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[15]N-amino sugar stable isotope probing (^{15}N-SIP) to trace the assimilation of fertiliser-N by soil bacterial and fungal communities

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ABSTRACT

Although amino sugars represent a major component of soil organic nitrogen (ON), the assimilation of nitrate (NO_3^-) and ammonium (NH_4^+) into amino sugars (AS) by soil bacteria and fungi represents a neglected aspect of the global N cycle. A deeper knowledge of AS responses to N fertiliser addition may help enhance N use efficiency (NUE) within agricultural systems. Our aim was to extend a sensitive compound-specific ^{15}N-stable isotope probing (SIP) approach developed for amino acids to investigate the immobilization of inorganic N into a range of amino sugars (muramic acid, glucosamine, galactosamine, mannosamine). Laboratory incubations using ^{15}N-ammonium and ^{15}N-nitrate applied at agriculturally relevant rates (190 and 100 kg N ha^{-1} for $^{15}NH_4^+$ and $^{15}NO_3^-$, respectively) were carried out to obtain quantitative measures of N-assimilation into the AS pool of a grassland soil over a 32-d period. Using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) we found that δ^{15}N values for individual AS reflected differences in routing of the applied ammonium and nitrate. The contrasting N-assimilation dynamics of bacterial and fungal communities were demonstrated through determinations of percentage ^{15}N incorporation into diagnostic AS. N-assimilation dynamics of the bacterial community were altered with the applied substrate whilst fungal N-assimilation dynamics were unaffected. Rates and fluxes of the applied N-substrates into the bacterial AS pool reflected known biosynthetic pathways for AS, with fungal glucosamine appearing to be biosynthetically further from the applied substrates than bacterial glucosamine due to different turnover rates. This sensitive and specific compound-specific ^{15}N-SIP approach using AS, building on existing approaches with amino acids (AA), enables differentiation of N-assimilation dynamics within the microbial community and assessment of microbial NUE with agriculturally relevant fertilisation rates.

1. Introduction

The soil microbial community (SMC) plays a key role in providing ecosystem services, such as the storing, filtering and transforming nutrients, pollution mitigation and food provisioning (Brussaard, 2012). In an agricultural setting, the stabilisation of fertiliser-N by assimilation into the SMC (i.e. into the organic nitrogen pool; ON) is of importance to reduce loss of applied fertilisers to the wider environment (and associated negative environmental impacts) and to maintain a continued supply of N to plants (Schulten and Schnitzer, 1998). Therefore, understanding N transformations in soil is important to understand both the global N cycle and to work towards improved nitrogen use efficiency (NUE) and sustainable land management practices in agriculture.

Amino sugars (AS) account for 5 to 12% of the ON pool and can be used as specific indices for the role of bacterial and fungal communities in the soil N-cycle due to source specificity (Amelung, 2001; Appuhn and Joergensen, 2006; Joergensen, 2018; Schulten and Schnitzer, 1998). Glucosamine (GlcN) is the most abundant AS and is the major component of chitin, a polymer of N-acetyl-GlcN in fungal cell walls (GlcN$_F$). There is also a bacterial contribution to the GlcN pool (GlcN$_B$) and it is found in cultured soil bacteria in a 5-to-1 mass ratio with muramic acid (MurN), which is solely of bacterial origin from peptidoglycan (Gavrichkova and Kuzyakov, 2008; Glaser et al., 2004; He et al., 2011a,b). This ratio estimates GlcN$_B$ contributions alongside MurN to living biomass and necromass, where other ratios (e.g. 1-to-1 and 2-to-1) may neglect other sources of GlcN$_B$, necromass GlcN and faster MurN degradation rates (Amelung, 2003; Engelking et al., 2007; Glaser et al., 2004; He et al., 2011b). GlcN$_B$ is also found in bacterial extracellular polymeric substances (EPS) and in teichoic acids

of Gram-positive bacteria (Appuhn and Joergensen, 2006; Mikusová et al., 1996). There are other minor contributions to the soil GlcN pool from soil invertebrates (Engelking et al., 2007). Galactosamine (GalN), the second most abundant AS in soil, and with mannosamine (ManN), are of microbial origin, present in fungal and bacterial extracellular polymeric substances (EPS) although their role is not fully understood (Amelung, 2001; Engelking et al., 2007; Glaser et al., 2004). The AS pool in soil is thought to be stable and representative of both living and dead microbial biomass in soils (Amelung, 2001; Glaser and Gross, 2005; Guggenberger et al., 1999; He et al., 2011a). Therefore, ^{15}N-tracer techniques, which can differentiate newly formed AS from the stabilised (non-living) AS pool, are required. Such techniques would also provide insight into microbial assimilation pathways for N fertilisers and provide quantitative insights of N-assimilation dynamics for bacterial and fungal communities (Charteris et al., 2016; Joergensen, 2018).

A ^{15}N-tracer approach has previously been applied to investigate temporal responses within the microbial community to substrate addition and the stability of AS linked to carbon (C) availability (He et al., 2011a,b, 2006). Observed substrate-induced shifts in the microbial community were attributed to C demand and the form of N applied (He et al., 2011a). [15]N assimilation of substrate ^{15}N into the AS pool was achieved following preparation of aldononitrile derivatives followed by gas chromatography-mass spectrometry (GC-MS; He et al., 2006, 2011a, 2011b). Due to the low precision (± 0.01 atom %) of GC-MS for isotopic determinations it was necessary to use high ^{15}N-enrichments and high substrate application rates, both of which can perturb the system and result in ^{15}N isotopic discrimination (Mathieu et al., 2007; Tang and Maggi, 2012). Therefore, observed N-assimilation dynamics and substrate-induced SMC shifts may be artefacts of the experimental design. Critically, the soils may have become C limited due to the high application rates of inorganic fertiliser.

