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The soil microbial community and plant biomass differentially contribute to the retention and recycling of urinary-N in grasslands

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ABSTRACT

Urine patches in grazed systems are hotspots for nitrogen (N) cycling and losses to the wider environment. Retention and subsequent recycling of urinary-N is key to minimise losses and increase ecosystem nitrogen use efficiency. Biosynthesis into the microbial organic N pool is an important N pathway but this has not been directly quantified in a urine patch. Herein, we present the results of a time course experiment using soil mesocosms sown with perennial ryegrass (*Lolium perenne* L.) and treated with ^{15}N-labelled sheep urine to determine partitioning of the applied N between plant, soil biomass pools and leaching losses following simulated rainfall events. ^{15}N-tracing used bulk and compound-specific ^{15}N-stable isotope probing (SIP) to determine the fate of urinary N. Initial high leaching losses (233 kg N ha^{-1}) were comprised of native soil N, ammonium and nitrate derived from urine by urea hydrolysis and nitrification, respectively. Leaching subsequently decreased whilst uptake into plant biomass and microbial biosynthesis increased during periods of low rainfall. Uptake into above and belowground plant biomass was the largest fate of urinary-^{15}N after 94 d (42%), although assimilation into microbial biomass dominated for *ca.* 1 month after urine deposition (34%). Compound-specific ^{15}N–SIP of amino acids and amino sugars revealed immobilisation of urinary-N following mineralisation was the dominant pathway for biosynthesis, with incorporation into bacterial organic N pools more rapid than into the fungal biomass. There was also intact utilisation of glycine derived from urine. This study provides clear evidence that direct assimilation of urine-derived N into microbial organic N pools is an important process for retaining N in a urine patch, which will subsequently support plant N supply during microbial turnover.

1. Introduction

There are *ca.* 23.3 million sheep and lambs in the UK alone (DEFRA, 2018), which excrete 75–95% of ingested nitrogen (N) meaning urine and dung are major N inputs into grasslands (Oenema and Tamminga, 2005; Huhtanen and Hristov, 2009). Of this excreted N, urine accounts for 27–61%, with N loading in a urine patch ranging between 203 and 2283 kg N ha^{-1} (Cheng et al., 2013; Marsden et al., 2020). The nature of a urine patch makes it a hotspot of N cycling, with high N loading of water soluble low molecular weight organic N compounds, which are readily available to both biota, and for leaching (Selbie et al., 2015). Minimising N losses to the wider environment is central to future agricultural sustainability goals, including the UN Resolution on Sustainable Nitrogen Management (UNEP/EA.4/Res.14). Subsequently, consideration of how N is retained in agricultural systems, particularly in areas of high N loading, such as urine patches, is crucial. The role of the soil microbial community in retaining, recycling, and supplying N to plants is frequently neglected in current nutrient balances, which must be expanded to manage livestock systems in a holistic manner (Oenema et al., 2003; Robertson and Swinton, 2005; Heijden et al., 2008).

N losses beneath a urine patch are of great concern, with potential negative impacts on human health from nitrate (NO_3^-) in drinking water (Di and Cameron, 2002; Galloway and Cowling, 2002) and on freshwater ecosystems, including eutrophication, acidification and biodiversity loss (Anderson et al., 2002). ^{15}N tracing approaches have been widely applied to quantify N losses from a urine patch. For example, Di and Cameron (2007) found around a quarter of urinary-N was leached in a high N loading urine patch equivalent to 1000 kg N ha^{-1}. A wide range

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of leached N from urine patches has been observed (3–368 kg N ha^{-1} (Cameron et al., 2013; Maheswaran et al., 2022)). This is due to the number of factors influencing N transformations and subsequently N losses in a urine patch, including plant nitrogen use efficiency (NUE) (Woods et al., 2017), soil type (Clough et al., 1998), soil pH (Marsden et al., 2018), season and prevailing weather conditions (Buckthought et al., 2015). Furthermore, fertilisation alongside urination of grazing animals serves to further increase N losses via leaching, due to high N loading above plant and microbial demand (Silva et al., 2005; Cameron et al., 2013). Leached-N is not limited to urinary-N, with osmotic stress due to high salt concentrations in a urine patch resulting in leaching of the contents of lysed microbial and root cells (Ambus et al., 2007; Lambie et al., 2012). Additionally, changes in soil pH following urea hydrolysis to ammonium (NH_4^+) results in native soil organic matter (SOM) solubilisation, which is subsequently susceptible to leaching (Shand et al., 2002; Bertram et al., 2012; Anderson et al., 2018).

While there is clear evidence for N losses, how urinary-N is retained and recycled within the soil pool by the microbial community is poorly understood. The importance of retention in soil has been confirmed, with between 14 and 60% of urinary-N retained across a range of studies with sheep urine (e.g. Sørensen and Jensen, 1996; Clough et al., 1998; Bosshard et al., 2008; Buckthought et al., 2015; Woods et al., 2017). However, indicators of microbial processing have largely been indirect, such as respiration (Kelliher et al., 2005), enzyme activity (Rooney et al., 2006), or via difference between inorganic and total soil ^{15}N retained in ^{15}N tracing studies, ranging between 11 and 50% (Bronson et al., 1999; Ambus et al., 2007). Wachendorf and Joergensen (2011) directly quantified incorporation of ^{15}N-labelled cow urine into microbial biomass via chloroform fumigation and showed that this pool accounted for 7–17% of applied ^{15}N after 6 months. These indirect, or bulk approaches do not allow direct quantification of urinary-N uptake, nor insight into the biosynthetic pathways of assimilation. Immobilisation by the microbial community is central to fully assess N losses and recycling in a grazed grassland. Thus, revealing the biosynthetic fate of urinary N, in combination with plant uptake and leaching, is essential.

Compound-specific ^{15}N-stable isotope probing (^{15}N–SIP) has confirmed microbial assimilation of relevant organic-N compounds into newly biosynthesised protein in soil via compound-specific isotope analyses of amino acids (AAs), including ^{15}N-urea (Charteris, 2019) and ^{13}C,^{15}N-glycine (Knowles et al., 2010). While the assimilation of these N constituents of urine into the soil microbial community have been shown in separate ^{15}N–SIP studies, these have not reflected the complexity and concentration of urine as an N input, thus cannot be used to extrapolate microbial N assimilation in a urine patch. Furthermore, recent development of compound-specific ^{15}N–SIP to amino sugars (ASs) can now provide insights into bacterial and fungal N assimilation, alongside assimilation into the soil protein pool (Reay et al., 2019a, 2019b). Thus, the application of compound-specific ^{15}N–SIP to a urine patch will provide previously unavailable insights into microbial processing of nitrogenous compounds in urine, influenced by realistic conditions in a urine patch.

In this study, we traced the partitioning of ^{15}N-labelled sheep urine applied to laboratory mesocosms containing *Lolium perenne* L. We combined ^{15}N-urine produced in a ^{15}N-labelled feeding study (Reay et al., 2023), alongside compound-specific ^{15}N–SIP to reflect the complexity of N in a urine patch and partitioning of all urinary-N pools into newly biosynthesised microbial organic N (ON) pools. This approach targets both proteinaceous-N, via amino acids, the largest ON pool in soil, alongside amino sugars, where differences in bacterial and fungal N assimilation can be investigated. Furthermore, following quantification of plant uptake and leaching losses, a partial ^{15}N mass balance approach was used to reflect the relative importance of these pools during a period of high potential for leaching (i.e., rainfall events) at different time points after urine deposition. It was hypothesised that large leaching losses would be observed immediately after urine application in a rainfall event. Subsequently, rapid assimilation into the microbial community, largely following mineralisation of urinary-N, will reduce susceptibility to leaching. It is also hypothesised while microbial uptake will be rapid, plants will outcompete microbes for urinary-N and be the major fate of N in a urine patch when not lost via leaching.

2. Materials & methods

2.1. ^{15}N urine

^{15}N-labelled sheep urine was collected from Welsh Mountain sheep ($n = 5$) fed with ^{15}N-labelled ryegrass (4.41 ± 0.18 atom% ^{15}N) for 7 days (Reay et al., 2023). Urine collected between days 5 and 7, corresponding to the maximum ^{15}N enrichment observed, were defrosted, filtered and stored at 4 °C. The composition of the urine is shown in Table 1. The bulk urine was applied to mesocosms within 24 h of defrosting. The ^{15}N-enrichment was 2.041 ± 0.009 atom% ^{15}N and the total N (TN) concentration was 9.98 ± 0.01 g N l^{-1}. Ureido-N accounted for 84.6% of the TN pool, and amino acids accounted for 4.8%, primarily as glycine (Gly, 4.4% of TN; Fig. S1).

2.2. Mesocosm set-up

Soil was collected (0–15 cm) from the Henfaes Research Station, Abergwyngregyn, Wales (53°14′N, 4°, 01′W), which is run by Bangor University. It is a Global Farm Platform (Rivero et al., 2021; https://globalfarmplatform.org/henfaes) and has a commercial sheep flock of *ca.* 1350 pure bred Welsh Mountain ewes. Soil was collected from the lowland area of the platform. The sward is dominated by *L. perenne* and previously received inorganic N fertiliser at rates between 100 and 130 kg N ha^{-1} y^{-1} alongside potassium (K), phosphorus (P) and lime (target pH 6.5). The soil was classified as a freely draining Eutric Cambisol (FAO) with a sandy clay loam texture. Soil was sieved to 5 mm to remove large stones, earthworms, and vegetation, but minimise the disruption of fungal structures present in soil. The soil was transported from the field site to University of Bristol in the dark and stored at 15 °C in ventilated bags until use.

Soil was packed into the glass columns (diameter 7.5 cm) to a depth of 15 cm and to a density of 1.1 g cm^{-3}, consistent with field conditions, with a small hole at the base plugged with glass wool to allow leaching and to maintain aerobic conditions (Fig. S2). The soil was adjusted to 50% water holding capacity (WHC) using artificial rainwater reflecting composition at the site (Table S1). *L. perenne* L. (Cotswolds Seed Company, Moreton-in-Marsh, UK) was sown at a density equivalent to 25 kg ha^{-1} and allowed to establish to between the 2nd and 3rd leaf stage of maturation, which is used to indicate when pasture is ready to be grazed; this took 6 weeks. Throughout this period the soil columns were maintained at 50% WHC by weight by adding artificial rainwater. The soil columns were kept in a temperature-controlled greenhouse, maintained at 18 °C and held in acrylic holders, covered with foil to ensure dark conditions for the soil (Fig. S2).

