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Combining field and laboratory approaches to quantify N assimilation in a soil microbe-plant-animal grazing land system

Michaela K. Reay [a,*], Karina A. Marsden [b], Sarah Powell [a], David R. Chadwick [b], Davey L. Jones [b,c], Richard P. Evershed [a,*]

[a] Organic Geochemistry Unit, School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, UK
[b] Environment Centre Wales, Bangor University, Bangor, Gwynedd LL57 2UW, UK
[c] SoilsWest, Centre for Sustainable Farming Systems, Food Futures Institute, Murdoch University, 90 South Street, Murdoch WA 6105, Australia

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ABSTRACT

Efficient fertiliser nitrogen (N) management is critical to global food production and ecosystem health. Considering sheep grazing systems as whole ecosystems, and quantifying key ecosystem services provided by the soil microbial community, including plant N supply and N pollution mitigation, is essential in assessments of N use efficiency (NUE). Using a systems approach, we disassembled a low-intensity sheep (>5 ewe ha^{-1}) grazed grassland, dominated by *Lolium perenne*, into a series of interlinked ^{15}N-tracer experiments in North Wales during a summer growing season to assess fertiliser-N partitioning. ^{15}N was traced into soil microbial protein-N via compound-specific amino acid ^{15}N-stable isotope probing, with subsequent integration to provide a whole-system perspective. Retention of feed-N into sheep was low (11 %), despite high grass ^{15}N-fertiliser uptake (58 %). The majority of grazed-N re-entered the soil N-cycle as excreta (47 % of total ^{15}N) during the peak growing season. Quantifying ^{15}N-assimilation into soil microbial protein (0–15 cm) demonstrated the central role soil microbes occupy in capturing excess fertiliser (16 %) and urinary-N (8 %) of the total ^{15}N-fertiliser applied, thereby reducing N losses and subsequently supporting plant N supply. This approach emphasises how future management of moderate intensity grazing systems should target sheep NUE, alongside the role of the soil microbial community to retain, and later recycle N, for plant supply, optimising essential ecosystem service provisioning.

1. Introduction

Globally, nitrogen (N) is the most important limiting nutrient in agriculture, therefore, N management has a critical role in world food production (Mueller et al., 2012). The livestock sector is worth over $1.4 trillion y^{-1} to the global economy, and this will continue to grow with increasing demand for livestock products, fuelled by population and income increases, and changing diets (Oenema et al., 2005; Thornton, 2010). Much of this food production and economic value relies on synthetic N fertiliser inputs, which support 48 % of the global population (Erisman et al., 2008). However, poor management of N inputs into, and within livestock systems, has large impacts on ecosystem and human health, including greenhouse gas emissions (GHGs), NH_3 volatilisation and redeposition, particularly in N sensitive environments. These include freshwater eutrophication, stratospheric ozone depletion (O_3) and tropospheric O_3 production (Galloway et al., 2013; Galloway and Cowling, 2002; Gerber et al., 2013; Vitousek et al., 2009). It is projected that an additional 70–100 % more food will need to be produced by 2050, and a major challenge associated with this is doing so in an environmentally sustainable way (Godfray et al., 2010).

Conversion of feed N to animal protein is a key ecosystem service, however, it is important to consider other beneficial ecosystem services in agricultural systems (Heijden et al., 2008). These include those provided by the soil microbial community (SMC), which support plant N supply and mitigate N pollution, alongside disease protection and carbon sequestration (Bardgett et al., 2008; King, 2011). Immobilisation into the microbial community is an essential process after fertiliser application, to utilise N initially above plant N demand, and during microbial turnover, subsequently release N for plant uptake on a longer timescale. N retention and 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).

* Corresponding authors.
E-mail addresses: michaela.reay@bristol.ac.uk (M.K. Reay), r.p.evershed@bristol.ac.uk (R.P. Evershed).

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Therefore, livestock production systems must be managed holistically as ecosystems, rather than for one ecosystem service (e.g., yield) (Robertson and Swinton, 2005). There are several whole system nutrient balances at farm and supply chain level, which confirm low feed-N conversion to animal protein (Galloway and Cowling, 2002; Oenema et al., 2003; Rugoho et al., 2018; Schröder et al., 2003). While these nutrient balances are efficient tools to assess nutrient losses and nitrogen use efficiency (NUE), such assessments neglect the role of the SMC (Heijden et al., 2008; Oenema et al., 2003). Furthermore, N returned as sheep urine patches, equivalent to 800–1100 kg N ha^{-1}, is much greater than plant and microbial demand and is thus susceptible to loss (Marsden et al., 2020a; Wachendorf et al., 2005; Whitehead, 1995). Considering the transformations of "new" N inputs, alongside the microbial role in the soil N cycle, is essential to manage livestock systems in a holistic manner (Robertson and Swinton, 2005).

Addressing the livestock production system as a whole ecosystem is a multifaceted question, due to the complexity of the N cycle and associated N transformations and transfers between N pools. Thus, to address this question, ^{15}N-tracing techniques are a powerful tool to quantify the fate of N inputs into an ecosystem. ^{15}N-stable isotope probing (^{15}N-SIP) allows determination of ^{15}N at a bulk level (e.g., grass, animal tissue and soil pools) and at a molecular level. This has provided previously unattainable insights into the role of the SMC in the fate of N fertiliser, and the routing of N through the microbial community (Charteris et al., 2016; Knowles et al., 2010; Reay et al., 2019). The application of this novel approach to the livestock system will allow a more holistic view of N fate, accounting for the role of the SMC in providing ecosystem services. Furthermore, different labelled N tracers can be used to represent standard agricultural practices i.e., fertilisation, and recycling of N inputs (e.g., grass, urine), giving a whole system perspective of sinks for N fertiliser.