Consequently, an alternative approach is required that allows investigation of N microbial assimilation dynamics in ^{15}N-incubation studies with minimal perturbation to the soil system.

A highly sensitive ^{15}N-stable isotope probing (SIP) approach has been developed and applied to investigate the soil protein pool to elucidate N-assimilation dynamics at relatively low ^{15}N-enrichments (Knowles et al., 2010; Charteris et al., 2016). These studies used gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), which is highly sensitive and has precision of 0.0002 to 0.0008 atom %, enabling the use of agriculturally relevant N application rates (DEFRA, 2010) and relatively low ^{15}N enrichments (10 atom % ^{15}N) to minimise system perturbations (Mathieu et al., 2007; Tang and Maggi, 2012). This approach has also been used to investigate microbial 'N mining' under contrasting N and C availabilities (Redmile-Gordon et al., 2015). These studies have elucidated N-assimilation dynamics for the whole SMC due to the ubiquitous nature of amino acids (AA). Differentiating N-assimilation of the bacterial and fungal communities in agricultural systems is vital to understanding role of soil microbes in the global N-cycle to inform land management strategies to improve agricultural NUE.

Herein, we report the use of our recently developed GC-C-IRMS method for the analysis of ^{15}N-enriched AS (as alditol acetate derivatives) in a ^{15}N-SIP investigation of the dynamics of N fertiliser assimilation by soil microorganisms (Reay et al., 2019). Due to their greater source specificity compared to AA, it will be possible to relate N-assimilation dynamics of individual AS to the role of bacteria and fungi in N-fertiliser processing within the soil ON pool. The investigation uses soils from the ^{15}N-SIP experiments conducted by Charteris et al. (2016) in which agriculturally relevant application rates and low ^{15}N-enrichments of inorganic fertilisers were employed. This previous study provided a detailed insight into estimates of AA biosynthesis and differentiated incorporation dynamics of inorganic fertilisers associated with fundamental differences in their biochemical nature (Charteris et al., 2016). The ^{15}N-

incorporation into the three most abundant soil AS (GlcN, MurN and GalN) will be discussed in relation to bacterial and fungal N-assimilation dynamics in response to the different N-substrates. These new determinations of AS N dynamics will then be compared with N dynamics of the AA pool from the same experiments to provide a comprehensive of assessment of the microbial N use efficiency of inorganic fertilisers in relation to the two largest ON pools in soil (Charteris et al., 2016).

2. Materials and methods

2.1. Soil incubations with ^{15}N-labelled fertiliser

Soil incubations were carried out as described in Charteris et al. (2016). In short, the soil (Typic haplaquept; 0-10 cm depth; clay texture; Harrod and Hogan, 2008) was randomly sampled from under an improved grassland at the Rowden Moor experimental site (plot six at the North Wyke Research Station near Okehampton, Devon, UK). After collection, the soil was homogenised, air-dried and sieved (2 mm). Subsequently, the soil was adjusted to 50% water holding capacity (WHC) using double-distilled water (DDW) and left for 96 h to equilibrate. Incubations were carried out (10 g of soil) under aerobic conditions in the dark at 20 °C after the addition of either ^{15}N-ammonium chloride ($^{15}NH_4^+$ treatment, 10 atom % ^{15}N, 400 µg in 200 µl DDW), ^{15}N-potassum nitrate ($^{15}NO_3^-$ treatment, 10 atom % ^{15}N, 400 µg in 200 µl) or DDW (200 µl) for the control. The application rate is equivalent to 190 and 100 kg N ha^{-1} for $^{15}NH_4^+$ and $^{15}NO_3^-$ respectively (based on a soil depth of 0.1 m). Substrates were introduced by injection to achieve an even distribution and microcosms were maintained at 50% WHC throughout. A time course experiment was run with incubations halted by immersion of individual replicates ($n = 3$) in liquid N$_2$ at 12 h and 1, 4, 16 and 32 days after ^{15}N-fertiliser addition. All samples were stored at −20 °C prior to freeze-drying.

2.2. Amino sugar extraction and derivatisation

Freeze-dried and finely ground soil (800 mg) was extracted using 6 M HCl (5 ml) under a N_2 atmosphere at 100 °C for 6 h (Zhang and Amelung, 1996). Myo-inositol (400 µg ml^{-1} in 0.1 M HCl) was added as an internal standard (IS). Hydrolysates were collected by centrifugation, dried under a stream of N_2 at 60 °C and stored at −20 °C in 0.1 M HCl. AS were isolated using ion exchange chromatography (acidified DOWEX 50WX8 200-400 mesh). The hydrolysates were desalted using DDW and the total hydrolysable AS (THAS) eluted using 2 M HCl. This was followed by conversion of the AS to their alditol acetate derivatives (method adapted from Whiton et al. (1985) and described in detail in Reay et al. (2019)

2.3. GC-FID analyses of amino sugars

AS were quantified as their alditol acetate derivatives by comparison to the IS (myo-inositol) using gas chromatography (GC) as described in Reay et al. (2019). An Agilent Technologies 7890B GC (Agilent Technologies Inc., Santa Clara, CA, USA) fitted with a VF-23ms column (60 m x 0.32 mm i.d., 0.15 µm film thickness; Agilent Technologies Inc.) and a flame ionisation detector (FID) was employed for quantification by comparison with the IS. AS derivatives were identified using their known elution order and by comparison with amino sugar standards. The carrier gas was helium (He) at a flow rate of 2.0 ml min^{-1} and 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). Data was acquired and analysed using Agilent OpenLab Control Panel (version 1.0; Agilent Technologies Inc.). A typical chromatogram of alditol acetate derivatives of AS extracted from soil is shown in Fig. 1.