Urine (40 ml) was applied to 44 soil columns as a stream 5 cm above the soil surface, which was equivalent to 900 kg N ha^{-1}, within the wide range of urinary N inputs for Welsh mountain ewes (Marsden et al.,

Table 1
Properties of sheep urine applied to mesocosms. Values indicate means ± SE ($n = 3$). THAA = total hydrolysable amino acids.

Urine properties	
^{15}N-enrichment	2.041 ± 0.009 atom %^{15}N
Total N	9.98 ± 0.01 g N l^{-1}
Total organic C	13.8 ± 0.1 g l^{-1}
Ureido-N	8.44 ± 0.11 g N l^{-1}
NH_4^+	0.091 ± 0.003 g N l^{-1}
NO_3^- + NO_2^-	8.67 ± 0.05 mg N l^{-1}
THAA	0.476 ± 0.018 g N l^{-1}

2016a, 2016b, 2020). Artificial rainwater (40 ml) was applied to the control treatment (Table S1). Soil columns were sacrificed at 0, 3 and 6 h and 1, 3, 7, 16, 31, 65 and 94 d ($n = 4$, total = 80). At each timepoint, a storm event was simulated using artificial rain equivalent to 45 mm h^{-1} over 2 h. This allowed leachate discharge to equilibrate to a constant rate of leaching, based on pre-experiment trials. Leachate was collected for 30 min following the end of rainfall, the volume determined and then frozen. Above ground biomass was cut just above the soil, roots were removed from the soil at two depths (0–5 and 5–15 cm) and rinsed with deionised water (DI). The grass, leachate, soil and roots were stored at −20 °C prior to lyophilisation and grinding. The mass of aboveground biomass at each timepoint was used to account for grass growth and adjust the soil to 50% WHC by weight.

2.3. Analyses

2.3.1. Leachate composition

The NO_3^-, NH_4^+, phosphate (PO_4^{3-}) and ureido-N concentrations of soil leachate were determined using a continuous flow analyser (Skalar San^{++} system, Skalar Analytical B.V., The Netherlands), with a Series 1050 sampler. TN was converted to NO_3^- following digestion (MARS Microwave digester, CEM corporation, UK) with sodium hydroxide and potassium persulfate. NO_3^- and TN were determined using a modified hydrazine reduction method (Kempers and Luft, 1988; ISO/TC 147/SC 2, 1996). NH_4^+ was determined using a method based on the modified Berthelot reaction (Krom, 1980). Urea and ureido-N compounds were detected using a modified diacetyl monoxime (DAM) method (Reay et al., 2019c; Sullivan and Havlin, 1991). PO_4^{3-} and TP were determined following reaction with ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and potassium antimony (III) oxide tartrate ($K(SbO)C_4H_4O_6.3H_2O$) under acidic conditions (Boltz and Mellon, 1948). TOC was determined as non-purgeable organic carbon (TPOC) using a Shimadzu TOC-L Series analyser (Shimadzu Corporation, UK).

2.3.2. Amino acids and amino sugars extraction, isolation and derivatisation

The total hydrolysable amino acid (THAA) portion of soil (200 mg) was extracted, isolated and derivatised to their *N*-acetyl, *O*-isopropyl (NAIP) derivatives as previously outlined (Corr et al., 2007; Charteris et al., 2016). Norleucine (Nle; 100 μl of 400 μg ml^{-1} in 0.1 M HCl) was added as an internal standard. Total hydrolysable amino sugars (THASs) in soil (800 mg) were extracted, isolated and derivatised to alditol acetate derivatives (Reay et al., 2019a). Myo-inositol (100 μl of 400 μg ml^{-1} in 0.1 M HCl) was added as an internal standard.

2.3.3. Total carbon (TC) and total nitrogen (TN) content

Soil, roots and grass percentage total carbon (%TC) and nitrogen (% TN) were determined using a Thermo EA1110 elemental analyser (EA; Thermo Electron Corporation, MA, USA). Data was acquired and processed in Clarity (Version 2.6.2; DataApex, Prague, Czech-Republic). % TN and %TC of soil, grass and roots were determined using aspartic acid as a calibration standard and a soil NC standard as a quality control (CE Instruments Ltd, Wigan, UK). Values were only accepted when the calibration had r^2>0.99 and the QC was within ±0.02% and ±0.14% for %TN and %TC, respectively.

2.3.4. Bulk nitrogen isotopic composition

$\delta^{15}N$ values were determined for soil, leachate and plant biomass (sufficient mass to yield 0.2 mg N) using EA-IRMS. Soil, grass, roots and leachate were analysed as dried solids, while urine was analysed as a liquid on an inert absorbent material. A Flash EA1112 Series NC Analyser was coupled to a ThermoFinnigan Delta^{Plus} XP (Thermo Electron Corporation) via a Conflo III interface. Data were acquired and analysed using IsoDat NT 3.0 (Thermo Electron Corporation) and determined atom% values were corrected using a two-point linear normalisation using bracketing and QC standards traceable to IAEA-305 and IAEA-311.

2.3.5. Quantification of amino acids and amino sugars

THAA and THAS derivatives were analysed separately using an Agilent Technologies 7890B GC-FID (Agilent Technologies, CA, USA). Data was acquired and analysed using Agilent OpenLab CDS Chemstation (Rev C.01.07; Agilent Technologies). The analysis for THAAs was as follows: using a DB-35 coated capillary column (60 m × 0.32 mm i.d., 0.5 μm phase thickness) and He carrier gas at a constant flow of 2.0 ml min^{-1}, the temperature programme was 70 °C (2 min) to 150 °C at 15 °C min^{-1}, to 210 °C at 2 °C min^{-1} and a final temperature of 270 °C (8 °C min^{-1}; 10 min). For THASs analyses, the GC was fitted with a VF-23ms column (60 m × 0.32 mm i.d., 0.15 μm phase thickness) and the carrier gas (He) flow rate was 2.0 ml min^{-1} in constant flow mode. The temperature programme was 70 °C (1 min hold) to 210 °C (30 °C min^{-1}) to 260 °C (10 °C min^{-1}; 18 min hold). An external standard of amino acids and amino sugars were used to monitor instrument performance, identify AAs and ASs, and calculate individual compound response factors for quantification. The external standard for AAs comprised of 14 AAs (alanine; Ala, aspartic acid; Asp, glutamic acid; Glu, Glycine; Gly, hydroxyproline; Hyp, leucine; Leu, lysine; Lys, Nle, phenylalanine; Phe, proline; Pro, serine; Ser, threonine; Thr, tyrosine; Tyr and valine; Val) in 0.1 M HCl. The external standard for amino sugars comprised of myo-inositol, muramic acid; MurN, glucosamine; GlcN, galactosamine; GalN and mannosamine; ManN.

2.3.6. GC-C-IRMS

The ^{15}N content for individual AAs, as NAIP derivatives, and ASs, as alditol acetate derivatives, were determined using a ThermoFinnigan Trace 2000 GC coupled to a DeltaPlus XP IRMS via a Combustion III interface (Thermo Electron Corporation), as previously outlined (Reay et al., 2019b; Chiewattanakul et al., 2022). Data was acquired and analysed using IsoDat NT 3.0 (Thermo Electron Corporation). All analyses were completed in duplicate. Instrument performance was confirmed by use of in-house AS and AA standards, traceable to secondary standards, and values were only accepted when standard values were within ±1 SD. Additionally, ^{15}N values determined for individual AS were corrected using a two-point linear normalisation, as detailed in Reay et al. (2019a).

2.4. Calculations

All data processing, linear regressions and statistical analyses were performed in R version 4.1.0 (R Core Team, 2021). Normality of the data was determined by Shapiro-Wilk test ($p > 0.05$) and checked visually (Q-Q plots). Homogeneity of variance of the data was also confirmed prior to statistical analyses. ANOVAs were used to determine if there was a significant difference between the control and ^{15}N-urine treatment across the experimental period, with a significance value set at $p < 0.05$.

The contribution of bacterial and fungal sources to the GlcN pool were estimated using previously observed 5:1 ratio of bacterial GlcN to MurN in cultured soil bacteria (Glaser et al., 2004; He et al., 2011), which has previously been applied to compound-specific analyses of amino sugars (Reay et al., 2019a).

^{15}N enrichments of plant tissues, soil, and THAA and THAS pools are reported as atom fractions, and as percentage incorporated (%I) into each pool as derived from applied ^{15}N-urine at $t = 0$ following:

$$\%I = \frac{mol^E\left(^{15}N\right)_p}{mol\left(^{15}N\right)_g} 100$$

where $mol(^{15}N)_g$ is the ^{15}N in applied ^{15}N-labelled urine and $mol^E(^{15}N)p$ is the total amount of ^{15}N in excess (above background/ambient concentration) in the analysed pool, calculated from the pool size, N content and atom fraction excess ($x^E(^{15}N)$):

$$x^E\left(^{15}N\right) = x\left(^{15}N\right)_p - x\left(^{15}N\right)_C$$

where $x(^{15}N)_p$ is the atom fraction of ^{15}N in the pool at each sampling timepoint, and $x(^{15}N)_C$ is the atom fraction of ^{15}N in the pool before urine application ($t = 0$). The calculated incorporation into each pool reflected both uptake/assimilation/retention, and turnover of ^{15}N in the pool at that timepoint (Li et al., 2021). Hence, rates of incorporation determined, particularly from the microbial THAAs and THASs should be considered net incorporation, reflecting concurrent losses from the pools during turnover, or transfer to a more stable pool, alongside biosynthesis. Initial rates of incorporation are calculated during periods of increase of ^{15}N incorporation, when assimilation of urinary-^{15}N dominates over turnover from the pool, to better reflect uptake dynamics.