Here, we used a combined ^{15}N-tracer approach to quantify the transfer and transformation of a ^{15}N-fertiliser input in a livestock system. The study used a series of interlinked ^{15}N-tracing experiments which separated the system into discernible parts (fertilisation, grazing and excretion). Disassembling these components of a grazed-grassland plant-soil microbe-animal ecosystem allowed both a detailed study of N partitioning in key aspects of the grassland cycle, with reassembly to provide a whole system perspective. Partitioning of applied N in each of these components was addressed, using pool level ^{15}N-SIP, and immobilisation by the microbial community via novel compound-specific amino acid (AA) ^{15}N-SIP approaches. The interlinked nature of the studies allows an overall mass balance of the ecosystem. This detailed study aimed to provide insight into the other fates of N inputs (other than livestock products), which provide important ecosystem services. We hypothesised that the largest sink of applied N fertiliser will be plant biomass, followed by the soil microbial pool, while incorporation into animal biomass will be relatively low, with the majority of ingested N returned to the soil N cycle via excretion. Providing such a holistic mass balance of N fertiliser has the potential to inform future indicators of agricultural productivity including the central role of the SMC in N cycling, thereby improving nitrogen modelling at the farm level.

2. Methods

We employed a ^{15}N-tracing approach using a series of three interlinked studies based on a grassland at the Henfaes Research Station (Bangor University, Aberwyngregyn, Wales, 53°14′N, 4°, 01′W) during a summer growing season (July-September). The partitioning of fertiliser and grass ^{15}N was conducted in 2017, while the urine partitioning experiment was conducted in 2018, replicating the field conditions from the 2017 season. The site has a temperate oceanic climate, and the annual precipitation is 1250 mm. It has an average altitude of 12.1 m and is a semi-permanent sheep-grazed grassland used for year-round grazing at moderate stocking density (>5 ewe ha^{-1}) and silage production since April 2009. The sward is dominated by *Lolium perenne* L. and previously received inorganic N fertiliser at rates between 100 and 130 kg N ha^{-1} y^{-1} alongside potassium (K) and phosphorus (P). The soil was classified as a freely draining Eutric Cambisol (FAO) with a sandy loam texture.

2.1. Partitioning of ^{15}N-fertiliser in a field setting

An area (14 m × 7 m; Fig. S1) was fenced off in March 2017 and did not receive any N-inputs until application of ^{15}N-fertiliser in July 2017. Three SDI− 12 TDT sensors (Acclima Inc., Meridian, ID) were installed within the field plot at depths of 10, 30 and 60 cm to record soil moisture and temperature. An in-field MiniMet weather station (Skye Instruments Ltd, Llandrindod Wells, UK) within 50 m of the field plot monitored hourly rainfall and air temperature. Six adjacent plots (1 × 10 m^2) were was treated with $(^{15}NH_4)_2SO_4$ at a rate equivalent to 70 kg N ha^{-1} (16.73 atom % ^{15}N), within the range of typical fertiliser at this site (DEFRA, 2010), when the grass was at the jointing stage. The fertiliser was spray applied and subsequently watered to simulate a rainfall event of 5 mm to minimise foliar uptake of the applied ^{15}N. The plot did not receive any further addition of N during the collection period. An artefact of the experiment meant the grass biomass was allowed to grow past the stage it would normally be grazed at for this site. This was done to allow a realistic fertilisation rate at sufficient ^{15}N enrichment to follow through each stage of this experiment. It would be prohibitively expensive to fertilise a field with the ^{15}N enrichment used and use the standard stock density at this site (>5 ewe ha^{-1}). Hence while the stocking density for this experiment is higher than typically used at this site, and it was assumed losses on a larger scale would be comparable, given comparable fertilisation rates and grazed dry matter.

Soil (0–5 and 5–15 cm depth), using a 1 cm diameter corer, and grass was collected ($n = 6$) over a 90-day period ($t = 0$, 3 and 6 h, 1, 3, 7, 14, 21, 28, 44, 90 d). Soil was sieved to remove roots (2 mm) and then immediately frozen at − 20 °C, prior to freeze drying and grinding to a fine powder. Due to restrictions in soil collection to minimise disturbance and ensure sufficient grass for the subsequent feeding experiment, root biomass was calculated from standing biomass elsewhere in the field at 90 d at the same growing stage as in the plot (747 kg dry weight ha^{-1}) (Carswell et al., 2022).

2.2. ^{15}N-feeding study with sheep

Five barren Welsh Mountain ewes (*Ovis aries* L.) were used in the ^{15}N-feeding experiment. This breed of sheep is representative of the breed utilised to graze the field site. Further information on the sheep is shown in Table S2. Prior to the feeding experiment, the sheep were accustomed to the type of diet subsequently used (*L. perenne*). The urine collection pens were approved by Bangor University College of Natural Sciences Ethics Committee (Ethics approval code CNS2016DC01) (Marsden et al., 2018, 2017). The feeding experiment was conducted over a total of 12 days from 21st August 2017–1st September 2017. The first day was used as $t = 0$ and the ^{15}N-feeding period was from 9:00 am on 22nd to 9:00 am on 29th August 2017. After this time, natural abundance *L. perenne* was supplied for four subsequent days. The ewes were housed indoors for the duration, with access to ample food (equivalent to 1 kg DM $sheep^{-1}$ day^{-1}), which was cut fresh daily from the ^{15}N plots, and carried to the sheep, and water was provided throughout. A sub-sample of grass was taken daily for each sheep to determine ^{15}N enrichment and account for variability in grazed ^{15}N. Grass for the unlabelled periods was collected from the same field, at least 10 m away from the ^{15}N labelled plots. The mean daily time in the urine collection pens was 8.4 ± 0.4 h and housed indoors with ^{15}N grass overnight. Pen time was limited to ensure animal welfare, and uncollected excreta was estimated as outlined in Section 2.6.

Urine was collected from plastic trays beneath the urine collection pens within 10 min of urination, and the volume determined. It was then filtered to remove debris and frozen within 20 min of excretion

(Charteris et al., 2021). Prior to analysis, urine was filtered (0.45 μm combusted GF). Faeces were collected within 15 min of deposition, weighed and frozen within 30 min. Blood was collected daily from the jugular vein in the sheep necks into 5 ml vacutainer blood collection tubes containing no additives. Wool was shaved from the neck of the ewes prior to ^{15}N-feeding, which allowed fresh wool growth to be collected at 8 days after the start of ^{15}N feeding. Grass feed was also collected daily, and all samples (except urine) were freeze dried and ground prior to analysis.