2.4. GC-C-IRMS analyses

The $\delta^{15}N$ values of alditol acetate derivatives of AS were determined using GC-C-IRMS as described in Reay et al. (2019). The instrument comprised a ThermoFinnigan Trace 2000 gas chromatograph coupled via a ThermoFinngan GC-III interface to a ThermoFinnigan Delta[Plus] XP isotope ratio mass spectrometer (Thermo Electron Corp., Waltham, MA, USA) with a GC Pal autosampler (CTC Analytics, Zwingen, Switzerland) for sample introduction via a programmable temperature vaporisation (PTV) inlet (Thermo Electron Corporation). The GC temperature programme (same column as for GC analyses) was from 70 °C (1 min hold) to 200 °C (30 °C min^{-1}) to 260 °C (12 °C min^{-1}; 23 min hold). The oxidation reactor was composed of copper (Cu) and nickel (Ni) wires (OEA Laboratories Ltd, Callington, UK) and maintained at 1030 °C. The reduction reactor was composed of Cu wires and maintained at 650 °C. All analyses were run in duplicate and determined $\delta^{15}N$ values were corrected using a two-point normalisation (dynamic range between −3.30 and 46.8 ‰ using GlcN standards) with instrument performance monitored with a quality control standard (natural abundance mixture of MurN, GlcN, GalN and ManN; Reay et al., 2019).

2.5. Calculations

The glucosamine pool was separated into the bacterial and fungal contributions using the mass ratio of bacterial glucosamine and muramic acid of 5-to-1 in cultured soil bacteria during steady state growth (Appuhn and Joergensen, 2006; Glaser et al., 2004; He et al., 2011a,b). The concentration of bacterial glucosamine (GlcN$_B$) and fungal glucosamine (GlcN$_F$) was calculated as follows:

$$[GlcN_B] = 5 \, [MurN] \quad (Eqn. \ 1)$$

$$[GlcN_F] = [GlcN] - [GlcN_B] \quad (Eqn. \ 2)$$

Where [AS] is the concentration of the AS with [MurN] and [GlcN] determined by GC (section 2.4).

The total hydrolysable AS pool is assumed to be representative of the soil AS pool and any ^{15}N enrichment observed in the THAS pool is newly synthesised AS. The percentage of applied ^{15}N incorporated into each AS can be calculated as shown in Charteris et al. (2016) and Knowles et al. (2010) for the AA pool and accounted for the ^{15}N-abundance in the AS pool present at t=0. The percentage of applied ^{15}N incorporated into the AS pool is used rather than the percentage ^{15}N incorporated from the retained ^{15}N due to high retention of the ^{15}N label for the incubations used in this study (Charteris et al., 2016). Furthermore, the percentage ^{15}N incorporation of applied ^{15}N-label also accounts for competing processes for the applied ^{15}N-substrate (i.e. loss pathways). It was assumed the GlcN$_F$ and GlcN$_B$ pools had the same ^{15}N enrichment.

2.6. Statistical analyses

All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc.). For all statistical analyses and regressions, data was tested for normality (Shapiro-Wilk) and homogeneity of variance (Brown-Forsythe). The significance level was set at $P \leq 0.05$ for all statistical analyses.

Quantitative information regarding initial rates of change (zero-order rate constant k_0) for amino sugar Δ^{15}N (i.e. change at time t relative to the control treatment) and percentage incorporated of applied ^{15}N over time (t) were applied to the initial period of increase, as shown in Eqn. 3. Non-linear regression was applied to time course plot to obtain a quantitative measure of overall rate change (first order rate constant, k_1) and plateau (P_1) in AS δ^{15}N values and percentage ^{15}N incorporations, with the equation of fitted regression in the form of (4). The goodness of fit was evaluated using the standard error to the estimate (S) and considered a good fit when 95% of data points fall within the 95% confidence intervals. When non-linear

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regressions in the form of (4) was a very poor fit, a linear regression (3) was used to obtain the overall rate of change across the incubation period.

$$\Delta^{15}N = k_0 t \text{ (Eqn. 3)}$$

$$\Delta^{15}N = \Delta^{15}N_0 + P_1(1 + e^{-k_1 t}) \text{ (Eqn. 4)}$$

Un-paired t-tests were performed to compare percentage ^{15}N incorporations between treatments for individual AS.