3. Results

3.1. Plant biomass N

Total aboveground and belowground (0–5 and 5–15 cm) biomass across the experimental period is shown in Fig. S3. Following urine addition, root biomass was lower compared to the control in both soil depths (Fig. S3). It remained lower than the control treatment until 31 d in the top soil layer, while it was consistently lower following urine deposition in the deeper soil layer. There was no significant two-way interaction, but a significant treatment effect was observed ($p < 0.001$). Above-ground biomass was consistently higher in the urine treatment, with a significant two-way interaction (time × treatment, $p < 0.001$). Above ground biomass increased across the experimental period to 860 ± 24 g m^{-2} in the urine treatment, compared to 256 ± 25 g m^{-2} for the unamended control at the end of the experimental period (Fig. S3c).

The N content of all biomass pools increased after urine addition compared to the control treatments and were consistently higher than the control treatment throughout the experiment (Fig. 1). For roots at both depths, there were significant two-way interactions (time × treatment, both $p < 0.001$), while for above ground biomass, after an increase to day 16 (51.9 ± 1.6 mg N g^{-1}), it gradually decreased (35.0 ± 1.7 mg N g^{-1} at 94 d). There was no significant two-way interaction (time × treatment, $p > 0.05$), however, there was a significant treatment effect ($p < 0.001$).

3.2. Soil N content

Similar to plant biomass, following urine addition, the N content of soil increased up to day 16 (Fig. 1) and subsequently plateaued. Soil TN was significantly higher in the top (treatment, $p < 0.001$) and lower soil layers (time × treatment $p = = 0.004$), compared to the control. The THAA concentration within soil rapidly increased following urine application to 3 d, followed by a second phase of increase to 16 d for 0–5 cm (15.3 ± 0.8 mg g^{-1}), and 31 d for the deeper soil (17.0 ± 1.0 mg g^{-1}; Fig. 2a). While THAA concentration subsequently declined to 94 d (9.8 ± 0.7 mg g^{-1}), it remained above pre-application levels ($p < 0.01$). Within individual AAs, all AAs followed a similar trend in concentration changes after urine application (Fig. S4). THAAs accounted for 40.8% of the soil TN pool prior to urine application, and a maximum of 48.9% 6 h after application. While the THAA concentration remained higher than $t = 0$, it reached a comparable portion of the TN pool at 94 d (38.1%) to pre-urine application.

Similarly, there was a rapid increase after urine application only to 6 h for AS (Fig. 2b). After a drop at 1 d, THAS concentration increased for both soil depths until 31 d (2.0 ± 0.2 mg g^{-1} and 1.8 ± 0.2 mg g^{-1}). At the end of the experimental period, THAS concentration and that of the

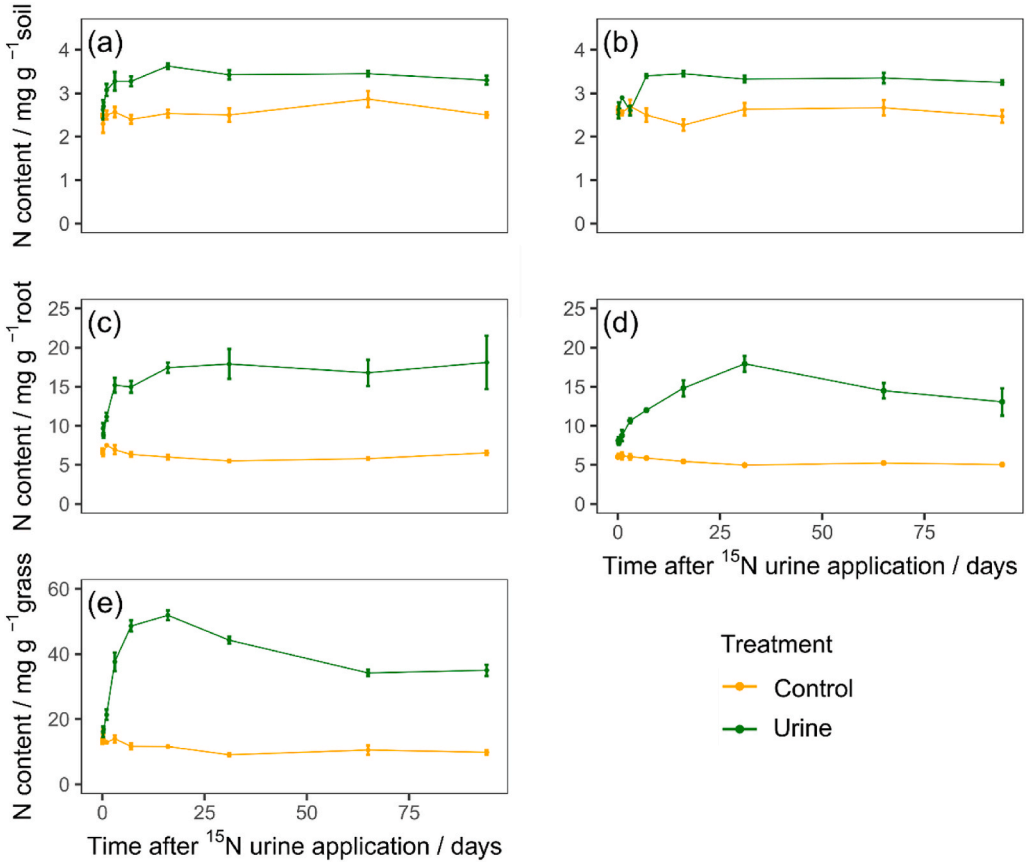

Fig. 1. Nitrogen content for (a) soil 0–5 cm, (b) soil 5–15 cm, (c) root 0–5 cm, (d) root 5–15 cm and (e) aboveground biomass following urine application. Values are mean ($n = 4$) ± SE.

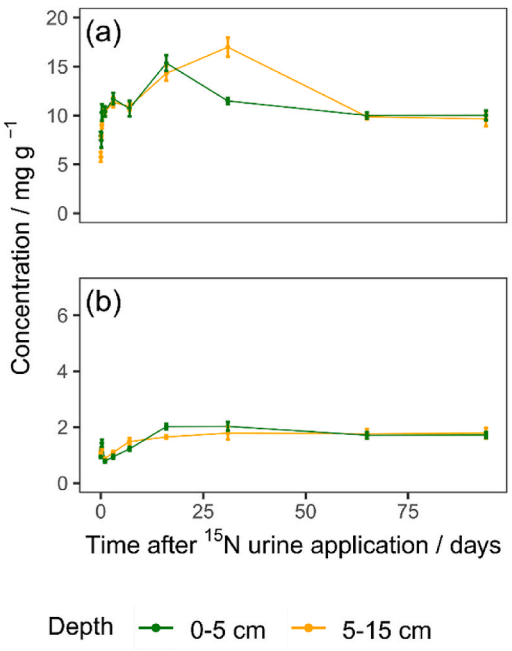

Fig. 2. Total hydrolysable (a) AA and (b) AS concentrations extracted from 0 to 5 cm and 5–15 cm soil after ^{15}N-urine application. Values are mean ± SE (n = 4).

individual AS (Fig. S5) remained higher than pre-urine application ($p <$ 0.01). The THAS pool accounted for 4.7 and 5.1% of the total soil N pool for the two depths at t = 0, with variable contribution across the experimental period. At 94 d, AS-N was a larger pool relative to total soil N than t = 0 (6.3% and 6.9%, respectively).

3.3. Leachate composition

The N leached during the experimental period is shown in Fig. 3. For the control treatment, the total N leached at each storm event was 1.08 ± 0.08 mg N, with no change across the experimental period. This was largely organic N (0.94 ± 0.08 mg N: 86.6 ± 1.0%), with small contributions from ureido-N (0.015 ± 0.001 mg N), NH_4^+ (0.05 ± 0.01 mg N) and NO_3^- (0.090 ± 0.001 mg N). After urine addition, there was a rapid increase in TN leached, which gradually decreased across the remainder of the experiment (Fig. 3a). It remained above the control throughout (4.3 ± 0.5 mg N at 94 d). ON followed the same pattern as total N, peaking at 82 ± 8 mg N (3 h), although within this pool, ureido-N, was comparable to the control at 94 d (0.033 ± 0.003 mg N). The proportion of ON relative to the TN pool decreased to 31 d (11.7 ± 1.0%). Initially, this was due to increased NH_4^+ leaching to 3 d (peak at 1 d; 21.0 ± 1.1 mg N), and subsequent increases in NO_3^- leached to 31 d (19.6 ± 1.0 mg N). For all determined N pools, there was a significant two-way interaction (time × treatment all $p < 0.001$), except ON and NO_3^-, where there was a significant treatment effect (both $p < 0.001$). The TP concentration of leachate did not vary between the control and urine treatments (Fig. S6, $p = 0.797$), although the composition was altered by urine addition, with higher OP leached by 3 d ($p = 0.013$). The leached C concentration increased after urine deposition and was at a maximum 3 h after urine addition (31.6 ± 2.3 mg l^{-1}: Fig. S6d). It was significantly higher than the control treatment for 7 d ($p < 0.01$), and C concentrations were subsequently comparable (all timepoints, $p > 0.05$).

