peak emissions in this study were ca.  $100 \mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$ , whereas Hoefl et al. (2012), report peak emissions of  $556 \mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$  for a sheep urine patch and  $1921 \mu\text{g N}_2\text{O-N m}^{-2} \text{ h}^{-1}$  for a cattle urine patch.

### 4.3. Were $\text{N}_2\text{O}$ emissions limited by low nitrification rates in acid soil?

We hypothesised that  $\text{N}_2\text{O}$  emissions might be low due to low rates of nitrification under acidic soil conditions. Traditionally nitrification has been assumed to be low in acidic soils due to i) autotrophic nitrifiers isolated from acid soils failing to oxidise  $\text{NH}_4^+$  in cultures of  $< \text{pH } 5.5$  and ii) the substrate for the ammonia mono-oxygenase enzyme (which catalyses the first step of the nitrification process) is  $\text{NH}_3$  gas (Ward, 2013), the availability of which would be lower under acidic soil conditions, as it is present in the ionised form of  $\text{NH}_4^+$  (De Boer and Kowalchuk, 2001; Li et al., 2018). Although  $\text{NH}_3$  may be limited under acidic soil conditions this does not rule out active transport of  $\text{NH}_4^+$  into the nitrifying cell and internal oxidation of  $\text{NH}_4^+$  to  $\text{NH}_3$ , alternatively urea can diffuse directly into nitrifying cells and be converted to  $\text{NH}_3$  via cytoplasmic urease (De Boer and Kowalchuk, 2001). More recent studies have shown nitrification in acid soils is possible, with ammonia oxidising archaea, rather than bacteria, playing an important role (Gubry-Rangin et al., 2010; Zhang et al., 2012; Li et al., 2018). In this study, nitrification clearly occurred in autumn, but the magnitude was very low in the spring applied urine, with values of extractable  $\text{NO}_3^-$  not becoming greater than the control at any time point. Rather than this suggesting that nitrification did not take place in spring, it could be that the rates of  $\text{NO}_3^-$  production were tightly coupled to  $\text{NO}_3^-$  immobilisation and plant uptake in spring, therefore, a build-up of  $\text{NO}_3^-$  was not observed in the extractable pool. The appearance of a smaller extractable  $\text{NH}_4^+$  pool following urea hydrolysis in spring compared to autumn also suggests removal of N at an earlier stage in the N cycle than the process of nitrification i.e. we suggest that low nitrification rates were not the only cause of the low observed  $\text{N}_2\text{O}$  emissions from the urine patches in this study.

#### 4.4. Potential fate of the spring-applied urine-N

The lack of ploughing and re-seeding in this upland grassland allows the development of a distinct litter layer (Fig. S2). This litter layer would have a high C:N ratio which may favour immobilisation of urinary-N and C, prior to it reaching the underlying organic or mineral soil horizons. A similar phenomenon is demonstrated for  $^{15}\text{N}$  labelled urea in N-limited tundra ecosystems (Barthelemy et al., 2018). Possible sinks of urea (before reaching the soil) include foliar absorption through pasture leaves (Bowman and Paul, 1992) or retention by bryophytes (Barthelemy et al., 2018). Volatilisation of  $\text{NH}_3$  may have been responsible for the small extractable  $\text{NH}_4^+$  pool in spring, but the magnitude of N loss via this pathway is generally considered to be low under acidic soil conditions (Cameron et al., 2013). Nevertheless,  $\text{NH}_3$  volatilisation has been demonstrated to have significant temperature and soil moisture interactions, with  $\text{NH}_3$  volatilisation increasing with higher temperatures and at lower soil moisture contents (McGarry et al., 1987). In this study the air temperature was warmer in spring (14 °C) compared to autumn (8 °C), and the soil was drier in spring compared to autumn, which may have promoted  $\text{NH}_3$  volatilisation. An increase in soil pH as caused by urine application may have occurred in soil microsites, creating favourable conditions for  $\text{NH}_3$  volatilisation. In this study, the bulk soil pH was measured, where overall changes were limited. Nevertheless, a greater pH may have been found in the litter layer which, unlike the bulk soil, is unlikely to be buffered. Sorption and protection of  $\text{NH}_4^+$  within organic matter may have also occurred, although this pool should be included within the extractable pool of  $\text{NH}_4^+$ , and this physical process is likely to have remained constant between seasons. Sorption of  $\text{NH}_4^+$  may have also been limited due to the amount of cations present within urine (e.g. potassium salts), which may have saturated exchange sites within the soil.