3. Results

3.1. Soil amino sugar content

Figure 1 shows a typical GC chromatogram of the THAS using our new alditol acetate approach (Fig. 1). Quantification based on the internal standard indicated there were changes in AS concentration across the incubation period for both treatments, as shown in Tables 1 and 2. MurN and $GlcN_B$ initially increased up to 4 d, but subsequently returned to concentrations comparable to $t = 0$. GalN concentration fluctuated across the incubation period, however, addition of $^{15}NH_4^+$ resulted in no observable trend across the incubation period, whilst there was a general decrease for the $^{15}NO_3^-$ treatment. ManN fluctuated around a mean of 0.16 ± 0.01 mg g^{-1} and 0.10 ± 0.02 mg g^{-1} for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively. Whilst there were changes in individual AS concentration across the incubation period, the relative contribution of each AS did not change for either treatment during the incubation period. GlcN was the most abundant AS, accounting for $60 \pm 1.4\%$ and $63 \pm 1.5\%$ for the $^{15}NO_3^-$ and $^{15}NH_4^+$ treatments, respectively. The bacterial contribution to the GlcN pool was calculated from the 5-to-1 mass ratio of MurN to $GlcN_B$ in cultured soil bacteria. $GlcN_B$ accounted for $58 \pm 1.8\%$ (2.47 mg g^{-1} soil) and $63 \pm 2.1\%$ (2.39 mg g^{-1} soil) of the GlcN pool for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively, with the remainder of the GlcN pool of fungal origin. The second most abundant AS was GalN which accounted for $32.1 \pm 1.3\%$ and $28.1 \pm 2.5\%$ for $^{15}NH_4^+$ and

$^{15}NO_3^-$ treatments, respectively, across the incubation period. MurN accounted for $7.2 \pm 0.6\%$ and $8.6 \pm 0.9\%$ in the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively, with ManN contributing less than $2.7 \pm 0.32\%$ for both treatments.

3.2. Amino sugar $\delta^{15}N$ values

3.2.1 Trends in $\delta^{15}N$ values of AS pools

Figure 2 shows the determined $\delta^{15}N$ values for the AS from the incubations with $^{15}NH_4^+$ and $^{15}NO_3^-$ (10 atom % ^{15}N), with no observable change in $\delta^{15}N$ values for individual AS in the control treatment. The $^{15}NH_4^+$ treatment (Fig. 2a) showed an initial rapid period of ^{15}N-incorporation for GlcN and MurN for days 0 to 4. GlcN then had a second, slower period of increase in $\delta^{15}N$ values over the remainder of the incubation period. MurN plateaued after day 4 for the remainder of the incubation period of the $^{15}NH_4^+$ treatment. The $\delta^{15}N$ value of GalN fluctuated initially at 12 h but then returned to $t = 0$ pre-incubation values and was subsequently relatively constant over the remainder of the incubation. In the $^{15}NO_3^-$ treatment (Fig. 2b) the $\delta^{15}N$ value of GlcN rapidly increased up to 1 d, then exhibited a second slower period of increase up to 32 days of incubation. MurN fluctuated in the first 4 d, with a dip at day 1 and a relative plateau in ^{15}N-enrichment after day 4. GalN showed the same trend in $\delta^{15}N$ values as for the NH_4^+ treatment.

3.2.2 Quantitative changes in $\delta^{15}N$ values of AS pools

The initial and overall rates of change in $\delta^{15}N$ values (compared to the control $\delta^{15}N$ values; $\Delta^{15}N$) for individual AS is summarised in Table 3. In the $^{15}NH_4^+$ treatment, the initial rate of increase in $\delta^{15}N$ values was MurN > GlcN, whilst GlcN > MurN for the overall rate of change in $\delta^{15}N$ values observed across the incubation period (Table 3). The reverse was

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observed for the initial rate of change in the $^{15}NO_3^-$ treatment, GlcN > MurN in the initial 4 day period (Table 3). The non-linear regression achieved a very poor fit for the determined $\delta^{15}N$ values of GlcN over 32 days for the $^{15}NO_3^-$ treatment, therefore, it was not possible to determine overall rates of change and plateau $\delta^{15}N$ values (Table 3). A linear regression across the 32-day incubation was a better fit for determining overall rate of change in $\Delta^{15}N$ values (overall zero-order rate $k_0 = 0.36$ ‰ day^{-1}, r$^2 = 0.70$). The regressions used did not fit the GalN $\Delta^{15}N$ values for either treatment or a horizontal line was the best fit across the incubation period.

3.3. Percentage ^{15}N incorporation of applied ^{15}N substrates into the AS pool

3.3.1. Trends in ^{15}N incorporation into bacterial and fungal AS pools

The percentage ^{15}N incorporation of the applied $^{15}NH_4^+$ and $^{15}NO_3^-$ are shown in Fig. 3 indicating the total proportion of the applied ^{15}N incorporated into the bacterial (MurN and GlcN$_B$) and fungal communities (GlcN$_F$) in the soil. The relative contribution from bacterial and fungal sources to the GalN pool is not fully understood, therefore, GalN has been excluded from these analyses. In both treatments, the amount of ^{15}N incorporation into bacterial AS pools had two-phases of incorporation, with a rapid increase in ^{15}N incorporation to day 4, followed by a relative plateau in ^{15}N incorporation (Fig. 3). Within this pool, incorporation into the GlcN$_B$ pool was higher than MurN for both the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, as indicated by the overall rate of ^{15}N incorporation and plateau ^{15}N incorporation (Fig. 4). Incorporation of applied ^{15}N into the fungal AS pool also had two phases of incorporation, an initial rapid increase within 1 d of application, followed by a second, slower period of increase over the remainder of the time course for both treatments (Fig. 3).