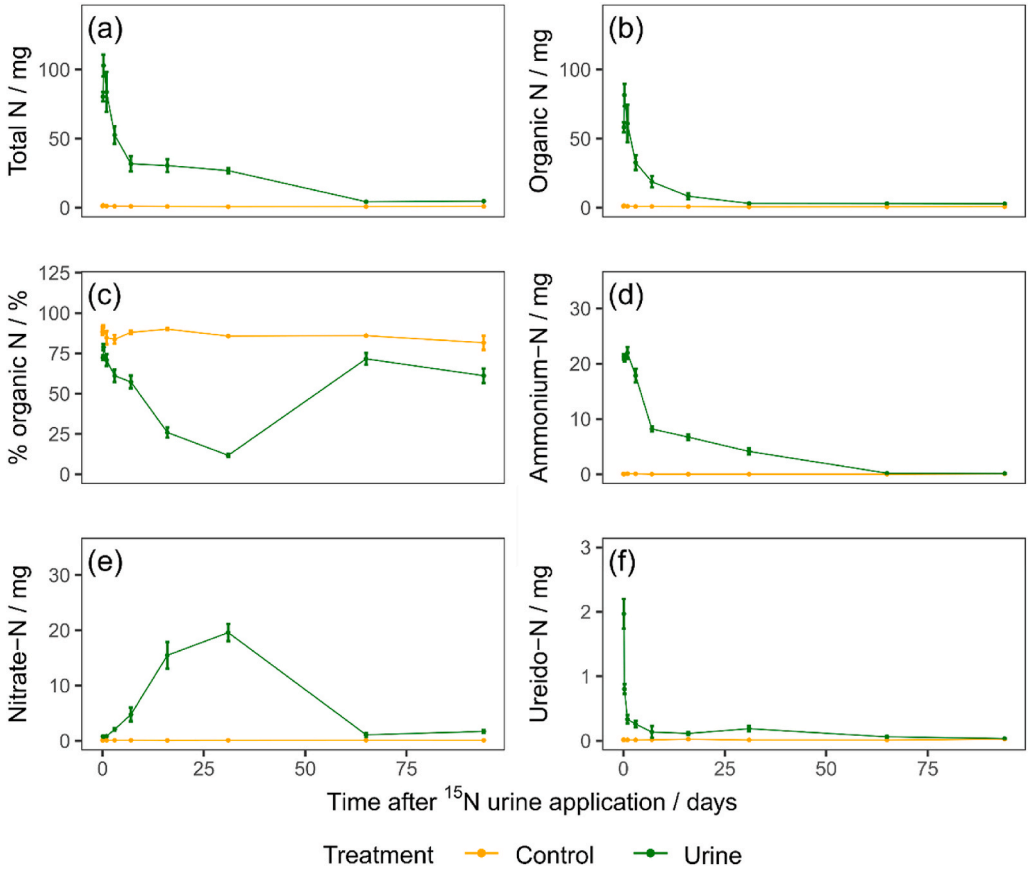

Fig. 3. Leached (a) total nitrogen (b) organic nitrogen, (c) percentage organic nitrogen, (d) ammonium, (e) nitrate and (f) ureido-N following ^{15}N-urine application. Values are mean ± SE (n = 4).

3.4. Bulk ^{15}N partitioning

The relative partitioning of ^{15}N-urine across the experimental period is shown in Table 2. Across the time course period, the two soil depths were the largest fate of ^{15}N for all timepoints, accounting for between 54.9 ± 3.8% (94 d) to 76.4 ± 3.8% (7 d). The top soil layer initially had high retention (55.4 ± 5.4% at 3 h), compared to a slower increase for the deeper soil layer (maximum 34.8 ± 2.0% at 7d), following transport of ^{15}N down the soil column. Leachate was initially a major fate for applied ^{15}N-urine, accounting for a maximum of 34.4 ± 2.6% at 6 h, but the proportion of ^{15}N leached during the simulated storm event at each sampling point decreased for the remainder of the experiment (to 1.08 ± 0.07% at 94 d). In terms of plant biomass, uptake into roots increased to 7 d (0.69 ± 0.13%) and 31 d (5.64 ± 0.49%) at 0–5 and 5–15 cm depths, respectively, and total root ^{15}N content accounted for 4.0 ± 0.5% of applied ^{15}N-urine at 94 d. Transport into above ground biomass was rapid up to 3 d (12.4 ± 1.9%), with a slower increase to 94 d (38.4 ± 1.6%). The overall ^{15}N retained in the determined pools was high throughout the experimental period and was between 96% (3 h and 94 d) and 99% (1 d), fluctuating within this range at the other time points, likely due to variation between replicates. The unaccounted for ^{15}N was assumed to be gaseous losses, as all other pools were quantified, and therefore, gaseous losses were between 1.2% and 4.1%.

3.5. Microbial ^{15}N incorporation

The ^{15}N enrichment of individual AAs and ASs are shown in Figs. S7 and S8, respectively, and alongside concentrations, were used to calculate incorporation of the applied ^{15}N-urine (Fig. 4) to account for pool size. To allow comparison of initial incorporation rates, pool size, and total net incorporation over 94 d, relative differences between individual AA and AS are presented in Fig. 5. For the THAA pool in the top soil layer (Fig. 4a), there was a rapid increase to 3 d (2.87% day^{-1}, r^2 = 0.682), and after a second period of slower increase to 16 d (0.60% day^{-1}, r^2 = 0.740) and a subsequent plateau for the remainder of the experimental period (13.7 ± 0.58%). A similar trend of increase was observed in the deeper soil layer, although delayed relative to the top soil (to 7 d 1.24% day^{-1}, r^2 = 0.526: to 31 d 0.49% day^{-1} r^2 = 0.860). The maximum incorporation in this soil layer was 19.1 ± 1.8% at 31 d, although it subsequently decreased to 15.1 ± 2.9% at 94 d. The relative routing through individual AA pools is graphically represented in Fig. 5, combining pool size, rates of incorporation, and incorporation at 94 d. The highest incorporation was observed for the most abundant AAs (Ala and Gly, 3.34 ± 0.14% at 65 d and 3.79 ± 0.48% at 16 d, respectively). AAs central to AA biosynthesis also had high incorporation (Glx and Asx 2.25 ± 0.15% and 1.82 ± 0.16%, respectively). Comparatively, AAs which are further away in terms of biosynthetic proximity (e.g., Phe, Lys, Hyp and Tyr) had the lowest ^{15}N incorporation over the time course. Similarly, rates of incorporation into these pools (Table S2) via linear regression reflected the influence of pool size (e.g., Ala k_0 = 0.732% day^{-1}, r^2 = 0.676 and Gly k_0 = 0.615% day^{-1}, r^2 = 0.315) and biosynthetic proximity (Glx k_0 = 0.426% day^{-1}, r^2 = 0.462), compared to less proximal AAs (e.g. Hyp k_0 = 0.011% day^{-1}, r^2 = 0.778 and Tyr k_0 = 0.022% day^{-1}, r^2 = 0.616).

In the deeper soil layer, individual AAs largely exhibited the same hierarchical trends as for the top soil layer, with high incorporation linked to abundance (e.g., Ala 3.51 ± 0.26% at 31 d), and those central to biosynthesis of AAs (Asx 3.98 ± 0.58% at 31 d; Glx 2.71 ± 0.22% at 31 d). This was also reflected in initial incorporation rates into individual AA in soil (Fig. 5b, Table S2), which were consistently lower than those observed in the top soil layer. One key difference was Gly, which accounted for 2.77 ± 0.46% of applied ^{15}N at 3 h but had rapidly decreased by 6 h (1.03 ± 0.07%), and subsequently followed the same trend as other AAs. Gly was the most abundant AA in urine (Fig. S1), hence, it is likely this is reflecting urinary-derived Gly, which could not be distinguished from newly biosynthesised ^{15}N-labelled Gly.

The proportion of urinary-N incorporated into the THAS pool was lower than THAAs, reflecting the smaller pool size. In the top soil layer, the percentage of urine assimilated into the THAS pool showed two periods of increase 0–3 d (0.495% day^{-1}, r^2 = 0.612) and 3 d–31 d (0.090% day^{-1}, r^2 = 0.888), to 3.86 ± 0.34%, followed by a gradual decrease over the remainder of the time course (3.28 ± 0.29%). ^{15}N incorporation into the THAS pool at 5–15 cm increased to 7 d (0.56 ± 0.08%, 0.089% day^{-1}, r^2 = 0.644) and a second slower period of increase to 65 d (1.63 ± 0.19%, 0.019% day^{-1}, r^2 = 0.802). As with the THAA pool, the response of the deeper soil layer was delayed relative to the top soil layer. Bacterial AS (MurN and bacterial GlcN (GlcN-B)) exhibited similar trends in incorporation in the top soil layer, with increases to 16 d (0.29 ± 0.1%, 0.015% day^{-1}, r^2 = 0.861) and 31 d (2.20 ± 0.15%, 0.065% day^{-1}, r^2 = 0.861), respectively, prior to a decline for the remainder of the experiment. GalN showed a similar trend to GlcN-B, increasing to 0.25 ± 0.07% (0.007% day^{-1}, r^2 = 0.698). GlcN-F incorporation continued to increase to 65 d to 1.56 ± 0.19% (0.023% day^{-1}, r^2 = 0.870). The trend in the overall flux into the individual AS pools, indicated by plateau ^{15}N incorporation, was $GlcN_F \approx GlcN_B > MurN > GalN$ (Fig. 5b, Table S3). Trends for all individual AS were similar in the

Table 2
Bulk percentage ^{15}N retention in soil at two depths (0–5 cm and 5–15 cm), roots at two depths (0–5 cm and 5–15 cm), above ground biomass (grass) and leachate across the 94-d time course following ^{15}N-urine application. Values are mean (n = 4), and SE is standard error of the mean.