The extensification of grassland management typically results in a shift in microbial community composition to one with a higher fungal:bacterial ratio. Our microbial PLFA data supports this and is similar to that observed in unimproved grasslands by Grayston et al. (2004). De Vries et al. (2012) observed a greater N retention in extensive compared to intensive grassland systems after  $^{15}\text{N}$  labelled  $\text{NH}_4\text{NO}_3$  application, and attributed this to greater plant root uptake (associated with greater fungal biomass e.g. mycorrhizal symbioses) and microbial immobilisation. In this study, the pasture had developed a thick root mat (Fig. S2), where undisturbed hyphal networks have had the opportunity to develop, some of which are symbiotrophs (e.g. arbuscular mycorrhizas and dark septate endophytes). The small pool of soil  $\text{NH}_4^+$  appearing following urine application in spring was probably the result of rapid plant acquisition and microbial immobilisation of the applied N, under a time of faster plant growth and microbial activity. Indeed, the foliar N content of the pasture biomass was higher after the first biomass cut in spring, but not in autumn, suggesting a greater capture of urine-N by the plant biomass with less appearing in the extractable mineral N pools. This may have limited the supply of  $\text{NH}_4^+$  available for nitrification and subsequent denitrification in spring, resulting in the low observed  $\text{N}_2\text{O}$  emissions in the spring-applied urine. Although unmeasured, we assume  $\text{NO}_3^-$  leaching losses were limited in spring, due to the lack of appearance of a  $\text{NO}_3^-$  pool and the relatively shallow area of influence of sheep urine (e.g. compared to the larger volume of urine deposited by cattle).

#### 4.5. Potential fate of the autumn-applied urine-N

Nitrification clearly took place in this acidic upland soil in the autumn, providing the substrate for denitrification. This did not, however, translate into noticeably higher  $\text{N}_2\text{O}$  emissions from this trial, despite the soil WFPS also being at an optimum for denitrification (mean of 80% WFPS across the high intensity sampling period in autumn). If complete denitrification to  $\text{N}_2$  had occurred, this may have resulted in low  $\text{N}_2\text{O}$  emissions, however, there is a negative correlation of the

$\text{N}_2\text{O}:\text{N}_2$  ratio with soil pH, due to the assembly of  $\text{N}_2\text{O}$  reductase being impaired at low pH (Liu et al., 2010, 2014). Fungi (and many bacteria) also possess a truncated denitrification pathway, where they are unable to produce  $\text{N}_2\text{O}$  reductase (Phillipot et al., 2010).