3.3.2. Quantitative assessments of ^{15}N incorporation into bacterial and fungal AS pools

Quantitative information regarding ^{15}N incorporation into bacterial and fungal AS pools was obtained using Eqn. 3 and 4 and presented in Table 4. Initial rates of incorporation for the $^{15}NH_4^+$ treatment were $GlcN_B$ > MurN and the same trend was observed in the $^{15}NO_3^-$ treatment. The initial rate of ^{15}N-incorporation (k_0) was significantly higher for these AS pools in the $^{15}NH_4^+$ treatment than the $^{15}NO_3^-$ treatment (t-test P = 0.014 and P = 0.009 for $GlcN_B$ and MurN, respectively; **Error! Reference source not found.**). The fit for $GlcN_F$ was very poor, therefore, this pool was excluded in comparisons of k_0. This was the same trend ($GlcN_B$ > MurN) for the overall rate of change (k_1), however, ^{15}N-incorporation for $GlcN_F$ did not fit the selected regression. The plateau percentage ^{15}N incorporation for $GlcN_B$ was 0.61% (SE=0.08%) compared to 0.19% (SE=0.03%) for MurN in the $^{15}NH_4^+$ treatment (Table 4). Plateau percentage ^{15}N incorporation was lower for the $^{15}NO_3^-$ treatment for both $GlcN_B$ (P_1=0.12% SE=0.01%) and MurN (P_1=0.017% SE=0.004%). For initial rates of change, overall rates of change and plateau percentage ^{15}N incorporation, the trend was consistently NH_4^+ > $^{15}NO_3^-$ for individual bacterial AS. This was also observed for the bacterial AS pool as a whole and the initial rate of ^{15}N incorporation for the $^{15}NH_4^+$ treatment was 0.205% d^{-1} (r^2=0.534), compared to 0.135% d^{-1} (r^2=0.815) for the $^{15}NO_3^-$ treatment. The same trend was observed for the overall rate of incorporation (k_1=1.13 d^{-1} (SE=0.15 d^{-1}) and 0.69 d^{-1} (SE=0.065 day^{-1}) for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively) and a higher plateau was observed for $^{15}NH_4^+$ (P_1=0.74 ‰ SE=0.09 ‰) compared to $^{15}NO_3^-$ (P_1=0.38 ‰ SE=0.07 ‰).

The non-linear regression was a very poor fit to evaluate the overall rate of ^{15}N incorporation into the fungal AS pool and the best fit was obtained using a linear regression over the 32-d incubation period, following examination of residual plots. The overall rate of change observed for the two treatments were k_0=0.0127% d^{-1} (r^2=0.46), and k_0=0.0093% d^{-1} (r^2=0.73) for $^{15}NH_4^+$ and $^{15}NO_3^-$, respectively, and there was no significant difference in the rate of incorporation for the two treatments (t-test, P=0.059).

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4. Discussion

4.1. Application of the method for studying AS dynamics in soil

Our new analytical approach achieves effective separation of THAS allowing qualitative and quantitative assessments of fungal and bacterial contributions to this important soil organic N pool to be reliably assessed (Reay et al., 2019). The method has advantages over the commonly used aldononitrile derivatisation approach as it avoids the introduction of exogenous N, thereby eliminating correction errors in determinations of high precision δ^{15}N values, critical for subsequent kinetic, flux and pool size calculations. The results reported above allow a number of hitherto unattainable insights into fertiliser N cycling to be revealed.

4.2. Agriculturally relevant N application rates did not alter contribution of the AS pool to soil N in a short-term incubation

The average THAS content of the soil on average was 8.2% (SE 0.3%) and 7.3% (SE 0.4%) of the total N pool for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively, which is consistent with the expected contribution (Indorf et al., 2011; Schulten and Schnitzer, 1998; Stevenson, 1982). Changes occurred in the concentration of AS pools across the incubation period, including an initial increase in MurN concentration, and associated increased GlcN$_B$ concentration, and decreased fungal contribution to the GlcN pool. This increase in biosynthesis was possibly due to a priming effect resulting from addition of $^{15}NH_4^+$ and $^{15}NO_3^-$.Whilst there were changes to the source of GlcN, the composition of the AS pool did not change during the incubation and the composition of the pool was consistent with the expected abundance of individual AS in soil (GlcN > 50%; GalN between 30 to 50% and MurN < 20%; Appuhn et al., 2004; Indorf et al., 2011; Joergensen, 2018).

The small priming effect observed for THAS concentration was higher in the $^{15}NH_4^+$ treatment than the $^{15}NO_3^-$, linked to the higher application rate of $^{15}NH_4^+$. However, the maximum increase in THAS concentration treatments was over 4 times higher in the $^{15}NH_4^+$ treatment than the $^{15}NO_3^-$ treatment, whilst the application rate was only *ca.* 2 times higher. This indicated the size of the priming effect induced by the added N was influenced by both the concentration of added N and the form. The lower priming effect observed for $^{15}NO_3^-$ may arise as this form of N must be reduced to NH_4^+ prior to assimilation into the soil protein pool, which is an energy intensive process (Burger and Jackson, 2003; Puri and Ashman, 1999). Furthermore, the changes in the size of the THAS pool was of short duration, indicating N-application did not result in a sustained shift in the SMC. This was supported by the lack of stimulation of protein biosynthesis above native rates, with a mean concentration of 14.1 ± 0.2 mg g^{-1} and 13.3 ± 0.2 mg g^{-1} observed for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively, across the incubation period (Charteris et al., 2016).