Time		Grass	Leachate	Root		Soil		Total
				0–5 cm	5–15 cm	0–5 cm	5–15 cm	
0.125	*mean*	0.6	26.5	0.07	0.50	55.4	13.0	96.0
	SE	0.1	2.3	0.01	0.16	5.4	0.6	8.5
0.25	*mean*	1.5	34.4	0.06	1.02	46.1	14.4	97.4
	SE	0.2	2.6	0.00	0.11	2.6	1.5	7.0
1	*mean*	2.7	28.0	0.25	0.51	40.5	26.9	98.8
	SE	0.5	4.3	0.04	0.06	1.7	1.4	8.0
3	*mean*	9.3	20.6	0.56	1.71	41.8	23.9	97.8
	SE	1.5	1.6	0.06	0.23	3.3	2.2	8.9
7	*mean*	12.4	7.9	0.69	1.18	41.4	34.8	98.3
	SE	2.0	3.0	0.13	0.11	1.7	2.0	9.0
16	*mean*	16.4	9.6	0.50	3.55	36.7	31.8	98.6
	SE	3.8	1.6	0.04	0.78	2.6	1.5	10.2
31	*mean*	17.6	7.6	0.43	5.64	32.7	34.8	98.7
	SE	1.8	0.4	0.14	0.49	1.4	2.6	6.7
65	*mean*	35.0	0.9	0.22	4.90	29.1	28.3	98.4
	SE	0.3	0.2	0.04	0.59	0.8	2.0	3.9
94	*mean*	38.4	1.1	0.25	3.77	25.0	25.8	95.9
	SE	2.6	0.1	0.05	0.49	1.8	0.5	5.5

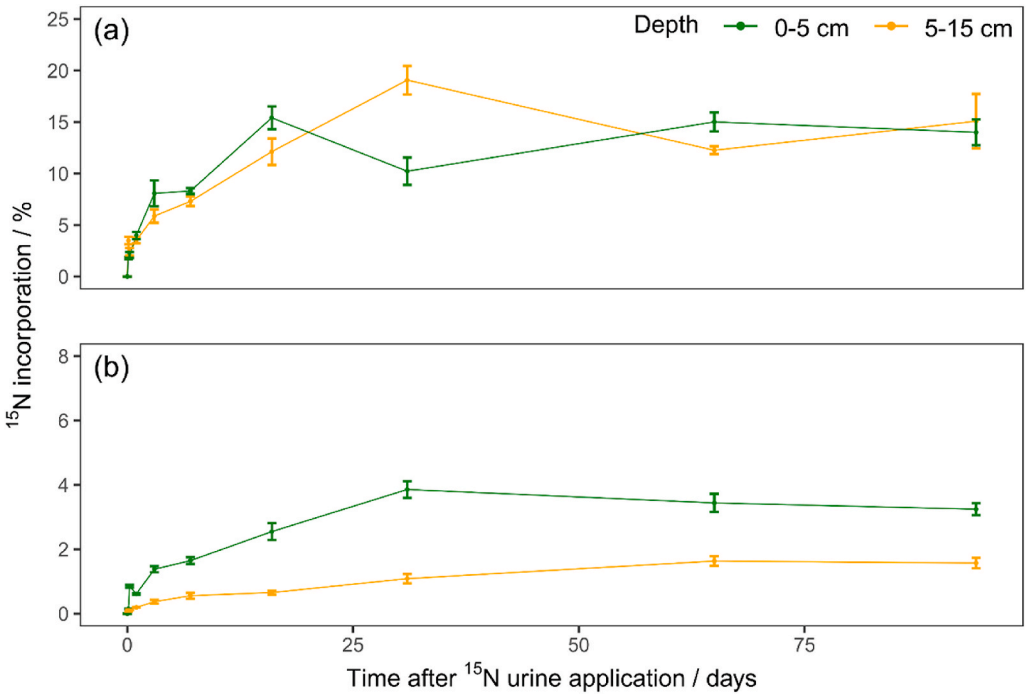

Fig. 4. Percentage of applied ^{15}N-urine into (a) THAA at 0–5 cm and 5–15 cm and (b) THAS at 0–5 cm and 5–15 cm. Values are mean ± SE (n = 4).

deeper soil layer, where incorporation was lower for all individual AS (Fig. 5b, Fig. S10).

4. Discussion

4.1. High N leaching losses in urine patches

Each urine patch was a hotspot of N cycling, with N concentrations above that of plant and microbial N demand. This was confirmed by high N leaching losses (233 kg N ha^{-1} at 6 h) observed immediately after urine deposition, the composition of which indicated the dominant processing pathways of urinary N. This supported our hypothesis of immediate large leaching losses. As leaching was only determined at 15 cm soil depth, this represents potential leaching to deeper soil layers, prior to leaching below rooting depth. Despite urea being the main form of N added (equivalent to 764 kg N ha^{-1}), this was rapidly hydrolysed to NH_4^+, and was subsequently leached. Subsequent increases in NO_3^-, due to nitrification, peaking at 44 kg N ha^{-1}, were consistent with previous observations of leaching beneath sheep urine patches (20–74 kg N ha^{-1}; Di and Cameron, 2007; Hoogendoorn et al., 2017; Maheswaran et al., 2022). Potential leaching of ON also increased relative to the control after urine application due to ON pools in urine (e.g., AAs, allantoin, hippuric acid, creatine; *ca.* 14% of urine-N pool in sheep, up to 28% in cattle (Chadwick et al., 2018; Marsden et al., 2020)). There was also evidence of native soil N leaching and cell lysis, arising from osmotic stress and rapid change in pH (Ambus et al., 2007; Bertram et al., 2012; Lambie et al., 2012). Assuming no isotopic fractionation of applied urine and no additional native soil N loss above that leached in the control, the theoretical enrichment of the leachate was 1.99 atom%^{15}N. The lower enrichment (1.48 ± 0.01 atom% ^{15}N at 3 h) of leached N indicated dilution with native soil N accounting for *ca.* 25% of leached N. Some variation may have also arisen due to differential processing and retention of urinary-N pools, which were not expected to be homogenously labelled given the relatively short (7 days) feeding period and varying biosynthetic sources of urinary-N pools (Sørensen et al., 1994). The quantity of leached ON in this study indicated this is a major source of N loss and was higher than nitrate until *ca.* 7 d after urine deposition. Hence, ON must be considered when quantifying N losses from a urine patch (van Kessel et al., 2009), given the potential for processing of this N in deeper soil (Jones et al., 2018), and in watercourses (Mena-Rivera et al., 2022).

While urinary-N initially indicated high potential for leaching, it was a minor fate (1%) after 94 d. In previous studies, there has been a wide range of reported amounts of leached N beneath a urine patch in comparable pasture and soil type using ^{15}N tracing, from 6% (Di et al., 2002; Silva et al., 2005) to 64% (Wachendorf et al., 2005) over 1 year. The lower leaching rate from a sandy loam soil was comparable to leaching between 7 and 31 days, with high initial leaching consistent with that observed by Di and Cameron (2007). Studies in the same soil and pasture type have not been conducted in summer, although low losses (7%) have been observed in a *L. perenne* pasture over loam soil (Decau et al., 2003). In this study, the low rainfall conditions reflected those observed during the preceding growing season at the site (13.5 mm between July 01, 2017 to September 30, 2017). The subsequent simulated storm event indicated that the timing of prevailing weather following urine deposition was a key control on leaching (Buckthought et al., 2015), although low losses may have also been influenced by the laboratory nature of this study. In periods of low rainfall, uptake and assimilation into plants and soil microbes mitigated leaching losses. The precipitation pattern simulated in this study reflected period of droughts, and increased frequency of extreme rainfall, associated with climate change (Allen and Ingram, 2002). The findings herein suggest there may be significant changes in N cycling in a urine patch due to future changes in precipitation, with longer period of droughts increasing availability to microbes and plants, or other N loss pathways.

Gaseous losses were estimated via difference from the quantified pools. Losses via N_2O, N_2 and NH_3 were relatively low, accounting for up to 4% of applied ^{15}N, in-line with IPCC emission factor values for N_2O, although much lower than the default emission factor for NH_3 (20%) (IPCC, 2019). While previous studies have found up to one third of applied urinary-N lost as NH_3 (Bronson et al., 1999; Nichols et al., 2018; Adhikari et al., 2020), NH_3 volatilisation from this site are low, especially at pH values < 7 (Jones et al., 2012), and were likely minimised under the controlled greenhouse conditions. Emission factors of N_2O at this site have also been found to be lower than default values used for IPCC (Marsden et al., 2016a; Chadwick et al., 2018; IPCC, 2019). While

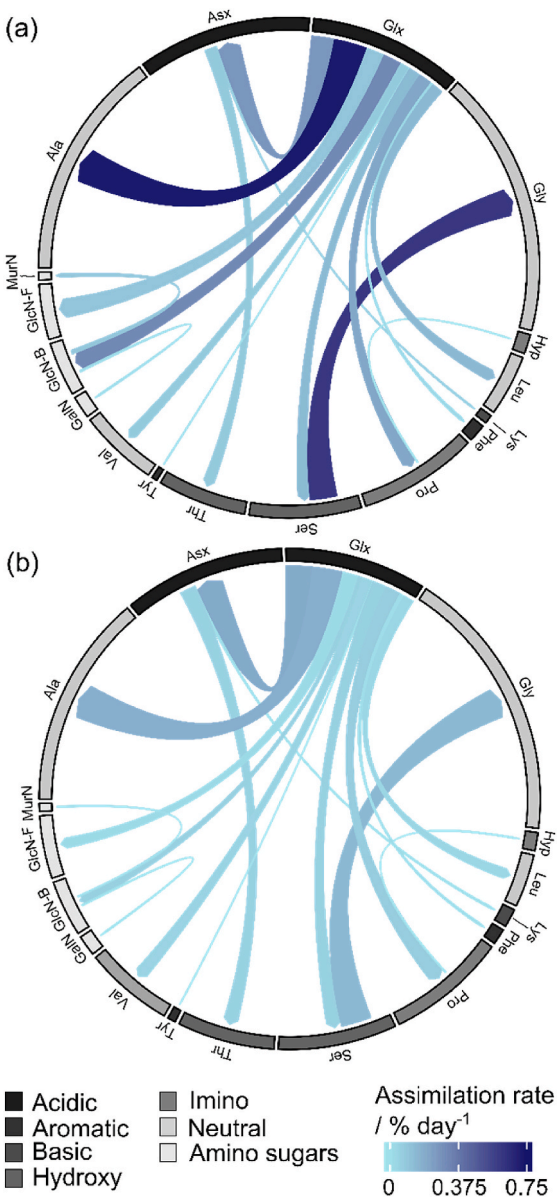

Fig. 5. Rates and percentage of ^{15}N-urine assimilation into individual proteinaceous AAs and individual ASs in soil at (a) 0–5 cm and (b) 5–15 cm. The circle segment colour reflects the class on amino acid/amino sugars, the circle segments represent the average composition of the pool of THAA and THAS in mol %, and the colour of the segment indicates the compound class. The colour of the arrow represents the incorporation rates (% day^{-1}) after 3 d (a) and 7 d (b). The arrow width is proportional to the percentage incorporation of ^{15}N-urine after 94 d, and the direction indicates the transfer of N according to primary biosynthetic pathways of each AA and AS.

losses were dominated by leaching, there is potential for increases in gaseous losses via denitrification in deeper soil from this leached pool.