2.3. Partitioning of ^{15}N-urine in grassland mesocosms

Soil was collected from the same field at Henfaes Research Station and sieved to 5 mm to remove large stones and vegetation, whilst preserving fungal structures (Jones and Willett, 2006). Mesocosms were prepared (diameter 7.5 cm, depth 15 cm) with a density of 1.1 g cm^{-3} (Fig. S10). *L. perenne* L. was planted 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. The mesocosms were maintained at 50 % water holding capacity throughout the incubation, using a synthetic rain solution representative of rain at the field site (Table S4) (Jones and Murphy, 2007). This limited leaching, which reflected the dry conditions of the preceeding summer season when the field experiment was conducted (Fig. S2b). Thus, this low rainfall was used to mimic these field conditions. The average temperature for incubation was 19.5 ± 0.1 °C (Fig. S11). The mesocosm experiment reflected the field site as closely as possible, however, there will be no lateral transport of ^{15}N, no root uptake below 15 cm, and controlled conditions may influence volatilisation.

^{15}N-labelled sheep urine was obtained from Welsh Mountain ewes (n = 5) between days 5–7 after the start of ^{15}N feeding and the chemical composition is shown in Table S5. ^{15}N-labelled urine (40 ml) was applied to the whole surface of each mesocosm (44 cm^2), based on average urination volume during ^{15}N-feeding experiment, urine patch size (Doak, 1952) and mesocosm surface area. This was equivalent to 904 kg N ha^{-1}. Mesocosms were sampled 94 d after urine application, with a simulated storm event equivalent to 45 mm rain over 2 h. Leachate was collected, frozen and freeze dried. Soil (0–5 and 5–15 cm), grass and roots were frozen within 10 min of the end of the storm event, freeze dried and finely ground.

2.4. N and C content and bulk δ^{15}N analyses

CN content, and ^{15}N enrichment of soil, plant material and leachate from the fertiliser and urine partitioning studies were all determined in the same way. Dried samples (sufficient mass for 50–100 μg N) were sealed into tin capsules, and urine was added to an inert liquid sample absorbent (Chromosorb®, a purified diatomaceous earth) in a tin capsule prior to analysis via EA and EA-IRMS for C and N content, and ^{15}N value determination. N content and bulk δ^{15}N values of faeces, blood, wool, and grass feed were determined in the same way at the Lancaster Node of the NERC Life Sciences Mass Spectrometry Facility (LSMSF, Lancaster, UK). N and C content was determined using a Thermo EA1110 elemental analyser (EA) using aspartic acid as a calibration standard (0–10 mg; r^2>0.99) and a soil standard for quality control (QC; values within ± 0.02 % for %TN; CE Instruments Ltd., UK). ^{15}N values were all determined using a Flash EA1112 Series NC Analyser, coupled to a ThermoFinnigan Delta^{Plus} XP (Thermo Electron Corp.) via a Conflo III interface. Standards (^{15}N enrichment range from −8.4 ± 0.4 ‰ to 2.05 ± 0.2 atom %^{15}N) and samples (sufficient mass to yield 0.2 mg N) were analysed and ^{15}N values were corrected using a two-point linear normalisation and secondary reference material traceable to AIR-N2 (Paul et al., 2007).

2.5. Compound-specific AA analyses

Extraction, isolation, and derivatisation of AAs was conducted as outlined in Charteris et al. (2016) on soil following both fertiliser and urine addition. Freeze-dried soil (200 mg) was hydrolysed with 6 M HCl (5 ml; 100 °C for 24 h) under an N_2 atmosphere. Norleucine (Nle; 100 μl of 400 μg ml^{-1}) was added as an internal standard. AAs were isolated from hydrolysates using acidified DOWEX 50WX8 200–400 mesh ion exchange resin and derivatised to *N*-acetyl, *O*-isopropyl (NAIP) derivatives (Corr et al., 2007). A GC-FID (7890B GC Agilent Technologies) fitted with a DB-35 coated capillary column (35 % phenyl-methyl polysiloxane; 60 m × 0.32 mm i.d., 0.5 μm phase thickness; Agilent Technologies) and a flame ionisation detector (FID) was used for AA quantification (Reay et al., 2022). The carrier gas was helium (constant flow, 2.0 ml min^{-1}) and the temperature programme was 70 °C (2 min) to 150 °C (15 °C min^{-1}), then to 210 °C (2 °C min^{-1}) and finally to 270 °C (5 min, 8 °C min^{-1}). Data was acquired and analysed using Agilent OpenLab Control Panel (version 1.0; Agilent Technologies Inc.).

The δ^{15}N values of individual AA as NAIP derivatives were determined using GC-C-IRMS as outlined in Charteris et al. (2016), except the oxidation reactor was comprised of high purity copper and nickel wires and held at 1030 °C. δ^{15}N values were determined relative to that of a monitoring gas with known N isotopic composition and in-house standards. AA δ^{15}N values were accepted when standard values were with ± 1σ of the duplicate analyses of the sample AA NAIP derivatives. Data was acquired and analysed using IsoDat NT 3.0 (Thermo Electron Corp.).

2.6. Calculations and statistics

Percentage ^{15}N incorporation for bulk and compound specific analyses throughout the experiments was calculated using δ^{15}N values and N content of soil, plant biomass, leachate and animal tissues, and individual AA pools (Charteris et al., 2016; Knowles et al., 2010). Overnight excretion was not collected for animal welfare considerations, therefore, overnight excretion was estimated from previous studies with the same group of Welsh Mountain Sheep using remote sensing (Marsden et al., 2021, 2020b). This was combined with regression analyses of ^{15}N abundance and N content of collection excreta, and observed excretion frequencies in this study.

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 (qqnorm plots). Homogeneity of variance of the data was also confirmed prior to statistical analyses. T-tests 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 $p < 0.05$. Autocorrelation analyses were used to test for correlation of sub-plots for the field experiment.