The priming effect observed herein is much lower than previous studies, which used the less sensitive technique of GC-MS with fertiliser applied at rates between 8 to 20 times higher than this study (He et al., 2011a,b). The observed shift in the size and composition of the THAS pool in previous studies may be due to artefacts of the experimental design, with stimulated AS production due to excessively high N-application rates (He et al., 2011a,b). This emphasises the importance of minimising perturbations of SMC N-dynamics by applying ^{15}N-fertilisers at concentrations representative of real-world agricultural treatment rates. With the agriculturally relevant fertiliser treatment used in this study (190 and 100 kg N ha^{-1} for $^{15}NH_4^+$ and $^{15}NO_3^-$, respectively), no change was observed in the relative size of the AS pool and the priming effect was minimal. This emphasises the advantage of using GC-C-IRMS for ^{15}N-SIP studies, with its high sensitivity (0.0002 to 0.0008 atom %) enabling the use of low ^{15}N-labelled substrate application rates and relatively low ^{15}N-enrichments, in order to minimise microbial

population perturbations and ^{15}N isotopic fractionation effects (Charteris et al., 2016; Mathieu et al., 2007; Meier-Augenstein, 1999; Merritt and Hayes, 1994; Metges et al., 1996; Tang and Maggi, 2012). Therefore, the small priming effect observed reflected realistic N fluxes in this pool in an agricultural setting due to the use of agriculturally relevant application rates (DEFRA, 2010).

4.3. AS ^{15}N assimilation dynamics elucidated with δ^{15}N values represent the whole microbial pool

This study builds on the compound-specific ^{15}N-SIP approach applied to the AA pool by Charteris et al. (2016) to determine microbial N-assimilation dynamics, which were not discernible using bulk soil δ^{15}N values. Using incubations with agriculturally relevant fertilisers (hence the ^{15}N-glutamate treatment is not present herein) this study showed that the N-assimilation dynamics for both the AS and AA pools were similar. As shown in Charteris et al. (2016), all individual AA had an initial rapid period of increase in δ^{15}N values over 12 h and 4 d for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively, followed by a plateau as the incorporation of the applied ^{15}N reaches equilibration with loss from the pool. A similar trend was observed for the δ^{15}N values of MurN in both treatments, although the δ^{15}N values of GlcN continued to increase slowly across the 32-d incubation period, indicating an equilibrium between incorporation and loss of ^{15}N-fertiliser was not reached in this pool for both treatments (Fig 2; section 3.2). While trends were similar, it was not possible to directly compare δ^{15}N values between the two treatments due to different application rates, although it is possible to compare the hierarchy of δ^{15}N values, which reflect differences in N routing.

MurN had the highest initial rate increase and plateau δ^{15}N values in the $^{15}NH_4^+$ treatment, whilst GlcN had a higher initial rate of increase and plateau δ^{15}N values in the $^{15}NO_3^-$ treatment (Table 3). This difference was influenced by two factors: (i) ability of the fungal

community, included in the GlcN pool, to utilise secondary N sources, such as NO_3^- (He et al., 2011a; Högberg et al., 2003; Marzluf, 1997), and (ii) the dual role MurN plays in compensating for C requirements associated with application of NO_3^- (He et al., 2011a). MurN was previously shown to be degraded to a greater degree than GlcN in high N input systems, where available C was exhausted (Engelking et al., 2007; He et al., 2011b; McFarland et al., 2002; Six et al., 2006). Although the study presented here used lower N application rates, the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$ is promoted by C availability, indicating the SMC was C rather than N limited in the $^{15}NO_3^-$ treatment (Abaas et al., 2012; Burger and Jackson, 2003; Fazzolari et al., 1998; Puri and Ashman, 1999; Rütting et al., 2011). It was not possible to determine if N form impacted ^{15}N-routing into the GalN pool as there were no observable trends in the determined δ^{15}N values of the GalN. This indicated there was no detectable incorporation of the applied ^{15}N-label by the sensitive GC-C-IRMS analytical technique from either substrate, therefore, ^{15}N-incorporation was very low into the GalN pool in a short incubation timescale. Higher ^{15}N-application rates may be required to discern a significant change in GalN δ^{15}N values, as used in previous studies due to stimulation of AS production (He et al., 2011a,b).

4.4. Decoupling bacterial and fungal N assimilation dynamics

Decoupling the N assimilation dynamics of the bacterial and fungal pools in soil was possible using percentage ^{15}N incorporation for bacterial and fungal pools, accounting for the two origins of GlcN (Fig. 3). Key assumptions involved in these analyses were the $GlcN_B$ and $GlcN_F$ pools had the same ^{15}N-enrichment, and the mass ratio of $GlcN_B$ and MurN was 5-to-1 from cultured soil bacteria (Glaser et al., 2004). This ratio was used as it was advantageous to estimate mean $GlcN_B$ contributions during bacteria growth from both peptidoglycan and other sources to living biomass and necromass (Glaser et al., 2004). Whilst this may underestimate the contribution from fungal GlcN, the ratio was used alongside ^{15}N-incorporation into MurN

to elucidate bacteria-derived ^{15}N-incorporation and the fungal response to ^{15}N addition (Glaser et al., 2004; He et al., 2011a,b). Also, percentage ^{15}N incorporation into AS pools accounted for the differences in application rates between the two ^{15}N-fertilisers applied, enabling elucidation of the fate of applied ^{15}N, irrespective of initial soil N pools. While the dilution of the applied ^{15}N varied between the two treatments (theoretical enrichment of 8.7 atom % ^{15}N and 2.6 atom % ^{15}N for ^{15}NH$_4^+$ and ^{15}NO$_3^-$ treatments, respectively; Köster et al., 2015), the fate of the applied ^{15}N-fertiliser was determined as opposed to the inorganic N pools as a whole, allowing the treatments to be directly compared.