4.2. Plant utilisation of urinary-N reduced N leaching losses

Increases in plant uptake following urinary-N deposition offset leaching N losses. Initially, uptake into plant biomass was a minor fate of urinary-^{15}N, when N supply was higher than plant N uptake capacity. Decreased root biomass initially indicated potential toxic effects on roots due to high N and salt concentrations. This has previously been observed whereby increased dead root biomass beneath a synthetic urine patch was attributed to damage from high osmotic stress (Shand et al., 2002). With root biomass recovery, aboveground biomass increased (after *ca.* 16 d) and the uptake of ^{15}N-urine into plant biomass reduced N available for leaching. Incorporation into above ground biomass accounted for 42% of urinary-N after 94 d, supporting our hypothesis that plant biomass would be a major fate of urinary N on a longer time scale. This was comparable to *L. perenne* uptake in a sandy loam soil after dairy cow urine addition in previous studies, despite the shorter timescale presented herein (e.g. 39% (Silva et al., 2005), 38.8% (Di et al., 2002)). Other studies using sheep urine have found a range in plant uptake of applied urine but were comparable to this study following application in spring and summer. For example, Sørensen and Jensen (1996) found 30% of urine ^{15}N in shoots in a *Lolium multiflorum Lam* pasture in early spring after 2 months, increasing to 37% accumulated after 3 months. This high retention was likely due to low leaching, except at simulated storm events at sampling timepoints, and the low rainfall reflected that observed in the preceding summer season at the site. It may also be influenced by the constant soil moisture, and minimising of N losses in this laboratory mesocosm experiment. This high plant uptake during periods of low leaching is important in terms of recycling excreted N in a grazing system, with retention of excreted N in aboveground biomass subsequently available for grazing sheep and re-entering the grassland N cycle.

4.3. Microbial biosynthetic processing of urinary-N

High retention in soil was observed across the time course after urine deposition, accounting for between 52% (94 d) and 68% (3 h). This was higher than previous studies (e.g. 13–30% (Sørensen and Jensen, 1996; Clough et al., 1998; Ambus et al., 2007; Woods et al., 2017)), although higher soil retention has been observed in summer over sandy loam, similar to this study (63% in non-irrigated pasture (Pakro and Dillon, 1995)). While high retention may have arisen later in the experimental period due to the low simulated rainfall, even immediately after urine deposition, soil retention remained high, indicating potential abiotic retention, or rapid microbial utilisation. Incorporation into newly biosynthesised AAs and ASs accounted for up to 65% of urine-^{15}N retained in soil (34% of total applied urine). This was higher than quantified in microbial biomass following ^{15}N-labelled cow urine deposition after 12 weeks (12%), which increased 17% after 20 weeks (Wachendorf and Joergensen, 2011). Assimilation in this study was also higher than for synthetic sheep urine applied to mesocosms seeded with *Agrostis capillaris*, where only 6% was recovered at 7 days, compared to 18% herein (Williams et al., 2003). The different seasons (summer *vs.* winter) and conditions (greenhouse *vs.* field) which limited leaching, alongside differences in applied urine (real *vs.* synthetic) in this study may be responsible for the higher microbial N assimilation of urinary-^{15}N. This study serves to highlight the potentially large pool of urinary-N which was assimilated into microbial biomass, with further studies required to investigate controls on microbial uptake (e.g. soil and plant type (Clough et al., 1998; Woods et al., 2017)).

This study also presents detailed data regarding the evolution of ^{15}N-urine biosynthesised into the microbial ON pool. As discussed above for plant uptake, utilisation of urinary-derived N following deposition and stabilisation into the microbial N pool serves to highlight the role of the microbial community in reducing leaching losses. This is supported by a significant inverse correlation ($r^2 = 0.783$, $p < 0.001$) of decreasing ^{15}N leaching losses with increasing ^{15}N microbial assimilation, particularly during the first month. Microbial incorporation was initially rapid, and higher (for 31 d) than plant uptake, supporting our hypothesis that microbes initially would outcompete plants, while subsequently providing N for plant uptake during turnover. There was a clear priming effect on microbial biosynthesis, indicated by increased abundance with time of both THAAs and THAAs. Urine deposition only accounted for a minor contribution to the AA pool (1.3% of soil THAAs). A combination of C, N and P inputs, alongside solubilisation of SOM due to pH changes following urine deposition likely promoted microbial activity and resulted in a sustained increase in THAA and THAS concentration

(Blagodatskaya and Kuzyakov, 2008). High nitrate concentrations may have resulted in cell lysis of the existing microbial community, and depolymerisation and mineralisation of released THAAs and THASs (Charteris et al., 2016). Therefore, changes in concentrations reflect both production and turnover. While it is not possible to directly link microbial biomass to the quantified-ON pools, increases in this pool indicate increased microbial ON biosynthesis, and thus higher microbial activity. Previously derived indicators of microbial activity have yielded variable results and some (e.g., respiration (Kelliher et al., 2005)) have not indicated increased microbial activity. The differing approaches make direct comparison challenging between studies, thus further work to constrain the impact of urine deposition on microbial community size and activity is warranted. Regardless, the rapid assimilation of urinary-^{15}N into microbial-derived THAAs and THASs was higher than plant uptake for the first month after urine deposition. This likely mitigated N losses, and N assimilated into microbial biomass will subsequently support plant N supply following microbial turnover.

The application of compound-specific ^{15}N–SIP to ^{15}N-labelled urine directly revealed the flow of urinary-derived N through the largest microbial N pools. The rates, and degree of net incorporation into individual AA and AS pools reflected (i) pool size, (ii) biosynthetic proximity to urinary-N, and (iii) transport down the soil profile. Additionally, AS incorporation also revealed different rates of processing for bacterial and fungal pools within soil. Higher assimilation rates into bacterial AS (MurN and GlcN-B) suggested the bacterial community dominated processing of urinary-N initially, which is consistent with previous observations of a shift towards bacterial dominance in a urine patch via PLFA analyses (Nunan et al., 2006). Continued increases in GlcN-F are consistent with utilisation of secondary sources of N (e.g., ON) (He et al., 2011; Reay et al., 2019a). The source of GalN is unclear, however, trends in ^{15}N assimilation reflected that of other bacterial AS.

Rates of incorporation for both THAA and THAS pools are considered net change, given likely turnover of the pools across the experimental period. Rates were determined when assimilation dominated over turnover (i.e. increased incorporation), and differences reflect both varying rates of incorporation, and turnover of the pools. Given the high rate of incorporation into the Glx pool, via glutamate dehydrogenase or glutamine synthetase (Reitzer, 2004), it is suggested the majority of urinary-N was likely mineralised prior to incorporation as NH_4^+. Subsequently, biosynthesis largely reflected primary pathways and expected biosynthetic proximities to Glx (Fig. 5). AAs further in biosynthetic proximity (e.g. Hyp, Tyr) showed lower rates of incorporation, and total incorporation over 94 d (Fig. 5). AAs in the deeper soil showed lower rates, and total incorporation, reflecting the additional transport, and subsequent lower availability of urinary-N down the soil profile. Further, all AS showed slower relative rates of incorporation, and lower total incorporation than AAs (Fig. 5). This reflected the smaller pool size and potential slower turnover of the existing AS pool, or a larger inactive pool, compared to Glx, and other AAs in comparable biosynthetic proximity.

Gly and Ala showed elevated ^{15}N enrichment (Fig. 5), particularly in the deeper soil due to transport in the simulated storm event and appeared closer biosynthetically to Glx than previously observed for inorganic fertiliser (Charteris et al., 2016; Charteris, 2019; Reay et al., 2023). This was an artefact of Gly being the most abundant AA in the applied urine, and a rapid drop in the ^{15}N enrichment of this pool indicated microbial processing of urinary-derived Gly. The high incorporation rates into Ala (Fig. 5) indicated that intact urinary-derived ^{15}N-Gly underwent transamination with pyruvate to yield Ala, a secondary biosynthesis pathway for Ala, alongside the primary pathway from Glu (Keseler et al., 2009; Caspi et al., 2018). Higher incorporation into Ala was previously observed when ^{13}C,^{15}N-Gly was applied to a grassland soil (Knowles et al., 2010). While it is not possible to directly quantify the proportion of Ala produced by the primary and secondary biosynthetic pathways, there is clear evidence of intact AA processing alongside mineralisation-immobilisation in the urine patch.

4.4. Conclusions

The findings presented herein clearly revealed the dynamics of urine partitioning in grasslands following deposition in the active growing season. The timing of rainfall after urine deposition controlled the susceptibility to potential leaching of both urine-derived and native soil N, and the form of N leached. Periods of low rainfall ultimately increased microbial assimilation and plant uptake of urinary N, consistent with our hypothesis. With expected increases in extreme weather events (drought, precipitation) due to climate change, these changes in urine patch N-cycling are likely to be significant for grassland productivity, and loss pathways in future climates. Uptake into plant biomass was the largest fate of urinary-N, but the role of soil microbial processing in retaining N was clearly revealed via compound-specific ^{15}N–SIP, to a level of detail previously unavailable. There was a priming effect in the urine patch, with increased microbial biosynthesis of ON from urinary-N, via the mineralisation-immobilisation pathway, while intact processing of dominant urinary-AAs also occurred. Inclusion of amino sugars in this study also indicated bacterial assimilation initially dominated incorporation into newly biosynthesised ON. Further work investigating the potential impacts of differing soil, plant and/or weather conditions is required to fully constrain the role of the microbial community in retaining and recycling urinary-N in grazing systems.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2023.109011.