3. Results

Three interlinked ^{15}N-tracer experiments were undertaken to follow the fate of ^{15}N fertiliser following application in a field experiment, sheep grazing and subsequent re-application of collected urine in a mesocosm experiment. A mass balance for each individual experiment is first determined, using the input in each experiment (fertiliser, grass, urine) as the total available ^{15}N. Subsequently, through the interlinked design of the experiments, the relative importance of N pools and biochemical fate of ^{15}N throughout the grassland ecosystem was determined, where partitioning into pools were corrected for the available ^{15}N at each step (Fig. 1).

3.1. Plant and microbial uptake of ^{15}N-fertiliser

The average air temperature was 15 °C and the cumulative rainfall over the 3-month period was 16 mm, which was an exceptionally dry

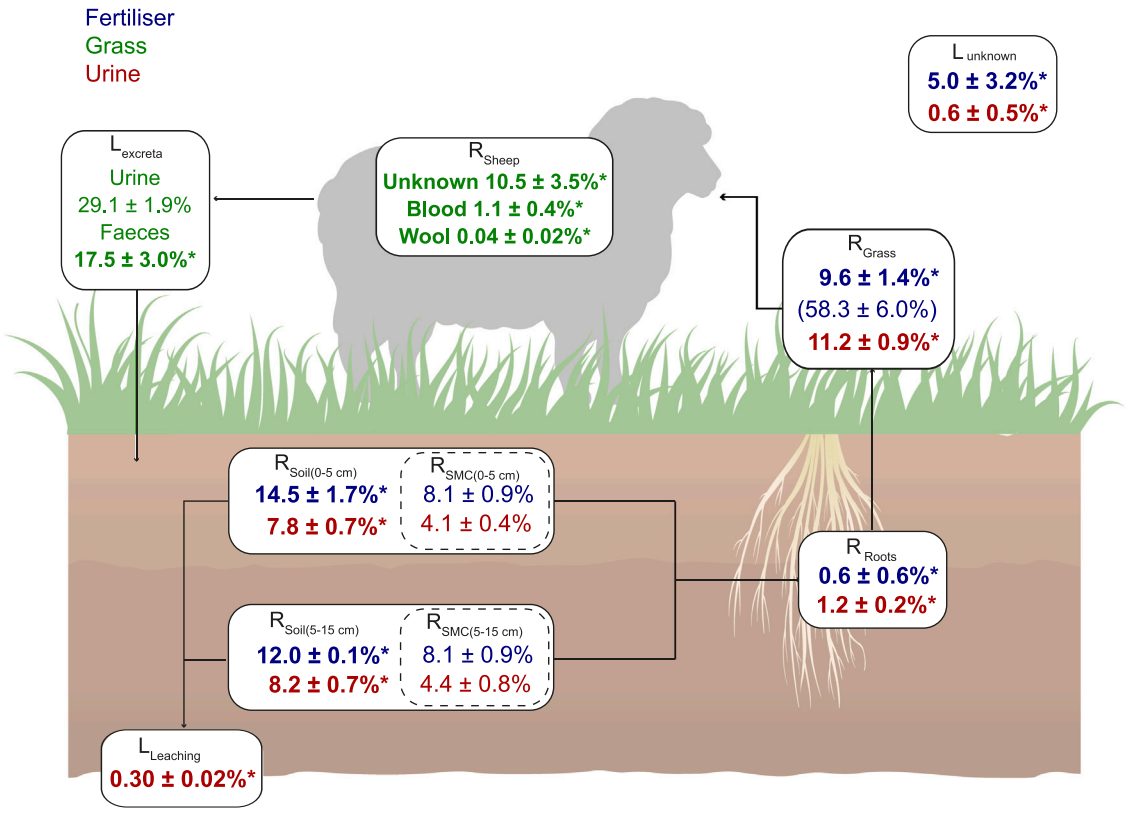

Fig. 1. Schematic showing partitioning of applied ^{15}N-fertiliser through the grassland N cycle (fertilisation, grazing and excretion). Colours indicate which experiment the pool was determined in. Blue indicates fertiliser application to field ($n = 6$, except THAA where $n = 3$). Two R_{grass} values are present to indicate grass grazed (in brackets), and residual grass in field (in bold). Green indicates grass 'grazing' by sheep ($n = 5$). Red indicates reapplication of urine to grass mesocosms ($n = 4$). All values are mean ± SEM. Values with an asterisk (*) and in bold were included in the final mass balance, with grazed grass and urine applied to mesocosms not included, alongside R_{SMC}, which is a sub-pool of R_{soil}.

year (Fig. S2). The average soil volumetric water content was 26 % and the average soil temperature was 16 °C at 10 cm depth (Fig. S3). Changes in percentage total carbon (%TC) and nitrogen (%TN) content in soil and grass are shown in Fig. S4. Soil %TC showed no variation over time, while grass C content showed a marked increase at day 44. Soil TN increased over time following fertiliser application for both soil depths across the 90-d period (0–5 cm $r^2 = 0.397$, $p < 0.01$; 5–15 cm $r^2 = 0.706$, $p < 0.001$). Grass N content increased following fertiliser application to day 7, followed by a decrease until harvest at day 44 (Fig. S4).

Within the soil pool, total hydrolysable amino acid (THAA) concentration rapidly increased after fertiliser application to day 14 and subsequently declined back to pre-application levels (Fig. S5a). Within the THAA pool, hierarchy of individual AA pool sizes were maintained across the 90-d period, with the most abundant AAs (Ala, Gly, Asx and Glx) showing the largest increases following ^{15}N-fertiliser application. The proportion of AA relative to TN also varied following ^{15}N-fertiliser application, where it increased after fertiliser application (33.9 ± 0.9 %), and subsequently declined to pre-application levels (Table S1).

Percentage ^{15}N retention in soil and plant biomass following ^{15}N fertiliser application are shown in Fig. 2a, based on pool size, N content of pools and ^{15}N values for soil and grass (Fig. S4 and S6). Autocorrelation analyses confirmed there were no trends within the sub-plots. Initially, soil retained the majority of applied ^{15}N fertiliser (e.g., 78 ± 14 % at 0–5 cm and 18 ± 1.1 % at 5–15 cm at 6 h), then declined until day 21, where retention in both soil depths plateaued (average 14.5 ± 4.2 % at 0–5 cm; 11.3 ± 1.9 % at 5–15 cm).