The trends in ^{15}N incorporation for the bacterial AS pool was as observed for the δ^{15}N values, with an initial rapid period of incorporation followed by a relative plateau (Fig. 3). The same trend was also observed in the THAA pool (Charteris et al., 2016). Whilst the observed trend in ^{15}N-incorporation was the same in the bacterial pool, lower initial and overall rates of ^{15}N incorporation and a lower plateau level for percentage ^{15}N incorporation was observed for the ^{15}NO$_3^-$ treatment compared to the ^{15}NH$_4^+$ treatment (Table 4). NH$_4^+$ is known to be the preferred N source for the SMC, directly assimilated into the ON pool via the GDH (glutamate dehydrogenase) and GS-GOGAT (glutamine synthetase-glutamine oxoglutarate aminotransferase) pathways (Caspi et al., 2018; Geisseler et al., 2010; Meers et al., 1970; Puri and Ashman, 1999; Recous et al., 1990; Rice and Tiedje, 1989). N is subsequently assimilated into GlcN from glutamine with the transfer of an NH$_2$ group to fructose-6-phosphate to yield glucosamine-6-phosphate prior to further biosynthetic steps to produce MurN, ManN and GalN (Kottom et al., 2017; Leloir and Cardini, 1953). NO$_3^-$ is incorporated into GlcN via the same biosynthetic pathway but must first be reduced to NH$_4^+$, an energy intensive process, hence, the lower observed ^{15}N incorporation of applied ^{15}NO$_3^-$ (Burger and Jackson, 2003; Puri and Ashman, 1999). This fundamental biochemical difference in the nature of the added N-substrates was also reflected in the ^{15}N incorporation into the THAA pool, shown by lower

plateau ^{15}N incorporation for the $^{15}NO_3^-$ treatment (P_1=1.82%) compared to the $^{15}NH_4^+$ treatment (P_1=13.6%; Charteris, 2016; Charteris et al., 2016).

The fate of the applied $^{15}NH_4^+$ and $^{15}NO_3^-$ was dominated by the bacterial pool immediately after application (up to 4 days), while fungal assimilation of applied ^{15}N was of increasing importance in the later portion of the incubation, shown by the continued increase in percentage ^{15}N incorporation for GlcN$_F$ (Fig. 3) up to 32 days of incubation. This reflected the different temporal response of the fungal and bacterial AS pools and was consistent with observations of slower turnover of GlcN in fungal cell walls compared to bacterial cell walls (Amelung, 2001; Guggenberger et al., 1999; He et al., 2011b). Interestingly, there is no significant difference in the overall rate of ^{15}N incorporation between the two treatments for the fungal AS pool (Fig. 3). This is contrary to observed decreased percentage ^{15}N incorporations and lower rates of ^{15}N incorporation for both bacterial AS and THAA (Fig. 4 and Fig. 5) for the $^{15}NO_3^-$ treatment(Charteris et al., 2016). It has been previously suggested fungi are more active in soils with lower nutrient concentrations due to their ability to utilise different secondary N sources when required. Therefore, the fungal community may be better adapted to utilising NO_3^- than the bacterial community, an observation which warrants further investigation (He et al., 2011a; Högberg et al., 2003; Marzluf, 1997). Alternatively, as ^{15}N-assimilation into GlcN$_F$ continues across the incubation period, $^{15}NO_3^-$ may have been converted to other forms of N, predominantly by the bacterial community (e.g. NH_4^+ or ON) and subsequently utilised by the fungal community, without the high energy requirements of assimilating NO_3^- directly.

4.5. Biosynthetic pathways of AS reflected in ^{15}N-incorporation dynamics

^{15}N-incorporation dynamics relating to ^{15}N assimilation into individual AS were combined with known biosynthetic pathways for AS to construct biosynthetic maps indicating

biosynthetic proximity and fluxes into pools based on experimentally derived data outlined in section 3.3.2 (Fig. 5; Knowles et al., 2010; Kottom et al., 2017; Leloir and Cardini, 1953; Macechko et al., 1992). All parameters used to construct these biosynthetic maps were based on percentage ^{15}N incorporation into AS pools, which accounted for the different application rates of N. The pathways are based on known bacterial biosynthetic routes of AS, with fungal assimilation of GlcN$_F$ and GalN thought to occur by the same pathways (Milewski et al., 2006). Whilst the bacterial and fungal contribution to the GlcN pool has been previously investigated, the relative contribution of these sources to the GalN is not fully understood (Joergensen, 2018). Therefore, the GalN pool is represented with two biosynthetic pathways in Sankey diagrams in Fig 5. As negligible ^{15}N incorporation into GalN was observed for both treatments, it was assumed GalN was considerably biosynthetically further from applied ^{15}N, consistent with reported AS biosynthetic pathways (Kottom et al., 2017; Leloir and Cardini, 1953; Macechko et al., 1992).