References

Adhikari, K.P., Saggar, S., Hanly, J.A., Guinto, D.F., 2020. Urease inhibitors reduced ammonia emissions from cattle urine applied to pasture soil. Nutrient Cycling in

Agroecosystems 117, 317–335. https://doi.org/10.1007/S10705-020-10070-0/TABLES/4.
Allen, M.R., Ingram, W.J., 2002. Constraints on future changes in climate and the hydrologic cycle. Nature 2002 (419), 224–232. https://doi.org/10.1038/nature01092, 6903 419.
Ambus, P., Petersen, S.O., Soussana, J.-F., 2007. Short-term carbon and nitrogen cycling in urine patches assessed by combined ^{13}C and ^{15}N labelling. Agriculture, Ecosystems & Environment 121, 84–92. https://doi.org/10.1016/j.agee.2006.12.007.
Anderson, C.R., Peterson, M.E., Frampton, R.A., Bulman, S.R., Keenan, S., Curtin, D., 2018. Rapid Increases in Soil pH Solubilise Organic Matter, Dramatically Increase Denitrification Potential and Strongly Stimulate Microorganisms from the Firmicutes Phylum. PeerJ 2018. https://doi.org/10.7717/PEERJ.6090/SUPP-8.
Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704–726. https://doi.org/10.1007/BF02804901.
Bertram, J.E., Orwin, K.H., Clough, T.J., Condron, L.M., Sherlock, R.R., OCallaghan, M., 2012. Effect of soil moisture and bovine urine on microbial stress. Pedobiologia 55, 211–218. https://doi.org/10.1016/J.PEDOBI.2012.03.004.
Blagodatskaya, E., Kuzyakov, Y., 2008. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. Biology and Fertility of Soils 45, 115–131. https://doi.org/10.1007/s00374-008-0334-y.
Boltz, D.F., Mellon, M.G., 1948. Spectrophotometric determination of phosphorus as molybdiphosphoric acid. Analytical Chemistry 20, 749–751. https://doi.org/10.1021/ac60020a021.
Bosshard, C., Frossard, E., Dubois, D., Mader, P., Manolov, I., Oberson, A., 2008. Incorporation of nitrogen-15-labeled amendments into physically separated soil organic matter fractions. Soil Science Society of America Journal 72, 949. https://doi.org/10.2136/sssaj2006.0376.
Bronson, K.F., Sparling, G.P., Fillery, I.R.P., 1999. Short-term N dynamics following application of ^{15}N-labeled urine to a sandy soil in summer. Soil Biology and Biochemistry 31, 1049–1057. https://doi.org/10.1016/S0038-0717(99)00019-X.
Buckthought, L.E., Clough, T.J., Cameron, K.C., Di, H.J., Shepherd, M.A., 2015. Urine patch and fertiliser N interaction: effects of fertiliser rate and season of urine application on nitrate leaching and pasture N uptake. Agriculture, Ecosystems & Environment 203, 19–28. https://doi.org/10.1016/J.AGEE.2015.01.019.
Cameron, K.C., Di, H.J., Moir, J.L., 2013. Nitrogen losses from the soil/plant system: a review. Annals of Applied Biology 162, 145–173. https://doi.org/10.1111/aab.12014.
Caspi, R., Billington, R., Fulcher, C.A., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Midford, P.E., Ong, Q., Ong, W.K., Paley, S., Subhraveti, P., Karp, P. D., 2018. The MetaCyc database of metabolic pathways and enzymes. Nucleic Acids Research 46, D633–D639. https://doi.org/10.1093/nar/gkx935.
Chadwick, D.R., Cardenas, L.M., Dhanoa, M.S., Donovan, N., Misselbrook, T., Williams, J.R., Thorman, R.E., McGeough, K.L., Watson, C.J., Bell, M., Anthony, S.G., Rees, R.M., 2018. The contribution of cattle urine and dung to nitrous oxide emissions: quantification of country specific emission factors and implications for national inventories. Science of the Total Environment 635, 607–617. https://doi.org/10.1016/J.SCITOTENV.2018.04.152.
Charteris, A.F., 2019. ^{15}N tracing of microbial assimilation, Partitioning and Transport of Fertilisers in Grassland Soils. Springer Theses. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-030-31057-8.
Charteris, A.F., Knowles, T.D.J., Michaelides, K., Evershed, R.P., 2016. Compound specific amino acid ^{15}N stable isotope probing of nitrogen assimilation by the soil microbial biomass using gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 30, 1846–1856. https://doi.org/10.1002/RCM.7612.
Cheng, L., Nicol, A.M., Dewhurst, R.J., Edwards, G.R., 2013. The effects of dietary nitrogen to water-soluble carbohydrate ratio on isotopic fractionation and partitioning of nitrogen in non-lactating sheep. Animal 7, 1274–1279. https://doi.org/10.1017/S1751731113000311.
Chiewattanakul, M., McAleer, A.D.A., Reay, M.K., Griffiths, R.I., Buss, H.L., Evershed, R. P., 2022. Compound-specific amino acid ^{15}N-stable isotope probing for the quantification of biological nitrogen fixation in soils. Soil Biology and Biochemistry 169, 108654. https://doi.org/10.1016/j.soilbio.2022.108654.
Clough, T.J., Ledgard, S.F., Sprosen, M.S., Kear, M.J., 1998. Fate of ^{15}N labelled urine on four soil types. Plant and Soil 199, 195–203. https://doi.org/10.1023/A:1004361009708.
Corr, L.T., Berstan, R., Evershed, R.P., 2007. Optimisation of derivatisation procedures for the determination of $\delta^{13}C$ values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun. Mass Spectrometry 21, 3759–3771. https://doi.org/10.1002/rcm.3252.
Decau, M.L., Simon, J.C., Jacquet, A., 2003. Fate of urine nitrogen in three soils throughout a grazing season. Journal of Environmental Quality 32, 1405–1413. https://doi.org/10.2134/JEQ2003.1405.
DEFRA, 2018. Farming Statistics - Livestock Populations at 1 December 2017 United Kingdom.
Di, H.J., Cameron, K.C., 2007. Nitrate leaching losses and pasture yields as affected by different rates of animal urine nitrogen returns and application of a nitrification inhibitor—a lysimeter study. Nutrient Cycling in Agroecosystems 79, 281–290. https://doi.org/10.1007/s10705-007-9115-5.
Di, H.J., Cameron, K.C., 2002. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. Nutrient Cycling in Agroecosystems 64, 237–256. https://doi.org/10.1023/A:1021471531188.
Di, H.J., Cameron, K.C., Silva, R.G., Russell, J.M., Barnett, J.W., 2002. A lysimeter study of the fate of ^{15}N-labelled nitrogen in cow urine with or without farm dairy effluent in a grazed dairy pasture soil under flood irrigation. New Zealand Journal of Agricultural Research 45, 235–244. https://doi.org/10.1080/00288233.2002.9513514.
Galloway, J.N., Cowling, E.B., 2002. Reactive nitrogen and the world: 200 Years of change. AMBIO: A Journal of the Human Environment 31, 64–71. https://doi.org/10.1579/0044-7447-31.2.64.
Glaser, B., Turrion, M.-B., Alef, K., 2004. Amino sugars and muramic acid—biomarkers for soil microbial community structure analysis. Soil Biology and Biochemistry 36, 399–407. https://doi.org/10.1016/j.soilbio.2003.10.013.
He, H., Li, X.B., Zhang, W., Zhang, X., 2011. Differentiating the dynamics of native and newly immobilized amino sugars in soil frequently amended with inorganic nitrogen and glucose. European Journal of Soil Science 62, 144–151. https://doi.org/10.1111/j.1365-2389.2010.01324.x.
Heijden, M.G., Bardgett, R.D., Straalen, N.M. Van, 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters vol. 11, 296–310. https://doi.org/10.1111/J.1461-0248.2007.01139.X. YEAR-OF-BIODIVERSITY, 10.1111/(ISSN)1461-0248.
Hoogendoorn, C.J., Lambert, M.G., Devantier, B.P., Theobald, P.W., Park, Z.A., 2017. Nitrogen Fertiliser Application Rates and Nitrogen Leaching in Intensively Managed Sheep Grazed Hill Country Pastures in New Zealand, pp. 154–172. https://doi.org/10.1080/00288233.2017.1287100, 60.
Huhtanen, P., Hristov, A.N., 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. Journal of Dairy Science 92, 3222–3232. https://doi.org/10.3168/JDS.2008-1352.
IPCC, 2019. 2019 Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories.
ISO/TC 147/SC 2, 1996. ISO13395:1996 Determination of Nitrite Nitrogen and Nitrate Nitrogen and the Sum of Both by Flow Analysis (CFA and FIA) and Spectrometric Detection.
Jones, D.L., Magthab, E.A., Gleeson, D.B., Hill, P.W., Sanchez-Rodriguez, A.R., Roberts, P., Ge, T., Murphy, D.V., 2018. Microbial competition for nitrogen and carbon is as intense in the subsoil as in the topsoil. Soil Biology and Biochemistry 117, 72–82. https://doi.org/10.1016/J.SOILBIO.2017.10.024.
Jones, D.L., Willett, V.B., Stockdale, E.A., Macdonald, A.J., Murphy, D.V., 2012. Molecular weight of dissolved organic carbon, nitrogen, and phenolics in grassland soils. Soil Science Society of America Journal 76, 142. https://doi.org/10.2136/sssaj2011.0252.
Kelliher, F.M., Sedcole, J.R., Minchin, R.F., Wan, Y., Condron, L.M., Clough, T.J., Bol, R., 2005. Soil microbial respiration responses to repeated urea applications in three grasslands. Soil Research 43, 905. https://doi.org/10.1071/SR05068.
Kempers, A.J., Luft, A.G., 1988. Re-Examination of the determination of environmental nitrate as nitrite by reduction with hydrazine. Analyst 113.
Keseler, I.M., Bonavides-Martinez, C., Collado-Vides, J., Gama-Castro, S., Gunsalus, R.P., Johnson, D.A., Krummenacker, M., Nolan, L.M., Paley, S., Paulsen, I.T., Peralta-Gil, M., Santos-Zavaleta, A., Shearer, A.G., Karp, P.D., 2009. EcoCyc: a comprehensive view of *Escherichia coli* biology. Nucleic Acids Research 37, D464–D470. https://doi.org/10.1093/nar/gkn751.
Knowles, T.D.J., Chadwick, D.R., Bol, R., Evershed, R.P., 2010. Tracing the rate and extent of N and C flow from ^{13}C,^{15}N-glycine and glutamate into individual de novo synthesised soil amino acids. Organic Geochemistry 41, 1259–1268. https://doi.org/10.1016/j.orggeochem.2010.09.003.
Krom, M.D., 1980. Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. The Analyst 105, 305. https://doi.org/10.1039/an9800500305.
Lambie, S.M., Schipper, L.A., Balks, M.R., Baisden, W.T., Lambie, S.M., Schipper, L.A., Balks, M.R., Baisden, W.T., 2012. Solubilisation of soil carbon following treatment with cow urine under laboratory conditions. Soil Research 50, 50–57. https://doi.org/10.1071/SR11195.
Li, X., He, H., Zhang, X., Kazanci, C., Li, Z., Necpalova, M., Ma, Q., 2021. Calculation of fungal and bacterial inorganic nitrogen immobilization rates in soil. Soil Biology and Biochemistry 153, 108114. https://doi.org/10.1016/J.SOILBIO.2020.108114.
Maheswaran, S., Cranston, L.M., Millner, J.P., Horne, D.J., Hanly, J.A., Kenyon, P.R., Kemp, P.D., 2022. Effects of sheep grazing systems on water quality with a focus on nitrate leaching. Agriculture 2022 12, 758. https://doi.org/10.3390/AGRICULTURE12060758. Page 758 12.
Marsden, K.A., Holmberg, J.A., Jones, D.L., Chadwick, D.R., 2018. Sheep urine patch N_2O emissions are lower from extensively-managed than intensively-managed grasslands. Agriculture, Ecosystems & Environment 265, 264–274. https://doi.org/10.1016/j.agee.2018.06.025.
Marsden, K.A., Jones, D.L., Chadwick, D.R., 2016a. Disentangling the effect of sheep urine patch size and nitrogen loading rate on cumulative N_2O emissions. Animal Production Science 56, 265–275. https://doi.org/10.1071/AN15613.
Marsden, K.A., Jones, D.L., Chadwick, D.R., 2016b. The urine patch diffusional area: an important N_2O source? Soil Biology and Biochemistry 92, 161–170. https://doi.org/10.1016/j.soilbio.2015.10.011.
Marsden, K.A., Lush, L., Holmberg, J.A., Whelan, M.J., King, A.J., Wilson, R.P., Charteris, A.F., Cardenas, L.M., Jones, D.L., Chadwick, D.R., 2020. Sheep urination frequency, volume, N excretion and chemical composition: implications for subsequent agricultural N losses. Agriculture, Ecosystems & Environment 302, 107073. https://doi.org/10.1016/J.AGEE.2020.107073.
Mena-Rivera, L., Lloyd, C.E.M., Reay, M.K., Goodall, T., Read, D.S., Johnes, P.J., Evershed, R.P., 2022. Tracing carbon and nitrogen microbial assimilation in