De Vries et al. (2011) have shown that soils with a high fungal:bacterial ratio have been shown to have low  $\text{N}_2\text{O}$  emissions and rates of denitrification. Šimek and Cooper (2002) reviewed the effect of soil pH on denitrification, and found that total gaseous emissions ( $\text{NO}$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$ ) are often less in acidic compared to neutral or alkaline soils. This is postulated to be due to an indirect effect of pH reducing N mineralisation rates and C availability (Šimek and Cooper, 2002), rather than a direct effect of pH on denitrification enzymes. In these upland environments lower productivity and therefore lower C inputs could also contribute to lower  $\text{N}_2\text{O}$  emissions. Nevertheless, the application of urine should temporarily remove these limitations, due to the large quantities of dissolved C and N applied over a small area. Despite seeing an increase in total dissolved N, the extractable dissolved organic C pool did not peak following urine application in this study. This may have been the result of strong resource use efficiency by microbes in these grassland soils i.e. a rapid utilisation of labile C prior to the build-up of  $\text{NO}_3^-$ , resulting in temporal separation of available C and  $\text{NO}_3^-$ , and low emissions of  $\text{N}_2\text{O}$ . Leaching of  $\text{NO}_3^-$  cannot be ruled out in the autumn applied urine, however, the long residence time of the  $\text{NO}_3^-$  (> 100 d) would suggest otherwise. The use of  $^{15}\text{N}$  labelled urine would be useful to determine N-loss pathways in these upland soil and vegetation types, to determine the potential for other N losses (e.g. gaseous losses of  $\text{NH}_3$ ,  $\text{NO}$ ,  $\text{N}_2$ , and leaching of  $\text{NO}_3^-$  to watercourses) and the potential consequences for indirect  $\text{N}_2\text{O}$  emissions.

#### 4.6. Wider implications of the results

Considering the wider implication of these results, livestock movement and behaviour in the uplands can cause differences in emissions in areas subjected to a greater frequency of excretal deposition and compaction, such as sheltered low-lying areas (Betteridge et al., 2010a, b). Livestock movement and topography have an interactive effect, causing the redistribution of nutrients from high to low slopes (Luo et al., 2013). Indeed, nitrification and denitrification enzyme activities were greater in lower slopes compared to medium and high slopes in New Zealand (Zhong et al., 2016). An increased nitrification potential has also been found for sheep camping areas in hill grazing systems in New Zealand (Letica et al., 2006), creating a higher potential for  $\text{N}_2\text{O}$  emissions and  $\text{NO}_3^-$  leaching from these areas.

Whilst our study provides high temporal resolution in terms  $\text{N}_2\text{O}$  emissions from upland sheep urine patches, especially during the automated measurement period, the spatial variability in  $\text{N}_2\text{O}$  emissions are less well represented. Gaining a mechanistic understanding of the interacting factors which can produce elevated  $\text{N}_2\text{O}$  emissions in these upland environments would be useful in order to model emissions across these spatially complex landscapes. Previous efforts to assess the C footprint of lamb production tend to use default IPCC  $\text{N}_2\text{O}$  emission factors for excretal deposition to soil (Jones et al., 2014), yet the C footprint of lamb produced in the uplands could be lower if using hill grazing-specific urine patch emission factors. To calculate this, further work would also be necessary to determine  $\text{N}_2\text{O}$  emissions from highly organic soils (e.g. peats) and shallow mineral soils (e.g. rankers) typical of mountainous terrain at higher altitudes. In addition,  $\text{CH}_4$  emissions from the rumen could also differ substantially in upland systems, and a recent study by Zhao and Yan (2017) suggest  $\text{CH}_4$  emissions are lower than the default for upland hill ewes fed an upland diet.

## 5. Conclusions

Sheep urine patch  $\text{N}_2\text{O}$  emission factors from an extensively grazed upland grassland were much lower than current IPCC default values derived from intensively grazed grassland. The results presented herein

supports the importance of disaggregating excretal N emission factors by grazing area types. Further work is necessary to: i) elucidate the mechanisms of reduced N<sub>2</sub>O production in these upland soils (to assess other potential N loss pathways), ii) explore spatial variability in N<sub>2</sub>O emissions which may vary as a function of livestock movement and behaviour, iii) corroborate findings across a greater number of upland regions, and iv) assess whether emissions are also of a low magnitude from organic soils, typical of extensive grazing lands at higher altitudes than that studied here. Our results could be used to provide a more accurate C footprint for lamb produced in the uplands, but for full C accounting, quantification of CH<sub>4</sub> emissions from the rumen in such extensively grazed systems would also be necessary.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.agee.2018.06.025>.

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