Within the soil N pool, incorporation into the THAAs increased linearly up to day 3 ($r^2 = 0.879$; Fig. 2b), and subsequently plateaued (16.2 ± 0.9 %). After 90 d, ^{15}N-derived from fertiliser incorporated into the THAA pool accounted for 61 % of ^{15}N retained in the soil N pool. Within the THAA pool, Glx, Asx, Ala and Gly pools had the highest ^{15}N incorporation (Fig. S8), while Tyr, Lys, Hyp and Phe had the lowest ^{15}N incorporation. ^{15}N uptake by plant biomass (above and below ground) increased up to day 21 (71 ± 5.6 % and 2.7 ± 0.8 %, respectively) and subsequently decreased in above ground biomass until harvest (day 44, 58 ± 6.0 %). Overall, above ground biomass was the largest sink for ^{15}N-fertiliser (68 ± 7.4 %) at 90 d, of which 58 ± 6.0 % was harvested at day 44, and subsequently used in the sheep feeding experiment and "grazed", with the remainder (10 %) considered residues not grazed, e.g., due to field rotation. Overall partitioning of ^{15}N fertiliser 90 days after application is shown in blue in Fig. 1. Losses, determined by difference from total ^{15}N applied and accounted for in soil and plant biomass, at 90 d were 5 % of total applied ^{15}N-fertiliser, although it should be noted, across the experimental period, N losses appeared higher than observed at day 90 (Fig. S9).

3.2. *Feeding of ^{15}N-labelled grass*

Partitioning of consumed grass, provided by cutting and carrying from the field plot, between animal tissues and excreta is shown in Fig. 1. The mean TN concentration for urine ($n = 191$) and faeces ($n = 148$) was 13.7 ± 0.5 g N l^{-1} and 2.5 ± 0.1 %, respectively. Faecal N content decreased with time, which was linked to lower TN of grass during ^{15}N feeding (2.4 ± 0.1 %) compared to field used for accustoming sheep to the diet (3.1 ± 0.1 %). TN content for blood ($n = 50$) was 14.8 ± 0.1 %, and the TN content of sheep wool ($n = 10$) was 15.7 ± 0.1 %. Additional information including N content of determined pools, and pool sizes are shown in Table S3.

Partitioning of ingested ^{15}N following a 7-day feeding period and

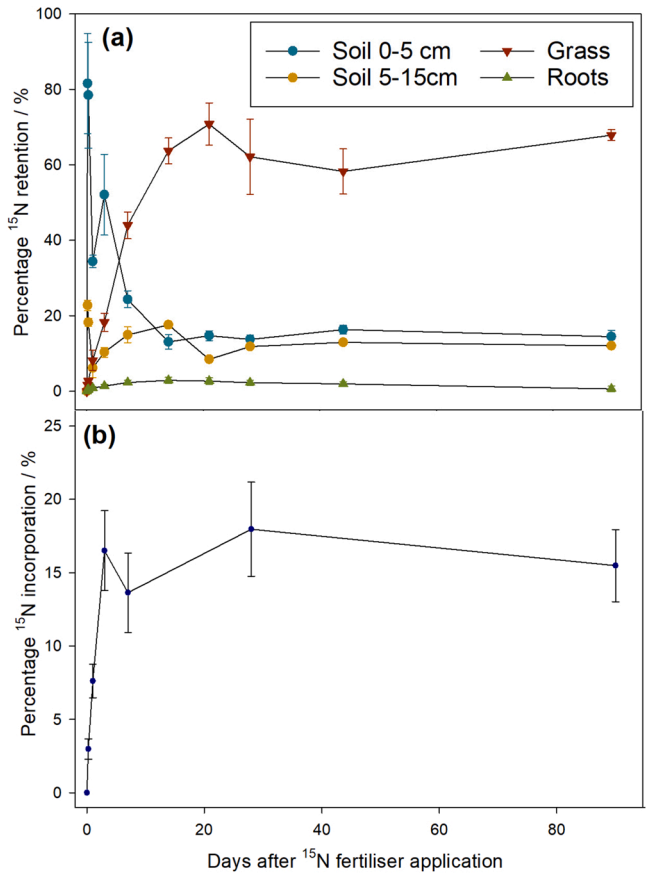

Fig. 2. ^{15}N-fertiliser retained in soil and plant biomass (a) and incorporated into soil THAA (0–15 cm) (b). Symbols indicate mean ((a) $n = 6$ and (b) $n = 3$) ± SEM. Note retention in grass at day 90 is total of harvested and in-situ aboveground biomass.

subsequent 4 day feeding period with natural abundance grass is shown in Fig. 3. The largest fate of ingested ^{15}N was urination, accounting for 28.9 ± 3.6 % during the daily collection period. An estimate of a further 22 % excreted overnight, based on urination volume, N content and frequency of urination overnight for this breed of sheep, with comparable ages and conducted at the same site (Marsden et al., 2021). Further, the ewes used in this study were included in this previous remote sensing study. Excretion in faeces was also a major fate of ingested ^{15}N, with collected faeces accounting for 25.0 ± 5.2 %. Similar to urine, using observations of urination and defecation occurring simultaneously (ca. 70 % of events) in this study, and previous observations of urination frequency for the breed, an estimate of 15 % for overnight faeces was calculated. Wool was a minor fate of ^{15}N during the initial ^{15}N feeding period, accounting for 0.07 ± 0.03 % of ingested ^{15}N, and blood was also a minor fate (2.0 ± 0.7 %). Unknown fates of ingested ^{15}N include all animal tissues which were not sampled, and overnight faecal events which were not collected.