suspended particles in freshwaters. Biogeochemistry. https://doi.org/10.1007/S10533-022-00915-X.
Nichols, K.L., del Grosso, S.J., Derner, J.D., Follett, R.F., Archibeque, S.L., Delgado, J.A., Paustian, K.H., 2018. Nitrous oxide and ammonia emissions from cattle excreta on shortgrass steppe. Journal of Environmental Quality 47, 419–426. https://doi.org/10.2134/jeq2017.12.0463.
Nunan, N., Singh, B., Reid, E., Ord, B., Papert, A., Squires, J., Prosser, J.I., Wheatley, R.E., McNicol, J., Millard, P., 2006. Sheep-urine-induced changes in soil microbial community structure. FEMS Microbiology Ecology 56, 310–320. https://doi.org/10.1111/j.1574-6941.2006.00072.x.
Oenema, O., Kros, H., De Vries, W., 2003. Approaches and uncertainties in nutrient budgets: implications for nutrient management and environmental policies. European Journal of Agronomy 20, 3–16. https://doi.org/10.1016/S1161-0301(03)00067-4.
Oenema, O., Tamminga, S., 2005. Nitrogen in global animal production and management options for improving nitrogen use efficiency. Science in China - Series C: Life Sciences 48, 871–887. https://doi.org/10.1007/bf03187126.
Pakro, N., Dillon, P., 1995. Preferential flow, nitrogen transformations and ^{15}N balance under urine-affected areas of irrigated and non-irrigated clover-based pastures. Journal of Contaminant Hydrology 20, 329–347. https://doi.org/10.1016/0169-7722(95)00077-1.
R Core Team, 2021. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Reay, M.K., Charteris, A.F., Jones, D.L., Evershed, R.P., 2019a. ^{15}N-amino sugar stable isotope probing (^{15}N-SIP) to trace the assimilation of fertiliser-N by soil bacterial and fungal communities. Soil Biology and Biochemistry 138, 107599. https://doi.org/10.1016/j.soilbio.2019.107599.
Reay, M.K., Knowles, T.D.J., Jones, D.L., Evershed, R.P., 2019b. Development of alditol acetate derivatives for the determination of ^{15}N-enriched amino sugars by gas chromatography–combustion–isotope ratio mass spectrometry. Analytical Chemistry 91, 3397–3404. https://doi.org/10.1021/acs.analchem.8b04838.
Reay, M.K., Yates, C.A., Johnes, P.J., Arthur, C.J., Jones, D.L., Evershed, R.P., 2019c. High resolution HPLC-MS confirms overestimation of urea in soil by the diacetyl monoxime (DAM) colorimetric method. Soil Biology and Biochemistry 135, 127–133. https://doi.org/10.1016/J.SOILBIO.2019.04.015.
Reay, M.K., Marsden, K.A., Powell, S., Chadwick, D.R., Jones, D.L., Evershed, R.P., 2023. Combining field and laboratory approaches to quantify N assimilation in a soil microbe-plant-animal grazing land system. Agriculture, Ecosystems & Environment 346, 108338. https://doi.org/10.1016/j.agee.2022.108338.
Reitzer, L., 2004. Biosynthesis of glutamate, aspartate, asparagine, L -alanine, and D -alanine. EcoSal Plus vol. 1, 10.1128/ECOSALPLUS.3.6.1.3/ASSET/A6EF5AE4-4A4C-48F5-B1D0-1E8C24364311/ASSETS/GRAPHIC/3.6.1.3_IG_006.GIF.
Rivero, M.J., Evans, A.C.O., Berndt, A., Cartmill, A., Dowsey, A., Farruggia, A., Mignolet, C., Enriquez-Hidalgo, D., Chadwick, D., McCracken, D.I., Busch, D., Pereyra, F., Martin, G.B., Sanford, G.R., Sheridan, H., Wright, I., Brunet, L., Eisler, M. C., Lopez-Villalobos, N., Rovira, P., Harris, P., Murphy, P., Williams, A.P., Jackson, R.D., Machado, R., PT, S., Puech, T., Boland, T.M., Ayala, W., Lee, M.R.F., 2021. Taking the steps toward sustainable livestock: our multidisciplinary global farm platform journey. Animal Frontiers 11, 52–58. https://doi.org/10.1093/af/vfab048.
Robertson, G.P., Swinton, S.M., 2005. Reconciling agricultural productivity and environmental integrity: a grand challenge for agriculture. Frontiers in Ecology and the Environment vol. 3, 38–46. https://doi.org/10.1890/1540-9295(2005)003,0038:RAPAEI]2.0.CO;2.
Rooney, D., Kennedy, N., Deering, L., Gleeson, D., Clipson, N., 2006. Effect of sheep urine deposition on the bacterial community structure in an acidic upland grassland soil. Applied and Environmental Microbiology 72, 7231–7237. https://doi.org/10.1128/AEM.00926-06.
Selbie, D.R., Buckthought, L.E., Shepherd, M.A., 2015. The challenge of the urine patch for managing nitrogen in grazed pasture systems. Advances in Agronomy 129, 229–292. https://doi.org/10.1016/bs.agron.2014.09.004.
Shand, C.A., Williams, B.L., Dawson, S., Smith, S., Young, M.E., Dawson, L.A., Smith, S., Young, M.E., 2002. Sheep urine affects soil solution nutrient composition and roots: differences between field and sward box soils and the effects of synthetic and natural sheep urine. Soil Biology and Biochemistry 34, 163–171. https://doi.org/10.1016/S0038-0717(01)00163-8.
Silva, R.G., Cameron, K.C., Di, H.J., Jorgensen, E.E., 2005. A lysimeter study to investigate the effect of dairy effluent and urea on cattle urine N losses, plant uptake and soil retention. Water, Air, and Soil Pollution 164, 57–78. https://doi.org/10.1007/s11270-005-2249-7.
Sorensen, P., Jensen, E.S., 1996. The fate of fresh and stored ^{15}N-labelled sheep urine and urea applied to a sandy and a sandy loam soil using different application strategies. Plant and Soil 183, 213–220. https://doi.org/10.1007/bf00011436.
Sorensen, P., Jensen, E.S., Nielsen, N.E., 1994. Labelling of animal manure nitrogen with ^{15}N. Plant and Soil 162, 31–37. https://doi.org/10.1007/bf01416087.
Sullivan, D.M., Havlin, J.L., 1991. Flow injection analysis of urea nitrogen in soil extracts. Soil Science Society of America Journal 55, 109–113.
van Kessel, C., Clough, T., van Groenigen, J.W., 2009. Dissolved organic nitrogen: an overlooked pathway of nitrogen loss from agricultural systems? Journal of Environmental Quality 38, 393. https://doi.org/10.2134/jeq2008.0277.
Wachendorf, C., Joergensen, R.G., 2011. Mid-term tracing of ^{15}N derived from urine and dung in soil microbial biomass. Biology and Fertility of Soils 47, 147–155.
Wachendorf, C., Taube, F., Wachendorf, M., 2005. Nitrogen leaching from ^{15}N labelled cow urine and dung applied to grassland on a sandy soil. Nutrient Cycling in Agroecosystems 73, 89–100. https://doi.org/10.1007/s10705-005-8313-2.
Williams, B.L., Dawson, L.A., Grayston, S.J., Shand, C.A., 2003. Impact of defoliation on the distribution of ^{15}N-labelled synthetic sheep urine between shoots and roots of Agrostis capillaris and soil N pools. Plant and Soil 251, 269–278.
Woods, R.R., Cameron, K.C., Edwards, G.R., Di, H.J., Clough, T.J., 2017. ^{15}N recoveries from ruminant urine patches on three forage types. Plant and Soil 417, 453–465. https://doi.org/10.1007/s11104-017-3270-5.