The initial rates of ^{15}N incorporation into individual AS pools indicated biosynthetic proximity to the applied substrate (Fig. 5). Within the bacterial AS pool, biosynthetic proximity of individual AS was GlcN$_B$ > MurN, consistent with the biosynthetic pathways for AS assimilation in bacteria (Kottom et al., 2017; Leloir and Cardini, 1953; Macechko et al., 1992). GlcN$_B$ is biosynthesised first and following acetylation, subsequently converted to MurN by addition of a lactate group (Kottom et al., 2017; Leloir and Cardini, 1953). All AS were biosynthetically closer to the ^{15}NH$_4^+$ treatment than ^{15}NO$_3^-$ (section 4.2; Charteris et al., 2016; Knowles et al., 2010). This is consistent with the known microbial preference for NH$_4^+$, and observations from the THAA pool, as discussed in section 4.4. Higher incorporation of ^{15}NH$_4^+$ compared to ^{15}NO$_3^-$ was also supported by higher ^{15}N-flux, reflected by plateau ^{15}N incorporation, which was consistently higher in the ^{15}NH$_4^+$ treatment compared to the ^{15}NO$_3^-$ treatment for bacterial AS. Furthermore, MurN was relatively biosynthetically further away

from the added ^{15}N relative to $GlcN_B$ in the $^{15}NO_3^-$ treatment compared to the $^{15}NH_4^+$ treatment. As proposed in section 4.3, differences in routing of applied ^{15}N likely contributed to this as MurN has been observed to compensate for C requirements associated with application of NO_3^- (He et al., 2011a). Finally, $GlcN_F$ appeared biosynthetically further from the applied ^{15}N substrates compared to the $GlcN_B$, although biosynthesis of GlcN is the same in bacteria and fungi. Therefore, the apparent difference in biosynthetic proximity actually reflected lower turnover rates of GlcN in fungi than bacteria (Amelung, 2001; Guggenberger et al., 1999; He et al., 2011b; Kottom et al., 2017; Leloir and Cardini, 1953). The overall routing into the $GlcN_F$ pool, indicated by plateau ^{15}N-incorporation, was similar in the two treatments, as the fungal community is be better suited to utilising NO_3^- compared to the bacterial community (He et al., 2011a; Högberg et al., 2003; Marzluf, 1997).

4.6. Microbial nitrogen use efficiency

Building on the novel insights into fate of N amendments into the soil protein pool as undertaken in Charteris et al. (2016), the application of a compound-specific ^{15}N-SIP approach using AS elucidates N-assimilation dynamics for the two largest defined pools of ON in soil (Schulten and Schnitzer, 1998). This enables evaluation of the role of the SMC in assimilation of applied N-fertiliser into the ON pool, termed microbial NUE (Mooshammer et al., 2014). This is important due to the central role of the SMC in soil services, including supporting plant nutrient supply and pollution mitigation (Brussaard, 2012). When ^{15}N-incorporation plateaued (suggesting applied ^{15}N turnover is at equilibrium, or is immobilized in more recalcitrant pools), the percentage of the applied ^{15}N incorporated into the microbial pool was 14.6% (SE 1.4%) and 2.49% (SE 0.4%) for the $^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively (Charteris et al., 2016; Fig. 6). Within this pool, the flux into the AA pool accounted for the majority of the assimilated ^{15}N for both treatments (13.6 ± 1.3 % and 1.81 ± 0.34 % of applied ^{15}N for the

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$^{15}NH_4^+$ and $^{15}NO_3^-$ treatments, respectively; Charteris, 2016; Charteris et al., 2016). This is due to the higher proportion of AA comapred to AS and therefore, higher incorporation into this pool compared to AS (Schulten and Schnitzer, 1998).The higher microbial NUE determined for the $^{15}NH_4^+$ treatment compared to $^{15}NO_3^-$ can be related to microbial preference for NH4+, due to the additional biosynthetic processing required for NO_3^- assimilation. Microbial NUE can be used to determine the impact of soil management strategies (e.g. N-form, application rates and timings) on the ability of the microbes to stabilise applied N and subsequently act as a source of N for plants in a field setting, thereby improving the NUE of the system.

5. Conclusions

The extension of a compound-specific ^{15}N-SIP approach to the source specific AS pool using experimentally derived flux and initial rate parameters allowed deconvolution of N-assimilation dynamics of the bacterial and fungal communities in soil in particular:

(i)   Application of environmentally relevant rates of ^{15}N-fertilisers did not alter the composition of the AS pool, however, a small priming effect was observed. This was not proportional to the difference in N application rates, indicating the effect was associated with the form of N applied.

(ii) $\delta^{15}N$ values revealed differences in the relative routing of ^{15}N between the two treatments, due to C demand associated with NO_3^- assimilation, and the capacity of fungi to use secondary N sources. This was also supported by ^{15}N-incorporation estimates, which accounted for differences in N application rates.

(iii)   Percentage ^{15}N incorporation into individual AS enabled separation of the bacterial and fungal ^{15}N-assimilation dynamics. Following ^{15}N-fertiliser addition, observed ^{15}N-assimilation dynamics were initially dominated by N-fluxes associated with the bacterial community (up to *ca.* 4 days), however, N-

fluxes into the fungal community became more significant later in the incubation period (up to 32 days). This provided insights into the temporal scale on which members of the SMC incorporate applied ^{15}N-fertilisers. Assimilation into individual AS pools also reflected known biosynthetic pathways of AS, and the slower turnover of fungal pools, relative to bacterial pools. .

(iv)   The microbial preference for NH_4^+ was reflected by higher flux of applied ^{15}N into the AS pools, which accounted for the difference in application rate of the two substrates (Geisseler et al., 2010; Meers et al., 1970; Puri and Ashman, 1999; Recous et al., 1990; Rice and Tiedje, 1989).

These hitherto unavailable N-dynamic factors, combined with N-assimilation dynamics of the AA pool (Charteris et al., 2016) can be used to evaluate the microbial NUE of different N-substrates, working towards informing soil management strategies using insights from the microbially-mediated soil N-cycle.

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