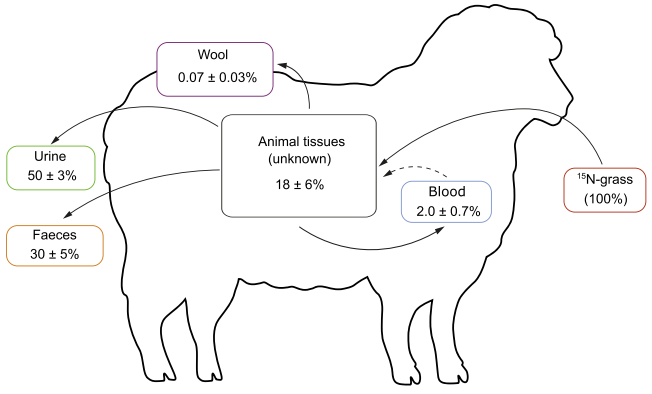

Fig. 3. Partitioning of ingested ^{15}N-grass in Welsh Mountain ewes. Values are mean ± SEM ($n = 5$). The unknown portion was calculated by difference.

3.3. Plant and microbial uptake of excreted ^{15}N-urine in grassland mesocosms

The total carbon and nitrogen content of soil and above and below ground biomass is shown in Table S6. The soil C and N content was significantly higher following urine application compared to the control (t-test, $p = 0.008$ and $p = 0.006$, respectively). Both above and below ground C content did not vary compared to the control after 94 d, while N content did significantly increase for roots at both soil depths ($p = 0.012$) and aboveground biomass ($p = 0.006$). Leached C beneath a urine patch during a simulated storm event 94-d after urine application was comparable to the control treatment at 94 d (5.0 ± 0.4 mg C l^{-1}; Table S6). Leached N was significantly higher than the control treatment at 94 d after urine application (t-test, $p < 0.01$). Soil THAA concentration (Table S6) was significantly higher at both soil depths than the control treatment (t-tests, $p = 0.008$ and $p = 0.014$, for 0–5 cm and 5–15 cm, respectively). Within the THAA pool, increases in concentration of individual AAs reflected hierarchy of pool sizes at $t = 0$ and Ala, Gly, Asx and Glx were the most abundant AAs (Table S8).

Partitioning of applied ^{15}N-urine between pools across the experiment is shown in Table 1, determined from N pool size (Table S6) and ^{15}N enrichment (Table S7). Retention in the soil was the largest fate, accounting for a total of 55 ± 3.7 % of ^{15}N-urine in both soil depths. Within the soil pool, incorporation of applied ^{15}N-urine into the THAA pool (Table 1) was a major fate for applied ^{15}N, with 28 ± 2.8 % of urine ^{15}N biosynthesised into microbial protein at 94 d in the two soil depths. This equated to 62 ± 3.4 % of the total retained ^{15}N in soil at the end of the experiment period. Incorporation into individual AAs (Table S8) was largely within the most abundant AAs (Ala, Gly, Pro), and those central to AA biosynthesis (Asx, Glx). Uptake into plant biomass was the major fate, with 38.4 ± 1.6 % in aboveground biomass, and a further 4.0 ± 0.6 % in roots at both depths. Leaching was a minor fate during a period of low rainfall, only accounted for 1.08 ± 0.07 % of applied ^{15}N urine and unknown losses, attributed to gaseous emissions were also low (1.5 %).

3.4. Mass balance of applied ^{15}N

Fig. 1 shows the overall fate of applied ^{15}N-fertiliser following ^{15}N-fertiliser application, sheep grazing and subsequent re-application as ^{15}N-urine. Overall, aboveground plant biomass was the largest sink for added ^{15}N with 67.9 ± 7.4 % captured following fertiliser application

Table 1
^{15}N partitioning for urine patch 94 d after urine application. Values represent mean ± SEM ($n = 4$). THAA assimilation in *italics* are a subpool of total soil and were not included in the final mass balance. The unknown pool was determined by difference.

Pool	Percentage^{15}N retention / %
Soil	
0–5 cm total	26.7 ± 1.8 %
0–5 cm THAAs	13.7 ± 0.6 %
5–15 cm total	28.3 ± 1.9 %
5–15 cm THAAS	15.1 ± 2.9 %
Plant	
Shoots	38.4 ± 1.6 %
Roots (0–5 cm)	0.3 ± 0.05 %
Roots (5–15 cm)	3.8 ± 0.5 %
Losses	
Leachate	1.1 ± 0.1 %
Unknown	1.5 %

and 13.8 ± 0.9 % captured after urine application. Belowground plant biomass was a relatively minor fate of applied ^{15}N, accounting for 0.6 ± 0.6 % and 1.2 ± 0.2 % following fertiliser and urine application, respectively. Soil was also a major fate of applied fertiliser and urine (26.5 ± 1.8 % and 16.0 ± 1.4 %, respectively), with over 60 % of soil ^{15}N incorporated into the THAA pool for both N forms, equating to 24.7 ± 2.4 % of the applied ^{15}N fertiliser. Overall, N losses, as leaching or gaseous losses, accounted for around 6 % of total applied ^{15}N. A total of 101 % was recovered in the pools contributing to the final mass balance (grazed grass and applied urine was not included). This overestimation is likely due to errors associated with the number of pools contributing to this total mass balance.

4. Discussion

The complexity of transfer, transformation and recycling following N fertiliser application to a pasture, and recycling as feed-N and urine-N has meant these aspects are rarely integrated within a single ecosystem. Therefore, this study aimed to bridge this gap by following ^{15}N tracers through sequential experiments, to provide a holistic overview of the fate of different N inputs. This included microbial capture via immobilisation into the total soil protein pool. This whole system perspective is essential to inform future agricultural best practices, and to incorporate indicators of agricultural productivity and ecosystem health, including the role of the microbial community.

Application of ^{15}N-fertiliser in a field setting was representative of a mid-summer fertiliser application for this low intensity grazing setting, with grazing 44 d after fertiliser application. Plant biomass captured 58 % of ^{15}N fertiliser prior to simulated grazing, and a further 10 % after grazing with the 90-d period. This high uptake by aboveground biomass was likely due to (i) application during peak growing season, and (ii) N demand for re-growth after grazing, resulting in high N demand during low nutrient availability (Mooshammer et al., 2014) as plant N uptake tends to be below 50 % (Di and Cameron, 2002; Godfray et al., 2010; Goulding et al., 2008).

Soil was also a major fate of ^{15}N-fertiliser, although initially very variable. This was attributed to an uneven spatial distribution of added ^{15}N in the heterogeneous soil environment, with subsequent mixing (e.g., bioturbation, diffusion, and mass flow) resulting in stabilisation of soil retention around 27 % of total applied ^{15}N fertiliser. Within the soil pool, incorporation of ^{15}N into the THAA pool indicated rapid microbial capture of ^{15}N-fertiliser and increased THAA pool size, outcompeting the plant community for applied ^{15}N within the first 24 h (Harrison et al., 2007). Subsequently, a plateau ^{15}N incorporation of 16 % into the THAA pool resulted from an equilibrium between incorporation and loss, and immobilisation into more recalcitrant pools. The trend and proportion of ^{15}N-fertiliser assimilated into the THAA pool is comparable to that observed for other grasslands following fertiliser application (Charteris et al., 2016). Hierarchical trends observed for ^{15}N incorporation into individual AAs provides an additional level of insight into the biochemical fate of fertiliser. Higher ^{15}N incorporation was observed both in the more abundant AAs (e.g., Ala, Gly) and into AAs associated with proximity to NH_4^+ incorporation into AA biosynthesis pathways (e.g., Glx and Asx) (Caspi et al., 2018; Knowles et al., 2010). Ultimately, assimilation into microbial biomass was a major fate of ^{15}N, accounting for 61 % of the total ^{15}N soil-retained in the soil pool, 16 % of total applied fertiliser. This likely reduced susceptibility of fertiliser to losses to the environment and will subsequently support plant N availability following turnover of microbial N pools.

Overall, ^{15}N retention in the grassland varied initially, due to initial heterogenous distribution. It was assumed once N was lost from the system, it could not return, therefore, the variation in losses across the experiment were likely due to (i) uneven distribution of N due to preferential transport/water infiltration pathways, resulting in hot spots of ^{15}N, and (ii) uptake into unmeasured pools e.g., deeper soil where it subsequently captured by plant biomass. Subsequent low losses at 90 d (5 % of applied ^{15}N fertiliser) were attributed to redistribution, optimised timing of N application and favourable weather conditions. This minimised volatilisation of NH_3 immediately after NH_4^+ application, alongside dry conditions which did not promote denitrification and leaching (Bouwman et al., 2002; Carswell et al., 2019; Di et al., 2014). Furthermore, losses of NH_3 from this soil were previously shown to be low, especially at pH< 7 (Jones et al., 2012). Combined leaching and gaseous losses were comparable to previous studies, where losses via leaching were lower in dry periods compared to wetter years (e.g., ca. 9 % compared to 19 %) (Jenkinson et al., 2004), and low intensity grassland systems, where N_2O and leaching losses were negligible (Ammann et al., 2009; Jenkinson et al., 2004). There was potential for increased losses outside of the growing season, which was beyond the scope of this study. The partitioning of applied ^{15}N fertiliser was representative of application in a low intensity grassland system during peak growing season, which resulted in high retention in the plant soil system, and minimised N losses.

Animal grazing represents the largest recycling of fertiliser N after application, and ultimately, N partitioning in this portion of the grassland N cycle controls the overall efficiency and proportion of fertiliser-N entering the food supply chain. The dominant fate of grass-^{15}N was excretion, returned as urine (50 %) and faeces (30 %), relative to 20 % retained by sheep. This includes estimates of overnight urination and defecation, informed by remote sensing the Welsh Mountain sheep and observations herein (Marsden et al., 2021), alongside daytime collection of excretion, which captured the most frequent periods. Direct comparison of N partitioning with other studies were undertaken in consideration of differences in breed, life stage of animal and feed variations (Jonker et al., 2015; Stergiadis et al., 2015; Wilkerson et al., 1997). Partitioning of N into excreta was influenced by dry matter and N content of grass, which is the largest predictor of urine and faecal N excretion (Patra, 2010). Retention in sheep tissue pools (including muscle, bone etc.) was comparable to previous studies (e.g., 5–20 % in meat and milk) (Castillo et al., 2000; Jonker et al., 2015; Patra, 2010). Within this, 2 % was recovered in blood and wool, and low incorporation in wool reflected the slower growth time of this pool (Zazzo et al., 2008). In a commercial sheep flock, there would be greater variation in age and life stage (e.g., pregnancy, number of lambs, lactating) and this variability could not be factored into the scale. Little variation in the proportion of feed-N excreted in dry and lactating sheep has been observed, with increased N intake rather than improved NUE to meet increased N demands for pregnant and lactating sheep (Decandia et al., 2011). Irrespective of life stage, return to the pasture as urine and faeces will be the major fate of feed-^{15}N. Targeting this portion of the grassland N cycle, to improve sheep incorporation, by improved digestibility and grazing practices via animal suitability and feed optimisation, would minimise return of N to the grassland.

The partitioning of ^{15}N returned as urine was targeted in this study as it represents a hot-spot of N cycling grasslands, with the high concentration of dissolved N susceptible to loss and above plant and microbial demands (Chadwick et al., 2018; Cowan et al., 2015). Previous studies have found high leaching losses in comparable soil-plant systems over a 1 year period (between 6 % and 64 %; (Di and Cameron, 2007; Maheswaran et al., 2022)). However, with low rainfall, reflecting that observed in the field experiment, leaching was a minor fate of urinary-^{15}N in this study. This is representative of leaching potential during a dry summer season. It is suggested cumulative leaching may increase as rainfall increased in the autumn and winter seasons, consistent with lower observations of previous studies. Another route for loss, determined by difference, was gaseous losses, which were also minor. Both NH_3 losses via volatilisation and N_2 and N_2O losses via denitrification at this site have previously been found to be low (Jones et al., 2012; Marsden et al., 2016), consistent with observations in this study.

Meanwhile, the low losses from the mesocosm enabled high plant uptake, accounting for 42 % of urinary-^{15}N, which would subsequently be available for grazing sheep. This was comparable to plant uptake for

urine deposited in a spring/summer season (Sørensen and Jensen, 1996), and other *L. perenne* systems (Di et al., 2002; Silva et al., 2005). Plant utilisation of urinary-N has the potential to further increase the NUE of the system, following subsequent grazing and incorporation into sheep. Based on findings from the prior field and feeding experiment, is suggested ca. 7 % of urinary N could be incorporated into sheep following grazing, equating 4 % of the originally applied ^{15}N fertiliser.

Soil was the largest fate of urine-^{15}N, and was higher than observed for previous studies (e.g., 13–30 % (Ambus et al., 2007; Clough et al., 1998; Woods et al., 2017)). This higher retention was unsurprising, given the low leaching losses observed herein, although retention in the soil pool was also higher than for fertiliser. While this may be an artefact of the mesocosm vs. field experiments, there was also evidence of priming of the soil microbial community. The THAA pool was elevated 94 d after urine application, while increases following fertiliser application were transient (Charteris et al., 2016). This was likely due to priming of the microbial community, from increased N and C availability from urine, and native soil pools due to changes in pH (Lambie et al., 2012). Due to this increase in newly biosynthesised microbial protein, 30 % of ^{15}N urine was assimilated into this pool, higher than previous observations for cow urine (7 −17 %; (Wachendorf and Joergensen, 2011)). Given this previous study had lower soil retention, and higher leaching losses, the higher microbial assimilation herein is attributed to longer-term N availability in soil due to low leaching losses. The microbial community thus played a key role in retaining urinary-N in dry periods, and during microbial turnover, would support longer term plant N supply while mitigating losses on the timescale of this study.

The interlinked design of the experiments allowed an ecosystem approach to determine the fate of applied ^{15}N fertiliser, following experiments simulating fertiliser application, sheep grazing and urine excretion (Fig. 1). The overall mass balance assumed the differing scale did not influence the ^{15}N partitioning, as all other portions (e.g., soil type, plant biomass) reflected the field system. Furthermore, as it was not feasible to fertilise a sufficient area for grazing with ^{15}N, grass was grown in a smaller area to a higher standing stock than would typically be grazed at the site. It was assumed that this did not alter N dynamics at this stage of the N cycle, and it was comparable to sheep grazing on a wider area, or grazing the same area following regrowth. Further, a mesocosm approach was utilised for the urine application, to enable the same rainfall conditions as the field experiment, however, this will have influenced lateral leaching losses, alongside leaching/root uptake below 15 cm, hence leaching should be considered as potential leaching losses here.

Incorporation into plant biomass was the largest fate, accounting for 68 % as fertiliser and 11 % after re-application as urine, of total ^{15}N applied. This was a contrast to the relatively low retention in the grazing sheep (10 %). Further losses in the supply chain, with a third of food produced wasted before consumption (Galloway and Cowling, 2002), mean the overall efficiency of this system would be further reduced below 10 %. Beyond yield, other ecosystem services, notably the role of the soil microbial protein pool in retaining and recycling N have now been incorporated via this systems approach. ^{15}N assimilation into the soil microbial protein pool accounted for 16 % and 10 % of the initial fertiliser input, when applied as fertiliser and urine, respectively, and 60 % of ^{15}N retained in soil. This accounted for a quarter of total ^{15}N-fertiliser, confirming, as hypothesised, this pool as an important store of surplus N, reducing potential N losses, and, following microbial turnover, would subsequently be available for plant N uptake (Heijden et al., 2008). Higher relative incorporation by microbial community of urine than fertiliser, based on total ^{15}N applied in each experiment, reflected the enhanced priming effect in a hot spot for N cycling (Wachendorf and Joergensen, 2011; Williams et al., 1999).

Losses from the ecosystem were minimised by low rainfall, efficient plant and microbial N capture, and low losses of NH_3 and N_2O associated with the soil (Jones et al., 2012; Marsden et al., 2016), but increase when further losses within the supply chain for animal products are considered. The partitioning found in this summer season was likely strongly influenced by the dry conditions, alongside controls of soil and plant type (Clough et al., 1998; Woods et al., 2017). Given predicted changes in precipitation events, with increased frequency of summer droughts and likelihood of extreme rainfall events (Allen and Ingram, 2002), this study highlights potential differences in N partitioning in a grassland during low rainfall periods which may become more frequent.

The novel approach adopted in this study integrated a series of discrete ^{15}N-tracer experiments, to assess the overall partitioning of N fertiliser within the grazed-grassland, plant-soil, animal-microbe N cycle. This dissembling followed by subsequent reassembly into the grassland N cycle provided hitherto unavailable insights into the partitioning of different N forms (fertiliser, grass, urine) into plant biomass, grazing animals, and, importantly, the often-neglected soil microbial pool. The relative importance of this pool was identified via the application of compound-specific amino acid ^{15}N-SIP, which confirmed rapid microbial N assimilation into the microbial protein pool, likely capturing excess N when it exceeded plant N demand (e.g., following urination). This pool of N represents a source of N for plant uptake following turnover, and was central to reducing N losses from the grassland, a key ecosystem service provided by the soil microbial community. This holistic assessment of N fertiliser partitioning demonstrates the importance of not just providing nutrient mass balances, but also quantifying microbial biosynthesis. Such factors and pools can subsequently be used to assess future management strategies to improve production efficiency and contribute to improved nutrient modelling at the farm-level, benefitting from detailed insights into N partitioning throughout the complex grassland ecosystem.

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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Supplementary Information

Amino acid contribution to soil pool; background sheep data; sheep excretion properties; artificial rain composition; sheep urine properties for mesocosm application; field and mesocosm experiment design; field

and mesocosm temperature and rainfall data; field soil temperature and moisture content; field and mesocosm soil TC and TN content; field and mesocosm soil amino acid concentrations; field and mesocosm bulk $\delta^{15}N$ values for soil and grass; field and mesocosm individual $\delta^{15}N$ values for amino acids; field and mesocosm percentage ^{15}N incorporation into individual AAs; total ^{15}N retention following fertiliser application; mesocosm leachate TOC and TN concentration.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.108338.

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